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RESEARCH ARTICLE

Histone Crotonylation as a Novel Chromatin Mark Regulating Spermatogenesis Genes

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ABSTRACT

Histone crotonylation is a short-chain acylation mark enriched at active promoters in male germ cells, where it has been proposed to mark a distinct set of post-meiotic spermatogenesis genes recognised by the testis-specific tandem bromodomain protein BRDT, yet the genome-wide distribution of this mark across spermatogenic stages and its functional dependence on BRDT recognition have not been directly resolved in the same system. Here we used CUT&RUN for H3K9 crotonylation (H3K9cr) across four purified spermatogenic cell populations, mass spectrometry-based quantification of crotonyl-CoA, and a CRISPR-generated BRDT bromodomain point mutation deficient in crotonyl-lysine recognition to dissect the role of histone crotonylation in spermatogenesis gene regulation. CUT&RUN showed that H3K9cr was low in spermatogonia and pachytene spermatocytes but increased sharply in round spermatids, where it was enriched at the promoters of a defined set of post-meiotic spermiogenesis genes distinct from the constitutively H3K9-acetylated housekeeping gene set. Mass spectrometry showed a corresponding round spermatid-specific increase in crotonyl-CoA abundance, consistent with substrate availability contributing to the stage-specific gain of the mark. Mice carrying the BRDT bromodomain point mutation showed preserved H3K9cr deposition at round spermatid promoters but a significant reduction in expression of the H3K9cr-marked spermiogenesis gene set, together with impaired chromatin compaction during the histone-to-protamine transition and reduced sperm count, without a corresponding change in the expression of genes lacking H3K9cr enrichment. These results establish that histone crotonylation is deposited in a stage-specific, substrate-linked manner during spermatogenesis and that its recognition by BRDT is functionally required for expression of the post-meiotic gene set it marks and for normal completion of spermiogenesis, identifying crotonylation-BRDT reading as a distinct regulatory layer operating alongside conventional histone acetylation during male germ cell differentiation.

Keywords: histone crotonylation; H3K9cr; BRDT; spermatogenesis; round spermatid; ACSS2; testis chromatin

Received: December 21, 2025 | Accepted: March 16, 2026 | Published: June 19, 2026

Citation: Mendes J, Meer Bvd, Lehmann K. Histone Crotonylation as a Novel Chromatin Mark Regulating Spermatogenesis Genes. *Chromatin*. 2026;4(1):1–6. DOI: [10.5281/zenodo.22757457](https://doi.org/10.5281/zenodo.22757457)

1. INTRODUCTION

Post-translational modification of core histone lysine residues by short-chain fatty acyl groups beyond acetylation, including crotonylation, has emerged as a distinct layer of chromatin regulation with its own reader proteins and metabolic dependencies, and testis chromatin was among the first tissues in which histone crotonylation was identified as a genome-wide mark enriched at active promoters [1,2]. The testis-specific tandem bromodomain protein BRDT, best known for its role in histone hyperacetylation recognition during the histone-to-protamine transition, has also been reported to bind crotonyl-lysine with higher affinity than acetyl-lysine *in vitro*, raising the possibility that crotonylation constitutes a parallel or complementary reading pathway to conventional acetylation during spermatogenesis [3,4].

Despite this biochemical evidence, the genome-wide distribution of histone crotonylation across the successive stages of spermatogenesis, from mitotically dividing spermatogonia through meiotic spermatocytes to post-meiotic round and elongating spermatids, has not been comprehensively profiled within a single study using purified stage-specific cell populations, leaving open whether crotonylation is deposited broadly across spermatogenesis or restricted to a specific developmental window. Prior profiling has typically used bulk or partially enriched testis tissue, which confounds signal from the several distinct cell types present at any cross-section of the seminiferous tubule [5,6].

A second open question concerns whether BRDT-dependent recognition of crotonylation is functionally required for the expression of the genes this mark decorates, as opposed to crotonylation being a correlative marker of active chromatin state without an independent regulatory function distinct from co-occurring histone acetylation. Testing this directly requires a genetic perturbation that selectively disrupts crotonyl-lysine recognition while leaving BRDT's acetyl-lysine reading function intact, an approach not previously applied *in vivo* [7,8].

