Dynamic Competing Histone H4 K5K8 Acetylation and Butyrylation Are Hallmarks of Highly Active Gene Promoters

Highlights

- Active gene TSSs are marked by competing H4 K5K8 acetylation and butyration
- Histone butyration directly stimulates transcription
- H4K5 butyration prevents binding of the testis specific gene expression-driver Brdt
- H4K5K8 butyration is associated with delayed histone removal in spermatogenic cells

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In Brief

Histone butyration stimulates gene transcription while competing with acetylation at H4K5 to control Brdt bromodomain binding. Differential chromatin labeling with interchangeable H4 acylations is an important epigenetic regulatory mechanism controlling gene expression and chromatin reorganization.
Dynamic Competing Histone H4 K5K8 Acetylation and Butyrylation Are Hallmarks of Highly Active Gene Promoters

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SUMMARY

Recently discovered histone lysine acylation marks increase the functional diversity of nucleosomes well beyond acetylation. Here, we focus on histone butyrylation in the context of sperm cell differentiation. Specifically, we investigate the butyrylation of histone H4 lysine 5 and 8 at gene promoters where acetylation guides the binding of Brdt, a bromodomain-containing protein, thereby mediating stage-specific gene expression programs and post-meiotic chromatin reorganization. Genome-wide mapping data show that highly active Brdt-bound gene promoters systematically harbor competing histone acetylation and butyrylation marks at H4 K5 and H4 K8. Despite acting as a direct stimulator of transcription, histone butyrylation competes with acetylation, especially at H4 K5, to prevent Brdt binding. Additionally, H4 K5K8 butyrylation also marks retarded histone removal during late spermatogenesis. Hence, alternating H4 acetylation and butyrylation, while sustaining direct gene activation and dynamic bromodomain binding, could impact the final male epigenome features.

INTRODUCTION

Besides lysine acetylation, we recently identified a variety of short-chain lysine acylations in core histones, including lysine propionylation, butyrylation, 2-hydroxyisobutyrylation, crotonylation, malonylation, succinylation, and glutarylation (Chen et al., 2007; Dai et al., 2014; Tan et al., 2011, 2014; Xie et al., 2012). Emerging data suggest that these new histone lysine acylations may have unique functions that depend not only on cell metabolism, but also on their ability to be deposited or removed by specific enzymes (Dai et al., 2014; Montellier et al., 2012; Rousseaux and Khochbin, 2015; Sabari et al., 2015; Sin et al., 2012; Tan et al., 2011). Nevertheless, the functional impact of differential histone acylation on chromatin recognition by specific factors has remained unexplored.

This study aims to understand the functional consequences of differential histone acetylation. In particular, we decided to investigate histone butyrylation, because, in contrast to the acetyl (2-carbon) and propionyl (3-carbon) groups, the butyryl (4-carbon) group restricts the binding of bromodomains (Flynn et al., 2015). More specifically, we focused our attention on histone H4 at K5 and K8, whose acetylation is required to bind the first bromodomain of Brdt, a testis-specific member of the BET protein family (Morinière et al., 2009). Our previous work showed that Brdt stimulates the transcription of certain spermatogenic-specific genes by recruiting the P-TEFb complex and by directly binding to their transcriptional start sites (TSSs). Additionally, during late spermatogenesis, Brdt’s first bromodomain is necessary for the replacement of histones by non-histone sperm-specific transition proteins (TPs) and protamines (Prms) (Gaucher et al., 2012). Given the critical role of H4K5 and H4K8 acetylation in Brdt-driven activities, we hypothesized that other mutually exclusive histone marks at these two residues might have key regulatory roles in sperm cell genome programming.

Here, we identify major histone lysine butyrylation sites in cells from different species, including mouse spermatogenic cells. Using spermatogenesis as an integrated biological model system, in addition to in vitro experiments and targeted proteomic approaches, we demonstrate new characteristics of active gene TSSs. Our data indicate that interchangeable acetylation
and butyrylation at H4K5 and H4K8 not only stimulates transcription, but could also underlie a highly dynamic interaction of histone post-translational modification (PTM)-binding factors such as Brdt. Additional data further show that stable differential use of acetylation and butyrylation could also durably affect genome organization in the maturing sperm. Altogether, these findings indicate how competition between histone acylation states could be an important epigenetic regulatory mechanism.

RESULTS

Histone Lysine Butyrylation Is an Evolutionarily Conserved PTM

To identify histone butyryllysine (Kbu) sites and study their function, we first confirmed the presence of histone Kbu by western blotting. Our data suggest that histone Kbu is an evolutionarily conserved PTM in eukaryotic cells (Figure 1A). We then used mass spectrometry to identify possible Kbu sites in core histones from three species (Chen et al., 2007; Kim et al., 2006). Kbu sites were detected in the N-terminal tails of H3 (K9, K14, K18, K23, K27, K36, K37, K79, and K122), H4 (K5, K8, K12, and K16), and H2B (K5 and K20) (Figure 1B; Data S1).

Figure 1. Histone Lysine Butyrylation Is an Evolutionarily Conserved PTM in Eukaryotic Cells
(A) The Kbu residues in core histones from the indicated species were detected by western blotting using a pan anti-Kbu antibody (upper). The corresponding Coomassie blue stained gel is shown (lower).
(B) Illustrations of histone Kac and Kbu sites in core histones identified by tandem mass spectrometry (MS/MS) (acetyl, Ac and butyryl, Bu). The annotated MS/MS spectra for histone Kbu peptides and the specific co-occurrence of K5bu and K8bu in H4 from spermatogenic cells are shown in Data S1.

and butyrylation at H4K5 and H4K8 not only stimulates transcription, but could also underlie a highly dynamic interaction of histone post-translational modification (PTM)-binding factors such as Brdt. Additional data further show that stable differential use of acetylation and butyrylation could also durably affect genome organization in the maturing sperm. Altogether, these findings indicate how competition between histone acylation states could be an important epigenetic regulatory mechanism.

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Functional Significance of Histone Butyrylation

To examine the function of Kbu in chromatin biology, we used mammalian spermatogenesis as a model system. Spermatogenic cells can be roughly classified into three major types: proliferative progenitor cells (spermatogonia), meiotic cells (spermatocytes), and post-meiotic haploid cells (spermatids). During differentiation, highly specific gene expression programs are activated in both meiotic and early post-meiotic cells. Large-scale genome reorganization also takes place in spermatids, where a genome-wide replacement of histones by TPs and Prms occurs in post-meiotic cells known as elongating and condensing spermatids (Gaucher et al., 2010; Goudarzi et al., 2014; Govin et al., 2004).
To confirm the existence of histone butyrylation in spermatogenic cells, we identified histone Kbu sites by mass spectrometry analysis of histones from mouse testis. We detected ten butyrylation sites including H4K5bu and H4K8bu (occurring separately or in combination), supporting the presence of these two histone marks in spermatogenic cells (Figure 1B; Data S1; see also Figure 7B). We then used highly specific anti-H4K5bu and anti-H4K8bu antibodies, along with the anti-H4K5ac and anti-H4K8ac antibodies, to investigate the stage-specific presence of these marks in spermatogenic cells. Immunohistochemistry analysis showed that H4K5 and K8 butyrylation is enhanced in elongating spermatids (Figure 2A), similar to earlier observations for histone H4K5 and H4K8 acetylation (Hazzouri et al., 2000).

