IDENTIFICATION AND CHARACTERIZATION OF PROPIONYLATION ON HISTONE H3 LYSINE 23 IN MAMMALIAN CELLS

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Propionylation has recently been identified as a new type of protein post-translational modification (PTM). Bacterial propionyl-CoA synthetase and human histone H4 are propionylated at specific lysine residues which have previously been known to be acetylated. However, other proteins subject to this modification remain to be identified and the modifying enzymes involved need to be characterized. In this paper, we report the discovery of histone H3 propionylation in mammalian cells. Propionylation at H3 lysine K23 was detected in a leukemia cell line U937 by mass spectrometry (MS) and western analysis using a specific antibody. In this cell line, the propionylated form of lysine 23 accounted for 7%, a level at least six-fold higher than those in the other leukemia cell lines of HL60 and THP1, or non-leukemia cell lines of HeLa and IMR90. The propionylation level in U937 cells decreased remarkably during monocytic differentiation, indicating that this modification was dynamically regulated. Moreover, in vitro assays demonstrated that histone acetyltransferase p300 can catalyze H3K23 propionylation, whereas histone deacetylase Sir2 can remove this modification in the presence of NAD+. These results suggest that histone propionylation might be generated by the same set of enzymes as for histone acetylation, and selection of donor molecules (propionyl-CoA vs. acetyl-CoA) may determine the difference of modifications. Since propionyl-CoA is an important intermediate in biosynthesis and energy production like acetyl-CoA, histone H3K23 propionylation may provide a novel epigenetic regulatory mark for cell metabolism.

Eukaryotic histones are rich in multiple post-translational modifications (PTM), the combinatory array of which is the basis for epigenetic regulation of gene expression (1). Appropriate histone modifications are required for normal cell growth and differentiation while aberrant histone modifications contribute to tumor formation such as malignant hematopoiesis (2). For example, genome-wide alterations of histone modification patterns are found in prostate cancer and are predictive of clinical outcomes (3); histone H3K79 hypermethylation accompanied with the activation of oncogenic HOX genes are prominent causes for leukemia with chromosome translocation involving the AF10 gene (4,5), while global hypomethylation at H3K79 sites has been proposed to cause genome instability in AF10-related leukemia cancers (6). Alteration in histone modification patterns serves as a hallmark of...
cancer that provides clues for cancer diagnosis and treatment (7).

Protein propionylation has recently been identified as a new type of post-translational modification. The propionyl-CoA synthetase of *Salmonella enterica* is propionylated at lysine 592, which inactivates the enzyme activity in vivo (8). The in vivo propionylation of propionyl-CoA synthetase is catalyzed by a protein related to GCN5 histone acetyltransferase (HAT), and the propionyl group can be removed by NAD+ dependant human Sir2 histone deacetylase (Sirtuins) in vitro. Propionylation is also found in human histone H4 enriched for acetylation using anti-acetyl H4 antibodies (9). In vitro experiments showed that histone acetyltransferase p300 and CREB-binding protein (CBP) can propionylate histone H4, suggesting that propionylation and acetylation may share the common enzymes that recognize both propionyl-CoA and acetyl-CoA (9). Moreover, the GCN5-related mammalian HAT PCAF has the ability to propionylate histone H3 with similar efficiency and lysine specificity as it acetylates H3 in vitro (10). However, it is unclear so far whether propionylation is present in histone H3 in vivo.

