Review

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Histone acylations and chromatin dynamics: concepts, challenges, and links to metabolism

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Abstract

In eukaryotic cells, DNA is tightly packed with the help of histone proteins into chromatin. Chromatin architecture can be modified by various post-translational modifications of histone proteins. For almost 60 years now, studies on histone lysine acetylation have unraveled the contribution of this acylation to an open chromatin state with increased DNA accessibility, permissive for gene expression. Additional complexity emerged from the discovery of other types of histone lysine acylations. The acyl group donors are products of cellular metabolism, and distinct histone acylations can link the metabolic state of a cell with chromatin architecture and contribute to cellular adaptation through changes in gene expression. Currently, various technical challenges limit our full understanding of the actual impact of most histone acylations on chromatin dynamics and of their biological relevance. In this review, we summarize the state of the art and provide an overview of approaches to overcome these challenges. We further discuss the concept of subnuclear metabolic niches that could regulate local CoA availability and thus couple cellular metabolisms with the epigenome.

Keywords acylation; chromatin; histones; metabolism; microdomains
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See the Glossary for abbreviations used in this article.

Introduction: Histone lysine acylations and metabolism

Genomic information is stored in the nucleus of eukaryotic cells in a structure referred to as chromatin which consists of DNA and proteins (Olins & Olins, 2003). The smallest building blocks of chromatin are nucleosomes, which are composed of 147 base pairs of DNA wrapped around a histone octamer. Such a histone octamer is formed by one histone H3-H4 tetramer and two histone H2A-H2B dimers. The flexible N-termini as well as the globular core domain of these four core histones can be heavily covalently modified (Kornberg, 1974; Luger *et al*, 1997). The best studied histone post-translational modifications (PTM) are currently acetylation (Kuo & Allis, 1998), methylation (Kouzarides, 2002), and phosphorylation (Oki *et al*, 2007). It has been suggested that these PTMs could form a so-called histone code (Strahl & Allis, 2000). If it is indeed a "code" is still controversial, but it becomes more and more convincing that the complex diversity of PTMs enables fine tuning of chromatin structure and function (Rando, 2012). Since chromatin regulates DNA accessibility, changes in chromatin structure influence DNA-dependent processes such as transcription (Tropberger & Schneider, 2013; Tessarz & Kouzarides, 2014). How chromatin architecture is regulated by histone PTMs can currently be best explained for histone lysine acetylation (Kac).

Positively charged lysine residues, for instance in the histone H4 tail, can interact with negatively charged DNA or neighboring nucleosomes. Acetylation of the ε-amino group of lysines occurs on many lysines in histone tails and globular domains and is catalyzed by histone acetyltransferases (HAT). Acetylation neutralizes the positive charge of lysines and thus can weaken the interactions between histones and DNA, contributing to a more open chromatin state (Fig 1A). Higher DNA accessibility enables, for example, transcription factor binding and can promote transcriptional activity (Bannister & Kouzarides, 2011). In addition, to these direct effects, Kac can also be directly bound by specific interactors (so-called "reader" proteins), such as bromodomain-containing (BRD) transcription factors that promote gene expression (Wang *et al*, 2007). Genome-wide distribution studies show a positive correlation between enrichment of histone acetylations at transcriptional start sites (TSS) and gene expression (Hebbes *et al*, 1988; Mikkelsen *et al*, 2007). Transcriptional regulation via Kac seems to be critical for cellular function, as aberrant acetylation is linked to cancer development, neurological disorders, and also metabolic diseases (Timmermann *et al*, 2001; Zhong & Kowluru, 2010; Sheikh, 2014).

Kac on the histone tails has a dynamic turnover and is dependent on the availability of the acetyl donor, acetyl-CoA, which is a key metabolic intermediate. Acetyl-CoA can diffuse from the cytoplasm to the nucleus or can be locally produced in the nucleus (preprint: Kafkia *et al*, 2020). Cellular changes in the acetyl-CoA concentration can be reflected in the levels of histone acetylation (Sivanand *et al*,

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Glossary

AcKRS	Acetyl-lysyl-tRNA synthetase
ACLY	ATP citrate lyase
ACSS2	Acyl-coenzyme A synthetase 2
AUC	Analytical ultracentrifugation
BRD	Bromodomain
CATNIP	CoA/Acetyltransferase interaction profiling
CDYL	Chromodomain Y-like
ChIP-seq	Chromatin immunoprecipitation sequencing
CRAT	Carnitine acetyltransferase
EPL	Expressed protein ligation
FRET	Fluorescence resonance energy transfer
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IDR	Intrinsically disordered region
ITC	Isothermal titration calorimetry
IVT	*In vitro* transcription
Kac	Lysine acetylation
Kbhb	Lysine β-hydroxybutyrylation
Kbu	Lysine butyrylation
Kbz	Lysine benzoylation
Kcr	Lysine crotonylation
KGDH	α-ketoglutarate dehydrogenase
Kglu	Lysine glutarylation
Khib	Lysine 2-hydroxyisobutyrylation
Kibu	Lysine isobutyrylation
Kla	Lysine lactylation
Kma	Lysine malonylation
Kpr	Lysine propionylation
Ksucc	Lysine succinylation
LLPS	Liquid–liquid phase separation
MS	Mass spectrometry
NCL	Native chemical ligation
NMR	Nuclear magnetic resonance
PDC	Pyruvate dehydrogenase complex
PPPS	Polymer–polymer phase separation
PTM	Post-translational modification
SDH	Succinate dehydrogenase
SIRT1-7	Sirtuins
TSS	Transcription start site

2018). Intriguingly, metabolic enzymes that produce acetyl-CoA such as the pyruvate dehydrogenase complex (PDC), ATP citrate lyase (ACLY), acyl-coenzyme A synthetase 2 (ACSS2), or the carnitine acetyltransferase (CRAT) can localize in the nucleus, which can lead to increased nuclear Kac levels (Boukouris *et al*, 2016). Additionally, ACLY as well as the ACSS2 homolog in *Saccharomyces cerevisiae* were shown to be involved in the regulation of histone acetylation levels (Takahashi *et al*, 2006; Wellen *et al*, 2009). More recently, Mews *et al,* (2017) linked reduced ACSS2 protein amounts with decreased nuclear acetyl-CoA concentrations and decreased histone acetylation levels as well as reduced gene expression (Mews *et al*, 2017). Such findings provide insights into how cellular metabolism, via cofactors required by chromatin modifiers, can "talk" to the epigenome and gave rise to the new field of metaboloepigenomics. How exactly this local acetyl-CoA production is established and how local acetyl-CoA pools are maintained is still one of the major questions in the field (Katada *et al*, 2012).

