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K16-biotinylated histone H4 is overrepresented in repeat regions and participates in the repression of transcriptionally competent genes in human Jurkat lymphoid cells☆

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Abstract

Holocarboxylase synthetase (HCS) catalyzes the binding of biotin to lysine (K) residues in histones H3 and H4. Histone biotinylation marks are enriched in repressed loci, including retrotransposons. Preliminary studies suggested that K16 in histone H4 is a target for biotinylation by HCS. Here we tested the hypotheses that H4K16bio is a real histone mark in human chromatin and that H4K16bio is overrepresented in repressed gene loci and repeat regions. Polyclonal rabbit anti-human H4K16bio was generated and affinity purified. An extensive series of testing with synthetic and natural targets confirmed that this new antibody is specific for H4K16bio. Using anti-H4K16bio and chromatin immunoprecipitation assays, we demonstrated that H4K16bio is overrepresented in repeat regions [pericentromeric alpha satellite repeats and long terminal repeats (LTR)] compared with euchromatin promoters. H4K16bio was also enriched in the repressed *interleukin-2* gene promoter in human lymphoid cells; transcriptional activation of the *interleukin-2* gene by mitogens and phorbol esters coincided with a depletion of the H4K16bio mark at the gene promoter. The enrichment of H4K16bio depended on biotin supply; the enrichment at LTR22 and promoter 1 of the sodium-dependent multivitamin transporter (SMVT) was greater in biotin-supplemented cells compared with biotin-normal and biotin-deficient cells. The enrichment of H4K16bio at LTR15 and SMVT promoter 1 was significantly lower in fibroblasts from an HCS-deficient patient compared with an HCS wild-type control. We conclude that H4K16bio is a real phenomenon and that this mark, like other biotinylation marks, is overrepresented in repressed loci where it marks HCS docking sites.

Keywords: Biotin; Histone; Holocarboxylase synthetase; Human

1. Introduction

Histones mediate the folding of DNA into chromatin [1]. Amino acid residues in the N-terminal tails and, to a lesser extent, other domains of histones are targets for covalent modifications such as acetylation and methylation [2]. These posttranscriptional modifications play important roles in gene regulation, chromatin remodeling, mitosis and DNA repair. For example, lysine (K)-9 acetylated histone H3 (H3K9ac) and K4-trimethylated histone H3 (H3K4me3) are overrepresented in transcriptionally active euchromatin, whereas hypoacetylated histones and K9-dimethylated histone H3 (H3K9me2) are overrepresented in heterochromatin and repressed genes.

Hymes et al. [3] provided evidence that histones are also modified by covalent attachment of the vitamin biotin, based on *in vitro* studies with purified histones and the histone biotinyl ligase, biotinidase. Subsequently, biotinylated histones were detected in chromatin from human and other metazoans by using radiotracers, streptavidin, anti-biotin, biotinylation site-specific antibodies and mass spectrometry [4–10]. The majority of biotinylation takes place at K4, K9, K18 and perhaps K23 in histone H3 [11,12], and K8 and K12 in histone H4 [6], but a weak biotinylation signal can also be detected in histone H2A [4,13]. These previous studies also suggest that holocarboxylase synthetase (HCS) is more important than biotinidase for catalyzing biotinylation of histones *in vivo* [7,12,14]. Phenotypes of HCS knockdown include a short life span and low heat survival in *Drosophila melanogaster* [7] and aberrant gene regulation in humans [15,16].

Biotinylation of histones is a fairly rare event, i.e., less than 0.1% of histones in bulk extracts are biotinylated in samples of human origin [10,17]. However, the comparably low level of global histone

Abbreviations: ADH, aldehyde dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3K4me3, K4-trimethylated histone H3; H3K9ac, K9-acetylated histone H3; H3K9bio, K9-biotinylated histone H3; H3K9me2, K9-dimethylated histone H3; H3K18bio, K18-biotinylated histone H3; H4K8bio, K8-biotinylated histone H4; H4K12bio, K12-biotinylated histone H4; H4K16bio, K16-biotinylated histone H4; HCS, holocarboxylase synthetase; IL-2, interleukin-2; K, lysine; KLH, keyhole limpet hemocyanin; LTR, long terminal repeats; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase; qRT-PCR, quantitative real-time polymerase chain reaction; SMVT, sodium-dependent multivitamin transporter.

