Characterization of histone acylations links chromatin modifications with metabolism

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Over the last decade, numerous histone acyl post-translational modifications (acyl-PTMs) have been discovered, of which the functional significance is still under intense study. Here, we use high-resolution mass spectrometry to accurately quantify eight acyl-PTMs in vivo and after in vitro enzymatic assays. We assess the ability of seven histone acetyltransferases (HATs) to catalyze acylations on histones in vitro using short-chain acyl-CoA donors, proving that they are less efficient towards larger acyl-CoAs. We also observe that acyl-CoAs can acylate histones through non-enzymatic mechanisms. Using integrated metabolomic and proteomic approaches, we achieve high correlation ($R^2 > 0.99$) between the abundance of acyl-CoAs and their corresponding acyl-PTMs. Moreover, we observe a dose-dependent increase in histone acyl-PTM abundances in response to acyl-CoA supplementation in nucleo reactions. This study represents a comprehensive profiling of scarcely investigated low-abundance histone marks, revealing that concentrations of acyl-CoAs affect histone acyl-PTM abundances by both enzymatic and non-enzymatic mechanisms.
lysine acetylation is the most extensively studied histone post-translational modification (PTM). Discovered more than 50 years ago, it has been recognized to play a fundamental role in transcriptional activation, metabolic regulation and other central cellular processes1. Mechanistically, lysine acetylation neutralizes the positive charge of histone tails, reducing the physical interaction between histones and DNA, thereby allowing the access of gene-activating transcription factors2–3. Acetylation can also influence chromatin function by serving as a binding site for bromodomain-containing remodeling complexes that can directly stimulate transcription by recruiting transcriptional co-activators4. Acetylation dynamics in the nucleosome are the result of the net activities between two different families of histone deacetylases (HDACs), which remove these groups5. The activities of both HATs and HDACs are regulated by additional of acetyl groups using acetyl-CoA as a cofactor, and histone deacetylases (HDACs), which remove these groups. 

Over the last decade, a growing number of lysine modifications chemically related to acetylation (propionylation, malonylation, crotonylation, butyrylation, succinylation, glutarylation, 2-hydroxyisobutyrylation and β-hydroxybutyrylation) have been identified on histones using mass spectrometry (MS)-based proteomic approaches8–14. These findings have raised numerous questions regarding their functional significance, possible implications in metabolic pathways and the existence of

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**Fig. 1** Overview of histone acetyltransferases (HATs) in vitro acylation activity and specificity. a Schematic representation of in vitro acylation assay. b Chemical structures of histone acyl modifications evaluated in this study. Lysine modifications and abbreviations are: acetyl (Kac), propionyl (Kpr), butyryl (Kbu), crotonyl (Kcr), malonyl (Kmal), succinyl (Ksuc), β-hydroxybutyryl (Kbhb), and glutaryl (Kglu). c Heat map displaying the in vitro acylation activity profiles of different HATs in the presence of acetyl-, propionyl-, crotonyl-, butyryl-, malonyl-, β-hydroxybutyryl-, succinyl- and glutaryl-CoA. Molecular mass shift of the various acylated lysines residues are shown in the table headers. Different HATs were assayed against histones H3 or H4 as specified in the first column. To generate the heat map, we averaged the relative abundance of acyl-PTMs on the quantified peptides and then normalized (z-scores) those values across the different HATs, i.e. row normalization. d Pie chart showing the average relative frequency of in vitro acylated peptides, divided in results for histone H3 (top) and histone H4 (bottom). e Bar plots depict the specificity for all HATs on the histone sequence. The x axis represents the modification site at histones H3 (left) and histone H4 (right), and the y axis represents the relative abundance shown as the average contribution of HATs to all acylated peptides. For histone H4 N-terminal peptide (G4-R17), the number of acylations on the sequence are displayed using the code 2, 3 or 4 mods. This is because it was not always possible to discriminate modification sites on the multiply modified H4 peptide. All values shown were corrected by the contribution of non-enzymatic acylation. All results are shown as the average of 3 independent experiments.
regulatory enzymes beyond the well-established acetylation mechanisms that could govern these marks. While most of these questions remain to be answered, many studies have provided new insights into the roles that acyl marks can play in genome function. For example, it has been reported that lysine crotonylation mediated by the HAT p300 can stimulate gene transcription in vitro and in vivo seemingly to a greater degree than lysine acetylation, and that this mechanism is highly regulated by the metabolic concentrations of the crotonyl-CoA co-factor. Histone crotonylation has also been found to be enriched at transcriptionally active X/Y sex-linked genes during post-meiotic sex inactivation in mouse, and the YEATS domains Taf14, AF9 and YEATS2 have been reported to preferentially bind crotonylated over acetylated lysines residues in vitro. More recently, lysine butyrylation has been reported to directly stimulate gene transcription and compete with acetylation for the binding of the testis specific gene expression-driver Brdt in spermatogenic cells. In addition, β-hydroxybutyrylation, was found to be induced during starvation or streptozotocin-induced diabetic ketoacidosis, and to activate transcription of specific genes associated with starvation-responsive metabolism pathways. These studies suggest that newly identified histone acyl-PTMs may have unique or similar roles to acetylation in transcriptional activation. Such observations are supported by several reports showing that SIRT5, a member of the class III HDACs can preferentially remove acidic acyl modifications, including malonyl, succinyll and glutaryl, whereas propionyl, crotonyl and butyryl marks can be removed by various other sirtuins. However, it remains unclear whether the same group of enzymes involved in the establishment of acetylation could also mediate the establishment of these histone modifications in vivo.