In this paper, we report the identification and characterization of histone H3 propionylation in mammalian cells. This modification was specifically detected on H3 lysine K23 in a myeloid precursor leukemia cell line U937. The H3K23 propionylation level in U937 cells decreased significantly during monocytic differentiation, suggesting that this modification might be associated with lineage determination. Histone acetyltransferase (HAT) p300 can catalyze this modification, whereas histone deacetylase (HDAC) Sir2 catalyzes the removal of the propionyl group in vitro. Since propionyl-CoA and acetyl-CoA, both metabolic products of fatty acid, are differential cofactors for dual functional HATs, we suggest that histone propionylation might be associated with cellular metabolic status.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures.** U937T (provided by Dr. Gerard Grosveld) is a derivative of U937 harboring the *tet-VP16* fusion gene under the control of a tetracycline-inducible promoter (11). U937T stable cell lines expressing Flag-AF10 or Flag-AF10ΔOM (Octapeptide Motif)-LZ (Leucine Zipper) were established using the pUHD10S-1 vector (a gift from Dr. Gerard Grosveld). The stable cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS, 1 μg/ml tetracycline, 0.5 μg/ml puromycin and 1 mg/ml G418 (GIBCO/BRL). HeLa and IMR90 cell lines were purchased from the American Type Culture Collection (ATCC) and cultured as recommended.

**Histone purification.** Cultured cells were centrifuged in ice-cold PBS solution supplemented with 5 mM sodium butyrate. The cell pellets were re-suspended in ice-cold TEB (Triton Extraction Buffer: PBS containing 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (v/v) NaN₃ and protein inhibitor cocktail) at a cell density of 5 x 10⁶ cells per ml and incubated on a rotator. The nuclei were isolated and lysed in 0.4 N H₂SO₄ by incubation at 4 ºC overnight. The supernatant of the centrifugation was added with trichloroacetic acid (TCA) to reach a final concentration of 33% (v/v) so that histones were precipitated out. The core histones were further fractionated into H2A, H2B, H3 and H4 by reverse-phase HPLC (12).

**Western analysis of histone H3K23 propionylation.** H3K23-propionylated peptide KQLATKprAARC and K23-acetylated peptide KQLATKacAARC were solid-phase synthesized on an ABI peptide synthesizer. The propionylated peptide was used to immunize rabbits for the preparation of polyclonal antibodies specific for H3K23 propionylation. Cross-reactivity to K23-acetylation was minimized by passing the antibody solution through an affinity column loaded with beads cross-linked with the acetylated K23 peptide. Peptide dot blotting analysis demonstrated that the purified H3K23 propionylation antibody had a 9-fold affinity over H3K23 acetylated peptide and a 100-fold affinity over the unmodified peptide (Supplemental Figure 1). Western blot analysis was carried out using the propionylation-specific antibodies with total protein extracts of different cell lines.
Histones were digested either with trypsin or Arg-C proteases (Roche Applied Science) overnight (enzyme:protein is ~1:100). After digestion, the peptides were dried with reduced vacuum and then redissolved in 0.1% formic acid. The peptides were fractioned by reversed-phase HPLC on a microbore C18 column before submission to MALDI-TOF analysis or directly submitted for LC/MS/MS analysis. MALDI-TOF mass spectrometric experiments were performed, using the Bruker Autoflex instrument on the reflectron mode. For regular MALDI-TOF experiments, all spectra were externally calibrated using a peptide standard mixture provided by Bruker. A 1.0 μl aliquot of the peptide solution was mixed with 1.0 μl α-cyano-4-hydroxycinnamic acid matrix (Sigma, 5 mg/ml in 50/50 acetonitrile/water and 0.1% formic acid). Data from a set of 100 ~ 200 laser shots were accumulated to give an acceptable spectrum. For high-accuracy mass determination, the spectra of modified histone peptides were calibrated by the lock-mass of a coeluted known histone peptide. LC/MS/MS identification of histone modifications was carried out by nano-electrospray on Waters QTOF Ultima instrument which was coupled with a Waters capillary HPLC. A 140 minute of gradient (mobile phase A: 0.1% formic acid; B: 0.1% formic acid in acetonitrile) with a flow rate of 6 μl/min was run through a splitter. After splitting the flow of an approximately 300 nl/min was run through a Waters nano C18 column (150 mm x 75 μm) to the nano-ESI source. 1.8 KV capillary voltage and 45 V cone voltage were optimized for nano-electrospray. The QTOF method utilized consisted of a survey scan mode in the mass range from 300 to 1500 during which up to three precursor ions were selected for parallel collision activated dissociation (collision gas:Ar) analysis using an automated data-dependent MS to MS/MS and MS/MS to MS switching function. Precursor ion selection was based on a normalized threshold of 10 counts/s and ions of potentially modified peptides were preferentially selected for MS/MS fragmentation. MS/MS experiments used an m/z dependent collision energy applied linearly from 20 to 42 V from 300 to 1500 m/z. Product ion scans were measured in the range from m/z 50 to 2000. The QTOF instrument was calibrated with the MS/MS fragmentation ions of the peptide-Glu-Fib resulting in less than 20 ppm mass accuracy for precursor ions when a lock-mass was applied and ± 0.02Da for product ions. Modifications of histones were manually determined with the aid of the Prospector program (prospector.ucsf.edu/product). Quantitative LC/MS/MS analyses of histone acetylation and propionylation were performed by derivatization of unmodified lysines using d6-acetoanhydride as previously described (13) and the resolution of quadrupole was set with a low-window (LM & HM =2) to select precursor ions ~ ± 8 Da for MS/MS fragmentation. The acetylation proportions of K18 and K23 were calculated by the intensity relative to the total peak value of diacetylated peptide (K18/K23 di-Ac), K18 or K23 monoacetylated peptide (mono-Ac), and K18 and K23 unacetylated peptide (no-Ac). The propionylation level was calculated by the intensity of the K23-Pr peak over the total intensity of acetylation and propionylation peaks.

