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K12-biotinylated histone H4 is enriched in telomeric repeats from human lung IMR-90 fibroblasts☆

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Abstract

Covalent modifications of histones play a role in regulating telomere attrition and cellular senescence. Biotinylation of lysine (K) residues in histones, mediated by holocarboxylase synthetase (HCS), is a novel diet-dependent mechanism to regulate chromatin structure and gene expression. We have previously shown that biotinylation of K12 in histone H4 (H4K12bio) is a marker for heterochromatin and is enriched in pericentromeric alpha satellite repeats. Here, we hypothesized that H4K12bio is also enriched in telomeres. We used human IMR-90 lung fibroblasts and immortalized IMR-90 cells overexpressing human telomerase (hTERT) in order to examine histone biotinylation in young and senescent cells. Our studies suggest that one out of three histone H4 molecules in telomeres is biotinylated at K12 in hTERT cells. The abundance of H4K12bio in telomeres decreased by 42% during telomere attrition in senescent IMR-90 cells; overexpression of telomerase prevented the loss of H4K12bio. Possible confounders such as decreased expression of HCS and biotin transporters were formally excluded in this study. Collectively, these data suggest that H4K12bio is enriched in telomeric repeats and represents a novel epigenetic mark for cell senescence.

Keywords: Biotin; Chromatin; Histone; Biotinylation; Human; Telomere

1. Introduction

Telomeres, the heterochromatic end caps of linear chromosomes, are composed of thousands of TTAGGG repeats in human chromatin and bind proteins such as TRF1 (telomeric repeat binding factor 1), TRF2 and tankyrase [1–3]. Telomeres play an important role in chromosome stability by preventing the loss or mutations of genes and the fusion of chromosomes [1,4,5]. Due to the inability of DNA polymerases to replicate the end of chromosomes, telomere length is shortened by approximately 50–150 bp with each cell division [6]. Division ceases when telomeres reach a critical threshold length in senescent cells [6–8]. In germline cells, immortalized cell lines and cancer cells, telomere shortening is prevented by telomerase, which is composed of two major components, the telomerase reverse transcriptase and telomerase RNA template. Telomerase helps to maintain telomere length by adding TTAGGG repeats to the chromosome ends during replication [9–13].

Chromatin structure and gene expression are regulated by covalent modifications of histones H1, H2A, H2B, H3 and H4 [14,15]. DNA and histones form the nucleosomal core particles, which consist of 146 bp of DNA wrapped around an octamer of core histones (one H3–H3–H4–H4 tetramer and two H2A–H2B dimers) [16]. Histone H1 binds to the DNA in between two nucleosomal core particles, completing the nucleosomal assembly [16]. The amino-terminal tails of histones that protrude from the nucleosomal surface are subjected to various covalent modifications [17,18]. Acetylation, phosphorylation and methylation of histone tails play a major role in regulating telomere attrition by controlling telomerase activity [19–21].

Recently, it was shown that biotinylation of lysine (K) residues in histones, a novel diet-dependent histone modification, plays a role in gene regulation [22–24]. Biotinylation is mediated by holocarboxylase synthetase (HCS) [23,25]. Human histone biotinylation sites include K9, K13, K125, K127 and K129 in histone H2A [26]; K4, K9, K18 and perhaps K23 in histone H3 [27,28]; and K8 and K12 in histone H4 [29].

We have shown that K12-biotinylated histone H4 (H4K12bio) is enriched in repeat regions such as pericentromeric alpha satellite repeats and retrotransposons and also participates in gene repression [24,30]. Here, we tested the hypothesis that H4K12bio is a general marker for repeat regions and, therefore, is enriched in telomeric repeats. We propose that decreased abundance of H4K12bio is an epigenetic mark for cell senescence.

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In this study, we used primary human IMR-90 lung fibroblasts as a model to investigate histone biotinylation in aging cells. IMR-90 cells were chosen because they undergo telomere attrition and enter a state of irreversible growth arrest, designated cellular senescence, after about 50–55 population doublings (PDs). IMR-90 cells over-expressing human telomerase reverse transcriptase (hTERT cells) do not undergo senescence [31] and were used as immortal controls. Our goals were to determine (a) whether H4K12bio is enriched in telomeric repeats in human cells, (b) whether the abundance of H4K12bio decreases with aging and (c) whether overexpression of telomerase prevents loss of H4K12bio.