Findings that other acyl-CoAs besides acetyl-CoA can occur in the nucleus and function as acyl donors for various histone-modifying enzymes, added additional complexity to the panel of histone modifications (Pietrocola *et al*, 2015; preprint: Trefely *et al*, 2020a). In line with this, metabolic enzymes involved in the generation of these additional acyl-CoAs have been recently detected in the nucleus. For example, the nuclear α-ketoglutarate dehydrogenase (KGDH) complex can increase the concentration of nuclear succinyl-CoA (Wang *et al*, 2017). Due to the development of increasingly sensitive mass spectrometry (MS) techniques, also novel types of histone lysine acylations and new acylation sites have been identified (Fig 1B). These lysine acylations include 2-hydroxyisobutyrylation (Khib) (Dai *et al*, 2014), β-hydroxybutyrylation (Kbhb) (Xie *et al*, 2016), benzoylation (Kbz) (Huang *et al*, 2018c), butyrylation (Kbu) (Chen *et al*, 2007), isobutyrylation (Kibu) (Zhu *et al*, 2021), crotonylation (Kcr) (Tan *et al*, 2011), glutarylation (Kglu) (Bao *et al*, 2019), lactylation (Kla) (Zhang *et al*, 2019a), malonylation (Kma) (Xie *et al*, 2012), propionylation (Kpr) (Chen *et al*, 2007) and succinylation (Ksucc) (Xie *et al*, 2012). Since the acyl-CoAs required for these modifications are derived from different metabolic pathways, specific histone acylations could act as sensors of the metabolic state of a cell and fine tune chromatin architecture and thus gene expression according to cellular needs (Simithy *et al*, 2017; Trefely *et al*, 2020b).

The versatility of the different histone lysine acylations is determined by their distinct chemical properties. Only acidic modifications like Ksucc, Kmal, and Kglu change the positive lysine charge to a negative one. The branched 4-carbon and polar modifications Khib and Kbhb contain hydroxyl groups and enable modified lysines to form additional hydrogen bonds. Hydrophobic acylations include Kcr, Kbu, Kbz, and Kpr. Kpr (linear) is a 3-carbon, Kbu (linear), and Kcr (planar) are 4-carbon modifications. Kbz is currently the only described histone acylation with an aromatic acyl group. Increasing the acyl chain length further increases the hydrophobicity and also the steric hindrance potential of modified lysines (Sabari *et al*, 2017; Dai *et al*, 2020). This short summary highlights the diversity of acylations that can occur on histone lysines.

Multiple studies have suggested functional differences for some of these acylations and have been excellently reviewed elsewhere (Sabari *et al*, 2017; Li *et al*, 2018; Barnes *et al*, 2019; Boon *et al*, 2020; Dai *et al*, 2020; Haws *et al*, 2020; Trefely *et al*, 2020b). However, important questions still need to be addressed to fully understand the impact of metabolism on cellular function via chromatin-based mechanisms. For this, additional mechanistic insights into the effect of acylations on chromatin structure and function will be necessary. In the last years, new highly sensitive and well-controllable assays have been developed that can help to mechanistically understand the role of additional lysine acylations in gene expression, and how different acylations compare to each other (Cuvier & Fierz, 2017). In this review, we aim to provide an overview of different strategies that can be used to deepen our mechanistic insight into lysine acylations and to discuss the findings gained with these approaches. We will further focus on the principle

Box 1: Histone modification mimics and designer chromatin

Site-specific mutations of histones have been used to mimic modifications or the unmodified state. One advantage of such mimics is that they can be used *in vitro* and *in vivo*. For example, histone point mutants have been used to study the function of acylations in *Saccharomyces cerevisiae*. The advantage of lower eukaryotes is that it is relatively simple to replace all endogenous histones with the mutated ones. For example, H2BK37E (as a K37succ mimic), H2AK119E (as a K119mal mimic), and H4K91E (as a H4K91glu mimic) mutant yeast strains were used to investigate chromatin structure changes *in vivo* and complemented existing *in vitro* data nicely (Ishiguro *et al*, 2018; Jing *et al*, 2018; Bao *et al*, 2019). Nevertheless, no mimic can entirely resemble the actual modification and close structural similarity of some short-chain lysine acylations such as Kac, Kpr, or Kbu makes it difficult to mimic acylations specifically (Tropberger *et al*, 2013; Zorro Shahidian *et al*, 2021). Furthermore, these mimics are constant alterations of histones, which is in contrast to the rather dynamic nature of endogenous acylations (Katan-Khaykovich, 2002). "Genetically encoding" acetyl-lysine can enable the generation of proteins acylated at defined sites (Fig 2A). For example, the amber (stop) codon suppression by an orthogonal acetyl-lysyl-tRNA synthetase (AcKRS)/ tRNA pair allows for the incorporation of noncanonical amino acids (e.g., acetylated lysine) into proteins (Neumann *et al*, 2008). A high yield of site-specifically acylated histones can be successfully obtained, e.g., by their expression in *E. coli*. Another common approach to obtain site-specific acylated nucleosomes is via a native chemical ligation (NCL) or expressed protein ligation (EPL), in which peptides containing C-terminal thioesters are fused to an N-terminal cysteine from another peptide (Dawson *et al*, 1994; He *et al*, 2003). For example, histone tails with acylated lysines can be generated by peptide synthesis and truncated histones can be recombinantly expressed and used for the chemo-selective reaction (Fig 2A) (Shogren-Knaak, 2006). This semi-synthetic method allows for the incorporation of multiple modifications on the same histone tail, as well as of different types of acylations. Alternatively, complete chemical synthesis of histones allows multiple distinct acylations to be added, but remains elaborative and inefficient in comparison with the amber system or NCL/EPL approaches (Zorro Shahidian *et al*, 2021). Elsässer *et al* (2016) demonstrated that site-specific histone acetylation can be genetically encoded in mammalian cells by stable integration of the components of the amber system and deposited into chromatin. However, cellular deacetylases can act quickly on these sites resulting in deacetylation, a problem that most likely also applies to other types of acylations. Recently, Fujiwara *et al* (2021) demonstrated that site-specific acylations can also be introduced *in vivo* via a protease-resistant nucleosome-binding catalyst and a cell-permeable acetyl donor. Subsequent development of this exiting method might enable us to study the effects of histone acylations on nucleosome assembly *in vivo*.

of metabolic microdomains in the nucleus as well as the current challenges to study metaboloepigenomics *in vivo*.

Impact of histone acylations on chromatin structure

Chromatin accessibility and compaction can be fine-tuned by an interplay of various histone PTMs including different types of acylations (Trefely *et al*, 2020b). However, our mechanistic understanding of the direct or indirect effects of acylations on chromatin is still limited. In order to gain more insight, *in vitro* assays have been used, many of which require so-called "designer" chromatin. Designer chromatin with specific histone acylations facilitates highly controllable *in vitro* studies on the direct effects of these acylations. To generate acylated nucleosomes or chromatin, several different approaches have been applied (Müller & Muir, 2015). More details on the generation of such chromatin are provided in Box 1 and Fig 2A. Although most of the previous studies focused on histone acetylation, we still discuss some of them here to encourage similar mechanistic analyses for other types of acylations.