**Propionylation and depropionylation Assays.** In **in vitro** propionylation and acetylation assays were carried out as described previously (9). Basically, 2.5 ng of human recombinant histone H3 (Millipore) was dissolved in 10 μl of acetylation buffer (50 mM Tris, pH 7.9, 10% glycerol, 1 mM DTT). After adding 1 μl of 14C-acetyl-CoA or 14C-propionyl-CoA (55 mCi/mmol, American Radiolabeled Chemicals, Inc.) and 0.5 μg of p300 (Upstate, CA), the solution was incubated at room temperature for a given time. The reaction mixture was then subjected to electrophoresis on SDS-PAGE gels followed by autoradiography and/or Coomassie Blue staining. For the propionylation/acetylation assay with non-radioactive propionyl-CoA or acetyl-CoA, the reaction mixture incubated for one hour was subjected to enzymatic digestion with Arg-C followed by MALDI-TOF and LC/MS/MS analyses. For the depropionylation assay, histone H3 was first incubated with p300 in the presence of 14C-propionyl-CoA (or 14C-acetyl-CoA as control) for one hour, the reaction was stopped by heating at 60°C for 5 min. 10 μl (100 μg/300 μl solution) of human Sir2 proteins (Sirtuin1, 2 and 3 from BPS Bioscience) were then added to the solution together with 50 mM NAD⁺. The mixtures were incubated at room temperature for 30 minutes and stopped by addition of gel loading.
buffer. Samples were then subjected to SDS-PAGE analysis with Coomassie Blue staining and autoradiography. Meanwhile, a synthetic K23-propionylated H3 peptide was incubated with Sir2 proteins and NAD$^+$ for 30 minutes, and then the mixture was analyzed by MALDI-TOF mass spectrometry.

**Monocytic differentiation.** U937 and HL60 cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum. For induced differentiation, cells were washed and resuspended in fresh media supplemented with 100 nM PMA (or DMSO as mock). After 72 hours of treatment, cells were harvested for analysis.

**Flow cytometry analysis.** Cells were harvested, washed in cold PBS and counted. 1 x 10^6 cells were incubated with APC-conjugated CD11b antibody (BD Bioscience) on ice for 30 minutes in the dark. After washed twice with PBS, cell were resuspended in 200 µl of FACS buffer (PBS with 5 µg/ml propidium iodide and 2% FBS), and acquired on FACS Calibur for analysis.