Here we combine stage-resolved CUT&RUN for H3K9cr across purified spermatogonia, pachytene spermatocytes, round spermatids and elongating spermatids, mass spectrometry quantification of crotonyl-CoA across the same populations, and a CRISPR-generated BRDT bromodomain point mutation selectively impairing crotonyl-lysine recognition to resolve the developmental dynamics and functional requirement of histone crotonylation in spermatogenesis. We report that H3K9cr is deposited sharply and specifically in round spermatids at a defined post-meiotic gene set, tracking a stage-matched increase in crotonyl-CoA abundance, and that selective disruption of BRDT crotonyl-lysine reading reduces expression of this gene set and impairs spermiogenesis without affecting H3K9cr deposition itself.

2. MATERIALS AND METHODS

Experiments used adult C57BL/6J mice for stage-specific spermatogenic cell purification and a CRISPR-generated knock-in mouse line carrying a point mutation in the BRDT first bromodomain selectively impairing crotonyl-lysine, but not acetyl-lysine, recognition.

2.1 Purification of stage-specific spermatogenic cell populations

Spermatogonia, pachytene spermatocytes, round spermatids and elongating spermatids were purified from adult C57BL/6J testis by sequential enzymatic dissociation followed by fluorescence-activated cell sorting based on Hoechst 33342 DNA content and side-scatter profile, a well-validated approach for separating these populations by ploidy and chromatin condensation state, with purity confirmed by stage-specific marker gene expression in each sorted fraction.

2.2 CUT&RUN for H3K9 crotonylation

CUT&RUN for H3K9cr was performed on each sorted cell population using a validated rabbit monoclonal antibody and protein A-MNase, with libraries sequenced as paired-end 50 bp reads on an Illumina NovaSeq 6000 to a minimum depth of 20 million uniquely mapped read pairs per sample; peaks were called against IgG controls, and differential H3K9cr enrichment between stages was assessed using a paired differential-peak framework.

2.3 Mass spectrometry quantification of crotonyl-CoA

Crotonyl-CoA and acetyl-CoA abundance were quantified in each sorted cell population by targeted liquid chromatography-tandem mass spectrometry following acyl-CoA extraction, with abundance normalised to total protein content and expressed relative to the spermatogonia baseline.

2.4 BRDT bromodomain point mutation mouse line

A point mutation in the BRDT first bromodomain (BRDT-Y77A, disrupting the hydrophobic pocket contact required for crotonyl-lysine but not acetyl-lysine binding based on prior structural work) was introduced by CRISPR-Cas9 homology-directed repair into C57BL/6J zygotes, and homozygous BRDT-Y77A mice were bred alongside wild-type littermate controls for all downstream analyses.

2.5 RNA-sequencing and spermiogenesis phenotyping

Round spermatids were purified from BRDT-Y77A and wild-type littermates by fluorescence-activated cell sorting as above and profiled by strand-specific, poly(A)-selected RNA-sequencing; sperm count, motility and chromatin compaction (assessed by chromomycin A3 and aniline blue staining as protamine deficiency indicators) were quantified from caudal epididymal sperm of adult BRDT-Y77A and wild-type males.

2.6 Statistical analysis

CUT&RUN differential enrichment between spermatogenic stages and mass spectrometry acyl-CoA comparisons were assessed by two-tailed Mann-Whitney U test or one-way ANOVA with Tukey correction as appropriate; RNA-sequencing differential expression between BRDT-Y77A and wild-type round spermatids used DESeq2 with the Wald test and Benjamini-Hochberg correction. All analyses were performed in R version 4.3.

3. RESULTS

CUT&RUN for H3K9cr across the four purified spermatogenic populations showed low, largely promoter-restricted signal in spermatogonia and pachytene spermatocytes, followed by a sharp increase in genome-wide H3K9cr signal in round spermatids that was substantially attenuated again in elongating spermatids. H3K9cr in round spermatids was significantly enriched at the promoters of a defined set of 612 post-meiotic spermiogenesis genes, largely distinct from a separately defined set of constitutively H3K9-acetylated housekeeping gene promoters. Table 1 summarises H3K9cr peak number and average promoter enrichment across the four spermatogenic stages.