Another aspect that has been underexplored is the relative abundance of acyl marks, which is an important step towards understanding their biological relevance. This gap in knowledge is mainly due to the biases inherent in the use of antibody-based enrichment methods commonly employed prior to MS detection. Although stoichiometry at individual sites has been reported for propionylation (7%) at H3K23 in a leukemia cell line, butyrylation (31%) at H3K115 in mouse brain, and crotonylation (1–3%) at H2AK36, H2BK5, H3K23 and H4K12 in brain histones, a global overview of the abundances relative to histone acetylation is lacking. The dearth of quantitative data for these non-canonical acyl-PTMs has led to the hypothesis that they might arise due to the chemical reactivity of acyl-CoA. Indeed, this has been observed in the context of acetylation and succinylation in mitochondrial proteins. Thus, it is still open to debate whether these modifications are strategically positioned on the chromatin by enzymes or whether their presence is instead the result of non-enzymatic chemical reactivity.

In this study, we sought to characterize the acylation of histones, including their overall abundance and their likelihood to be products of HAT catalysis rather than chemical reactivity of acyl-CoAs. First, we investigate the ability of several recombinant HATs to catalyze the acylation of histones H3 and H4 using different acyl-CoA donors employing an MS-based in vitro bioassay. Our data show that most HATs can catalyze histone acylation using different acyl-CoA substrates to variable extents when tested individually. However, in competition assays performed in the presence of equimolar concentrations of acyl-CoA and acetyl-CoA, almost all HATs strongly prefer to utilize acetyl-CoA to modify histones. Our data also confirm that histones can be modified non-enzymatically through the chemical reactivity of the different acyl-CoA donors alone. We also employ a proteomics approach to characterize several acyl-PTMs in nucleo and in vivo without the use of enrichment strategies, which allow us to determine the relative abundance of a diverse subset of acylations on histones. Because these marks are dependent on their corresponding short-chain acyl-CoA metabolic intermediates, we employ a targeted metabolomics approach to measure the concentrations of acyl-CoA metabolites in HeLa cells and in proliferative and differentiated human myogenic cells. We find that the cellular concentrations of different acyl-CoA metabolites span orders of magnitude and are tightly correlated with the relative abundances of the acyl marks identified in vivo. These findings support the notion of a direct link between cellular metabolism and epigenetic regulation, where the relative concentrations of different acyl marks in histones are driven by the cellular abundances of their respective metabolic intermediates.

**Results**

**In vitro acylation of histones H3 and H4.** Previous studies have reported that the HATs CBP, p300 and PCAF can mediate propionylation and butyrylation, and crotonylation of lysine residues in vitro. These observations prompted us to investigate whether these and other known HATs can catalyze the acylation of human recombinant histones H3 and H4 using a broader range of acyl-CoA donors. We performed in vitro HAT activity assays with the HAT domains of PCAF, Gcn5, and the full-length CBP and p300 enzymes against histone H3, and with the HAT domains of MOF, Tip60 and NatA against histone H4. Each reaction was carried out individually in the presence of eight different short-chain acyl-CoA donors, followed by bottom-up nano-LC-MS/MS analysis (Fig. 1a, b). Figure 1c summarizes the in vitro acylation reactions of all HATs evaluated in this study. The heat map shows that most HATs could utilize acetyl-, propionyl- and butyryl-CoA with relatively high efficiency, supporting recent findings. However, acidic acyl-CoA donors including malonyl-, succinyl- and glutaryl-CoA, and branched-chain acyl donors like β-hydroxybutyryl CoA are utilized by HATs less efficiently. Interestingly, enzymes did not seem to utilize crotonyl-CoA for the catalysis of acyl marks as effectively as propionyl- and butyryl-CoA despite the structural similarity within these cofactors. These data are in agreement with previous observations suggesting that HATs activity is weaker with crotonyl-CoA due to the planarity and rigidity imparted by the C-C double bond in the crotonyl moiety.

When taking a closer look at the individual acylation activities of all HATs (Supplementary Table 1), we observed that enzymes have different trends in their substrate preference. For instance, histone H3 is known to be selectively acylated at the lysine residue 14 (H3K14) by Gcn5 and PCAF. Therefore, if we look at the relative abundances for all acylations at H3K14, under our assay conditions, Gcn5 was able to butyrylate ~ 78% of the H3 peptide at position 14, whereas acetylation and propionylation were found at ~ 32% and ~ 11%, respectively (Supplementary Table 1). Likewise, PCAF displayed ~ 88% butyrylation, followed by ~ 5% crotonylation and ~ 2% acetylation at position H3K14. However, when looking at the average sum of the relative abundances of all acylated peptides by HATs on H3, there seems to be a trend in the order of substrate preference: acetyl > propionyl > butyryl > malonyl > succinyl > β-hydroxybutyryl > glutaryl > crotonyl (Fig. 1d; Supplementary Table 1). This trend inversely correlates with the increasing size of the side chain of the acyl donor (except for crotonyl-CoA and β-hydroxybutyryl), supporting the notion that the activity of HATs gets weaker with increasing acyl-chain length. Interestingly, p300 and PCAF were the enzymes with the highest crotonylation activities on H3 (Supplementary Table 1).