2. Methods and materials

2.1. Cell culture

Human lung fibroblast IMR-90 cells were purchased from American Type Culture Collection (Manassas, VA). These cells were passaged two to three times per week and replated at 60,000 to 80,000 cells in T75 cell culture flasks with 15 ml medium. IMR-90 cells were harvested at timed intervals. Young cells at PD 35 were used as baseline. IMR-90 cells were considered to be senescent when they did not need to be passaged for 2 weeks, and at this stage, they were at a PD of 50–55. hTERT cells were kindly provided by Dr. Judith Campisi (Lawrence Berkeley National Laboratory, California) at PD 55. hTERT cells at PD 60 were used as baseline and harvested at 5 or 10 additional PDs. Cells were cultured in Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, 0.1% nonessential amino acids, 100,000 U/L penicillin and 100 mg/L streptomycin.

2.2. β-Galactosidase (β-gal) staining

Senescent cells are known to express β-gal, which produces a blue color in the β-gal assay; the β-gal assay was performed as described previously [32].

2.3. Telomerase activity

Telomerase activity was detected by telomerase repeat amplification protocol (TRAP) assay [33] with slight modifications. In this assay, telomerase from cell extracts ligates synthetic TTAGGG repeats to the 3′ end of the telomerase substrate oligonuceotide "TS" (5′-AATCCGTCGAGCAGAGTT-3′). The extended product is then amplified by PCR using TS and the reverse primer CX (5′-CCTTACCCTTACCCTTACCC-TAA-3′), which generates a ladder of PCR products with size increasing in six-base increments starting at 50 nucleotides. Cell lysate [33] was incubated on ice for 30 min and centrifuged at 12,000×*g* for 20 min at 4°C. The protein concentrations in lysates were adjusted to 1 g/L by dilution with lysis buffer; 2 μl of lysate was used in TRAP reaction as follows. PCR amplification of telomerase extension products was performed in 50-μl TRAP reaction mixes containing 20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 68 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM dNTP, TS and CX primers at 1.8 mg/L and 2 U of *Taq* polymerase. Samples were denatured for 2 min at 94°C, followed by 30 cycles of PCR amplification at 94°C for 10 s, 50°C for 25 s and 72°C for 30 s in a thermal cycler. Samples were analyzed using 10% TBE gels (Invitrogen, Carlsbad, CA) with 0.5 mg/L ethidium bromide staining.

2.4. Telomere length assay using real-time PCR

Relative telomere lengths were determined as described previously [34] with slight modifications using the iCycler iQ multicolor real-time detection system (Bio-Rad, Palo Alto, CA). The relative telomere lengths were determined as the factor by which a genomic DNA sample differs from a reference sample in its ratio of telomere (*T*) repeat copy number to a single (*S*) copy gene number. The cycle threshold (Ct) values generated were used to calculate *T*/*S* values for each sample: $T/S=2^{-\Delta Ct}$ (where $\Delta Ct=Ct_{singlecopygene}-Ct_{telomere}$). Long telomeres produce low Ct values because more PCR product is generated due to many PCR initiation sites; this results in higher *T*/*S* ratios. Genomic DNA was extracted from IMR-90 (PDs 35 and 45) and hTERT cells (PD *n* and PD *n*+10). Standard curves were generated for telomeres and the single-copy gene; DNA concentrations in standards ranged from 10 to 100 ng/reaction. Triplicate PCR reactions were carried out in a 25-μl total reaction mix containing ABsolute QPCR SYBR Green fluorescein mix (ABgene, Rochester, NY). Primers for telomeres (T1a and T1b) and the single-copy gene 36B4 (acidic ribosomal phosphoprotein PO) were added to obtain final concentrations of 0.2 and 0.3 μM, respectively [34].