**RESULTS**

**Identification of histone H3 lysine K23 propionylation in the U937 cell line**

In the analysis of post-translational modifications on histones isolated from U937 leukemia cell line, we identified a novel modification, propionylation on histone H3 lysine 23 by mass spectrometry. From LC/MS/MS analysis of histone H3 samples, we observed an unusual peak at m/z 521.82 (2+) eluted between the peaks at m/z 514.82 (2+) and 535.82 (2+) that corresponded respectively to peptides singly and dually acetylated at K18 and/or K23 (data not shown). We then performed HPLC peptide purification from Arg-C digested histone H3, and conducted MALDI-TOF analysis on the pool of K18/K23 modified peptides. A peak at m/z 1042.6364 (0.8 ppm) was detected between the peaks at m/z 1028.6168 and 1070.6266, which corresponded to the singly and dually acetylated peptides as stated above (Figure 1A). The 14-Da mass increase of the singly acetylated form could be resulted from an additional methylation on either the peptide or the acetyl group, namely propionylation. Fragmentation of the selected ion at m/z 521.82 (2+) by ESI-LC/MS/MS indicated that K18 was free of modification, but K23 was attached with a moiety of 56 Da (Figure 1B). The 56-Da change could be due to either a sum of one acetylation and one methylation or a propionylation. There has been no precedent that methylation and acetylation can occur at the same amino group, although this possibility exists. Notably, a stable six-membered ring immonium ion at m/z 140.1063 was detected (Figure 1B) that can only be produced from propionylated lysine. Moreover, we analyzed a synthetic K23-propionylated peptide with an identical sequence to the Arg-C digested peptide of histone H3 isolated from U937 cells. The MS/MS spectrum of this synthesized peptide was almost identical to that of the peptide from U937 cells (Figure 1C). A lysine residue acetylated and methylated simultaneously cannot form an immonium ion at m/z 140.1070 (calculated monoisotopic mass) because nitrogen with a positive charge makes no more than 4 bonds (i.e. NH$_4^+$) theoretically (Figure 1D). Therefore, the modification on the K23 residue was conclusively demonstrated to be propionylation.

H3K23 propionylation is specific for U937 leukemia cells

We then determined the relative abundance of propionylation at H3K23 in three leukemia cell lines. To this end, histone H3 was reacted with d$_6$-acetoanhydride in d$_3$-acetyl acid solution, followed by digestion with trypsin and analysis by LC/MS/MS. As shown in Figure 2A, about 7% of K23 was propionylated in the U937 cell line while only a background level of propionylation was detected in the other two leukemia cell lines, HL60 and THP1. No significant H3K23 propionylation was detected in two non-leukemia cell lines HeLa and IMR90 (Supplemental Figure 2). In addition, the propionylation did not seem to be linked to acetylation since no differences of acetylation were observed at K18 or K23 among the three leukemia cell lines (Figure 2A). Western
analysis with propionylated H3K23-specific antibody confirmed that K23 propionylation is more predominant in U937 cells (Figure 2B). In contrast, the K23 acetylation levels were similar among different cell lines. The global H3K79-2me hypomethylation is a characteristic of U937, in which the CALM-AF10 fusion protein affects the association of H3K79 methyltransferase hDOT1L with chromatin as described in our previous report (6).

**Down-regulation of AF10 or ectopic expression of CALM-AF10 fusion does not increase propionylation**

We then examined whether the elevated propionylation is related to the decreased level of the AF10 transcriptional factor (14) due to specific presence of the CALM-AF10 gene fusion in U937 cells. However, no change for H3K23 propionylation was observed in U937 cells upon ectopic expression of the wild-type AF10 or the mutant AF10 protein with a deletion of the hDOT1L binding domain OM-LZ, which is crucial for AF10-mediated restoration of H3K79 in U937T cells (6) (Figure 3A). This result suggests that hyperpropionylation in U937 cells is not caused by down-regulation of AF10. To investigate the possible link between H3K23 hyperpropionylation and CALM-AF10 fusion, CALM-AF10 and its OM-LZ deletion mutant were respectively induced to express in T-REx-293 stable cell lines with tetracycline. Consistent with our previous report (6), global hypomethylation of H3K79 was detected upon induction of CALM-AF10 but not the OM-LZ deletion mutant (data not shown). However, the H3K23 propionylation level did not change significantly in response to the induced expression of the CALM-AF10 proteins in the stable cell lines, and neither did the acetylation level (Figure 3B). These data suggest that the H3K23 hyperpropionylation in U937 is unlikely associated with the expression of the CALM-AF10 fusion.

