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# Regulation of post-meiotic male genome programming by NUT

Hitoshi Shiota

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## THÈSE

Pour obtenir le grade de

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Arrêté ministériel : 7 août 2006

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1209 – CNRS UMR 5309 Institut pour l'Avancée des  
Biosciences**  
dans l'École Doctorale Ecole Doctorale Chimie et Sciences du  
Vivant

## **Regulation of post-meiotic male genome programming by NUT / Régulation de la programmation post- méiotique du génome mâle par NUT**

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# Table of Contents

<b>Abstract</b> .....	<b>6</b>
<b>Résumé</b> .....	<b>7</b>
<b>List of Figure</b> .....	<b>8</b>
<b>List of Abbreviations</b> .....	<b>10</b>
<b>I. Introduction</b> .....	<b>11</b>
I.1. Chromatin and epigenetics .....	11
I.1.1. Nucleosome: a basic unit of chromatin .....	12
I.1.1.1. Core histones .....	12
I.1.1.2. Linker histones .....	14
I.1.2. Epigenetics: what is it? .....	15
I.1.2.1. DNA methylation .....	16
I.1.2.2. Histone modifications.....	18
I.1.2.3. Histone variants .....	24
I.1.3. Nucleosome dynamics.....	28
I.1.3.1. Histone chaperones .....	28
I.1.3.2. Chromatin remodelers .....	31
I.1.4. Functional aspects of epigenetics:	
examples of histone acetylation .....	33
I.1.4.1. Writers: histone acetyltransferases (HATs) .....	33
I.1.4.2. Readers: bromodomain and extraterminal domain family	
(BETs) .....	40
I.1.4.2.1. BET family members .....	41
I.1.4.2.2. BET inhibitors .....	45
I.1.4.3. Erasers: histone deacetylases (HDACs).....	47
I.2. Epigenetics of male germ cells.....	49
I.2.1. Mission of the male germ cell and its unique genome compaction ...	49
I.2.2. Mouse spermatogenesis .....	51
I.2.2.1. Pre-meiotic .....	52
I.2.2.2. Meiotic.....	53
I.2.2.3. Post-meiotic .....	55

I.2.3.Histone-to-protamine replacement during spermiogenesis .....	58
I.2.3.1. Histone hyperacetylation wave.....	59
I.2.3.2. BRDT: a testis specific BET.....	60
I.2.3.3. Histone variants in male genome reorganization .....	63
I.3. Nuclear Protein in Testis (NUT) .....	67
I.3.1.NUT Midline Carcinoma (NMC).....	67
I.3.2.BRD4-NUT: a strong enhancer of HAT activity.....	70
I.3.3.“Megadomain” formation by BRD4-NUT.....	72
<b>II. Objectives .....</b>	<b>74</b>
<b>III.Materials and Methods.....</b>	<b>76</b>
III.1. Mouse model .....	76
III.2. RNA purification and expression analysis.....	76
III.3. Purification of male germ cells.....	77
III.4. Spermatogenic cell preparation for immunofluorescence .....	78
III.5. Histological and immunohistochemical analysis .....	79
III.6. Histone preparation for PTM analysis .....	80
III.7. Protein sample preparation and Western blot.....	80
III.8. Immunoprecipitation, pull-down experiments.....	81
III.9. Antibody generation and purification.....	82
III.10. <i>In vitro</i> HAT assay .....	83
III.11. Proteomic-based analysis of histone PTMs .....	84
III.12. Transcriptomic analysis.....	85
III.13. Cell culture and transfection .....	87
III.14. Antibodies .....	87
III.15. PCR primers.....	88
<b>IV.Results .....</b>	<b>89</b>
IV.1. Expression of <i>Nut</i> Starts after completion of meiosis .....	89
IV.2. Generation of <i>Nut</i> knockout mice .....	94
IV.3. NUT is essential for sperm production.....	97
IV.4. NUT is essential for histone-to-protamine replacement .....	99
IV.5. Potential interaction between NUT and BET factors in spermatids .....	102