Chromatin dynamics can be shaped by nucleosome stability, which can be directly affected by acetylations on the core domain of histones (Tropberger & Schneider, 2013). To reveal the influence of acylated lysines in the histone core region on nucleosome stability, Bao *et al,* (2019) investigated histone H4 glutyrylation at lysine 91. During octamer purification via size-exclusion chromatography, they observed different elution profiles for H4K91glu octamers and unmodified octamers, hinting toward inefficient octamer assembly upon H4K91glu. This destabilization of nucleosomes provided mechanistic insight into the role of H4K91glu at highly expressed genes (Bao *et al*, 2019). Furthermore, they showed that H4K91glu facilitates H2A/H2B dimer dissociation from nucleosomes using a fluorescence resonance energy transfer (FRET) assay. Such FRET assays are well suited to study nucleosome stability but also to analyze folding of nucleosomal arrays (Fig 2B). Via a FRET approach, a destabilization effect on nucleosomes upon succinylation of H3K122, again located within the globular domain, was revealed (Zorro Shahidian *et al*, 2021). Likewise, H4K77succ was shown to decrease nucleosome stability while nucleosomal DNA accessibility increased (Jing *et al*, 2020). In addition to site-specific acylations, global Kac, Kpr, Kbu, Kmal, and Ksucc of histones can weaken nucleosome stability, histone-DNA interactions and promote nucleosome sliding. Of these modifications, Ksucc has the longest acyl-CoA chain and imparts a negative charge, which might explain why it was able to destabilize nucleosomes to the greatest extent in an optical tweezer approach using 12-mer nucleosomal arrays (preprint: Smestad *et al*, 2020). In general, single-molecule force spectroscopy techniques, such as optical or magnetic tweezers, can be used to study the stability of clamped mononucleosomes and nucleosomal arrays by applying an accurate force and measuring motions (Fig 2C) (Neuman & Nagy, 2008).

For studies on the impact of acylations on chromatin structure, the histone H4 tail can serve as a convenient starting point. The H4 tail plays an important role in this regard, since it can directly interact with the acidic patch (created between H2A/H2B) of the neighboring nucleosome (Luger *et al*, 1997). Kac in the H4 tail is one of the few histone modifications that has been shown to disrupt internucleosomal interactions and has a direct impact on chromatin structure (Shogren-Knaak, 2006; Allahverdi *et al*, 2011). This structural effect is not solely caused by charge neutralization of H4K16 and reduced interaction with the neighboring nucleosome. Collepardo-Guevara *et al,* (2015) observed that upon K16 acetylation, histone H4 tail flexibility decreases. This loss of flexibility could additionally limit internucleosomal interactions (Collepardo-Guevara *et al*, 2015) and thus provide another perspective on how H4K16ac opens up chromatin. This insight was based on nuclear magnetic resonance (NMR) spectroscopy, which enables analysis of nucleosome structure and tail dynamics in combination with computational simulation studies (Musselman & Kutateladze, 2021). Similar approaches could be used to systematically compare the effect of non-acetyl acylations on histone tail dynamics for all core

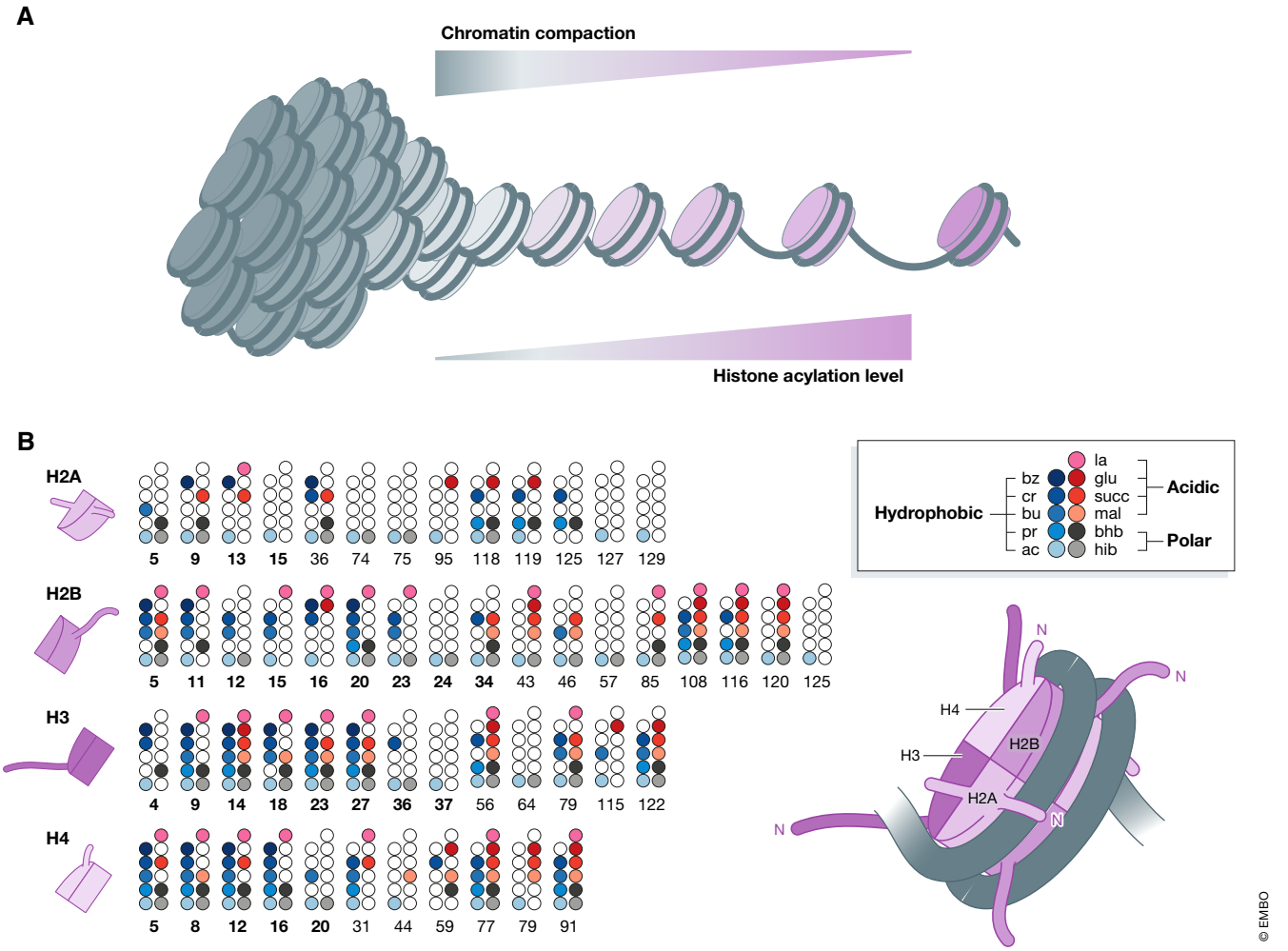

Figure 1. Overview of lysine acylations on core histones and their role in chromatin compaction.