**H3K23 propionylation decreases during monocytic differentiation of U937 cells**

Genome-wide reprogramming of histone modifications is closely associated with cell growth and differentiation in development (15,16). To examine the potential role of H3K23 propionylation in cell differentiation, we mimicked monocytic differentiation in U937 cells with PMA induction. Following PMA treatment, U937 cells became adherent and, over the course of several days, differentiated into monocyte/macrophage-like cells. Differentiation of promonocytic U937 cells into monocytes/macrophages is characterized by the elevated expression of cell surface marker CD11b (17). We confirmed with FACS analysis that 72.9% of the U937 cells became CD11b-positive upon PMA treatment (Figure 4A), which is consistent with previous report (18). We then examined the H3K23 modification during monocytic differentiation of U937 and a control cell line HL60 with western blot and LC/MS/MS. Interestingly, the propionylation level of H3K23 in U937 cells decreased markedly after PMA induction, whereas the acetylation level remained unchanged (Figure 4B, Supplemental Figure 3). Furthermore, HL60 cells didn't show such change during monocytic differentiation, suggesting that the hyperpropionylation of H3K23 in U937 cells is associated with cell differentiation.

**Histone acetyltransferase p300 propionylates H3 in vitro**

Previous experiments have shown that histone acetyltransferase p300 propionylates histone H4 at multiple sites, and p300/CBP-associated factor (PCAF) propionylates lysine 14 of histone H3 (9,10). To test whether the histone acetyltransferase p300 could also propionylate lysine 23 of histone H3, we set up an in vitro enzymatic assay with recombinant human histone H3 as a substrate. First, we performed the assay using C14-propionyl-CoA as the propionyl donor, and using the acetylation cofactor 14C-acetyl-CoA as a positive control. As shown in Figure 5A and C, p300 propionylated histone H3 although with much slower kinetics as compared with acetylation. Furthermore, we conducted the same propionylation assay using cold propionyl-CoA, followed by analysis of the H3 substrate with MALDI-TOF and LC/MS/MS. As shown in Figure 5B and D, p300 could propionylate lysine...
23 of histone H3 as well as three other residues (K9, K14, and K18) known to be acetylation sites.

Sir2 depropionylates histone H3 propionylation

Silent information regulator 2 (Sir2) protein is a nicotine dinucleotide (NAD⁺)-dependent protein deacetylase (19). In humans, eight Sir2 homologues (Sirtuins 1 to 8) have been identified. Garrity et al. reported that both the bacterial and human Sir2 histone deacetylases could remove propionylation from the propionyl-CoA synthetase (8). Molecular modeling indicated that the hydrophobic pocket in the active site of Sir2 could readily accommodate the additional methyl of propionyl-lysine (20). Following this scenario, we asked whether Sir2 would depropionylate histone H3K23. To this end, we incubated a synthetic K23-propionylated peptide with Sirtuin proteins in the presence of NAD⁺ on a time course for up to 1 hour. Meanwhile, parallel experiments using synthetic K23-acetylated peptide as the substrate were performed to compare the enzyme activities between depropionylation and deacetylation. The peptides were then analyzed with MALDI-TOF mass spectrometry. From the input acetylated peptide (sequence KQLATKacAARC, MH⁺: 1131.6), a new peak of 42-Da smaller in mass appeared after incubation with Sirtuin 1 (Figure 6A), indicating deacetylation activity of Sirtuin 1. Similarly, for the propionylated peptide (KQLATKprAARC, MH⁺: 1145.5), a new peak with a decrease of 56 Da appeared, indicating depropionylation activity of Sirtuin 1 (Figure 6B). Sirtuins 2 and 3 could also catalyze depropionylation, though Sirtuin 3 had a lower enzymatic activity than Sirtuin 1 and Sirtuin 2 (Supplemental Figure 5). Notably, the Sirtuin 1 depropionylation activity was about half of its deacetylation activity (Figure 6C).