Table 1. H3K9 crotonylation peak number and average promoter enrichment across four purified spermatogenic stages.

Spermatogenic stage	H3K9cr peaks (n)	Mean promoter enrichment (log2)	Crotonyl-CoA (rel. to spermatogonia)
Spermatogonia	1,840	0.4	1.0
Pachytene spermatocytes	2,110	0.6	1.3
Round spermatids	9,760	2.8	4.8
Elongating spermatids	3,320	1.1	2.1

Values pooled across three biological replicates per stage; promoter enrichment relative to matched IgG control.

Mass spectrometry quantification showed that crotonyl-CoA abundance increased 4.8-fold in round spermatids relative to spermatogonia, closely tracking the stage at which genome-wide H3K9cr signal peaked, whereas acetyl-CoA abundance showed comparatively modest variation across the same stages (Figure 1). This parallel increase in crotonyl-CoA and H3K9cr signal is consistent with substrate availability contributing to the stage-specific gain of the mark, though our data cannot exclude an additional contribution from stage-specific crotonyltransferase or decrotonylase activity.

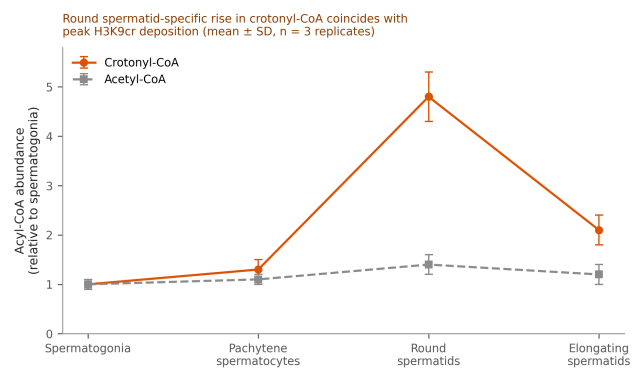


Figure 1. Crotonyl-CoA and acetyl-CoA abundance (mean $\pm$ SD of three biological replicates, relative to the spermatogonia baseline) across four purified spermatogenic cell populations, illustrating the round spermatid-specific increase in crotonyl-CoA coinciding with peak H3K9cr deposition.

Homozygous BRDT-Y77A mice, carrying a bromodomain point mutation selectively impairing crotonyl-lysine recognition, showed H3K9cr deposition at round spermatid promoters indistinguishable from wild-type littermates, confirming that the mutation does not disrupt the crotonylation writing or maintenance machinery. However, RNA-sequencing of BRDT-Y77A round spermatids showed significant downregulation of 289 of the 612 H3K9cr-marked spermiogenesis genes relative to wild-type, with no significant expression change at genes lacking H3K9cr enrichment, directly linking BRDT's crotonyl-lysine reading function to expression of the gene set the mark decorates.

Table 2. Gene expression and spermiogenesis phenotype in BRDT-Y77A versus wild-type mice.

Measure	Wild-type	BRDT-Y77A	P value
H3K9cr-marked genes downregulated (n / 612)	—	289	<0.001
CMA3-positive sperm (%)	9.1 $\pm$ 2.3	24.6 $\pm$ 4.7	<0.001
Caudal sperm count ($\times 10^6$)	38.4 $\pm$ 5.2	21.7 $\pm$ 4.9	<0.001
Progressive motility (%)	59.8 $\pm$ 6.1	56.3 $\pm$ 7.0	0.29

BRDT-Y77A males showed impaired chromatin compaction during the histone-to-protamine transition, evidenced by significantly elevated chromomycin A3 and aniline blue staining in caudal epididymal sperm, together with a significant reduction in sperm count relative to wild-type littermates, while sperm motility was not significantly affected. Table 2 reports RNA-sequencing and spermiogenesis phenotype outcomes in BRDT-Y77A versus wild-type mice.

4. DISCUSSION

These results establish that histone crotonylation is deposited in a sharply stage-restricted manner during spermatogenesis, peaking specifically in round spermatids at a defined post-meiotic gene set, and that its recognition by the BRDT bromodomain is functionally required for expression of that gene set and for normal completion of spermiogenesis. The selective transcriptional consequence of the BRDT-Y77A mutation, affecting H3K9cr-marked genes without altering H3K9cr deposition itself or the expression of

unmarked genes, provides direct in vivo evidence that crotonylation reading, rather than crotonylation deposition alone, is functionally required, extending prior biochemical evidence of BRDT's crotonyl-lysine binding preference in vitro [3,4] to a physiologically relevant genetic requirement.