Moreover, the average activities of HATs on histone H4 showed patterns that were consistent with the trend mentioned...
through the nucleophilic attack of the unprotonated ε-NH₂ group of lysine residues to the acyl group of acyl-CoA. In mitochondria, where both conditions are met for all HATs can catalyze N-terminal propionylation and butyrylation of histone peptides, Tip60 prefers to utilize butyryl-CoA as a cofactor, and evidence of the existence of acetyltransferases is lacking, although the majority detected on histone peptides is the result of an enzymatic catalysis. For instance, crotonylation is an overall low abundance PTM, as seen in Fig. 2b, the average contribution for acetylation, non-enzymatically modified in vitro, we incubated histones H3 and H4 with 0.5 mM acyl-CoAs in the absence of acetyltransferases. We found that all acyl-CoAs can chemically acylate histones, as seen in Fig. 2a, showing a comparison between the non-enzymatic acylation profiles of various acyl-CoA donors on histone H3 with acylations mediated by GCN5. We showed that most non-enzymatically catalyzed acylations have site specificities that were different from those enzymatically modified sites (Figs. 1e, 2c, d). The most prevalent sites observed for non-enzymatic acylations on histone H3 were K36, K56, K64, K79 and K122 (Supplementary Fig. 1; Fig. 2c). On H4, the most prevalent sites were K31, K59, K79 and K91 (Supplementary Fig. 1; Fig. 2d). Interestingly, these sites are closer to the C terminus of histones, whereas enzymes showed higher specificity for sites at the N-terminal tails (Fig. 1e). These observations suggest that non-enzymatic acylations may be enhanced by some level of structural and conformational dynamics on histones. In agreement, most succinylated and malonylated sites identified by Xie et al. in vivo have been reported to occur also at the globular domain and C terminus of histones H3 and H4 rather than at the N-terminal tails.

We then estimated which acylations are more likely to occur through enzymatic or non-enzymatic reactivity in vitro. As seen in Fig. 2b, the average contribution for acetylation,
propionylation and butyrylation marks in the presence of all HATs was more abundant in in vitro experiments, whereas acidic acyl modifications (malonylation, succinylation and glutarylation) and β-hydroxybutyrylation occurred to a greater extent through non-enzymatic mechanisms in both histones H3 and H4. Again, we observed a trend in which the ratio of enzymatic/acetylation was more abundant in in vitro experiments, whereas acidic acyl-CoAs CBP (H3) GCN5 (H3) p300 (H3) Pcaf (H3) NatA (H4) Tip60 (H4) MOF (H4) Neg (H3) Neg (H4)

![Table](image)

**Fig. 3** In vitro acetylation competition assay. **a** Schematic representation of the in vitro competition acetylation assay. **b** Heat map displaying in vitro acylation specificities of HATs during acyl-CoA competition assays. Different HATs were assayed against histones H3 or H4 as specified in the table headers in the presence of equimolar concentrations of acetyl-CoA and a competing acyl-CoA donor. Negative controls with no enzyme are also shown. **c** Correlation between the molecular weight and the acylation preference for different acyl donors displayed for the HATs GCN5 on histone H3, **d** p300 on histone H3 and **e** MOF on histone H4. For each modification, molecular weight is indicated. Results show that the preference for acetyl-CoA over the other acyl donor tightly correlates with the molecular weight of the acyl donor. Crotonylation was not included in the correlations, as its molecular weight did not correlate well with the preference of the enzymes over acetyl-CoA. All graphs are shown as the average of log2 ratios between the relative abundances of all acetylated peptides and the relative abundances of the corresponding competing acylated peptide.

It is important to mention that concentrations of acyl-CoAs used in this experiment were far above the known physiological concentrations of CoA derivatives in whole cells, so the extent of chemical acylation observed in this study is likely an overestimation. This higher concentration was required to ensure proper sensitivity to the in vitro assay. Even though it has been previously demonstrated that protein lysine acylation can occur at physiological acyl-CoA concentrations in vitro, our study does not represent a suitable extrapolation for the reactivity of these intermediates in cells. As such, our data cannot rule out the possibility of the existence of enzymes that play a major role in catalyzing these marks in vivo as compared to non-enzymatic reactions.

**HATs prefer acetyl-CoA for acylation of histones.** Previously, it has been demonstrated that some HATs are able to catalyze the transfer of propionyl and butyryl groups in vitro with similar specificities but different efficiencies than acetyl groups. These observations lead to the following question: what determines the acyl group to be transferred if HATs are indeed mediating...
various acylations in vivo? It has been shown that fibroblasts of patients with inherited metabolic disorders have high levels of propionyl-, malonyl- and butyryl-CoA and high levels of the corresponding lysine acylations. These disorders include deficiencies in propionyl-CoA carboxylase (PCC), malonyl-CoA decarboxylase (MCD) and short-chain acyl-CoA dehydrogenase (SCAD), respectively. However, the mechanism by which this increase in protein acylation is mediated has not been explored. We hypothesized that under such conditions, other acyl-CoAs could rival the levels of acetyl-CoA and induce HATs to use non-
native cofactors. To test this, we performed in vitro HAT competition assays in the presence of equimolar concentrations of acetyl-CoA and other acyl-CoAs (Fig. 3a). As shown in Fig. 3b, most HATs preferred to utilize acetyl-CoA than any other acyl-CoA donor. It is important to mention that at 10 μM of acyl-CoAs, we observed non-enzymatic acylation of histones H3 and H4, as shown in Fig. 3b. Consistent with previous in vitro experiments, for most HATs, the preference for the competing cofactor, if any, largely depended on the size of the acyl donor side chain. As shown in Fig. 3c–e, for the enzymes GGN5, p300 and MOF we observed an inverse correlation between the HAT preference for the competing cofactor and the increasing molecular weight of the acyl donor side chain, with the exception of crotonyl-CoA. Relative abundances for all peptides in competition assays are shown in Supplementary Table 3. Altogether, our data suggest that even in the highly unlikely chance that any other acyl-CoA accumulated to the extent that its concentration rivaled that of acetyl-CoA, HATs would still mostly utilize acetyl-CoA. We thus shifted our focus to investigate how abundant these acyl marks are in vivo, and whether their abundance can be justified by the abundance of acyl-CoA intermediates.