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biotinylation is not representative of the level of biotinylation in confined regions of chromatin. For example, about one of three histone H4 molecules in telomeric repeats is biotinylated at K12 [18]. Biotinylation of histones has important functions in gene repression and genome stability. Previous studies suggest that K9- and K18-biotinylated histone H3 (H3K9bio, H3K18bio) and K8- and K12-biotinylated histone H4 (H4K8bio, H4K12bio) are enriched in repeat regions and participate in gene repression [15,16,18–20]. The abundance of histone biotinylation marks depends on both biotin supply and HCS activity [15,16,20].

Preliminary studies provide evidence that histone H4 is also biotinylated at K16 (H4K16bio) [13]. Here, we tested the hypotheses that H4K16 is a true biotinylation mark in human chromatin and that H4K16bio, like other biotinylation signatures, is overrepresented in repeat regions and participates in the repression of transcriptionally competent chromatin.

2. Materials and methods

2.1. Cell culture

Jurkat human lymphoma cells (ATCC, Manassas, VA, USA) were cultured in commercial RPMI-1640 (Thermo Scientific, Waltham, MA, USA) using standard cell culture techniques [21]. Regular commercial RPMI-1640 contains 820 nmol/L of biotin, which is >3000 times the biotin concentration in human plasma [22]. In some experiments, cells were cultured in biotin-deficient (0.025 nmol/L biotin), biotin-physiological (0.25 nmol/L biotin) and biotin-pharmacological (10 nmol/L biotin) media for 12 days prior to analysis [21]. These concentrations represent levels observed in plasma from biotin-deficient individuals, biotin-normal individuals and individuals taking over-the-counter biotin supplements [22,23]. Biotin-defined media were prepared using customized RPMI-1640 and biotin-depleted fetal bovine serum [21]. Efficacy of treatment was confirmed by probing biotinylated carboxylases with IRDye-streptavidin [20]. Where indicated, expression of interleukin-2 (IL-2) was stimulated by treating Jurkat cells with phorbol-12-myristate-13-acetate (PMA) and phytohemaglutinin (PHA) [21] for 3 h.

Human WG2215 HCS-deficient skin fibroblast (Montreal Children's Hospital Cell Repository, Montreal, Canada) were cultured in Dulbecco's Modified Eagle's Medium containing 10 nmol/L biotin to investigate the effects of HCS deficiency on histone biotinylation [20]. Human HCS wild-type IMR-90 fibroblasts (ATCC) were used as controls. HCS activity in IMR-90 and WG2215 fibroblasts was assessed by using biotinylated carboxylases as markers in streptavidin blots [20].

2.2. Antibodies

Rabbit anti-human H4K16bio was generated as described [6]. Briefly, an H4-based, K16-biotinylated synthetic peptide (GGAK(bio)RHRKVLRD, Anaspec, San Jose, CA, USA) was conjugated to keyhole limpet hemocyanin (KLH, Pierce, IL, USA) and used as antigen for injection into New Zealand rabbits. Specificity tests revealed that the antiserum cross-reacted with K16-acetylated histone H4, using the synthetic peptide GGAK(ac)RHRKVLRD (H4K16ac) as a target (data not shown). To eliminate cross-reactivity with H4K16ac, the antiserum was affinity purified by using immobilized peptide H4K16ac and HiTrap NHS-Activated Sepharose High Performance Column (cat. no. 17-0717-01; GE Healthcare, Piscataway, NJ, USA) following the manufacturer's