(A) Increasing histone acylation levels can contribute to opening up chromatin. (B) Identified lysine acylation sites in the four core histones (Sabari *et al*, 2017; Barnes *et al*, 2019; Zhang *et al*, 2019a). Lysines within the N-terminal histone tail are in bold. Selected acylations and their chemical nature are depicted (hydrophobic: blue, polar: gray, acidic: red). Abbreviations: ac—acetylation, pr—propionylation, bu—butyrylation, cr—crotonylation, bz—benzoylation, hib—2-hydroxyisobutyrylation, bhb—β-hydroxybutyrylation, la—lactylation, mal—malonylation, succ—succinylation, glu—glutarylation.

histones (Fig 2D). A loss of nucleosomal array compaction upon H4K16ac was also observed in a more direct manner by using designer chromatin and analytical ultracentrifugation (AUC) sedimentation velocity experiments (Shogren-Knaak, 2006). Similar AUC assays could be useful for further comparison of the impact of different acylations on assembled chromatin (Shogren-Knaak, 2006; Funke *et al*, 2016). Interestingly, these effects seem to be specific to H4K16 modifications, since the acetylation of the nearby lysine H4K20 does not interfere with chromatin compaction (Wilkins *et al*, 2015). This could be explained by changes in the internucleosomal interactions caused by H4K16ac as demonstrated by Funke *et al*, (2016). For this, the authors developed an elegant method using a DNA origami-based force spectrometer (Fig 2E). This positioning device is based on a nanoscale folding of DNA with a flexible hinge region (stapler like) that allows for the targeted incorporation of mononucleosomes and the measurement of internucleosomal interactions strength (Funke *et al*, 2016). To assess the influence of histone acylation on chromatin structure *in vivo* is technically very challenging. Sidoli *et al*, (2019) provided a first step in this direction via a sophisticated approach to analyze the influence of histone PTMs on chromatin accessibility by MS and metabolic labeling. This method could also be useful to analyze acylation-dependent chromatin compaction *in cellulo* (Sidoli *et al*, 2019).

To summarize, many of these *in vitro* assays will be extremely useful for studies on the specific effects of histone acylation, beyond acetylation, in the future. A combination of different assays coupled with chromatin simulation approaches could help to precisely predict chromatin behavior upon specific histone acylation events (Moller & de Pablo, 2020). The ultimate goal is to study chromatin structure dynamics *in vivo*, as nucleosomal arrays do not resemble the complexity of chromatin in the nucleus. Recent advances in 3D super-resolution microscopy hold the promise to examine the

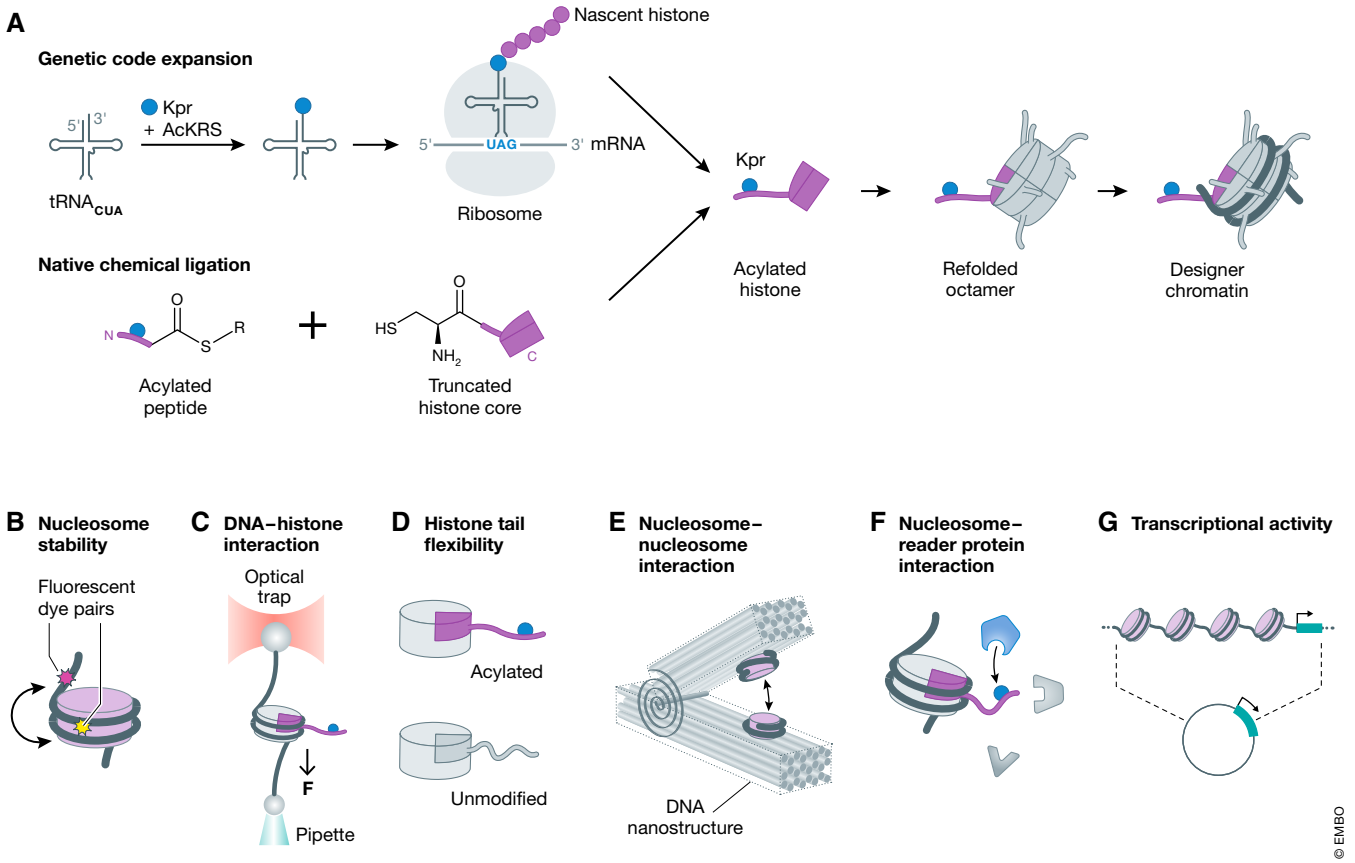

Figure 2. Overview of potential impacts of acylations on chromatin and exemplary *in vitro* approaches to study them.

(A) Generation of "designer" chromatin with site-specific acylations. The genetic code expansion approach uses an AcKRS/ tRNA$_{CUA}$ pair (top panel). The incorporation of an acyl-lysine (shown as example here: propionyl-lysine, Kpr) at site-specifically installed amber codons (UAG) leads to the synthesis of acylated histones. During native chemical ligation (bottom panel), the thioester of an acylated peptide (e.g., histone N-terminus) is linked to the cysteine of the truncated histone core. Acylated histones and remaining core histones can be used to refold octamers and assemble "designer" chromatin on DNA templates. (B) Impact on nucleosome stability. FRET approaches with fluorescent dye pairs, e.g., on the DNA (yellow and pink star) allow for the quantification of nucleosome disassembly. (C) Effects of histone acylations on DNA-histone interaction strength and nucleosome stability can be measured in an optical tweezer setup, where mononucleosomes or nucleosomal arrays can be clamped between two beads (light gray). Within an optical trap, a pulling force (F) on the lower bead displaces the upper bead in the optical trap. (D) Changes of histone tail flexibility upon lysine acylations can be studied, e.g., by NMR experiments assessing the conformation of the tails (shown here: acylated (Kpr) versus unmodified histone tail). (E) DNA origami (a nanoscale folding of DNA to create three-dimensional shapes such as tweezers) with a flexible hinge region can be used to study the interaction between attached nucleosomes. Nucleosome interaction is reflected in a closed conformation of the DNA origami and can be quantified. (F) Specific reader proteins. Schematic interaction study using an acylated mononucleosome as a bait. Interaction partners can be identified and quantified via MS. Their interaction affinity can further be analyzed by, e.g., isothermal titration calorimetry (ITC). (G) Role of acylation in transcription. Schematic of *in vitro* transcription assay in which chromatin is assembled on a plasmid. The direct and indirect effects of different lysine acylations on transcriptional efficiency can be studied in a well-controlled system.

influence of histone acylations on chromatin compaction *in vivo* (Otterstrom *et al*, 2019).