2.5. Chromatin immunoprecipitation (ChIP) assay

IMR-90 and hTERT cells were used to quantify the relative enrichment of proteins in telomeric repeats. ChIP assays were conducted as described previously [24]. The following antibodies were used for ChIPs: polyclonal antisera against H4K12bio [29] and antibodies against telomere protein TRF2 and the C-terminus in histone H3 (Abcam, Cambridge, MA). Efficiency of anti-H4K12bio in ChIP assays was tested by comparing nuclear extracts from ChIP assays before and after treatment with anti-H4K12bio; in these assays, supernatants from before and after immunoprecipitation were probed with anti-H4K12bio using Western blot analysis [29]. Chromatin immunoprecipitated with the preimmune serum of H4K12bio served as the negative control. Each immunoprecipitation was repeated three times, using 47 million cells per precipitation. Cross-links were reversed, and DNA was recovered for ligation-mediated PCR (LMPCR). For total DNA samples (denoted input DNA), aliquots corresponding to one sixth of the lysate volume used for immunoprecipitation were processed along with the rest of immunoprecipitated samples during the cross-link reversal step. DNA was amplified by LMPCR as described previously [35,36] with the following modifications. An asymmetric double-stranded T7 linker composed of a 25-mer (5′-GGCTAATACGACTCACTATAGGGAG-3′) annealed to a 13-mer (5′-CTCCCTATAGTGA-3′) was prepared by dissolving 670 pmol of each oligonucleotide in 100 μl of water, heating at 95°C for 5 min and cooling to room temperature. Blunt-end DNA was produced by mixing 25 ng of input or antibody-precipitated DNA and 5 U T4 DNA polymerase (Invitrogen). Blunt-end DNA was purified by using QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA) and resuspended in 20 μl of 10 mM Tris–Cl, pH 8.5. DNA was ligated to the T7 linker by incubating with 5 U T4 DNA Ligase (Ambion, Austin, TX) at 16°C overnight. Ligation products were purified by eluting with 20 μl of 10 mM Tris–HCl, pH 8.5, using QIAprep Spin Miniprep Kits. Ligation products were PCR amplified twice. First, 18-μl ligation products were amplified for 20 cycles by using 50 pmol 25-mer linker primer and 5 U *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 15 s, followed by a final extension at 72°C for 4 min. Ligation products were purified by eluting with 20 μl of 10 mM Tris–HCl, pH 8.5, using Miniprep Kits, and subjected to another 20 cycles of PCR under the same conditions as above. Ligation products were purified by eluting with 20 μl of ddH_2O using Spin Miniprep Kits.

Equal amounts of input and immunoprecipitated DNA were dot blotted onto a nylon membrane and fixed with UV cross-linking. Membranes were dried and hybridized using ^{32}P-labeled $(CCCTAA)_4$ as a probe for telomeric repeats. Intensities of dots were determined using Image J 1.38x software [37]. Dot blot analysis data (relative intensities of dots) were obtained for three independent blots. Input DNA represents the total telomeric DNA in cell extracts. The amount of telomeric DNA immunoprecipitated in each ChIP was calculated based on the signal relative to the corresponding total telomeric (input) DNA. ChIP data are reported as ratios of immunoprecipitated DNA to an equal amount of input DNA.

Co-immunoprecipitation experiments were conducted to confirm physical interaction between TRF2 and H4K12bio. In these experiments, nuclear extracts from ChIP assays were precipitated with anti-H4K12bio, followed by Western blot analysis with anti-TRF2 [29,38].

2.6. Abundance of biotinylated histones

Nuclear histones were extracted from IMR-90 and hTERT cells using 1 M HCl [22]. Histones were resolved on 18% Tris–glycine gels (Invitrogen). Transblots were probed with streptavidin peroxidase [22] and the following primary antibodies: rabbit anti-human H4K12bio [29], rabbit anti-human H3K9bio and rabbit anti-human H3K18bio [27]. Rabbit anti-human H4K20me3 (Abcam) is a known marker for aging [39] and was used as a control. Antibody and avidin blots were quantified using chemiluminescence and gel densitometry [40]. Equal loading of lanes and sample integrity was confirmed by using bicinchoninic assay (Pierce, Rockford, IL), densitometric quantitation of gels stained with Coomassie blue and Western blot analysis using an antibody to the C-terminus in histone H3 (Santa Cruz Biotechnology, Santa Cruz, CA).

2.7. Abundance of HCS protein and mRNA and biotin-dependent carboxylases

Abundance of HCS in whole-cell homogenates was quantified by Western blot analysis as described previously [41]. Histone H3 (Santa Cruz Biotechnology) was used as loading control [41]. Abundance of mRNA coding for HCS was quantified as described previously [41]. HCS is also responsible for catalyzing the attachment of biotin to acetyl-CoA carboxylase 1 and 2, pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC) and 3-methylcrotonyl-CoA carboxylase (MCC) [41]. Abundance of these holocarboxylases was quantified by streptavidin blotting as described previously [42]. Equal loading of protein was confirmed by gel densitometry after probing with anti-human histone H3.

2.8. Biotin transporter expression

The abundance of biotin transporter (SMVT) protein and mRNA was quantified as described previously [41]. Rates of biotin transport into IMR-90 cells were determined using [^{3}H]biotin at a physiological concentration of 475 pmol/L as described previously [43].

2.9. Statistics

Homogeneity of variances among groups was confirmed using Bartlett's test [44]. Significance of differences among groups was tested by one-way ANOVA. Fisher's