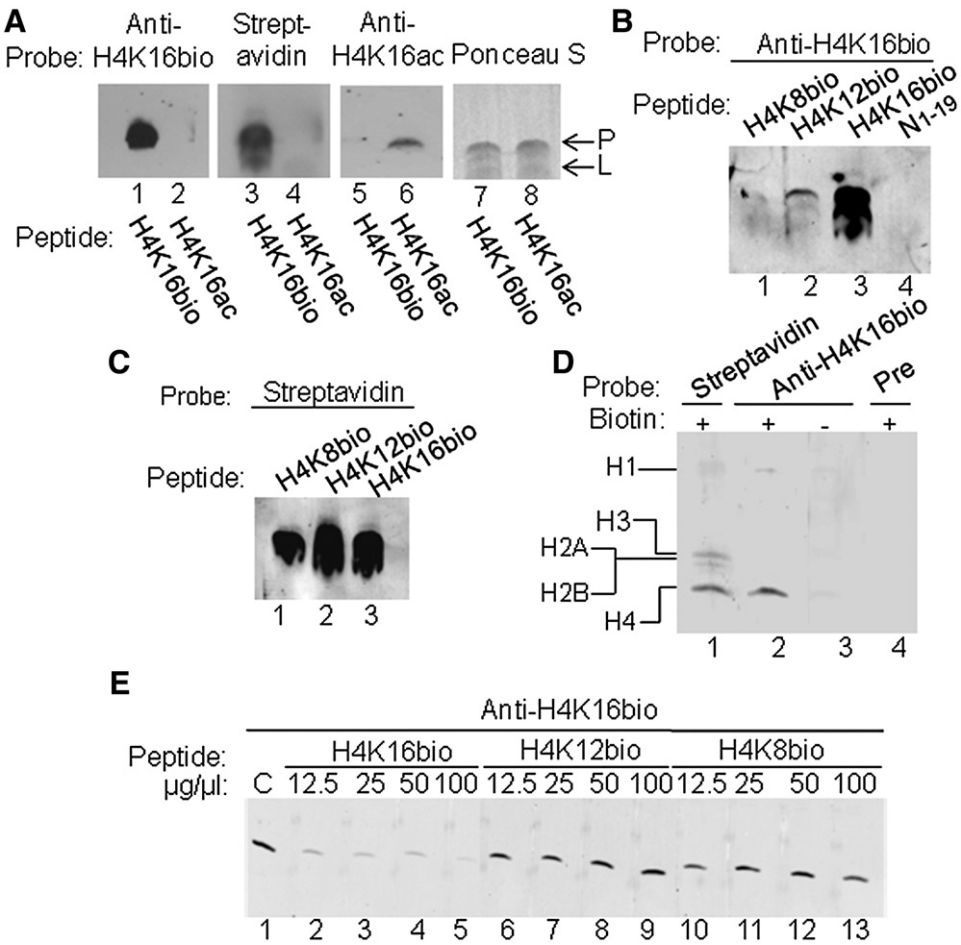

Fig. 1. Validation of anti-H4K16bio. (A) K16-biotinylated and -acetylated peptides were probed with anti-H4K16bio, IRDye-streptavidin, anti-H4K16ac and Ponceau S. (B) Synthetic H4-based peptides biotinylated at K8 (N_{6-15}bioK8), K12 (N_{6-15}bioK12) and K16 (N_{13-24}bioK16), and nonbiotinylated peptide N_{1-19} were probed with anti-H4K16bio. (C) Peptides H4K8bio, H4K12bio and H4K16bio from Panel (B) were probed with IRDye-streptavidin. (D) Bulk extracts of Jurkat cell histones were probed with IRDye-streptavidin, anti-H4K16bio and pre-immune serum; "—" denotes biotin-depleted histone samples. (E) Bulk extracts of Jurkat cell histones were probed with anti-H4K16bio in the presence of increasing amounts of synthetic peptides H4K16bio, H4K12bio and H4K8bio. The control sample ("C") was assayed in the absence of peptide competitors. Some gels were electronically rearranged to facilitate comparisons. L, Loading buffer; P, peptide.

protocol. Identity and purity of all synthetic peptides were confirmed by mass spectrometry and HPLC, respectively, by the manufacturer.

Biotinylation site specificity of anti-H4K16bio was confirmed by using synthetic histone-based peptides, histone bulk extracts, biotin-depleted histones and competition studies as described before [6]. Equal loading and transfer were confirmed by staining membranes with Ponceau S [20].

Polyclonal rabbit anti-human antibodies to H4K16bio, H4K12bio and H3K9me2 were generated in a commercial facility (Cocalico Biologicals, Reamstown, PA, USA) as described [6,11,20], while antibodies to the C-terminus in histone H3 (ab1791) and H3K9ac (ab10812) were purchased from Abcam (Cambridge, MA, USA). H3K9me2 and H4K12bio were used as gene repression and heterochromatin marks, whereas H3K9ac was used as a euchromatin mark. Rabbit polyclonal anti-human pyruvate carboxylase (PC) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and PC in transblots was probed as described before [20].