Acylation-specific readers, writers, and erasers

With the identification of novel types of histone acylations, the question arose whether canonical HATs are placing these diverse acylations or if additional, yet unidentified, acyltransferases exist. The same considerations hold true for the erasers, the histone deacetylases (HDAC). Also, many questions concerning acyl-specificity of "reader" proteins remain open (Allis & Jenuwein, 2016). So far, very few systematic screens have been performed to identify new acylation-specific writers, readers, or erasers, in an unbiased way (Fig 2F).

Most *in vitro* and *in vivo* studies have focused on the role of known HATs in mediating other types of acylations. (Zhao *et al*, 2018). For example, *in vitro* HAT assays revealed that several classical HATs such as KAT2A (GCN5), KAT3B (p300), Tip60, or MOF can use various acyl-CoAs (pr-/ bu-/ cr-/ mal-/ bhb-/ succ- and glu-CoA) as their cofactors, but for many of these enzymes the acyltransferase activity seems to decrease for bulkier acyl-CoAs. KAT3B, for example, seems to function as an acyltransferase "allrounder", being able to catalyze H3K18hib, H4K8hib (Huang *et al*, 2018a), H4K5bu, H4K8bu (Goudarzi *et al*, 2016), H3K14pr, H3K14bu (Kebede *et al*, 2017), H3/H4Kbhb (Kaczmarska *et al*, 2017), H3/H4Kibu (Zhu *et al*, 2021), and H3K122succ (Zorro Shahidian *et al*, 2021). Kaczmarska and colleagues showed that the KAT3B (p300) activity decreases with increasing acyl chain length since longer

acyl-CoAs interfere with histone lysine binding (Kaczmarska *et al*, 2017). Additionally, screenings of classical HATs for their activity toward different acylations also revealed that some HATs have a higher affinity toward other acyl-CoAs than acetyl-CoA. For instance, KAT2A (GCN5) has a higher binding affinity for succinyl-CoA than for acetyl-CoA (Wang *et al*, 2017). It should also be considered that *in vivo* additional cellular factors (absent in *in vitro* assays) could enhance the ability of, e.g., KAT3B to use specific acyl-CoAs, probably by inducing some structural rearrangements. Furthermore, the high reactivity of acyl-CoAs can also cause acylations on histones independently of enzymatic mechanisms (Trub & Hirschey, 2018) resulting in non-enzymatic covalent modifications (NECMs). These NECMs are an under-studied class of post-translational modifications that add further complexity to the control of histone acylations and need to be carefully considered for instance for *in vivo* studies (see next section) (Simithy *et al*, 2017). Similar to these findings with HATs, studies with HDACs have revealed that classical HDACs can have activity toward additional histone acylations. For example, class I HDACs can also act as histone decrotonylases *in vitro* and *in vivo* (Fellows *et al*, 2018). In addition, HDAC2 and HDAC3 were identified as the major enzymes to erase Khib (Huang *et al*, 2018a). Activity profiling on sirtuins (SIRT1-7) on multiple H3K9 acylations revealed that most sirtuins can remove different acylations (K9ac, K9bu, K9cr, K9bhb on H3). Interestingly, only SIRT2 and SIRT5 acted on H3K9succ. Furthermore, this profiling revealed SIRT1-3 and SIRT5 as novel de-β-hydroxybutyrylases (Zhang *et al*, 2019b).

Regarding reader domain specificity, most human bromodomains such as BRD2 or BRD4 seem to have higher affinities for Kpr than Kcr or Kbu. In contrast, bromodomains with larger binding pockets such as BRD9 and CECR2 were able to bind Kbu and only the second bromodomain of TAF1 was able to interact with Kcr (Flynn *et al*, 2015). Also, YEATS domains can bind to Kac, Kpr, Kbu, Kcr, and Ksucc (Barnes *et al*, 2019). Interestingly, the YEATS domain of the AF9 protein binds preferentially to Kcr (H3K9cr or H3K18cr) compared with Kac. The higher binding affinity toward Kcr (2.5- to 4.5-fold increase) results from an extended reader pocket of this YEATS domain into which crotonylated lysines perfectly fit (Li *et al*, 2016).

Surprisingly, to our knowledge, no reader, writer or eraser specific for only non-acetyl acylations has been discovered so far. Methods like the CoA/AcetylTraNsferase Interaction Profiling (CATNIP) enable high-throughput profiling of acyl-CoA/protein interactions and revealed additional specificities of known HATs toward different acyl-CoAs, but no novel non-acetyl specific interactor was identified (Levy *et al*, 2020). Such profiling approaches could expand our understanding of the enzymes adding or removing acylations or the proteins reading acylations, but several challenges for subsequent *in vivo* studies remain. Although cell-based studies confirmed *in vivo* many observations made through *in vitro* approaches, the redundancy in activity and specificity between different HATs (Kaczmarska *et al*, 2017; Simithy *et al*, 2017), the existence of multiple types of acylations on the same lysine residue (Tan *et al*, 2011) and the lack of systems for generating specific acylations at selected sites *in vivo* makes it challenging to draw unambiguous conclusions from many *in vivo* assays. In addition, the fact that some of the erasers and writers can also act on non-histone proteins, disrupting their function *in vivo* makes it hard to ascribe any cellular effect exclusively to changes in histone acylations (Zhang *et al*, 2019a). One example for such an acylated non-histone protein is p53, a transcription factor that is involved in the regulation of multiple target genes and tumor growth suppression (Vousden & Lane, 2007). P53 is activated upon acetylation, propionylation, and butyrylation mediated by p300/CBP (Chen *et al*, 2007; Cheng *et al*, 2009). Thus, changes in the acylation of p53 could alter transcriptional programs and cellular fate, and alterations in histone acylation could be merely a consequence p53 acylation (Liu *et al*, 1999). Another *in vivo* challenge is that writers as well as erasers and readers can act in concert with various other proteins and these interactions likely define their activity and specificity. Such interactors include metabolic enzymes that could provide locally specific CoAs used by acyltransferases. For example, the α-KGDH complex can bind to KAT2A (Wang *et al*, 2017). Also, ACSS2 was shown to interact with a HAT, namely KAT3A (CBP) (Mews *et al*, 2017). In addition, the enzymatic activity of HATs can also be shaped through direct binding of metabolites. For instance, the binding of free CoA or of HDAC1/2 to the same domain of CDYL (chromodomain Y-like protein) seems to impact its enzymatic function (Caron *et al*, 2003). Upon crotonyl-CoA binding, CDYL was shown to act as a crotonyl-CoA hydratase and to be involved in the reduction of histone Kcr levels (by converting crotonyl-CoA into β-hydroxybutyryl-CoA) (Liu *et al*, 2017) whereas the interaction of HDAC1/2 with CDYL prevents CoA binding. But in both cases, CDYL seems to play a part in transcriptional repression: in one scenario as a metabolic enzyme and in the other one as an epigenetic regulator (Caron *et al*, 2003; Liu *et al*, 2017). Findings like this highlight the tight links between metabolic processes and epigenetic mechanisms. We will discuss this crosstalk between histone acylation and cellular metabolism in more detail below.