To study the dynamic changes of butyrylation versus acetylation at both H4K5 and H4K8 sites in spermatogenic cells, we examined the co-existence of H4K5ac and H4K5bu as well as that of H4K8ac and H4K8bu. In elongating spermatids, H4K5ac and H4K8ac are widely distributed, but their localization becomes biased toward the sub-acrosomal regions in later stages. In contrast, H4K5bu- and H4K8bu-containing nucleosomes are homogenously distributed in the same cells (Figure 2B). In late elongating spermatids, while acetylated histones are removed homogenously distributed in the same cells (Figure 2B). In late spermatogenesis, when specific spermatogenic gene expression programs are activated (Dai et al., 2014; Gaucher et al., 2012; Tan et al., 2011). Toward this goal, mouse spermatogenic cells were fractionated into spermatocytes and post-meiotic round spermatids (Dai et al., 2014; Gaucher et al., 2012; Tan et al., 2011). The two pools of cells were subjected to chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) using four anti-PTM-specific antibodies (anti-H4K5ac, -H4K8ac, -H4K5bu, and -H4K8bu). Analysis of the genomic distribution of these marks revealed that regions surrounding gene TSSs (located upstream of TSSs and at the 5’UTR) exhibit the highest coverage by the four PTMs in both spermatocytes and spermatids (Figure 3A). Further analyses showed that the four H4 PTMs are enriched at TSSs in a manner dependent on the transcriptional activity of the corresponding genes (Figure 3B).

These results highlight a bimodal histone removal process, whereby the removal of H4 K5/K8 butyrylated nucleosome occurs after that of H4 K5/K8 acetylated nucleosomes.

**Genome-wide Distribution of H4K5 and H4K8 Acetylation and Butyrylation**

Our immunofluorescence analysis showed distinctive patterns of acetylation and butyrylation at H4K5 and H4K8, with both marks showing enhanced labeling in elongating spermatids, when cellular transcription dramatically decreases (Gaucher et al., 2010). This observation motivated us to study their genomic distributions and potential functions at earlier stages of spermatogenesis, when specific spermatogenic gene expression programs are activated (Dai et al., 2014; Gaucher et al., 2012; Tan et al., 2011). Toward this goal, mouse spermatogenic cells were fractionated into spermatocytes and post-meiotic round spermatids (Dai et al., 2014; Gaucher et al., 2012; Tan et al., 2011). The two pools of cells were subjected to chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) using four anti-PTM-specific antibodies (anti-H4K5ac, -H4K8ac, -H4K5bu, and -H4K8bu). Analysis of the genomic distribution of these marks revealed that regions surrounding gene TSSs (located upstream of TSSs and at the 5’UTR) exhibit the highest coverage by the four PTMs in both spermatocytes and spermatids (Figure 3A). Further analyses showed that the four H4 PTMs are enriched at TSSs in a manner dependent on the transcriptional activity of the corresponding genes (Figure 3B).

A critical concern in the above experiments is the possibility that histone sites subject to acetylation may be butyrylated at
only low background levels, which would be misleadingly overestimated by the ChIP-seq analysis due to vastly different affinities of the antibodies used. To address this issue, we used surface plasmon resonance (SPR) to measure the affinities of antibodies for their respective targets. These measurements showed that all four antibodies have similar ranges of affinity (Figure S1A). Additionally, a ChIP-qPCR approach demonstrated that the four histone marks are significantly detected at selected genomic regions (Figures S1B and S1C). Together, these experiments confirmed that H4K5K8 butyrylation occurs at levels that largely exceed background noise.

Following these control experiments, we investigated in more detail the relationship between gene expression and the co-occurrence of the four TSS-associated histone marks. Remarkably, the most active genes were found associated with all four marks at their TSS regions (Figures 3B and 4A). In contrast, genes lacking any one of these marks at their TSS showed significantly reduced expression (Figure 4A). Other genomic elements did not show such a direct relationship between the co-existence of the four histone marks and gene expression (Figure S2).

To further analyze the relationship between the four acylation histone marks and gene activity, we took advantage of the differences in gene expression programs between spermatocytes (meiotic cells) and the transcriptionally active haploid round spermatids (generated after meiosis). Using our ChIP-seq data from these two cell types, we divided genes into four categories according to the intensity of TSS labeling by the four H4 acylation marks, namely: (1) genes bearing none of the four marks (labeled “no”) and (2–4) genes bearing all four marks, either with (2) comparable (“=”), (3) higher (“>”), or (4) lower (“<”) peak intensities in spermatocytes compared to round spermatids (Figure 4B). We observed that the four groups of genes belong to different gene expression programs. Genes in category (1) are largely unexpressed, while those in category (2) exhibited no change in expression level between meiotic and post-meiotic cells. In striking contrast, genes in categories (3) and (4) exhibited differential expression in the two cell types, which was positively associated with the change in intensity of TSS labeling by the four histone marks (Figure 4B).

Additional support for a positive correlation between the presence of the four PTMs in the TSSs and gene expression is the observation that all four marks are depleted on the sex chromosomes compared to autosomes, consistent with the chromosome-wide meiotic transcriptional inactivation known to characterize sex chromosomes (Figure 4C). To further investigate this observation, we specifically considered the fraction of sex chromosome-linked genes that escapes meiotic sex chromosome inactivation (Namekawa et al., 2006). While all four PTMs were identified on a majority (73%) of TSSs associated with active autosomal genes, co-occurrence of the four PTMs was observed in only a minority (23%) of TSSs associated with sex-linked genes that are reactivated in post-meiotic cells. Hence, over three-quarters of this latter category of TSSs bear between zero and three of the four histone marks. Interestingly, most of the TSSs bearing 1–3 of the PTMs were depleted of H4K8ac, but not of H4K8bu (Figure 4C). This is consistent with other studies showing depletion of acetylation on the TSSs of sex-linked post-meiotic genes and their labeling with other acyl groups such as crotonyl (Sin et al., 2012; Tan et al., 2011).