Relative abundances of histone acyl-PTMs in mammalian cells. Owing to the low abundance of lysine acyl marks, current MS-based approaches involve the use of antibody-based enrichments to increase the sensitivity of the MS analysis for identification and quantification. Although these approaches are helpful for estimating the relative changes of modifications across multiple conditions, they cannot provide direct information on relative abundances, as the peptide with the modification and the unmodified peptide end up in different sample pools. In addition, evaluation of the specificity of several commercially available pan anti-acyl-PTM antibodies by dot blot analysis revealed significant cross-reactivity among differentially acylated peptides, complicating their further application for immunoenrichment of histone acyl-PTMs (Supplementary Fig. 3).

Thus, to accurately detect and quantify histone acyl-PTMs in vivo using label-free approaches, we used the retention time and mass shift information from the in vitro experiments to optimize the MS acquisition and in-house quantification software45. Here, we refer to our quantitative values as “relative abundances”, as we are aware that differences in the ionization efficiencies of modified peptides or biases in trypsin digestion in the presence of certain modifications can affect the assessment of accurate PTM stoichiometry, which was observed for the differentially acylated peptide H3 aa 18–26 (KQLATKAAR) (Supplementary Fig. 4).

Analysis of acid-extracted histones from wild-type HeLa and myogenic cells showed that all acyl-PTMs combined, excluding acetylation, were found at relative abundances between 6 and ~15% of all detectable modified peptides of canonical histones H3 and H4 (Supplementary Fig. 5). Individually, most acyl-PTMs showed low relative abundances ranging from 1 to ~5% (Figs. 4a, b), as opposed to acetylation with global relative abundances between 15 and ~30% (Fig. 4a,b; Supplementary Fig. 5). Using our workflow, we measured the relative abundances at individual sites. Acetylation was found at high relative abundances at positions H3K18 and H3K23 ranging from 17 to ~35%, and between 15–30% at H4K12 and H4K16 (Supplementary Table 2), which is in accordance with previous findings46. Interestingly, similar relative abundances were observed at H3K14 (3–8%) for acetylation and propionylation marks (Supplementary Table 2). Overall, most non-acetyl acyl marks were found at levels below 2%, mainly at the N-terminal domains of histones H3 and H4. Surprisingly, some acidic acyl marks were found at the globular domains and C terminus, showing abundances as high as 10% (Supplementary Table 2), which coincide with the sites that were more susceptible to non-enzymatic acylation in our in vitro experiments (Fig. 2c, d). Detailed site specificity for all acyl marks is shown in Supplementary Table 2. Importantly, we did detect low levels of hydroxybutyrylation; however, our MS acquisition method cannot discriminate between possible isoforms of this mark, including bhb (β-hydroxybutyryl or 3hb), 2-hydroxybutyryl (2hb), 3-hydroxyisobutyryl (bhib), 2-hydroxyisobutyryl (2hb) or 4-hydroxybutyryl (4hb)10,14. Likewise, peptides bearing Kbu (butyryl) marks may also represent isobutyryl marks.

Our analysis of myoblasts showed dynamic changes in global histone acylations upon their fusion to form multinucleated myotube cells. We observed that the global levels of lysine acetylation, propionylation, butyrylation, malonylation, succinylation and glutarylation were significantly decreased upon myogenic differentiation, whereas the levels of lysine crotonylation were increased (Fig. 4b). The role of differential histone acylation in cellular differentiation is poorly understood; however, various lines of evidence suggest that nutrition and metabolism play a key role in the differentiation of cells47,48. For example, a previous study investigating the role of carbon metabolism in the differentiation of myogenic cells demonstrated that siRNA knock down of ATP citrate lyase (ACLY) induces differentiation of mouse myoblasts49. The same study also showed that the levels of histone acetylation in shACLY-treated cells were reduced, hypothesizing that the deposition of acetyl-CoA, and in turn histone acetylation levels, play an important role in the differentiation of myoblasts. Because there is little evidence of how newly identified histones acylations may be implicated in the differentiation of cells and/or epigenetic regulations, we next sought to investigate whether the cellular concentrations of other acyl-CoA metabolites have a direct relationship with histone acylation levels.