2.3. Micro chromatin immunoprecipitation assay

The enrichment of H4K16bio in distinct loci in human chromatin was assessed by micro chromatin immunoprecipitation (μChIP) assay as described [20,24] with the following modifications. Chromatin from Jurkat cells was precipitated using 14 μl of anti-H4K12bio and anti-H3K9me2, and 28 μl of anti-H4K16bio sera.

2.4. Quantitative real-time PCR

The abundance of DNA in immunoprecipitated chromatin was quantified by quantitative real-time PCR (qRT-PCR) using Perfecta SYBR Green FastMix ROX (VWR, West Chester, PA, USA) and PCR primers for long terminal repeats (LTR) 15 and 22, pericentromeric alpha satellite repeats in chromosome 4, and promoters in genes coding for IL-2, sodium-dependent multivitamin transporter (SMVT), aldehyde dehydrogenase (ADH) 5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [20]. Amplicons were quantified using the cycle threshold values method [25]. GAPDH was used to normalize qRT-PCR data for amplification efficiency. The relative enrichment of histone marks in precipitated chromatin was calculated as percent of input DNA [26,27]. Enrichment data were normalized for nucleosomal occupancy by precipitating samples with an antibody to the (nonmodified) C-terminus in histone H3. Nonspecific rabbit IgG (Santa Cruz Biotechnology) was used as a control for background noise and precipitated negligible amounts of chromatin (<5% compared with anti-H4K16bio), consistent with previous observations [20].

The abundance of mRNA coding for IL-2, LTR22 and SMVT was quantified using Absolute QPCR SYBR Green fluorescein mix (ABgene, Rochester, NY, USA) as described [6,20].

2.5. Statistical analysis

Bartlett's test was used to test for homogeneity of variances [28]. Data were log transformed if variances were heterogeneous. Significance of differences among more than two groups was tested by one-way ANOVA and Fisher's Protected Least Significant Difference procedure for post hoc testing. Student's paired *t* test was used for pairwise comparisons of data from Jurkat cells before and after stimulation with PMA and PHA. μChIP data from WG2215 and IMR-90 fibroblasts were not normally distributed and were analyzed by using the Mann–Whitney *U* test. StatView 5.0.1 (SAS Institute, Cary, NC, USA) was used to perform all calculations. Differences were considered statistically significant if P was <.05. Data are expressed as mean±S.D.

3. Results

3.1. Specificity of anti-H4K16bio

Affinity-purified anti-H4K16bio is specific for its designated target, based on the following lines of observations. In a first series of experiments, we compared the affinity of anti-H4K16bio for the synthetic peptides H4K16bio and H4K16ac (Fig. 1A). Purified anti-H4K16bio produced a strong signal with peptide H4K16bio (Lane 1), but no signal with peptide H4K16ac (Lane 2). The presence of biotinylation and acetylation marks was confirmed by probing transblots with IRDye-streptavidin (Lanes 3 and 4) and anti-H4K16ac (Lanes 5 and 6). Equal loading and transfer were confirmed by staining transblots with Ponceau S (Lanes 7and 8). Note the nonspecific signal in the Ponceau S-stained membrane, caused by interference of the dye in the sample loading buffer (denoted "L").

In a second series of experiments, we assessed the biotinylation site specificity of anti-H4K16bio by comparing its binding to synthetic peptides biotinylated at K8 (N_{6-15}bioK8), K12 (N_{6-15}bioK12) or K16 (N_{13-24}bioK16), where subscripts denoted amino acid residues in histone H4. The nonbiotinylated peptide N_{1-19} was used as negative control. Anti-H4K16bio produced a strong signal with peptide H4K16bio, whereas the signals with peptides H4K8bio and H4K12bio were hardly detectable (Fig. 1B, Lanes 1–3). No signal was detectable with nonbiotinylated peptide (Lane 4). Biotinylation of synthetic peptides was confirmed by probing transblots with IRDye-streptavidin (Fig. 1C, Lanes 1–3). Loading of the nonbiotinylated peptide N_{1-19} was adjusted gravimetrically.