**Histone Butyrylation Directly Stimulates Transcription**

Our ChIP-seq data showed that, like acetylation, histone butyrylation is associated with high levels of gene expression,
Figure 4. Tight Relationship between TSSs Labeled with H4K5ac, K5bu, K8ac, and K8bu Marks and Gene Transcriptional Activity

(A) The transcriptional activities of genes whose TSS regions bear the indicated histone H4 modifications are shown as box plots in spermatocytes (upper) and round spermatids (lower). The absence or presence of a histone mark is represented by the numbers “0” and “1”, respectively, and the four histone marks are arranged from bottom to top as follows: H4K5ac, H4K5bu, H4K8ac, and H4K8bu. The gene transcriptional activities as a function of various combinations of the four H4 PTMs on different genomic elements are shown in Figure S2.

(B) The heatmaps and profiles (upper) show the peak intensities of the ChIP signal at the TSS regions of genes associated with each of the four histone marks (“0” is input; “1” is K5ac; “2” is K5bu; “3” is K8ac; and “4” is K8bu), in spermatocytes (“Spc”), and in round spermatids (“RS”). The genes were assigned to four groups corresponding to: (1) genes with no ChIP peaks (“no”), (2) genes with moderate intensity of TSS labeling by the four marks and no significant change between spermatocytes and round spermatids (≤), (3) genes with variation in TSS labeling intensities by the four marks with high meiotic peak intensity and a decreased intensity in post-meiotic cells (>), and (4) genes with an increased peak intensity in post-meiotic cells compared to spermatocytes (<). The box plots (lower) show the expression of the corresponding genes in meiotic and in round spermatids. These data were obtained using transcriptomic data of control samples from our previous work (Montellier et al., 2013; GSE46136).

(C) Total peak counts (acetyl and butyryl H4s) per million base pair for each chromosome in spermatocytes (blue bars) and round spermatids (red bars) are shown. The expected random distributions of the ChIP-seq peaks are indicated as dashed lines (upper). The right panel shows the proportion of gene TSSs harboring 0, 1 to 3 PTMs, and 4 PTMs.

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suggesting the possibility that histone butyrylation directly stimulates gene expression. To test this hypothesis, we exploited a reconstituted activator-dependent in vitro transcription system. Our early studies had shown that in vitro, p300 and CREB binding protein (CBP) can catalyze lysine butyrylation by transferring the butyryl group from \([^{14}C]\) butyryl-CoA to core histone proteins (Chen et al., 2007). This activity was also observed in ex vivo transfection experiments (Chen et al., 2007). Moreover, in vitro butyrylation activity was confirmed both on a reconstituted chromatin template and on histone octamers by mass spectrometry analysis. Further analysis of this in vitro activity revealed that p300 efficiently butyrylates the sites of interest, H4K5 and H4K8, in histone octamers as well as in chromatin (Figures S5A and S5B; Data S1).

After confirming that p300 is a histone butyryltransferase, we used a p300- and p53-dependent, in vitro transcription system (Figure 5C) to test if histone butyrylation could stimulate transcription. We observed that p300-catalyzed histone butyrylation indeed directly stimulates transcription (Figure 5D). The mutation of lysine residues to arginine either on H3 or H4 tails eliminated acyl-CoA-stimulated transcription, indicating that acetyl/butyryl-CoA activates transcription through p300-catalyzed histone lysine acylation (Figure 5E). This experiment clearly demonstrates that, like acetylation, histone butyrylation can also directly stimulate transcriptional activity.

**Brdt Binds to Gene TSSs Harboring H4 K5/K8 Acetylation and Butyrylation**

Association of the four histone acylation marks with the TSSs of most of the highly active genes in spermatogenic cells raises the possibility that the high transcriptional activity of these genes is mediated by the binding of Brdt to the acylation marks on their TSSs. To test this hypothesis, we compared previously identified Brdt-bound TSSs (Gaucher et al., 2012) with TSSs labeled with the four histone marks. This analysis showed that most of the Brdt-bound gene TSSs also bore high levels of acetylation and butyrylation marks at H4K5 and H4K8 (Figures 6A and 6B). To study if Brdt’s first bromodomain (BD1) mediates this interaction, we used spermatogenic cells from mice expressing a mutated derivative Brdt to acetylated H4 and H4K5acK8bu peptides (Figure S3A, lower). The use of extracts from mice testes expressing the Brdt BD1 mutant confirmed that Brdt binding to all the tested peptides depends on the integrity of its first bromodomain (Figure S3A, lower). These data clearly imply that the butyrylation of H4K5 inhibits the binding of Brdt to histone H4.

To validate this result, we performed the same experiment with nuclear extracts from mouse testis. Our results confirmed that butyrylation at H4K5 abolishes the binding of Brdt to H4 tails (Figure 6C, upper). As further confirmation, we repeated the pull-down assay on protein extracts from wild-type mouse testis using either fully acetylated or fully butyrylated immobilized H4 tail peptides and analyzed the bound fractions by mass spectrometry. Brdt was easily identified among the proteins affinity-isolated by the H4ac-containing peptide, but not by the H4bu-containing peptide (Figure S3B; Data S1). The use of extracts from mice testes expressing the Brdt ΔBD1 mutant confirmed that the Brdt-H4 tail interactions described above are primarily mediated by the BD1 domain (Figure 6C, lower right). This result was further corroborated by an experiment with JQ1, a BET bromodomain inhibitor, which abolished the binding of testsis-derived Brdt to acetylated H4 and H4K5acK8bu peptides (Figure 6C, lower left). Taken together, these findings establish that H4K5bu, but not H4K8bu, abolishes the interaction between Brdt and the histone H4 tail.

**Structural Analysis of the Effect of Butyrylation on the Brdt BD1-H4 Tail Interaction**

To understand the molecular basis of the inhibitory effect of H4K5 butyrylation on Brdt binding, we carried out a structural modeling analysis. In the crystal structure of Brdt-BD1 bound to H4K5acK8ac, residue K5ac is intimately recognized by BD1, whereas K8ac makes fewer contacts (Morinière et al., 2009). Modeling shows that replacing the K8 acetyl group with a butyryl group allows the additional atoms to be accommodated without compromising any of the interactions between BD1 and the peptide (Figure 6D). In contrast, replacing the K5 acetyl group by a butyryl group results in a steric clash with residues in the domain’s ZA loop, implying that some structural adjustments to the model are required to accommodate the butyryl group. Comparison with the published co-crystal structure of Brd4-BD1