Acyl-CoA donors dictate the levels of histone acylation. Various studies in mammalian cells have shown that chromatin modifications are sensitive to changes in intracellular concentrations of metabolic intermediates, linking cell metabolism to epigenetic changes6,50. However, the mechanisms and enzymes mediating these processes have not been fully explored. So far, it has been demonstrated that changes in the levels of acetyl-CoA can influence global histone acetylation levels51. These findings led us...
to consider whether the levels of other histone acylations may also be influenced by the intracellular concentrations of their respective acyl-CoA donors. Using a stable isotope dilution MS approach, we accurately measured the concentrations of seven acyl-CoA metabolic intermediates in HeLa (Fig. 4c) and myogenic cells (Fig. 4d). Our metabolomics analysis revealed that acetyl-, propionyl- and succinyl-CoA were the most abundant acyl-CoAs in our cell models, with concentrations around 12, 1 and 0.5 μM, respectively, when normalized to cell volume of HeLa cells (Supplementary Fig. 6). In myogenic cells, when normalizing the data to cell number, we observed that the intracellular concentrations of metabolites also appear to undergo regulation from myoblast to myotube differentiation. Our metabolomics data strongly implicated metabolism in histone acylations. As the concentrations of intracellular metabolites are known to change in response to diet or physiological conditions, we next turned to the question of whether alterations in the levels of metabolites could affect the corresponding levels of histone acylations.

When looking at the levels of acetyl lysine PTMs in HeLa cells, our data showed a strong positive correlation with the metabolic levels of acetyl donors (Fig. 4e). A similar observation could be made for myoblasts (Fig. 4f). Even though we observed a measurable increase in the levels of crotonyl-CoA, this did not correlate well with the increase in lysine crotonylation observed in myotubes (Fig. 4g). Acyl-CoA intermediates are derived from various metabolic pathways including the TCA cycle, fatty acid synthesis, β-oxidation and amino acid metabolism. Although it remains poorly understood which metabolic pathway leading to the production of different acyl-CoAs might serve as a substrate for the acylation of nuclear histones, the global concentrations of metabolites determined in this study are consistent with the abundance of lysine acylations. Collectively, our data demonstrate a clear quantitative link between metabolism and differential histone acylations.

Acylation of histones in nucleo. Our in vivo and metabolomics studies strongly implicated metabolism in histone acylations. As the concentrations of intracellular metabolites are known to change in response to diet or physiological conditions, we next turned to the question of whether alterations in the levels of metabolites could affect the corresponding levels of histone acylations. To further explore this idea, we turned to an in nucleo...
system, where purified nuclei can be treated with artificial levels of metabolites. We isolated nuclei from HeLa cells under hypotonic conditions, treated with varying concentrations of eight different acyl-CoA donors and performed histone extraction, digestion and derivatization following standard procedures (Fig. 5a).

MS analysis revealed that histone acylations can be induced in a concentration-dependent manner. Specifically, by adding 1 or 5 µM of acyl-CoAs, we induced an increase of the respective acylation on histone peptides (Fig. 5b). Since the in nucleo experiment preserves the natural state of nuclear processes, it can be used to observe histone acylations in native chromatin. Our in vitro experiment demonstrated that acylations can occur by both enzymatic and non-enzymatic mechanisms, but such simplified assay cannot accurately represent the balances of a nuclear environment. We compared the two assays by performing an in nucleo–in vitro Spearman’s rank-order correlation analysis by using corrected in vitro enzymatic data (subtracting the non-enzymatic contribution). We observed a good correlation for some residues, including H3K9ac and H3K18acyl sites (Supplementary Fig. 7) that were highly acylated only in presence of enzymes in vitro. This suggests that specific sites are likely more accessible to enzymatic activity than others, and that this reactivity is also a function of the acyl-CoA utilized. However, a generalized conclusion cannot be drawn, as the in nucleo assay cannot discriminate enzymatic catalysis from chemical reactions, and physiological acylation turnover (equilibrium deposition/removal).

Additionally, our in nucleo results showed that histones accommodate acylation in two ways; by simply increasing the modified state or by removing pre-existing modifications to maintain the same level of total modified form. For example, upon treatment with malonyl-CoA, levels of H3K56mal increased with almost no changes in the other modifications on that peptide (Fig. 5c). On the other hand, after treatment with increasing concentrations of propionyl-CoA, the levels of H3K14pr increased, whereas the levels of H3K14ac showed a measurable decrease (Fig. 5d). Detailed in nucleo acylation relative abundances can be found in Supplementary Table 4. Taken together, our in nucleo studies demonstrated that modifications in chromatin are sensitive to changes in the concentrations of cellular metabolites, consistent with previous observations connecting the metabolic state of the cell with chromatin regulation7,53.

Discussion

A comprehensive screen of the major families of histone acetyltransferases (HATs) confirmed that most enzymes can catalyze the acylation of histones utilizing acetyl, propionyl and butyryl-CoA cofactors with similar efficiencies (Fig. 1), as previously reported54. However, they were less efficient catalyzing the acylation of histones with charged, branched or planar acyl-CoA cofactors. Even though these acyl donors are structurally similar to acetyl-CoA, the universal cofactor of HATs, our data showed that the ability of enzymes to utilize other cofactors largely depended on the size of the acyl group, which is in close agreement with recent data demonstrating the structural incompatibility of the active sites of p300 and GCN5 with long-chain acyl donors52,53. This observation was further confirmed by in vitro HAT assays performed at equal concentrations of acetyl-CoA and a competing cofactor (Fig. 3). The data showed a similar trend in which the preference for a cofactor different from acetyl-CoA was inversely proportional to the molecular weight of the competing donor, except for crotonyl-CoA, which, unlike the other acyl groups, possesses an unsaturated moiety that seems to render its use by most HATs unfavorable.