Influence of histone acylations on transcription: mechanisms and correlations

The links between histone acylations and chromatin structure described above suggest that at least some acylations might be involved in transcriptional regulation. To investigate the effect of histone acylation on transcription, highly controllable *in vitro* transcription (IVT) assays on designer chromatin have been developed (Fig 2G) (Sawadogo & Roeder, 1985) using either nuclear extracts as the source for polymerases and cofactors or only purified, recombinant components. Applying such an IVT system, Kebede *et al,* (2017) showed that Kpr can enhance transcription (Kebede *et al*, 2017). Furthermore, Goudarzi and colleagues (2016) observed that Kbu can activate transcription to at least a similar extent as Kac (Goudarzi *et al*, 2016). Kcr has been suggested to stimulate transcription to a greater extent than acetylation (Sabari *et al*, 2015), while Khib (Huang *et al*, 2018b) and Kla (Zhang *et al*, 2019a) enhance transcription to a lesser extent than Kac. Designer chromatin has been used to demonstrate how two acylations, Kac (Tropberger *et al*, 2013) and Ksucc (Zorro Shahidian *et al*, 2021), on the same residue on the lateral surface of the histone octamer (H3K122) lead to a similar enhancement of transcription. To study the dynamics of transcription, a new innovative variation of the IVT assay, which enables real-time detection of the transcripts by fluorescence correlation spectroscopy, has recently been developed.

Via this approach, it was possible to demonstrate that tetra-acetylated H4 (acetylated at K5, K8, K12, and K16) stimulates transcription *in vitro* and to provide a model for the contribution of these acetylations to different steps of the transcription process (Wakamori *et al*, 2020). Overall, *in vitro* transcription assays are a powerful tool, which can dissect the precise impact of specific histone acylations on transcription.

In contrast to these more mechanistic *in vitro* approaches, intriguing insights into the correlations between acylations and transcription have emerged from genome-wide histone acylation enrichment studies (chromatin immunoprecipitation followed by high-throughput sequencing, ChIP-seq) in combination with gene expression profiling. However, these ChIP-seq approaches depend heavily on high-quality, ChIP-grade, site-specific antibodies, which are often limiting. The potentially high cross-reactivity of anti-acyl antibodies makes it essential to extensively verify their specificity (Simithy *et al*, 2017) (see Box 2). In many studies, "pan" antibodies have been used that recognize a specific acylation type but are not acylation site or protein specific. Nevertheless, such antibodies can help to circumvent the lack of antibodies that can site-specifically recognize acylations on histone lysines. For instance, Tan *et al,* (2011) performed ChIP-seq with a pan anti-Kcr antibody and revealed Kcr enrichment at promoter and enhancer regions, which strongly overlapped with Kac signals in a resting somatic human cell line. In post-meiotic male germ cells (spermatids), however, regions on sex chromosomes were enriched in histone Kcr and low in Kac. This occurred at X/Y-linked genes that are "active" in post-meiotic male germ cells. The authors suggest that a specific ratio of Kcr and Kac could contribute to an escape from inactivation and to maintain the genes active, despite the general transcriptional repression of sex chromosomes in these cells (Tan *et al*, 2011). In addition, ChIP with a pan-Ksucc antibody revealed that Ksucc peaks strongly correlate with peaks for histone modifications that mark actively transcribed chromatin, such as H3K4me3. Upon loss of the enzyme succinate dehydrogenase (SDH) in irradiated mouse embryonic fibroblasts, Ksucc levels both increased and decreased at distinct promoters. These changes in Ksucc correlated with gene expression changes, suggesting, a gene-specific transcriptional control by Ksucc (Smestad *et al*, 2018). These studies show that using pan-acyl antibodies can provide insights into the relationship between acylation and transcription. However, the insight is limited as the antibodies might recognize all proteins carrying a specific acylation, both histone and non-histone, rendering it difficult to identify the specific contribution of a given histone acylation. Furthermore, not all acylation sites are equally well recognized by these antibodies due to epitope preferences and thus preference toward specific proteins.

In contrast to pan-acyl antibodies, site-specific antibodies allow to focus on one specific acylation site within a specific protein. With this higher precision, it is possible to study the effect of metabolic changes on specific histone acylations at defined gene regions. For instance, in order to understand the microbiome-host interaction better, Fellows *et al,* (2018) performed ChIP-seq in murine colon epithelial crypts with an anti-H3K18cr antibody, and linked H3K18cr enrichment at TSS to transcriptional activation (Fellows *et al*, 2018). Further, microbiota depletion via antibiotics in mice caused short-chain fatty acid loss, which decreased H3K18cr levels in the colon. These findings suggest that Kcr levels in the colon could reflect short-chain fatty acid generation by the microbiota and provide an example of how changes in metabolism can influence the epigenome (Fellows *et al*, 2018). However, whether this crosstalk is direct or rather indirect requires further investigation. Another way to address the crosstalk between histone acylation and metabolism is via metabolic challenges such as fasting/ starvation experiments. For instance, H3K9bhb levels increased in livers of fasted mice and were linked with activated transcription of genes involved in the starvation response (Xie *et al*, 2016). This finding might indicate a fine tuning of transcriptional regulation via Kbhb during metabolic challenges. In addition, H3K14pr and H3K14bu were implicated in the expression of genes involved in lipid metabolism pathways in livers of fasted mice (Kebede *et al*, 2017). Interestingly, metabolic enzymes that are involved in the synthesis of CoAs have also been found to be present at the same genomic regions as the corresponding histone acylations. For example, H3K79succ enrichment profiles correlate with the presence of α-KGDH, suggesting a functional connection between α-KGDH, K3K79succ, and transcriptional activity. This is supported by the finding that α-KGDH interacts with the histone acyltransferase KAT2A that can succinylate histones, and that depletion of KAT2A results in a strong reduction of H3K79succ levels (Wang *et al*, 2017). In contrast to these examples of positive correlations between lysine acylations and gene expression, a noteworthy finding was recently made by

Box 2: ChIP-grade acylation type and site-specific antibodies: a challenge for the field

ChIP-seq depends strongly on the quality of the acylation type and site-specific antibodies. Thus, novel acylation-specific antibodies have to be validated thoroughly for their selectivity. A first step for this validation can be dot blot assays, in which the antibody is tested on immunoblot assays with serial diluted peptides carrying a comprehensive panel of different acylations. However, the many different types and sites of acylations on histones require the screening of a very high number of peptides and these assays only test for recognition of a peptide spotted on a membrane. In addition, a peptide competition assay with different "free" acylated peptides can be used to challenge target recognition by the antibody in immunoblots or immunoprecipitation. Histones or chromatin from cells with knockout of specific histone acyltransferases would be ideal to demonstrate antibody specificity. However, these enzymes are known to deposit various acylations on different sites (as described in the main text) and this is why mutant histones (e.g., expressed in cells) or site-specifically modified histones are often used instead as controls for selective binding of the antibody toward a specific site (e.g., in immunoblot, but also in immunoprecipitation).