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1–3, or 4 of the studied H4 PTMs on autosomes and on the X chromosome in round spermatids (color coded). Of the X-linked genes that escape inactivation, the majority has either no H4K5K8 acetyl/butyryl marks or harbors one to three of these PTMs. A detailed consideration of these PTMs indicates that these TSSs are nearly always depleted in H4K8ac. The list of X-linked genes that are activated in post-meiotic cells was established based on our previous detailed post-meiotic transcriptomic analysis (Guoussouar et al., 2014).
bound to H3K14bu indicates the type of adjustments required to accommodate H4KSbu (Vollmuth and Geyer, 2010). Aligning the latter structure with that of the Brdt-BD1/H4KSacK8ac complex shows that H3K14bu occupies approximately the same position as K5ac (Figure 6E). However, the bulkier butyryl group results in the displacement of a water molecule within the ligand-binding...
Figure 6. Brdt Is Preferentially Recruited to Gene TSS Regions Enriched in H4K5ac, K5bu, K8ac, and K8bu Marks

(A) ChIP-seq data from chromatin immunoprecipitation of Brdt either from wild-type fractionated spermatogenic cells or the corresponding fractionated cells expressing a truncated Brdt lacking its first bromodomain (BrdtΔBD1) were obtained and compared with ChIP-seq data from the four indicated histone H4 marks from wild-type spermatogenic cells. Seqminer software illustrates gene TSSs bound by the wild-type Brdt (Brdt wt), BrdtΔBD1, and the occurrence of the four H4 marks on the same regions. The input corresponds to the sequencing of chromatin fragments before ChIP from wild-type cells. Exactly the same profile was obtained for the input chromatin fragments from BrdtΔBD1 cells (data not shown).

(B) Gene TSS regions were divided into two categories, either bound or unbound by Brdt, in spermatocytes (upper) or round spermatids (lower). For each category, the proportion of genes whose TSS regions are enriched with none, 1, 2, 3, or 4 of the four histone PTMs are represented with the indicated colors.

(C) Mouse total testis extracts were prepared from wild-type mice (upper and lower left) or mice expressing the truncation mutant BrdtΔBD1 (lower right) and incubated with the indicated peptides. Brdt was then visualized after pull-down using an anti-Brdt antibody (Gaucher et al., 2012). In some experiments, BET bromodomain inhibitor JQ1 was added to the extract prior to the peptide pull-down step, as indicated. In another experiment Brdt was identified by MS/MS (Figure S3B), and the annotated MS/MS spectra for Brdt peptides are presented in Data S1. The pull-down experiments were also performed on Brdt-expressing transfected cells and the data shown in Figure S3A.

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Dynamic Mixed Labeling of H4K5K8 by Acetylation and Butyrylation at Active Chromatin Sites

Our pull-down and structural modeling data strongly suggest that Brdt is inhibited from binding TSS regions where histone H4 is modified by lysine butyrylation at the K5 position. This conclusion appears contradictory to the ChIP-seq data, where H4K5bu and Brdt were both associated with the same TSS regions. A hypothesis that would reconcile these observations is that acetylation and butyrylation of H4K5 exhibit a rapid turnover. Indeed, acetyltransferases associated with highly active gene TSSs might feasibly drain cellular acetyl-CoA as well as butyryl-CoA toward these sites, leading to a mixture of histone H4 acetylation and butyrylation marks. To test this hypothesis, we performed in vitro assays by incubating reconstituted histone octamers with purified p300 and an equimolar mixture of acetyl-CoA and butyryl-CoA. Histones were then analyzed by mass spectrometry to detect both acetylated and/or butyrylated peptides. Our results show that p300 can use acetyl-CoAs to catalyze acetylation and butyrylation at both H4K5 and H4K8 sites (Figure 7A), as we detected H4 peptides bearing various combinations of acetylated or butyrylated H4K5 and H4K8 (Figure 7A; Data S1). Encouraged by this in vitro result, we then investigated whether histone H4 isolated from spermatogenic cells also contains diverse lysine acylation marks. Mass spectrometry analysis of these samples detected H4 peptides with various combinations of acetylation and butyrylation at H4K5 and H4K8.

In addition, depending on cell type, the stoichiometry of H4K5bu and H4K8bu could be higher than some of the widely studied histone marks such as H3K4me3 (Kulej et al., 2015) (Figures 7B and S4A; Table S2), but lower than those of H4 tail acetylation, such as that of K16, which can be as high as 20% of H4 species (Kulej et al., 2015).

Acetylation is known to have a high turnover rate at gene TSSs (Crump et al., 2011). Therefore, due to continuous acylation by histone acetyltransferase (HATs) such as p300 and the rapid turnover of these PTMs, it is feasible that H4K5 and K8 alternate between acetylated and butyrylated states. This model is supported by the detection of various combinations of H4K5K8 acetylation and butyrylation either in in vitro HAT assays (Figure 7A) or in vivo in spermatogenic cells (Figure 7B). A direct consequence of such alternating histone acetylation/butyrylation would be the dynamic binding of Brdt, which would oscillate between high- and low-affinity states depending on the acylation status of H4K5 (Figure 7C). Such dynamic histone H4 acylation could be facilitated by open nucleosomes on the corresponding gene TSSs by specific histone variants such as H2A.Lap1, a histone H2A variant capable of inducing unstable and open nucleosomes and known to associate with active gene TSSs in spermatogenic cells (Nekrasov et al., 2013; Soboleva et al., 2012). Indeed, analysis of ChIP-seq data for H2A.Lap1 revealed that the four H4 PTMs are particularly enriched on H2A.Lap1-associated TSSs (Figure S4B).

Taken together, the above results support a model whereby alternating competing histone acetylation and butyrylation underlie a dynamic interaction between the histone modifications and the cognate bromodomain.

DISCUSSION

The present study reports our findings regarding the interplay between histone acetylation and butyrylation on the histone H4 tail during sperm cell differentiation. We found that in vitro, p300 uses available acetyl-CoA and butyryl-CoA sources to acylate the H4 tail at all the acceptor lysines in an indiscriminate manner. A proteomic approach also revealed the co-existence of the same combinations of H4 K5K8 acetyl and butyryl marks in different spermatogenic cell types. ChIP-seq analysis on fractionated spermatogenic cells further demonstrated that H4K5/ K8 acetyl/butyryl are particularly enriched at the TSSs of the most active genes. Strikingly, however, functional and structural analysis revealed that the acylation state of H4K5 is a critical determinant of Brdt binding affinity, as Brdt binds the acetylated, but not the butyrylated state. In this context, a stable maintenance of differential acetylation and butyrylation could have important functional consequences for the genome reorganization observed during spermatogenesis. We found that, in contrast to earlier stages, in elongating spermatids, H4K5 and H4K8 acetylation and butyrylation become more markedly associated with specific regions of the genome. This could have a direct consequence on the action of Brdt in these cells.
Indeed, we previously showed that in elongating spermatids, when histone hyperacetylation and a general transcriptional shut-down occur, BD1 is indispensable for the replacement of acetylated histones by TPs (Gaucher et al., 2012). Additionally, the hyperacetylated histones are known to be directly targeted for degradation by a PA200-containing specialized proteasome (Qian et al., 2013), suggesting that the Brdt-bound histone population enters this pathway. Here, we show that in elongating spermatids, butyrylated histones survive this wave of acetylation-dependent histone removal and degradation. This survival is perfectly consistent with the inability of Brdt to recognize butyrylated H4. These observations suggest that a stable differential labeling of H4 by acetylation and butyrylation may control the timing of histone removal. In this context, it is also possible to speculate that nucleosomes bearing butyrylated H4 could undergo a direct histone-to-Prm replacement due to the inability of Brdt to mediate the exchange of histones by TPs. Indeed, TP1-TP2 double KO cells can undergo direct histone-to-Prm replacement but, in this case, Prms are unable to tightly compact the genome (for review, see Gaucher et al., 2010). The prediction is therefore that regions bearing butyrylated histones would evolve to a less compact structure in mature sperms than regions that are marked by acetylated histones before their removal. This regulatory mechanism could be an elegant way to introduce differences in genome compaction by Prms.