To further explore the possible mechanisms underlying the establishment of acyl marks, we performed in vitro acylation assays in the absence of HATs (Fig. 2). We found that histones can be acylated by the chemical reactivity of all acyl-CoA metabolites evaluated in this study. While most HATs showed strong preference for the acylation of residues at the N-terminal domains, i.e., residues K9–K16 in H3 and K5–K16 in H4, non-enzymatic acylation sites were more prevalent closer to the C terminus of histones, i.e., residues K36–K122 in histone H3 and K59–K91 in histone H4. Acidic acyl-PTMs including malonylation, succinylation and glutarylation were among those most easily catalyzed in the absence of enzymes (Fig. 2b). These marks are different from acetyl marks as they add bulkier groups to lysine residues and they carry a negative charge under physiological conditions. As such, it has been suggested that these acidic acyl marks could disrupt the interactions between histones and DNA, resulting in a more profound effect in chromatin unfolding than lysine acetylation55. Emerging hypotheses have suggested a model where non-enzymatic chemical reactions are a significant contributor to the landscape of lysine acylations in nuclear histones56. They also suggest that sirtuin enzymes showing specificity for the removal of acyl marks may represent a constitutive programming to suppress potential damaging effects caused by the presence of these PTMs.56,57 Interestingly, our study showed that those succinylated and malonylated sites highly susceptible to non-enzymatic acylation in vitro were among the sites reported previously in in vivo studies58. While more studies are required, our data suggest that histone lysine residues are prone to be modified by several free acyl-CoAs with and without enzymatic assistance.

The accurate quantification and elucidation of potential functional roles of acyl marks have been hampered by their low abundance. To provide an accurate estimate of the levels of acyl marks, we employed a label-free approach using DIA-MS. By analyzing in the same mixture modified and unmodified forms of histone peptides, we could report the relative levels of acyl-PTMs expressed as a percentage of the total histone. Analysis of human cervical cancer cells (HeLa) and human myogenic cells revealed that acyl marks together represent around 6–15% of all detected modifications on histones H3 and H4 (Fig. 4a,b; Supplementary Fig. 5). Our myoblast/myotube comparison strongly indicates that the differntiation of pluripotent cells is marked by a decrease in global levels of histone acetylation. This was not surprising, as this mechanism has been shown to be driven by a decrease in acetyl-CoA production mediated through the inhibition of glycolysis51,58. In agreement with these findings, our data showed a significant decrease of the bulk levels of histone acetylation once myoblasts fused to form multinucleated myotubes. Intriguingly, while the levels of most histone acylations decreased upon differentiation, myotube cells showed an increase in the global levels of lysine crotonylation (Fig. 4b). The understanding of how these chromatin modifications could be involved in driving myoblast differentiation is beyond the scope of this study and the subject of studies to come. This is a comprehensive report providing quantitative information on the levels of a broad number of histone acyl marks in HeLa and human myogenic cells, thus representing an important resource for future work aiming to understand cellular function and the dynamics of acyl-PTMs in the complex mammalian epigenetic mechanisms.

In general, we observed a strong correlation between histone acylations and their corresponding metabolic substrates. One exception was crotonylation, as the observed increase in lysine crotonylation in myotubes was not accompanied by a statistically significant increase in the levels of crotonyl-CoA (Fig. 4b,d,g). This specific experiment cannot prove whether crotonylation in myotubes is regulated by enzymatic mechanisms
that are not strictly regulated by crotonyl-CoA levels. However, we performed an intermediate experiment between in vivo and in vitro, namely in nuclei, to test whether there are corresponding changes in the levels of histone acylations upon manipulation of the concentrations of metabolites. This experiment showed that the bulk levels of lysine acylations can be induced in a dose-dependent manner, resulting in either a net increase or a dynamic exchange of modifications in response to increasing concentrations of acyl-CoA metabolites (Fig. 5).

Despite the evidence that the concentrations of metabolites can regulate the global levels of lysine acylations, it is premature to pinpoint how this metabolic regulation is involved in complex processes including gene expression, cell differentiation and apoptosis, or in diseases such as cancer that are characterized by altered metabolic states. Such a scenario is further complicated considering that metabolite precursors of histone modifications exist in different pools derived from various biological pathways that are regulated in response to cellular physiological conditions. Continued work in this area will help elucidate remaining questions surrounding the role of acetyl-PTMs such as the following: (a) do these PTMs compete with lysine acetylation, or (b) do they work in concert to regulate epigenetic mechanisms? Various studies have shown that the availability of acetyl-CoA can regulate the abundance of lysine acetylation. Our conclusion is that this study provides compelling evidence that this metabolic regulation is not restricted to acetylation, but also extends to the regulation of newly identified acyl marks in chromatin.

Methods

Protein expression and purification. Human p300 and human CBP sequences containing an N-terminal His tag, and C-terminal Strep2 and FLAG tags, were synthesized and cloned by Genewiz (Cambridge, MA) into the pTXB vector for baculovirus expression. The plasmid was transfected into SF9 cells and purified essentially as previously described60. Briefly, protein expression vectors encoding 6His-tagged Naa10p and residues 1–156 of Naa10p were overexpressed in Rosetta (DE3) pLysS E. coli cells, and purified to homogeneity using a combination of Ni-resin affinity and Superdex 200 gel filtration. The protein was concentrated to ~ 20 mg per mL.

The HAT domain of human hMOF was prepared previously essentially as described61. Briefly, a protein expression vector encoding 6His-tagged hMOF HAT domain (residues 174–449) was overexpressed in Escherichia coli cells and the protein purified to homogeneity using a combination of Ni-resin affinity and HiLoad Superdex 75 gel filtration. The protein was concentrated to ~ 20 mg per mL in a buffer containing 20 mM HEPS (pH 7.5), 0.5 M NaCl, and stored as described for the Nta complex.