Even after thorough validation experiments, it is still difficult to completely exclude potential cross-reactivities based on *in vitro* assays, for example, toward Kac. Kac is often highly abundant whereas most non-acetyl acyl marks have a much lower abundance, and thus, even a slight cross-reactivity observed *in vitro* tests might result in the recognition of the much more abundant acetylation *in vivo* (Simithy *et al*, 2017). Ultimately, isotopic labeling combined with MS of, e.g., the immunoprecipitated chromatin is a highly sensitive approach, which overcomes many of the limitations and allows testing for epitopes recognized by an antibody under ChIP conditions. An alternative approach is the use of different designer chromatin for specificity checks. Promising alternatives to classical antibodies are DNA or RNA aptamers that can be engineered *in vitro* to gain enhanced binding affinities. To date, a DNA aptamer designed against H4K16ac was shown to be significantly more specific toward the modification than a commercial control antibody (Williams *et al*, 2009).

Gowans *et al,* (2019). They showed that H3K9cr was linked to a reduced expression of growth genes during low-nutrient periods in *Saccharomyces cerevisiae*. The authors speculate that this unexpected H3K9cr-mediated repression might derive from disruption or inhibition of transcriptional initiation via Taf14 recruitment (Gowans *et al*, 2019). An explanation for these results could be that the transcriptional regulation via histone acylations is defined by the relative abundance of non-acetyl acylations (in this case crotonylation) compared with acetylation. For instance, changes of the acylation/acetylation ratios in a given promoter region could determine the transcriptional activity through dynamic recruitment or repulsion of specific interactors. The observed transcriptional repression by Gowans and colleagues could therefore be caused by an acylation/acetylation ratio shifted toward Kcr and thus recruitment of Taf14 (Gowans *et al*, 2019). A similar model to explain the functional diversity of histone acylations has been proposed by Goudarzi *et al,* (2016). Their study revealed that although the Brdt protein, a testis-specific BET bromodomain factor, cannot bind H4K5bu *in vitro*, *in vivo* it was found at genomic sites where both H4K5ac (bound by Brdt) and H4K5bu co-exist (Goudarzi *et al*, 2016). The dynamic exchange of acylation marks and alterations of their ratios locally could allow for dynamic interactions of Brdt with chromatin. The importance of the acylation/ acetylation ratio is further supported by the fact that most of the described ChIP-seq datasets are pointing toward the genomic co-occurrence of various acylations with acetylation at active TSS. Overall, these observations suggest that acylation marks could act through their competing dynamic nature rather than through stable static action and should probably not be considered individually.

In the last years new insights into the genomic distribution of diverse acylations, their role in transcriptional regulation and potential links to metabolic pathways have been gained. The combination of correlating specific acylations with gene expression states, or monitoring their enrichments at specific genomic regions by ChIP-seq, and mechanistic *in vitro* assays using site-specific modified chromatin can provide a powerful toolset to increase our understanding of the mechanistic and physiological functions of histone acylations.

The concept of subnuclear metabolic niches/microdomains

As described, several recent studies suggested a link between cellular metabolism and histone acylations (Egervari *et al*, 2020). However, we are still in an early phase concerning this integration of metabolic signals into chromatin via histone acylations, due to technical limitations and conceptual challenges that will take some time to overcome. An interesting concept within the emerging field of metaboloepigenomics are the so-called (metabolic) subnuclear chromatin niches or microdomains (Katada *et al*, 2012). This model is based on the notion that transcription could be regulated by the local production and accumulation of metabolites that are then utilized as cofactors by chromatin modifiers. Site-specific recruitment of metabolic enzymes to genomic regions could facilitate the creation of such nuclear microdomains with, for example, elevated acyl-CoA levels contributing to the accurate control of gene expression (Boukouris *et al*, 2016). Indeed, metabolic enzymes such as ACLY, ACSS2, or α-KGDH were detected in the nucleus and could potentially be involved in the local generation of acyl-CoAs (Boukouris *et al*, 2016; Fang *et al*, 2021). It was further shown that such metabolic enzymes do not act in isolation, and that entire sections of the TCA cycle happen in the nucleus (preprint: Kafkia *et al*, 2020), producing locally available metabolites. How such local enrichment or pools of CoAs could be maintained is so far unknown.

Potential explanations for the generation of distinct nuclear acyl-CoA pools could be via the formation of nuclear condensates by liquid–liquid, polymer–polymer phase separation (LLPS/PPPS) or other mechanisms, which could lead to the accumulation of certain biomolecules (Erdel & Rippe, 2018; Sabari *et al*, 2020). LLPS describes the condensation of macromolecules into a concentrated liquid-like droplet that segregates from a less dense phase. Such a droplet could be induced by weak interactions between multivalent macromolecules that have multiple interaction domains, repeated structural domains, or intrinsically disordered regions (IDR). Phase separation could then support the dynamic creation of microdomains, in which specific proteins or molecules are concentrated and can act together (Sabari *et al*, 2020). Noteworthy, *in vitro* and *in vivo* evidence that LLPS is involved in the organization of the nucleus is accumulating (Larson *et al*, 2017; Strom *et al*, 2017). Histone acylations seem to be implicated in this process. Indeed, Gibson *et al,* (2019) showed an alteration of *in vitro* droplets formed by nucleosomal arrays upon their acetylation (Gibson *et al*, 2019). The addition of bromodomain-containing "reader" proteins to these acetylated nucleosomal arrays re-induced condensate formation. Intriguingly, these condensates were not able to fuse with droplets containing unmodified nucleosomal arrays. This finding might point toward a distinct regulation of chromatin regions marked by acylations, facilitated by LLPS (Gibson *et al*, 2019). However, potential effects of non-acetyl acylations on droplet formation still need to be investigated. In addition, the Kac "reader" BRD4 seems to form condensates regulating cell-identity genes in mouse embryonic stem cells, further suggesting a potential role of histone acylation in subnuclear LLPS (Sabari *et al*, 2018). Remarkably, a possible role of acetyl-CoA in LLPS processes was revealed recently by Houston *et al,* (2020). The depletion of acetyl-CoA *in cellulo* resulted in nucleolus remodeling and the activation of nucleolar stress responses (Houston *et al*, 2020). Thus, these changes induced by acetyl-CoA depletion might indicate a potential role of CoAs in LLPS (Mitrea *et al*, 2016; Zhu *et al*, 2019).