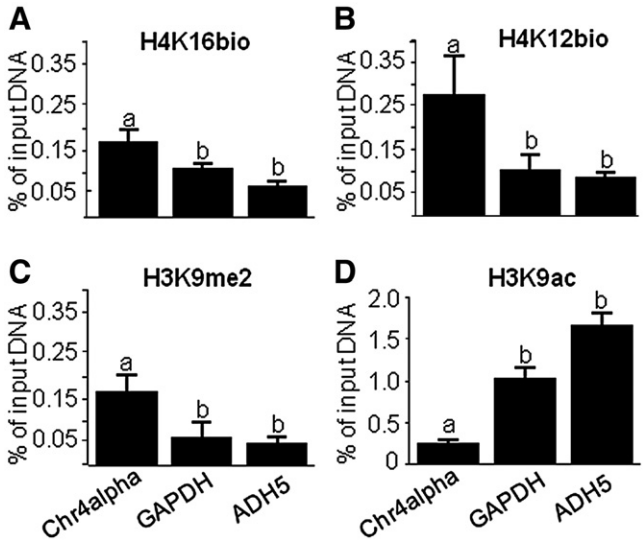

Fig. 2. H4K16bio is overrepresented in pericentromeric alpha satellite repeats (heterochromatin) compared with promoters in euchromatin. Chromatin was immunoprecipitated using antibodies to H4K16bio, H4K12bio, H3K9me2 and H3K9ac, and qRT-PCR was used to quantify the relative enrichment of alpha satellite repeats in chromosome 4 (Chr4alpha), and promoters of the *GAPDH* and *ADH5* genes. Bars without a common letter are significantly different (n=4, P<.05).

In a third series of experiments, we identified H4K16bio in histone bulk extracts from Jurkat cells. First, we probed histones with streptavidin to demonstrate that the majority of biotinylation marks reside in histones H3 and H4, and that biotinylation of histones H1, H2A and H2B is quantitatively minor (Fig. 1D, Lane 1), consistent with our previous results [4]. Equal loading and integrity of proteins were confirmed by staining with Coomassie blue (not shown). When histones were probed with anti-H4K16bio, the antibody produced a signal only with histone H4, but not with other classes of histones (Lane 2). This signal was specifically caused by the biotinylated fraction of histone H4, because biotin-depleted histones did not produce a signal (Lane 3). When transblots were probed with pre-immune serum, no signal was detectable (Lane 4).

In a fourth and final series of antibody testing, bulk extracts of Jurkat cell histones were probed with anti-H4K16bio in the presence of increasing amounts of synthetic peptides. As expected, peptide H4K16bio outcompeted histone H4 for binding by anti-H4K16bio compared with peptide-free control (Fig. 1E, Lanes 1–5). In contrast, peptides H4K12bio (Lanes 6–9) and H4K8bio (Lanes 10–13) did not compete for binding.

3.2. Relative enrichment of H4K16bio at selected genomic loci

H4K16bio is overrepresented in pericentromeric alpha satellite repeats (heterochromatin) in chromosome 4 compared to the promoters of the two euchromatin genes *GAPDH* and *ADH5* in Jurkat cells (Fig. 2A). The enrichment of H4K16bio in the promoter of *GAPDH* gene was similar to that of *ADH5*. The known repression marks H4K12bio and H3K9me2 [19,20] were used as positive controls and showed a pattern similar to that of H4K16bio (Fig. 2B and C). In

contrast, the known activation mark H3K9ac (negative control) was greatly enriched in euchromatin promoters compared with pericentromeric alpha satellite repeats (Fig. 2D).

H4K16bio was overrepresented in the transcriptionally repressed promoter in the IL-2 gene prior to stimulation with PMA and PHA compared with 3 h after activation with PMA and PHA (Fig. 3A). qRT-PCR was used to confirm the efficacy of de-repression of the *IL-2* gene by treatment with PMA and PHA (arbitrary units): 1.0±0.2 before stimulation vs. 28±2.7 after stimulation (n=4; P<.05). The controls showed the expected pattern. While the repression marks H4K12bio and H3K9me2 were removed from the IL-2 gene promoter in response to stimulation with PMA and PHA (Fig. 3B and C), the relative enrichment of the activation mark H3K9ac was greater after than before gene activation (Fig. 3D).