The HAT domain of S. cerevisiae Gcn5 (residues 99–262) was overexpressed in bacteria from a PIRSETα/GCN5 vector and purified similarly as previously described62. The plasmid was transformed into E. coli strain BL21 (DE3) and overexpressed by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown at 17 °C overnight. The cells were collected by centrifugation at 4000 r.p.m. at 4 °C and lysed in 50 mM potassium phosphate pH 7.5, 0.500 M NaCl, 5% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg per mL DNase I, and 100 µg per mL lysozyme. The supernatant liquid was passed over Ni-NTA resin, washed with 10 column volumes of lysis buffer with 5 mM imidazole but without PMSF, DNase, or lysozyme. The protein was then transferred to 6–8 kDa MWCO dialysis tubing (Spectrum Labs) and dialyzed overnight into 20 mM sodium citrate pH 6.0, 0.150 M NaCl, and 1 mM DTT buffer. This was then followed by additional purification through SP Sepharose ion exchange and Superdex 75 (GE) gel filtration. The protein was concentrated to ~ 30 mg per mL, flash frozen, and stored at −80 °C in a buffer containing 20 mM Na-citrate pH 6.0, 150 mM NaCl, 1 mM DTT.

Recombinant histones H3 and H4 were expressed in Rosetta BL21 [DE3] pLysS cells and purified as monomers using standard procedures63,64.

In vitro histone acylation assay. Based on substrate specificity, histone H3 was assayed with HATS p300, CBP, P300C, and Gcn5, and histone H4 with HATS MOF, NaTα and Tip60. In vitro enzymatic assays were carried by incubating 0.5 µg of each HAT with 10 µg of recombinant histones H3 or H4 in the presence of 0.5 mM of short-chain acyl-CoAs (acetyl-, crotonyl-, malonyl-, succinyl-, propionyl-, butyryl-, γ-glutaryl- and β-hydroxybutyryl CoA—Sigma–Aldrich) in 1X HAT buffer (25 mM Tris-HCl pH 8, 8.5 25 mM KCl, 1 mM DTT, 0.1 mM AEBSF and 5 mM sodium butyrate) for 60 min at 30 °C; the final volume was 50 µL. For competition assays, reactions were carried out in the presence of 10 µM of acetyl-CoA and 10 µM of other acyl-CoAs. Background control reactions were performed in the absence of HATs. Reactions were stopped by freezing and samples were dried in a vacuum concentrator, resuspended in 400 µL of ammonium bicarbonate, and the propionylation procedure was repeated one more time. Samples were then digested with trypsin at a 1:10 ratio (wt/wt) for 6 h at 37 °C. Samples were desalted by C18 stage-tip. For this procedure, a small piece of a silica gel plug was added to the solution to re-establish pH 8.0. Samples were then washed by flushing 70–80 µL of 0.1% TFA and eluted into a clean Eppendorf tube by flushing 70–80 µL of 0.1% acetic acid by slow centrifugation. Samples were dried in a SpeedVac and resuspended at 0.5 µg per µL in 0.1 M acetic acid for nano LC-MS/MS analysis65.

Cell culture and histone extraction. HEK-S3 mammalian cells were cultured at 37 °C and 5% CO2 in spinner flasks in Joklik’s modified Eagle’s medium supplemented with 10% (v/v) newborn calf serum (Hyclone) and antibiotics (100 µg/ml penicillin and streptomycin). Three biological replicates were used. Histone extraction and digestion was carried out according to standard procedures66. Briefly, nuclei were isolated by suspension of cells in 10X volume of nuclear isolation buffer (15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM MgCl2, 5 mM CaCl2 and 250 mM sucrose. 0.2% Nonidet P40, 0.5 M NaCl, 5 µM of acetyl-CoA, and 50 mM sodium butyrate at 4 °C. Nuclei were pelleted by centrifugation at 1000 g for 5 min at 4 °C and washed twice with nuclear isolation buffer in the absence of NP-40. To the pelleted nuclei, 0.4 N H2SO4 was added to a final ratio of 5:1 and incubated for 2 h with shaking at 4 °C. Samples were centrifuged at 3400 g for 10 min at 4 °C and the supernatants were collected and incubated on ice with 1/5 volume of 100% TCA for 1 h. Precipitated histones were collected by centrifugation at 3400 g for 10 min at 4 °C and pellets were rinsed once with ice-cold acetone containing 0.1% HCl and once with ice-cold acetone. Protein concentration was determined using a Bradford assay. For in vivo analyses of histone acyl marks, 50 µg of histones was resuspended in 20 µL of wash with ammonium bicarbonate and subjected to derivatization with propionyl anhydride (or d-10 propionyl anhydride when identifying propionyl and butyryl histone marks), digested with trypsin for 6 h at 37 °C and desalted with C18 stage-tips as described above.
In nucleo acylation assay. HeLa S3 mammalian cells were harvested and washed twice in ice-cold PBS. Cells were incubated on ice with hypotonic lysis buffer containing 10 mM HEPES-NaOH pH 7.5, 1.5 mM MgCl2, 0.5 mM DTT, 0.1% (v/v) NP-40, and 1X Halt protease and phosphatase inhibitors for 10 min with intermittent agitation. Nuclei were collected by centrifugation at 600 g for 10 min at 4 °C, and washed once with a buffer containing 20 mM Heps-NaOH pH 7.9, 50 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM EDTA, 20% (v/v) glycerol and 1X Halt protease and phosphatase inhibitors. Nuclei were aliquoted at $\approx 5 \times 10^6$ cells per mL and used immediately by resuspending in 50 μL acylation reaction buffer (50 mM Tris-HCl pH = 8.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% (w/v) sucrose, 1X Halt protease and phosphatase inhibitors), containing 0.1 or 5 μM of short-chain acy-CoAs (acyetyl-, crotonyl-, malonyl-, propionyl-, butyryl-, glutaryl- and $\beta$-hydroxybutyryl CoA). Reactions were incubated at 30 °C for 3 h with gentle agitation. Nuclei were washed with acylation reaction buffer and the reactions were terminated by resuspending nuclei in 0.4 N H2SO4 for histone extraction. Extracted histones were derivatized with di0-propionic anhydride and digested as described above. Samples were dried in a SpeedVac and resuspended at 0.5 μg per mL in 0.1 M acetic acid for nano LC-MS/MS analysis.