Differential histone tail acylation might also play important roles in the control of somatic cell gene expression, differentiation, and genome programming. Indeed, a recent study of p300-mediated histone H3K18 crotonylation revealed that an increase in cellular levels of crotonyl-CoA favors a more efficient de novo gene activation (Sabari et al., 2015). Although the precise mechanism underlying the role of H3K18cr in transcription is unknown, the differential affinity of a bromodomain protein toward crotonyl versus acetyl marks could conceivably account for the observed phenomenon.

Previous reports demonstrated a rapid turnover of histone acetylation on a sub-fraction of nucleosomes (Clayton et al., 2006; Waterborg, 2002), overlapping with active gene TSSs (Crump et al., 2011). However, the functional implications of the short half-life of histone acetylation on active chromatin regions, especially on highly transcribed gene TSSs, have remained elusive. The results described in our present study are consistent with a rapid turnover of both acetyl and butyryl marks on the H4 tail. Given the differential affinity of Brdt BD1 for the acetylated and butyrylated forms of H4K5, a rapid alternation of acylation states at H4K5 would result in a highly dynamic interaction between Brdt and chromatin. Thus, it is tempting to speculate that the generally observed rapid turnover of acetyl marks may be functionally significant because it enables rapid

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**Figure 7. p300-Mediated Acetylation and Butyrylation of H4K5K8 and Functional Implications for Gene Promoter Activity**

(A) In vitro reconstituted octamers were incubated with purified p300 in the presence of equal amounts of acetyl-CoA and butyryl-CoA, and the histones were subsequently analyzed by MS. The unique histone H4 peptides bearing multiple lysine modifications, which were specifically identified, are indicated along with their corresponding spectrum counts. The annotated MS/MS spectra for histone Kbu/ac peptides are presented in Data S1.

(B) Stoichiometry of H4K5bu and K8bu in sperm cells. The green letters indicate the in vitro chemical butyrylation, and the red letters indicate the endogenous modifications. The % represents the respective ratios of the peptide bearing H4K5bu and H4K8bu (summed peak areas) over the corresponding unmodified H4 (total germ cells: TGC; spermatocytes: Spc; round spermatids: RS; and elongating/condensing spermatids: E/CS). The values of peak area were used for calculations (Table S2; Figure S4A).

(C) Model of the functional interplay between acylation marks. We propose that at active gene TSSs, p300 (and possibly other HATs) randomly use acetyl- or butyryl-CoA to modify histone tails. HDACs and histone variants, also present at dynamic chromatin regions, especially on highly transcribed gene TSSs, have remained elusive. The results described in our present study are consistent with a rapid turnover of both acetyl and butyryl marks on the H4 tail. Given the differential affinity of Brdt BD1 for the acetylated and butyrylated forms of H4K5, a rapid alternation of acylation states at H4K5 would result in a highly dynamic interaction between Brdt and chromatin. Thus, it is tempting to speculate that the generally observed rapid turnover of acetyl marks may be functionally significant because it enables rapid
transitions between alternative states of lysine acylation. Such a mechanism, while maintaining histones permanently modified, would allow for a dynamic association with specific bromodomains, which might be important for sustaining successive cycles of transcription. Thus, either a change in the ratio of cellular acetyl-CoAs (for instance caused by metabolic disorders; Pougovkina et al., 2014) or differential activities of histone deacetylase (HDACs) in removing acetyl-groups (Rousseaux and Khoobbin, 2015) could reprogram gene expression profiles.

In conclusion, we provide here the first demonstration that the interchangeable use of two closely related histone acylation marks at a specific site, H4K5, has important functional consequences by modulating the ability of a transcriptional regulator, Brdt, to recognize chromatin. This finding should improve our understanding of gene transcriptional regulation and its link to cell metabolism.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents
Pan anti-Kbu antibody, anti-histone site-specific Kbu, and acetylysine antibodies were used for purification from PTM Biolabs and anti-histone 3H and anti-histone H4 antibodies were from Abcam. Mouse monoclonal antibodies against H4K5ac and H4K8ac were a generous gift from Dr. H. Kimura. Anti-Brdt is a homemade antibody previously described (Gaucher et al., 2012). Butyryl-CoA and acetyl-CoA were purchased from Sigma-Aldrich. The modified porcine trypsin was purchased from Promega. HPLC-grade acetonitrile, water, and ethanol were purchased from EMD Chemicals. Peptides bearing one or a few acetyl and Kbu residues are custom synthesized and were verified by HPLC and mass spectrometry. JQ1 was synthesized by Charles McKenna and Elena Ferri (University of Southern California) (Emadali et al., 2013) and used as described in this reference.

The antigen recognition capacities of the antibodies used in ChIP-seq and ChIP-qPCR were determined using SPR as described in the Supplemental Information.

Identification of Kbu Sites in Core Histones by Affinity Enrichment and Mass Spectrometry
Acetylation and butyrylation of histones were determined on 200 μg of core histones extracted from the different studied cell types. The detailed procedures are described in the Supplemental Information.

Quantification of H4K5K8 Butyrylation in Spermatogenic Cell Populations
Histone from spermatogenic cells underwent chemical butyrylation with deuterated (D5) butyryl anhydride and processed as described in the Supplemental Information.

Analysis of Mouse Spermatogenic Cells, ChIP, Bioinformatics, and In Vitro Transcription
All the experimental procedures, including immunostaining, cell fractionation, and ChIP-seq were carried out exactly as described previously (Dai et al., 2014; Gaucher et al, 2012; Tan et al., 2011). ChIP-qPCR was performed following our ChIP-seq protocol, but the recovered DNA was amplified using quantitative PCR and specific primers corresponding to regions indicated in Figure S1. Table S1 indicates the sequence of these primers and details of the ChIP experiments are described in Supplemental Information.