Based on the above, we hypothesize that droplets or condensates could function to increase local concentrations of metabolic enzymes that generate acyl-CoAs, the CoAs, and chromatin-modifying enzymes, thereby contributing to the establishment of distinct chromatin microdomains (Fig 3). This could explain how different acylation/ acetylation ratios are generated at distinct chromatin regions, despite being catalyzed by the same acyltransferases. For the validation of this hypothesis, such microdomains should be both observed in cells and subsequently disrupted and/or artificially created to test their impact on histone acylations and transcriptional regulation. A promising approach would be CRISPR-Cas9 facilitated loci-specific microdomain formation *in cellulo*. For example, Shin *et al,* (2018) used the CasDrop method that takes advantage of site-specific recruitment of dCas9 and the light-induced dimerization of target proteins to recruit proteins involved in LLPS to specific gene regions (Guntas *et al*, 2015; Shin *et al*, 2018). Similar approaches

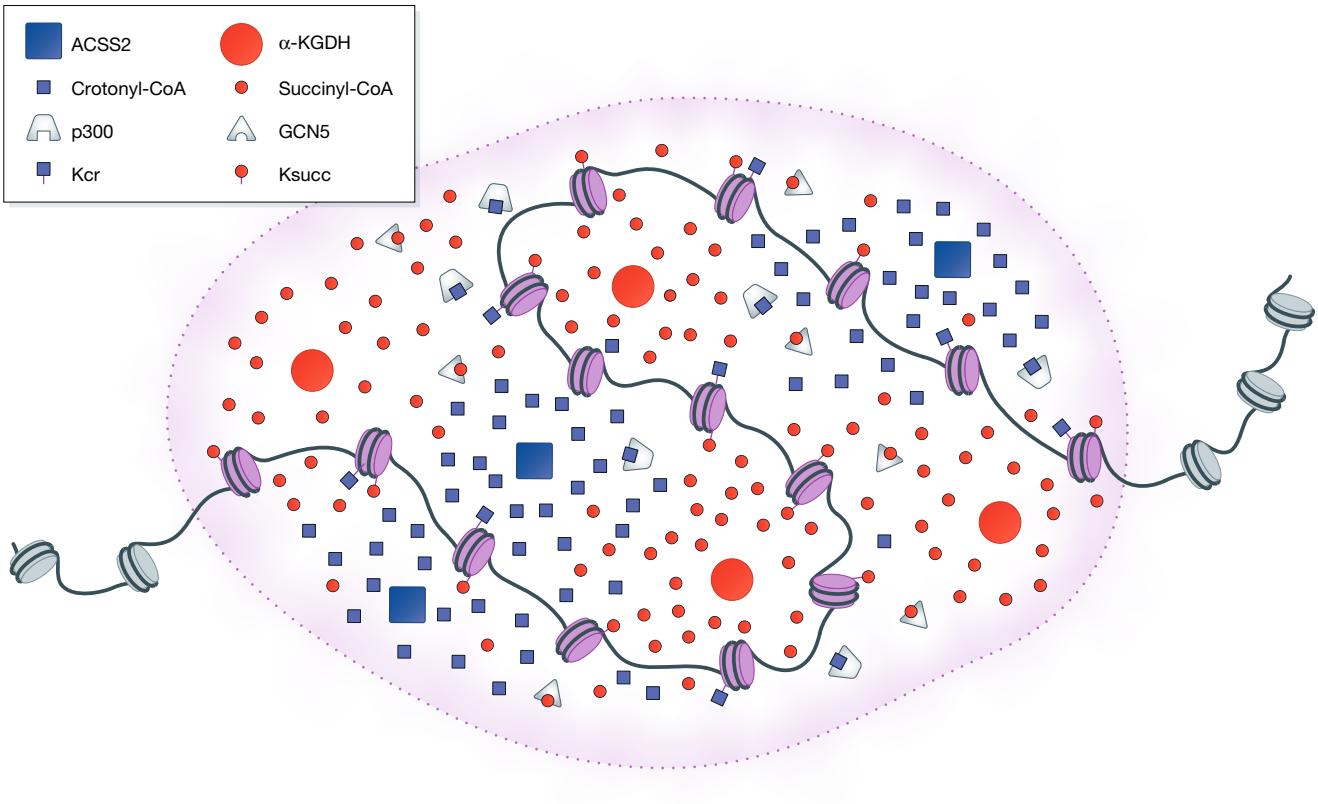

Figure 3. Metabolic subnuclear niches.

Droplet or aggregate formation within the nucleus could locally increase concentrations of metabolic enzymes, CoAs, and histone acyltransferases, thus promoting local histone acylations. The locus- or domain-specific microenvironment might further help to recruit (or retain) specific readers, transcription factors as well as the transcriptional machinery. Shown here is a putative metabolic niche (with symbols for nucleosomes, metabolic enzymes, and chromatin modifiers) in which crotonyl-CoA, succinyl-CoA, ACSS2, and α-KGDH are enriched. HATs such as GCN5 (KAT2A) or p300 (KAT3B) can use these CoAs to succinylate (Ksucc) and crotonylate (Kcr) histones (purple) within the niche. Unmodified exemplary nucleosomes outside the niche are shown in gray.

could be utilized to recruit metabolic enzymes and acyltransferases. MS imaging could be applied to visualize and quantify histone acylations and acyl-CoAs in such metabolic microdomains (Poté *et al*, 2013; Thomen *et al*, 2020). Additionally, locally increased cofactor concentrations could be visualized by novel RNA aptamers (Paige *et al*, 2012). In general, improvements in super-resolution microscopy and electron microscopy will help to observe droplet formation (Mitrea *et al*, 2018). Recent advances in these technologies will ultimately enable us to understand and reconstruct metabolic subnuclear chromatin niches and the role of different CoAs and acylations in their formation, maintenance, and function.

Conclusion

Histone acetylation was discovered nearly 60 years ago and since then its impact on chromatin dynamics and its biological role has been extensively studied. However, the complexity and combinatorial potential of histone modifications limit a full understanding of the histone acetylome. With the identification of additional short-chain lysine modifications, the so-called histone "code" has become even more enigmatic. So far evidence for distinct functions of histone acetylation and acylation is rather limited. This raises important questions: What is the specific functional significance of these histone acylations compared with acetylation, especially considering that they often colocalize? This is particularly important considering the low nuclear levels of many acyl-CoAs (compared to acetyl-CoA) and the fact that many of the known HATs are rather poor acyltransferases. How do histone acylations contribute to the epigenetic control of transcriptional processes? And last but not least: Do they reflect cellular metabolic fluctuations caused by specific diet, fasting, development, or even disease states and help to control cellular adaption? Many studies have started to address such questions and contributed to reveal a first glimpse into the functional differences between non-acetyl acylations on histones via biochemical *in vitro* studies but also *in vivo* work. Currently, technical limitations are the major challenge that prevent us from answering some of these central questions in more detail. However, progress in the presented methods will soon pave the way for a better understanding of histone acylations in particular and metaboloepigenomics in general. Studies on the interplay between epigenetic and metabolic players in metabolic subnuclear chromatin niches might unravel how CoAs can link the metabolic state to transcriptional response via epigenetic modifications.

In need of answers

i. Which additional lysine acylations do occur on histones? What are the writers, readers and erasers? Are there any acyltransferases that are not also acetyltransferases?
ii. How do non-enzymatic acylations contribute to chromatin function and do they functionally vary from enzymatic acylations?
iii. Do acylations have a distinct role during developmental processes or in various diseases and how do they compare with acetylation? What is their dynamic and their local abundance compared with acetylation?
iv. How does the acyl-CoA metabolism regulate gene expression programs and cell fate decisions via chromatin modifications?
v. How are metabolic niches in the nucleus formed and what are their functions? What is their role in metabolic diseases?

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Conflict of interest

The authors declare that they have no conflict of interest.

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