3.3. Biotin dependence

Previous studies suggest that the abundance of H3K9bio, H3K18bio, H4K8bio and H4K12bio at LTR15, LTR22 and *SMVT* promoter 1 depends on the concentration of biotin in culture media and dietary biotin intake in various model organisms including healthy adults [15,16,20]. Here, we observed a similar scenario for H4K16bio. The H4K16bio mark was 13-fold and 4-fold more abundant in the LTR22 locus in cells cultured in medium containing 10 nmol/L compared with cells cultured in 0.025 and 0.25 nmol/L biotin, respectively (Fig. 4A). Similar patterns were observed for the repression marks H4K12bio (Fig. 4B) and H3K9me2 (Fig. 4C), while the enrichment of H3K9ac was not affected by biotin (Fig. 4D). When the abundance of H4K16bio was low at LTRs, the transcriptional activity increased compared with cells where H4K16bio was high. The abundance of LTR transcripts originating in the U5 region [15] was 2.9±0.6 in cells cultured in medium containing 0.025 nmol/L, 0.9±0.06 in cells cultured in medium containing 0.25 nmol/L and 0.2±0.02 in cells cultured in medium containing 10 nmol/L (arbitrary units, n=4, P<.05 among all treatment groups).

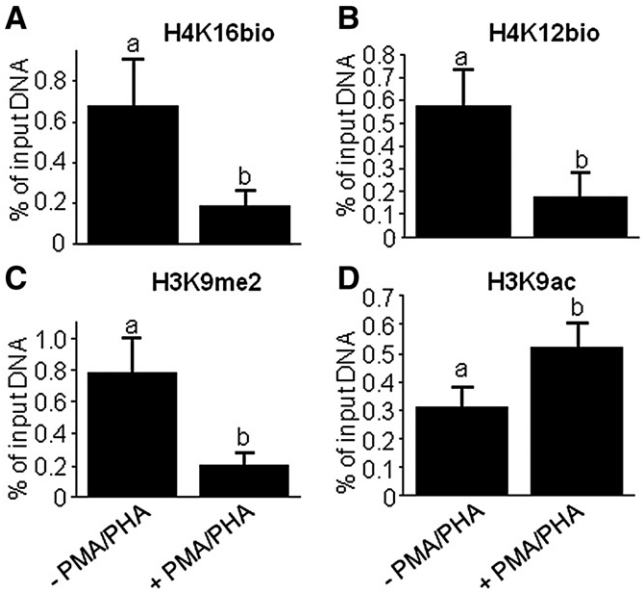

Fig. 3. Differential enrichment of histone marks in the *IL-2* gene promoter in Jurkat cells was stimulated with PMA/PHA for 3 h. Chromatin was immunoprecipitated with antibodies to H4K16bio, H4K12bio, H3K9me2 and H3K9ac before and after stimulation with phorbol-12-myristate-13-acetate (PMA) and phytohemagglutinin (PHA). qRT-PCR was used to quantify the relative enrichment of *IL-2* promoter sequences. Bars without a common letter are significantly different (n=5, P<.05).

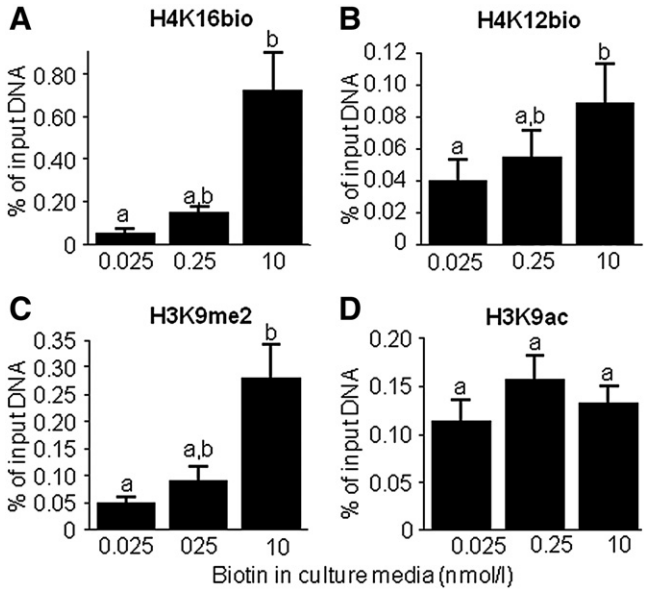

Fig. 4. The abundance of H4K16bio at the LTR22 locus depends on the concentration of biotin in culture media. Cells were cultured in biotin-defined media for 12 days, and chromatin was precipitated with antibodies to H4K16bio, H4K12bio, H3K9me2 and H3K9ac; qRT-PCR was used to quantify the relative enrichment of *LTR22* sequences. Bars without a common letter are significantly different (n=4–5, P<.05).