IV.6.	Potential interaction between NUT and p300 in spermatids .....	105
IV.7.	Round spermatid-specific pattern of histone PTM is regulated by NUT.....	106
IV.8.	Altered transcriptome in <i>Nut</i> <sup>-/-</sup> round spermatids .....	111
IV.9.	NUT-BRDT-regulated genes .....	113
<b>V.</b>	<b>Discussion .....</b>	<b>115</b>
V.1.	Precise assembly of male epigenome .....	115
V.2.	<i>Nut</i> expression is specific in post-meiosis.....	116
V.3.	Knockout of <i>Nut</i> specifically affects male fertility .....	116
V.4.	Post-meiotic arrest in <i>Nut</i> <sup>-/-</sup> male is due to defective histone replacement.....	117
V.5.	Regulation of histone PTMs by NUT .....	118
V.6.	NUT-dependent transcriptome .....	119
V.7.	Enhancement of cellular HAT activity by NUT .....	119
V.8.	NUT interaction with the proteins of BET family.....	120
V.9.	Comparison of the function of NUT in spermatids and NMC .....	121
V.10.	From cancer to sperm and from sperm to cancer.....	123
V.11.	Future works.....	124
V.11.1.	Identification of NUT interacting proteins.....	124
V.11.2.	Additional analysis of transcriptomic data .....	124
V.11.3.	Analysis of H4K5/K8 ac/bu of the genes regulated by NUT .....	125
<b>VI.</b>	<b>Annex.....</b>	<b>126</b>
<b>VII.</b>	<b>References .....</b>	<b>162</b>

## Abstract

During the late stages of spermatogenesis, post-meiotic male germ cells undergo a dramatic reorganization of their chromatin architecture involving the almost genome wide replacement of histones by protamines, creating highly condensed nuclei that are found in the mature sperm. During this process a key early event is known to be the wave of histone hyperacetylation, which precedes their replacement. Our team previously reported that the testis specific BET factor BRDT (BRomodoDomain Testis specific), which binds acetylated histones, is essential during this process. However, how this genome wide hyperacetylation occurs has remained one of the major questions in the field.

NUclear protein in Testis (NUT) is a testis specific factor whose physiological function in male germ cells was unknown. It has been found ectopically expressed in NUT Midline Carcinoma, a rare but highly aggressive cancer, in fusion with BRD4, resulting in a highly oncogenic fusion protein. In cancer cells, NUT is able to recruit and activate the histone acetyltransferase p300, hence contributing to the oncogenic activity of the BRD4-NUT fusion protein.

In my Ph.D. project, I investigated the original function of NUT by using *Nut* knockout mice that were generated by our team in collaboration with Mathieu Gerard (Saclay). The absence of NUT causes male sterility associated with a spermatogenic arrest during spermatids elongation/condensation, at a stage when histone replacement normally takes place. Additional experiments suggest that NUT could act through the regulation of epigenetic marks, including histone hyperacetylation. The mechanisms by which NUT interferes with the hyperacetylation wave and interacting factors, including BRDT were explored.

Altogether this study demonstrates the essential contribution of NUT to the epigenetic regulation and histone replacement during the post-meiotic maturation of male germ cells.



## Résumé

Pendant les derniers stades de la spermatogenèse, les cellules germinales mâles post-méiotiques subissent une réorganisation dramatique de l'architecture de leur chromatine, impliquant notamment le remplacement presque total des histones par les protamines, créant des noyaux fortement condensés que l'on trouve dans le sperme mature. Au cours de ce processus, un événement précoce clé est la vague d'hyperacétylation des histones, qui précède leur remplacement. Notre équipe a précédemment identifié le facteur d'expression testiculaire de la famille BET, Brdt (BRomoDomain Testis), qui se lie aux histones acétylées via ses deux bromodomains, comme essentiel au cours de ce processus. Cependant, les mécanismes aboutissant à l'hyperacétylation des histones à l'échelle génomique sont encore inconnus, ce qui reste l'une des questions majeures dans le domaine.