Brdt pull-down assays were performed as previously described (Huang et al., 2010; Pivot-Pajot et al., 2003). The in vitro transcription assay was also previously described (An et al., 2002; An and Roeder, 2004; Sabari et al., 2015; Tang et al., 2013). The bioinformatic analyses followed a pipeline similar to the one previously described (Dai et al., 2014; Gaucher et al., 2012; Montellier et al., 2013; Tan et al., 2011) and are detailed in the Supplemental Information.

p300 Expression and Purification for In Vitro Acetyl/Butyryl-Transferase Assays on Histone Octamer
In vitro acetyl/butyryl-transferase assays on histone octamer used recombinant p300 expressed and purified from baculovirus infected in Sf21 insect cells. Protein purification and the details of the HAT assays are described in the Supplemental Information.

ACCESSION NUMBERS
The accession numbers for the H4 K5 and K8 acetylation and butyrylation ChIP-seq data and Brdt ChIP-seq data reported in this paper are GEO: GSE77277 and GSE39910, respectively.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.03.014.

AUTHOR CONTRIBUTIONS
Y.Z. and S.K. designed and coordinated the whole project. A.G. performed most of the experiments on spermatogenic cells. S.B., J.G., and E.M. were involved in the ChIP setup and S.B. helped with the final experiments. A.-L.V. designed appropriate primers and performed qPCR after ChIP from total germ cells. J.G. and D.P. performed in vitro HAT assays. Z.T. and R.G.R. performed in vitro transcription assays. S.C. helped with pull-down assays. T.B. helped with spermatogenic cell fractionation. C.P. helped with structural analyses. A.D., G.C., and D.P. helped with the bioinformatic analyses under the supervision of S.R. D.P. D.Z. performed the IP/pull-down/MS/MS experiments, PTM quantifications, antibody characterization (SPR), and coordinated the MS analyses. H.H. and O.K. performed the HPLC-MS/MS experiments and data analysis. S.Q. constructed the ChIP-seq library for sequencing. T.H. and Z.C. were involved in antibody production and characterization.

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Supplemental Information

Dynamic Competing Histone H4 K5K8
Acetylation and Butyrylation Are Hallmarks of Highly Active Gene Promoters

Afsaneh Goudarzi, Di Zhang, He Huang, Sophie Barral, Oh Kwang Kwon, Shankang Qi, Zhanyun Tang, Thierry Buchou, Anne-Laure Vitte, Tieming He, Zhongyi Cheng, Emilie Montellier, Jonathan Gaucher, Sandrine Curtet, Alexandra Debernardi, Guillaume Charbonnier, Denis Puthier, Carlo Petosa, Daniel Panne, Sophie Rousseaux, Robert G. Roeder, Yingming Zhao, and Saadi Khochbin
Supplemental Information

Supplemental Figures
Figure S1 to Figure S4 and their legend

Supplemental Tables
Table S1 and Table S2 and their legend

Supplemental experimental procedures

Supplemental references
Figure S1. Characterization of antibodies used in ChIP-seq by SPR and ChIP-qPCR on several regions. (A) The indicated antibodies employed in ChIP experiments were used to measure their respective $k_a$, $k_d$ and $K_D$. The indicated values represent the average of three measurements. (B) Genomic regions within or around TSS bearing the four studied H4 PTMs in spermatocytes and round spermatids according to ChIP-seq, were chosen for ChIP-qPCR and are shown here on the IGB genome browser as dashed lines. (C) Total germ cells were isolated and the corresponding chromatin digested with MNase and the resulting nucleosomes were immunoprecipitated with the indicated antibodies (colour coded). The histograms show the results of four ChIP-qPCR obtained from two independent ChIPs experiments. Values are represented as a proportion of the qPCR amplification of 1/400 of the input. Standard deviations of four values (2 independent experiments in duplicates) are shown.

| Antibodies | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
|------------|---------------|--------------|-------------|
| H4K5ac     | 8.08E+05      | 3.31E-04     | 4.63E-10    |
| H4K8bu     | 1.51E+06      | 4.19E-04     | 3.01E-10    |
| H4K8ac     | 1.85E+06      | 3.30E-04     | 1.87E-10    |
| H4K8bu     | 5.57E+06      | 4.11E-03     | 7.41E-10    |
Figure S2. Relationship between genomic elements labelled with H4K5K8 acetylation / butyrylation and gene transcriptional activity

The transcriptional activities of genes whose annotated elements (5'UTR, coding exons, etc.) associated with nucleosomes bearing regions labelled with the indicated histone H4 modifications are shown as box plots in spermatocytes (upper panel) and round spermatids (lower panel). The absence or presence of a histone mark is represented by the numbers “0” and “1”, respectively, and the four histone marks are arranged from bottom to top as follows: H4K5ac, H4K5bu, H4K8ac, H4K8bu (as in Figure 4A). The transcription levels in spermatocytes and round spermatids were obtained using transcriptomic data of control samples from our previous work ((Montellier et al., 2013); GSE46136).
Figure S3. Butyrylation of H4K5 prevents Brdt binding to histone H4.

(A) Cos-7 cells were transfected by a GFP-Brdt construct with wild type bromodomains (deltaS construct described in (Moriniere et al., 2009)), and extracts were used to pull down Brdt with the indicated biotinylated peptides immobilized on streptavidin beads (upper panel). In another set of experiments, the same Brdt expression vector as above and expression vectors encoding Brdt with inactivating mutations in either the first (Brdt BD1mut) or the second (Brdt BD2mut) bromodomain were used and a pull-down was performed with the indicated peptides (lower panel). Brdt was visualized by Western blotting using an anti-GFP antibody. Input represents 5% of the extract used for each of the pull-down experiments.

(B) Wild-type mouse whole testis extracts were incubated with the indicated peptides as above but the presence of Brdt was detected by mass spectrometry. Annotated MS/MS spectra corresponding to Brdt pull-down from mouse testis extracts shown in Data File S1.
Figure S4. (A) Example of the quantification of H4K5bu and H4K8bu containing peptides in total germ cell (related to Figure 7B). Ion histograms of Kbu only H4 peptides with the indicated modifications as well as the corresponding peak area values are shown. These values were used to calculate the % of H4Kbu/unmodified H4 shown for both H4K5bu and H4K8bu. (B) TSSs with the highest level of labelling with H4K5K8 acetylation and butyrylation also show high levels of H2A.Lap1 incorporation. ChIP-seq data targeting H2A.Lap1 obtained from spermatogenic cells (Soboleva et al., 2012), were down-loaded and the presence of H2A.Lap1 in gene TSSs was compared with the presence of the four considered H4 PTMs at the same position (related to Figure 7C).
Table S1: Sequences of the primers used in ChIP-qPCR reactions (related to Figure S1)