The enrichment of H4K16bio, H4K12bio and H3K9me2 at the *SMVT* promoter 1 locus was comparable to that at LTR22, e.g., the H4K16bio mark was 1.7-fold and 1.4-fold more abundant in cells cultured in medium containing 10 nmol/L compared with cells cultured in 0.025 and 0.25 nmol/L biotin, respectively. However, the differences among the different biotin concentrations did not reach statistical significance for the SMVT promoter (P=.21).

The efficacy of biotin treatment was confirmed by probing biotinylated holocarboxylases with IRDye-streptavidin (Fig. 5, upper gel). Clearly, the abundance of holocarboxylases depended on the concentration of biotin in culture media. Equal loading of lanes was confirmed by using anti-PC as a probe for total PC (holocarboxylases and apocarboxylases; Fig. 5, lower gel). The levels of biotinylated acetyl-CoA carboxylases 1 and 2 are below the limit of detection on Jurkat cells, consistent with previous observations [21].

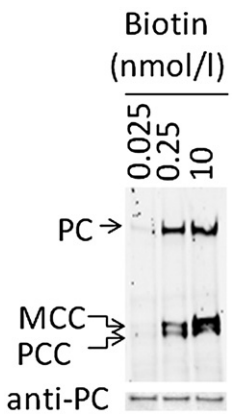

Fig. 5. Biotinylation of carboxylases depends on the concentration of biotin in culture media. Jurkat cells were cultured in biotin-defined media for 12 days, and carboxylase-bound biotin was probed with IRDye-streptavidin in pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) (upper gel). Equal loading of lanes was confirmed by using anti-PC as a probe (lower gel).

3.4. HCS deficiency

Consistent with a previous report [20], the enrichment of H4K16bio and the repression marks H4K12bio and H3K9me2 decreased at the *LTR22* locus in HCS-deficient fibroblasts (WG2215) compared with HCS-normal fibroblasts (IMR-90) (Fig. 6A–C). The same pattern was obtained if H4K16bio was studied at *SMVT promoter 1* and *LTR15* loci (data not shown). HCS activity in both types of fibroblasts was confirmed by probing transblots of biotinylated carboxylases with IRDye-streptavidin (Fig. 6D, upper gel). Biotinylated carboxylases were easily detectable in IMR-90 fibroblasts, whereas no signal was detectable in WG2215 fibroblasts. Anti-PC was used to confirm that the decrease of biotinylation signal in WG2215 cells was due to the inability of mutant HCS to biotinylate carboxylases rather than to low apocarboxylase expression or to differences in the loading or integrity of the proteins (Fig. 6D, lower gel).

4. Discussion

Here, we demonstrate that K16 in human histone is a target for covalent biotinylation, and we provide the first insights into possible biological functions of H4K16bio in pericentromeric heterochromatin and gene repression. A novel antibody was generated and its specificity for H4K16bio was demonstrated in an extensive series of testing. Importantly, we report that the abundance of the H4K16bio mark depends on both biotin supply and HCS activity.

H4K16bio seems to be a mark for gene repression, based on the following observations. First, the enrichment of H4K16bio in the *IL-2* gene promoter greatly decreases in response to gene activation with phorbol ester and mitogen. Second, the enrichment of H4K16bio at the retroelement LTR22 depends on the concentration of biotin in culture media. Depletion of H4K16bio coincides with an increased abundance of LTR transcript. This observation is particularly important given that previous in-depth studies of retroelement repression suggest an increase in chromosomal abnormalities and, therefore, cancer risk in biotin-depleted cells [15]. Third, the enrichment of H4K16bio in the *SMVT* gene promoter increases in response to biotin supplementation. In previous studies, we provided evidence that increased biotinylation of histones in the SMVT promoter is an important regulatory mechanism to repress the expression of the SMVT gene in biotin-supplemented cells [16]. It appears that the roles of histone biotinylation in gene repression are not specific for distinct biotinylation sites, as all biotinylation sites studies to date are overrepresented in transcriptionally repressed chromatin [19,20].