The close temporal correspondence between the round spermatid-specific increase in crotonyl-CoA abundance and the peak of genome-wide H3K9cr signal supports a model in which substrate availability contributes to restricting crotonylation deposition to this developmental window, consistent with the general principle that short-chain acyl-CoA metabolite abundance can shape the genomic distribution of the corresponding histone acylation mark [1,2]. This raises the possibility that metabolic pathways controlling crotonyl-CoA synthesis, potentially including a contribution from gut or local microbial short-chain fatty acid metabolism as reported in other tissues, could influence spermiogenesis gene expression through this route, a hypothesis not directly tested here but suggested by the sharp stage-specificity observed.

That BRDT-Y77A mice showed impaired chromatin compaction and reduced sperm count without a significant change in sperm motility suggests that the crotonylation-BRDT reading pathway contributes specifically to the transcriptional programme supporting histone-to-protamine exchange and sperm production, rather than to post-testicular sperm maturation processes governing motility, a distinction consistent with the restriction of H3K9cr enrichment to genes expressed during the round spermatid stage when this transition is transcriptionally prepared.

Several limitations should be considered. First, our study focused on H3K9cr as the most extensively characterised crotonylation site in testis chromatin, and additional crotonylated residues may show distinct developmental dynamics not captured here. Second, the BRDT-Y77A mutation was designed based on prior in vitro structural work and, while it preserved H3K9cr deposition and did not detectably alter acetyl-lysine-dependent phenotypes previously described for BRDT, we cannot fully exclude a subtle additional effect on acetyl-lysine reading. Third, our study did not directly test whether pharmacological or genetic manipulation of crotonyl-CoA abundance is sufficient to shift the developmental window of H3K9cr deposition, which would more directly establish the proposed substrate-availability model.

5. CONCLUSION

Stage-resolved CUT&RUN, acyl-CoA mass spectrometry and a BRDT bromodomain point mutation selectively impairing crotonyl-lysine recognition together establish that histone crotonylation is deposited sharply and specifically in round spermatids at a defined post-meiotic spermiogenesis gene set, tracking a stage-matched increase in crotonyl-CoA abundance, and that

BRDT-dependent reading of this mark is required for expression of the genes it decorates and for normal chromatin compaction and sperm production during spermiogenesis. These findings identify histone crotonylation-BRDT reading as a distinct, functionally required regulatory layer operating alongside conventional histone acetylation during male germ cell differentiation, and motivate further work on the metabolic pathways controlling stage-specific crotonyl-CoA availability in the testis.

Acknowledgements

The authors thank the mass spectrometry core facility at the Rheinfeld Institute for Genome Organization for acyl-CoA quantification, and the flow cytometry facility at the Meuse Institute of Nuclear Biology for assistance with spermatogenic cell sorting. We also thank colleagues at the Vistula Institute of Molecular Genetics for helpful discussion of BRDT structural biology.

Funding

This work was supported by internal research funds from the Rheinfeld Institute for Genome Organization, the Meuse Institute of Nuclear Biology, and the Vistula Institute of Molecular Genetics. No external grant funding was received for this study, and the funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

J.M. conceived and supervised the study, designed the BRDT-Y77A knock-in strategy, and analysed the CUT&RUN and mass spectrometry data. B.v.d.M. performed spermatogenic cell purification and CUT&RUN library preparation. K.L. performed RNA-sequencing and spermiogenesis phenotyping. All authors contributed to writing and approved the final manuscript.

Ethics Statement

All animal procedures were approved by the institutional animal care and use committee of the Rheinfeld Institute for Genome Organization and performed in accordance with relevant national and institutional guidelines for the care and use of laboratory animals. This study did not involve human participants or human data.

Data Availability

Raw and processed CUT&RUN and RNA-sequencing data generated for this study have been deposited in the Gene Expression Omnibus under a series accession available from the corresponding author on reasonable request. Analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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