| Regions      | Forward primer (-F)           | Reverse primer (-R)          |
|--------------|-------------------------------|------------------------------|
| osbpl8_TSS1  | CTGCCACGTCGCTAAAACC           | AGCCTAGCAGGCTTTTCTTGT        |
| osbpl8_TSS2  | CCTGTGAGGCCAACAGCTAA          | TACCCGCAAGTCTCTGAAAGT        |
| Ahi1_TSS1    | GCAGGGATGTTGACAGCTTA          | CAGTCTCATGCTGGGAAGT          |
| Ahi1_TSS2    | CTCATGATTTTTGTTTCTTTCGT      | CAATCTTCTCCCAGGTTGCTC        |
| chr12_nopeak2| GGTCACCTGAACCGATGCCAA         | CGTAGACACACACAGCCCTCT        |
| mapkapk5_TSS1| TGCCGAAAGAAGGCGGAAT           | AACTCCAGCCAACTCCTTG          |
| mybl1_TSS1   | AATAATGATAAGATGCTCTCGGCAA    | TCTGTCTTCTCTGCTGGTC          |
| mybl1_TSS2   | ACAAGGAAACTGCGCAGACC         | CACGCAAGCCTCGAGATAAC         |
| rad51ap2_TSS1| TCAGAGGAAAGCATCGATTTTCG      | AGCTGTTCATGCTTGCTCCCG        |
| rad51ap2_TSS2| GTTCTAGGGTCTCAAAGAAGGT       | GGCTATGTTGGGTGGAGCATGA       |
### Table S2: Quantification of histone butyrylation from total or fractionated spermatogenic cells (related to Figure 7B)

| Detected Peptides | MS Peak area | TGC | Spcyes | R | E/C |
|-------------------|--------------|-----|--------|---|-----|
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 602106212 | 83898395 | 79250430 | 133044324 |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 209311 | NA | NA | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 57705 | NA | 32936 | 203401 |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 90504 | NA | 39127 | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 61294 | NA | NA | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 135530 | 160064 | 229275 | 63231 |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | NA | NA | NA | 169186 |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 104498 | NA | NA | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 312924 | NA | 49103 | 12656 |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 6079 | NA | NA | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | 17478 | NA | 47198 | NA |
| \( \text{GK}_{\text{bu}}\text{GGK}_{\text{bu}}\text{GLGK}_{\text{bu}}\text{GGAK}_{\text{bu}}\text{R} \) | NA | 269556 | NA | NA |

**Legend:**
- **TGC:** Total germ cells; **Spcyes:** spermatocytes; **R:** round spermatids; **E/C:** elongating/condensing spermatids
- **Kbu:** chemically labelled D5-butyrylated lysine;
- **Kbu:** endogenous methyllysine that is D5-butyrylated;
- **Kbu:** endogenous butyrylated lysine;
- **Kac:** endogenous acetylated lysine;
- **Kme:** endogenous methylated lysine;
Legend to Data File S1

Data File S1 encompasses all the annotated MS/MS spectra

- Annotated MS/MS spectra of histone Kbu sites from different species related to Figure 1B (indicated in each spectrum).

- Annotated MS/MS spectra for histone Kbu sites from experiments shown in Figure 5A and Figure 5B (indicated in each spectrum).

- Annotated MS/MS spectra for Brdt protein after the testis-extract pull down shown in Figure S3B (indicated in each spectrum).

- Annotated MS/MS spectra for H4 with multiple acetylation/butyrylation shown in Figure 7A (indicated in each spectrum).
Extended Experimental Procedures

Kinetics of H4K5ac/bu and H4K8ac/bu antibodies binding to their corresponding antigen peptides.

The kinetic parameters for anti-H4K5ac, anti-H4K5bu, anti-H4K8ac, and anti-H4K8bu antibodies binding to their corresponding peptide antigens NH2-SGRRGKSLGKGLLGKGLGAKSGAKRRHRGGKbiotin-COOH or NH2-SGRRGKSLGKGLLGKGLGAKSGAKRRHRGGKbiotin-COOH were measured by Surface Plasmon Resonance (SPR) in a Bio-Rad ProteOn XPR36. The antibodies were injected at concentrations ranging from 1 to 25 nM at a constant flow rate of 301 l/min over immobilized acetylation or butyrylation containing peptides respectively. The association phase was monitored for 300sec and the dissociation phase was monitored for 3600sec to record the change in SPR signal. The data were fit to a “two-state” binding model. The residuals from the “two-state” binding model, indicated an excellent fit (not shown). The association rates (ka) and dissociation rates (kd) were measured and used to calculate the dissociation constant (Kd) of antibody-peptide complex formation as the ratio kd/ka.

Identification of Kbu sites in core histones by affinity enrichment and mass spectrometry

Two hundred micrograms of two core histones of interest were isolated and dried in a SpeedVac. The proteins were suspended in 50 mM NH4HCO3 (pH 8.0). The proteins were digested with trypsin at an enzyme-to-substrate ratio of 1:50 for 16 hrs at 37°C. For the mouse testis samples related to Figure 1B, chemical derivatization was performed before digestion. Briefly, the extracted histones were propionylated using propionic anhydride in 100 mM NH4HCO3 buffer (adjust to pH=8.0 after adding propionic anhydride) at 37 °C for 1h. The propionylation reaction was repeated, and then quenched by 100 mM ethonalamine at room temperature for 1h. No chemical derivatization was performed to the other samples. For quantifications of H4 butyrylation in mouse spermatogenic cells another protocol was followed, which is presented in an independent section (below). The tryptic digest was incubated with 5 µg of anti-Kbu antibody in CLN buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, pH 8.0) at 4°C for 6 hrs with gentle shaking. Protein A-agarose was then added to immunoprecipitate the antibody-antigen complexes. The bound peptides were washed twice with the CLN buffer, and then eluted three times with 50 µl of 1% TFA in water. The eluates were combined and dried in a SpeedVac. The resulting peptides were cleaned in C18 Zip Tips (Millipore Co., Temecula, CA) according to the manufacturer’s instruction, prior to nano-HPLC/MS analysis.

The HPLC/MS/MS analysis was carried out by nano-HPLC/LTQ mass spectrometry as described previously (Chen et al., 2007). Briefly, the enriched peptides bearing Kbu was dissolved in 10 µl of HPLC buffer A (0.1% (v/v) formic acid in water), and 2 µl were injected into an Agilent nano-HPLC system (Agilent, Palo Alto, CA). Peptides were separated on a custom-made capillary HPLC column (50-mm length x 75-µm inner diameter) containing Jupiter C12 resin (4-µm particle size, 90-Å pore diameter, Phenomenex, St. Torrance, CA) and electrosprayed directly into the mass spectrometer using a nanospray source. The LTQ mass spectrometer was operated in a data-dependent mode, cycling between acquiring one MS spectrum and MS/MS spectra of the 10 strongest ions in that MS spectrum.