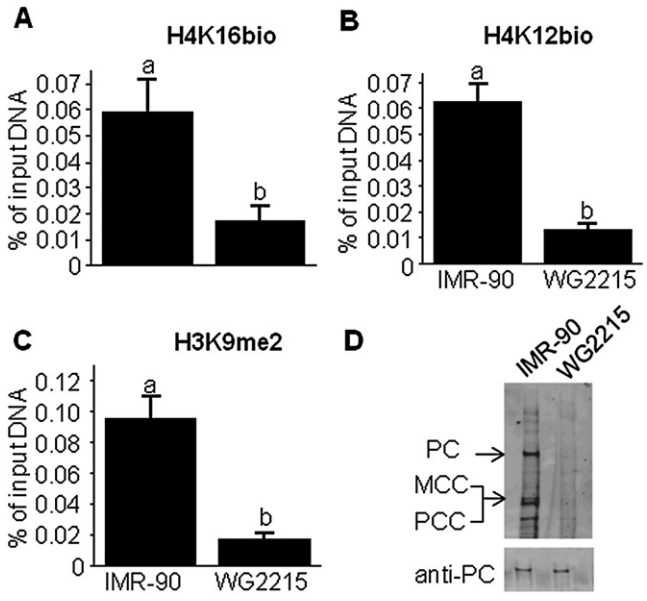

Fig. 6. The enrichment of H4K16bio at LTR22 depends on HCS in primary fibroblasts. (A–C) HCS-normal fibroblasts (IMR-90) and HCS-deficient fibroblasts (WG2215) were cultured in medium containing 10 nmol/L biotin. Chromatin was precipitated with antibodies to H4K16bio, H4K12bio and H3K9me2, and qRT-PCR was used to quantify the relative enrichment of LTR22. Bars without a common letter are significantly different (n=3–4, P<.05). (D) Biotin in PC, MCC and PCC was probed with IRDye-streptavidin (upper gel); equal loading was confirmed by using anti-PC as a probe (lower gel).

A recent report suggests that some of the commercial antibodies to biotinylated histones cross-react with acetylated histones [29]. In response to that report, we reexamined our in-house antibodies to H3K9bio, H3K18bio, H4K8bio and H4K12bio, and did not detect any cross-reactivity with acetylation marks [10,14]. We propose that these conflicting results are caused by the targets used for specificity testing. Healy et al. [29] used recombinant histones that were enzymatically acetylated or biotinylated; the extent of acetylation and biotinylation and the actual modification sites (e.g., K8 vs. K12) remained unknown. In contrast, in our studies, we used synthetic peptides as targets, where both the extent and location of the modification were unambiguous [10,14]. However, in contrast with the other in-house antibodies, the batch of polyclonal anti-H4K16bio tested here showed some cross-reactivity with H4K16ac. It seems prudent to individually validate each batch of polyclonal antibodies to biotinylated histones.

Our studies of histone biotinylation in HCS mutant fibroblasts lend further strength to the notion that histone biotinylation is a real phenomenon. The abundance of biotinylation marks was substantially lower in HCS mutant WG2215 fibroblasts compared with IMR-90 fibroblasts. Similar observations were made in previous studies [11,15,16], along with the observation that recombinant human HCS and its microbial ortholog BirA biotinylate histones *in vitro* [12,14]. There is still some level of uncertainty as to whether effects of HCS on gene regulation are mediated by biotinylation of histones or biotinylation of proteins such as carboxylases. However, the phenotypes of HCS knockdown are vastly different than phenotypes of carboxylase knockdown [7,30], suggesting that aberrant biotinylation of histones in HCS-deficient cells impairs gene regulation.

Biotinylation of histones is a rare event, occurring in <0.1% of histones [10,17]. One report suggested that histone biotinylation is an *in vitro* artifact [29], but that report has been rebutted [10]. Importantly, evidence is emerging that HCS is an integral part of a multiprotein gene repressor complex in human chromatin and that the rare biotinylation marks seen in the human epigenome are mere marks for HCS docking sites [10]. This theory would explain why all known histone biotinylations are enriched in repressed loci, including the H4K16bio mark reported here. Further mechanistic studies in whole organisms await the arrival of a conditional HCS knockout mouse, which is an ongoing effort in our laboratory.

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