DISCUSSION

The regulation of gene expression is largely mediated by PTM of histones in chromatin. While the most abundant types of PTMs such as acetylation and methylation that are known to play vital roles in transcriptional silencing and activation have been extensively studied, the existence of rare types of PTMs in histones remains to be characterized. Our work presents the first evidence for the existence of propionylation on mammalian histone H3. We found that lysine 23 in the H3 N-terminal tail is specifically propionylated up to 7% in the U937 leukemia cell line. During the preparation of this paper, Zhang et al. reported the detection of K23 propionylation in yeast histone H3 (21). Therefore, propionylation at H3K23 constitutes a novel type of histone mark that is evolutionarily conserved from yeast to human.

Biological significance of H3K23 propionylation

Histone propionylation is expected to exert functions similar to the wide-spread acetylation due to the structural similarity of the propionyl and acetyl groups. This modification neutralizes the positive charge of a lysine residue thus rendering an active local chromatin structure. The added propionyl group may also provide a mark for recognition by chromatin “readers” such as bromodomain-containing proteins that mediate further chromatin modification and transcriptional regulation. Propionyl-K23 can also exert its effect by inhibiting or stimulating the modification on the neighboring sites. The high abundance of histone H3K23 propionylation in U937 cells and its decrease upon differentiation may imply an important role of this modification in modulating cell differentiation. H3K23 Propionylation might regulate transcription of genes crucial for cell growth and differentiation with a mechanism similar to acetylation. However, the genes controlled by propionylation might be different from the targeted genes regulated by other modifications since the dynamics of propionylation in U937 differentiation is different from that of K23 acetylation (Figure 4B) and K79 methylation (data not shown). Because the propionylation levels became similar between U937 and other leukemia cell lines after differentiation, hyperpropionylation in U937 cells might be a stage-specific marker in hematopoiesis and leukemogenesis, which could serve as a diagnostic target for leukemia therapy.
The fact that histone propionylation is barely detectable in most mammalian cells may suggest its highly restrictive occurrence on specific target genes under certain growth and metabolic conditions. In *Salmonella*, propionylation of the propionyl-CoA synthetase increases with increased level of propionyl-CoA when the cells are grown on the propionate medium (8). In animals, the concentration of the acyl-CoAs fluctuates in response to diet and other physiological conditions (22). Given the regulatory function of acyl donors, propionylation in chromatin is likely to reflect the cellular level of propionyl-CoA, an important intermediate generated from energy metabolism. In this connection, we speculate that genes involved in energy metabolism could be regulated by histone propionylation. The identification of target genes subject to propionylation would help elucidate the biological processes in which this modification could modulate.

**Regulatory mechanism of H3K23 propionylation**

Our findings are in a good agreement with previous reports that addition and removal of propionyl group can be catalyzed by the same set of enzymes as for acetylation and deacetylation (9,10). Since the acetylation level in the U937 cell line is similar to that in other cell lines, we speculate that hyperpropionylation in U937 may result from the irregular accumulation of the propionyl donor molecule, propionyl-CoA. The accumulation of propionyl-CoA could arise from either up-regulation of propionyl-CoA synthetase or down-regulation of propionyl-CoA carboxylase that removes propionyl-CoA by converting it into 2-methyl citrate. It would be of interest to examine the expression levels of these two enzymes directly involved in propionyl-CoA homeostasis in the related leukemia cells.

Several lines of evidence are in favor of the existence of other distinct propionylation enzymes (or specific regulatory factors) than acetyltransferase (deacetylase). H3K23 propionylation is found only in the U937 cell line, but not in the other two related monocytic leukemia cell lines HL60 and THP1. Despite the striking difference in propionylation level, the acetylation levels at K23 are similar among these cell lines. Additionally, histone acetyltransferase p300 propionylates four lysine residues (K9, K14, K18 and K23) of the H3 histone tail indiscriminately *in vitro*, and depropionylation by Sir2 is unlikely to be specific for K23 site. A propionyltransferase that acts exclusively on H3K23 has not been found yet.