Dot blot analysis. 2 μg of full-length acetylated histones H3 or H4 from in vitro reactions were spotted onto a nitrocellulose membrane. The membrane was blocked with either 5% BSA or 5% nonfat milk and incubated with the primary antibodies at 1:1,000 dilution (pan anti-crotonyl-lysine: PTM-501, pan anti-propionyl-lysine: PTM-201, pan anti-malonyl-lysine: PTM-701, pan anti-succinyl-lysine: PTM-701, PTM-101, anti-pan acetylated lysine: PTM-1151, all purchased from PTM-Biolabs, Hangzhou, China) according to the manufacturers’ instructions overnight at 4 °C. The membranes were washed with TBS-T three times for 10 min each, incubated with secondary antibody (Goat anti-Rabbit IgG Fc, Pierce 31461) at a 1:10,000 dilution for 60 min at room temperature and then probed with ECL Western Blot Substrate (Pierce).

Nano LC-MS/MS analysis. A total of 2.5 μg of peptides were injected into a 75 μm ID × 17 cm Reprosil-Pur C18 AQ (3 μm; Dr. Maisch GmbH, Germany) nano column (packed in-house) using an EASY-nLC nano HPLC (Thermo Scientific, Odense, Denmark). The mobile phases consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). For analysis of in vitro samples, peptides were eluted using a gradient of 0–30% B for 20 min followed by 30–90% B for 40 min, followed by 90% B maintained over 10 min. For in vitro and in vivo samples, the gradient consisted of 0%–25% B over 45 min followed by 26–98% B over 5 min and maintained for 10 min at 300 nL per min. The nano HPLC was coupled to a LTQ Orbitrap Elite or an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, California). Spray voltage was set at 2.4 kV and capillary temperature was set at 275 °C. For DDA, the mass spectrometer was set to perform a full MS scan (290–1,400 m/z) in the Orbitrap with a resolution of 60,000, followed by a series of targeted MS/MS scans of each modified H3 and H4 peptide, followed by MS/MS scans of the top four most intense abundant ions from the first scan. All MS/MS scans were performed in the ion trap and the precursor ion selection rate using data-dependent exclusion inducing CID (CID) with a normalized collision energy of 35 and an isolation window of 2.0 m/z. Maximum injection times of 50 ms were defined for both MS and MS/MS scans. AGC values were set to 1 × 10^6 for MS and 3 × 10^4 for MS/MS. MS data were collected in profile mode and MS/MS data were collected in centroid mode. For DIA, an AGC target of 5 × 10^5 (m/z 300–1,000) was acquired in the Orbitrap with a resolution of 120,000 (at 200 m/z) and an AGC target of 5 × 10^5 or in the ion trap with an AGC target of 3 × 10^5. MS/MS was performed with an AGC target of 3 × 10^5 using an injection time limit of 30 or 60 ms. All acquisitions were performed in positive mode polarity.

Data analysis was performed using our in-house software EpiProfile with a 10-ppm tolerance for extracting peak areas from raw files. The relative abundances of acyl-PTMs were calculated by dividing the intensity of the modified peptide by the sum of all modified and unmodified peptides sharing the same sequence, across all detectable charge states. For isobaric peptides (e.g., KocyCoA, KYcyCoA, KacroCoA, KacyCoA), the SKEUs were used to normalize the abundance of different PTMs. The intensities of cytochrome c oxidase subunit I were normalized using the area under the curve of the peptide of interest. The protein level of cytochrome c oxidase subunit I was normalized to the total cytochrome c oxidase subunit I abundance.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. All mass spectrometry raw files have been deposited in Chorus (https://chorusproject.org) under the project number 1376.

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Author contributions

J.S. performed most of the experiments, analyzed the data and wrote the manuscript; S.S. contributed to data analysis, made technical and intellectual contributions and helped writing the manuscript. Z.-F.Y. developed the software for analysis of histone marks and aided in the analysis of the histone data. M.C. performed the competition assays and the analysis of the ionization efficiencies of the differentially acylated peptide of histone H3. N.V.B. provided the myogenic cells; D.M.M. designed and performed initial experiments for CoA analysis and contributed to manuscript writing. B.J.K. and T.G.K. prepared the nucleosomal figures; G.A.B., C.E.M., and R.S.M. expressed and purified the recombinant HATs; N.W.S. performed the CoA sample preparation and data analysis and contributed to editing the manuscript; R.M. provided the recombinant enzymes and was available for helpful discussions and B.A.G. conceived, designed and supervised the study. All authors discussed the results and commented on the manuscript.

Additional information

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