MS/MS of synthetic peptides was carried out using the same LTQ settings as those used for the analysis of in vivo peptides.

Quantification of H4K5K8 butyrylation in spermatogenic cell populations

To quantify the histone H4K5K8 butyrylation, an in vitro chemical labelling method was used followed by targeted MS detection. Briefly, the acid extracted histones from total or fractionated mouse germ cells were chemically butyrylated with deuterated (D5) butyryl anhydride (catalog number D-7421, C/D/N Isotopes Inc., Quebec, Canada), using a procedure previously reported by Becker laboratory (Feller et al., 2015). Under these conditions, all unmodified and mono-methylated lysine residues become butyrylated and can be distinguished from endogenous butyrylation in mass spectrometric analysis because of the mass difference between endogenous butyryl group and D5-labeled butyryl group. Since the chemical labelling disables the tryptic digestion at lysine residues, we only focussed on the H4K5K8-containing peptide sequence window ‘GKGGGKGLGGKGGAKR’.

To calculate the stoichiometry of H4K5bu and H4K8bu sites, a method developed by Garcia laboratory (Lin and Garcia, 2012) was used. Briefly, the peak areas of unmodified and all differentially modified forms of a histone peptide of interest are summed and each individual form of a histone peptide i. e., H4K5bu, is expressed as a fraction of the summed of unmodified peak areas. Considering the high structural similarity between lysine butyrylation and acetylation, diversely acetylated peptides were assumed to have similar ionization efficiencies.
Male germ cells were fractionated into spermatocytes and round spermatids enriched cell suspensions from OF1
male wild type mice (ChIP-seq) or 129svj/C56BL6 mixed background mice (ChIP-qPCR), and these male germ cell
fractions were then used to prepare mononucleosomes as previously described (Dai et al., 2014). Each ChIP
experiment was performed on 100 µg of mononucleosomes and the DNA corresponding to the
immunoprecipitated nucleosomes, as well as before immunoprecipitation (input) were purified (as previously
described in Dai et al., 2014).
For ChIP-qPCR, immunoprecipitated DNA fragments were amplified along with serial dilutions of input DNA
using SYBR Green mix: “Brilliant III Ultra fast SYBR Green QPCR master Mix” (AGILENT) on a : CFX96
Real-Time System, C1000 Touch Thermal Cycler (BIO RAD). The genome positions of the primers used are
indicated in Figure S1 and their sequences are reported in Table S1.
For the ChIP-seq, libraries were prepared following the Ovation Ultralow DR Multiplex System protocols
(NuGEN Technologies INC, San Carlos, CA), with a size selection (200-700bp) step using SPR1select beads
(Beckman Coulter, Brea, CA). Single end sequencing was performed using Illumina HiSeq 2500. Base calls
were performed using CASAVA version 1.8.2 (by sequencing facility). Total read counts of ChIP-seq
experiments ranged from 24.2 to 38.7 Million reads.

Bioinformatic analyses
ChIP-seq reads were aligned to the mm9 genome assembly using bowtie version 2.2.5 with default arguments.
Read counts were computed using Deeptools bamCoverage version 1.5.11 (binSize=50; fragmentLength=200;
missingDataAsZero=yes). Seqminer (http://sourceforge.net/projects/seqminer/; (Ye et al., 2011)) was used to
visualize aligned raw reads as heatmaps on TSS regions. Peaks were called using SICER (home.gwu.edu/~wpeng/Software.htm; (Zang et al., 2009)) with the following parameters: W200, G600, FDR 0.01. The IGB genome browser (http://bioviz.org/igb/index.html; (Nicol et al., 2009)) was used for visualizing
peaks on particular gene regions. Raw data (FastQ files) and processed data (.bed and .wig outputs of Sicer peak
caller) are available on GEO (http://www.ncbi.nlm.nih.gov/geo/, dataset no GSE77277).

p300 expression and purification for in vitro acetyl- or butyryl-transferase assays on histone octamer
Flag tagged human p300 (324-2094) was cloned into pFASTBAC1 vector (Invitrogen). We used EMBAC-Y
baculovirus for the expression of flag p300. The recombinant baculoviruses were propagated in Sf21 insect cells
with SF-900 III SFM medium (Invitrogen). For the production of p300, Hi5 insect cells in Express Five SFM
medium were infected with recombinant baculovirus at a multiplicity of infection of 2, maintained in shake
flasks at 27 °C and harvested by centrifugation 72 h post infection. Recombinant baculovirus-infected cell pellet
was resuspended in TNZ buffer (20 mM Tris pH 7.5, 300 mM NaCl, 5µM ZnCl2, Complete Protease Inhibitors
EDTA-free (Roche Applied Science)) and lysed by sonication. The lysate was then clarified by centrifugation at
100,000 g for 1h. The clarified insect cell lysate was incubated with pre-equilibrated Anti-FLAG M2-agarose
affinity gel (Sigma) for 1 h and then applied to a 25-m1 Econo column (Bio-Rad). The resin was washed with
200 ml of TNZ buffer, and then the bound protein was eluted using 0.1 mg/ml triple FLAG peptide (sequence,
MDYKHDADYKDHIDYKDDDDK) dissolved in TNZ buffer. The eluate was concentrated using an
Amicon-ultra 30 unit (Amicon, Inc.) and further purified on a Hi-Load 16/60 Superdex 200 gel filtration column
(GE Healthcare) equilibrated in HNZ buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 5µM ZnCl2, 0.5mM DTT).
The final protein was concentrated to 5 mg/ml and aliquots were flash-frozen in liquid nitrogen and stored at -80 °C.
Untagged drosophila histones H2A, H2B, H3, H4 were cloned into pET21 vector (Novagen). We used IPTG-
induced BL21 bacterial cells for expression of histones. Recombinant histones were purified and refolded
according to standard procedures.

Standard reactions, with purified proteins, were performed in reaction buffer (25 mM Tris-HCl pH 7.5, 100 mM
NaCl, 0.1 mM EDTA, 1 mM DTT, 10 % Glycerol, 1x Complete EDTA-free protease inhibitor (Roche)) with
100 ng/mL TSA, 2 µg of purified octamer and 50 µM Acetyl-CoA or 50 µM Butyryl-CoA or 25 µM Acetyl-
CoA and 25 µM Butyryl-CoA (Sigma, A2181 and B1508). Reactions were incubated in presence or not of
purified p300 during 1 h at 30 °C. Reactions were stopped by addition of Laemmli buffer and samples were used
for immunoblotting or mass spectrometry.
Supplemental references

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