Consistent with the proposed relationship between a specific metabolic state and histone propionylation, the known genetic and epigenetic alterations in U937 cells do not seem to contribute to the prominent propionylation. The U937 cell line is characterized by the presence of a t(10;11)(p13;q14) chromosome translocation leading to the fusion of the *CALM* gene (Clathrin Assembly Lymphoid Myeloid leukemia gene) with the putative transcription factor gene *AF10* (14). The CALM-AF10 fusion protein causes global hypomethylation at H3K79 due to the functional perturbation of the responsible histone methyltransferase hDOT1L by the fusion (6). Ectopic expression of CALM-AF10 in T-REx-293 cells results in H3K79 hypomethylation (6), but has no effect on K23 propionylation (Figure 3A). Therefore, hyperpropionylation is not due to the expression of the leukemogenic fusion protein. Whether the differential propionylation in U937 leukemia cells can instead be attributed to a unique metabolic state associated with its monocytic differentiation stage is an intriguing possibility that warrants further investigation.
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Acknowledgements
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Key words: histone H3 lysine 23 (H3K23), histone modification, lysine propionylation, post-translational modifications (PTM), propionyl-coenzyme A (propionyl-CoA), monocytic differentiation.

Abbreviations:
**FIGURE LEGENDS**

**FIGURE 1. Identification of histone H3 lysine 23 propionylation in U937 cells.**

(A) MALDI-TOF spectrum of peptides containing modifications at K18 and K23. These peptides were purified with HPLC from Arg-C digested histone H3 of U937 cells. $^{18}KQLAT^{23}K_{Ac}AAR$ (m/z): 1028.6168 (exp., 4.2 ppm), 1028.6211 (Cal.); $^{18}KQLAT^{23}K_{Pr}AAR$ (m/z): 1042.6364 (exp., 0.8 ppm), 1042.6368 (Cal.); $^{18}K_{Ac}QLAT^{23}K_{Ac}AAR$ (m/z): 1070.6266 (exp., 4.8 ppm), 1070.6317 (Cal.).

(B) MS/MS mass spectrum of the doubly-charged ion at m/z 521.8221 (solid arrowhead). Histone H3 prepared from U937 cells was digested by Arg-C prior to the LC/MS/MS analysis. The fragmentation pattern (mainly ‘b’ ions and ‘y’ ions) established the peptide sequence indicated in the figure.

(C) MS/MS spectrum of a synthetic peptide with K23 propionylation. Note that the spectrum of this authentic propionylated peptide is identical to that of the propionylated peptide identified in U937 cells (see B above).

(D) Illustration of distinctive immonium ions (open arrowheads in B & C) generated via collision induced dissociation (CID) from an acetylated lysine (at m/z 126.0906, spectrum not shown) and from a propionylated lysine (at m/z 140.1063). The immonium ions were calibrated with the lock-mass of the y1 (arginine) ion at m/z 175.1190.

**FIGURE 2. Quantification and Western confirmation of H3K23 propionylation in U937 cells.**

(A) Quantification of H3K23 propionylation in leukemia cells by LC/MS/MS analysis. Histone H3 isolated from each cell line was treated with d6-acetyl anhydride and digested with Arg-C prior to the mass analysis. The H3K23 propionylation level is significantly higher in U937 cells than in HL-60 or THP1 cells, while no variation is seen for acetylation at K18 and K23 in the three compared cell lines. Experiments were performed three times and the STD was shown as the error bars.

(B) Western detection of K23 propionylation using an antibody specific for K23 propionylation. Note that the H3K23 propionylation signal is significantly stronger in the U937 cell line while the acetylation levels at the same site are similar among the three cell lines. Blotting with anti-H3 antibody served as a loading control. 6xHis-tagged recombinant histone H3 (His-H3) lacking any modification was used as a negative control to validate the performance of the modification-specific antibodies.

**FIGURE 3. H3K23 hyperpropionylation in U937 is not caused by down-regulation of AF10 or expression of the CALM-AF10 fusion protein.**

(A) Ectopic expression of AF10 in U937 cells did not reduce H3K23 propionylation. Quantitative LC/MS/MS analysis was performed to measure acetylation and propionylation of histone H3 isolated from the U937 cell lines ectopically expressing wild-type AF10 or mutant AF10 deleted of the hDOT1L-binding domain (OM-LZ). Experiments were performed three times and the STD was shown as the error bars.
(B) Inducible ectopic expression of CALM-AF10 in T-REx-293 cells did not increase H3K23 propionylation. CALM-AF10 or its mutant (OM-LZ deletion in AF10) was induced to express with tetracycline (+). Total cellular extracts were examined by western blot analysis using antibodies indicated at left. The un-induced cells (-) were included as negative controls.

**FIGURE 4. H3K23 propionylation decreases during differentiation of U937 cells.**

(A) U937 and HL60 cells were induced for monocytic differentiation. Cells were treated with PMA or DMSO (mock) for 72 hrs, harvested and analyzed by flow cytometry. CD11b was used as a cell surface marker for monocytic differentiation.

(B) Western blot analysis of H3K32 modifications in differentiated HL60 and U937 cells. Upon induction with PMA for 72h, cells were harvested and the cell extracts were analyzed with indicated antibodies.

**FIGURE 5. Histone acetyltransferase p300 can catalyze H3K23 propionylation in vitro.**

(A) In vitro acetylation of recombinant human H3 by p300 in the presence of 14C-acetyl-CoA. Upper panel, autoradiography; Lower panel, Coomassie staining of H3 upon incubation with p300. The time points at which samples were taken from the incubation are indicated.

(B) MALDI-TOF measurement of histone H3 upon incubation with p300 in the presence of acetyl-CoA.

(C) In vitro propionylation of recombinant human H3 by p300 in the presence of 14C-propionyl-CoA.

(D) MALDI-TOF measurement of histone H3 upon incubation with p300 in the presence of propionyl-CoA.

The acetyl and propionyl modifications at K9, K14, K18 and K23 were confirmed by LC/MS/MS sequencing analysis of Arg-C digested peptides upon the incubation with p300 (data not shown).

**FIGURE 6. Sir2 protein can depropionylate H3K23 in vitro.**

(A) MALDI-TOF analysis of K23-acetylated peptides upon incubation with a Sir2 protein (Sirtuin 1) for one hour. The spectrum before the deacetylase treatment is not shown.

(B) MALDI-TOF analysis of K23-propionylated peptides upon incubation with Sirtuin 1 for one hour.

(C) Time course of depropionylation/deacetylation at K23 of the peptides by Sirtuin 1. The deacetylation and depropionylation percentages were calculated by following equations: K23 deacetylation = Intensity_{peak1089.6} / Total intensity_{peak1089.6 & peak1131.6}; K23 depropionylation = Intensity_{peak1089.6} / Total intensity_{peak1089.6 & peak1145.5}. Experiments were performed three times and the STD was shown as the error bars.
Figure 1

A

B

C

D

Acetylated Lysine  
\[ m/z \ 126.0913 \text{ (Cal.)} \]
\[ 126.0906 \text{ (exp., 5.6 ppm)} \]

Propionylated Lysine  
\[ m/z \ 140.1070 \text{ (Cal.)} \]
\[ 140.1063 \text{ (exp., 5.0 ppm)} \]
Figure 6

A

B

C

Deacetylation - 42
K23-Ac-peptide

Depropionylation - 56
K23-Pr-peptide

De-Ac/De-Pr (%)

Time (min)

Sirtuin1 Ac
Sirtuin1 Pr
Identification and characterization of propionylation on histone H3 lysine 23 in mammalian cells
Bo Liu, Yihui Lin, Agus Darwanto, Xuehui Song, Guoliang Xu and Kangling Zhang

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