La protéine NUClear in Testis (NUT) est un facteur spécifique testiculaire dont la fonction physiologique dans les cellules germinales mâles était inconnue. Cette protéine se trouve exprimée de manière ectopique dans un cancer rare mais très agressif, le carcinome de la ligne médiane (NUT Midline Carcinoma), en fusion avec BRD4, produisant ainsi une protéine de fusion hautement oncogène. Dans les cellules cancéreuses NUT est capable de recruter et d'activer l'histone acétyltransférase p300, contribuant ainsi à l'activité oncogénique de la protéine de fusion BRD4-NUT.

Mon projet de doctorat est d'explorer la fonction physiologique de NUT, en étudiant des souris knock-out pour *Nut* qui ont été générées par notre équipe en collaboration avec Mathieu Gérard (Saclay). L'absence de NUT provoque une stérilité mâle associée à un arrêt de la spermatogenèse lors de l'allongement et de la condensation des spermatides, au stade où normalement les histones sont remplacées. D'autres expériences suggèrent que NUT pourrait agir sur la régulation de marques épigénétiques, y compris l'hyperacétylation des histones. Les mécanismes par lesquels NUT interfère avec la vague d'acétylation et les facteurs en interaction, y compris Brdt, sont explorées.

Au total, cette étude démontre la contribution essentielle du NUT à la régulation épigénétique et au remplacement des histones au cours de la maturation post-méiotique des cellules germinales mâles.

# List of figures

## **Introduction:**

- Figure A. Chromatin fibers composed of Nucleosomes.
- Figure B. Nucleosome core particle.
- Figure C. Overview of epigenetic regulations
- Figure D. DNA methylation and oxidative demethylation
- Figure E. Histone tail modifications
- Figure F. Histone PTMs in the globular domains
- Figure G. Lysine acylations in histones
- Figure H. Members of the human BET family
- Figure I. BRD4's function during transcription
- Figure J. Structure of BD1 from BRD4 bound by JQ1
- Figure K. Inheritance of male genome through multiple chromatin reorganization
- Figure L. Organization of spermatogenic cells
- Figure M. Developmental stages of seminiferous tubules
- Figure N. Histone replacement by TPs and Prms
- Figure O. Hyperacetylation wave in late round and elongating spermatids
- Figure P. Preferential binding of BRDT's BD1 to H4K5ac and K8ac/ defective histone replacement in *BrdtABD1 mice*
- Figure Q. Histone variants during spermatogenesis
- Figure R. *BRD4-NUT* or *BRD3-NUT* fusion genes
- Figure S. Enhancement of HAT activity of p300 by BRD4-NUT
- Figure T. Feed-forward hyperacetylation by BRD4-NUT and p300 in NMC
- Figure U. Hyperacetylated megadomain formation by BRD4-NUT

## **Results:**

- Figure 1. Expression profile of *Nut* gene
- Figure 2. Expression of *Nut* in relative to a wave of histone hyperacetylation (histology)
- Figure 3. Expression of *Nut* in relative to a wave of histone hyperacetylation (cytology)
- Figure 4. Generation of *Nut* knockout mice (strategy)

- Figure 5. Confirmation of *Nut* knockout
- Figure 6. Phenotype of *Nut*<sup>-/-</sup>
- Figure 7. Defective histone replacement in *Nut*<sup>-/-</sup> spermatids
- Figure 8. Potential interaction between NUT and BRDT
- Figure 9. Potential interaction between NUT and other members of BET
- Figure 10. Potential interaction between NUT and p300 in the spermatogenic cells
- Figure 11. Ability of NUT to enhance HAT activity in vitro
- Figure 12. Histone PTMs that were detected in *WT* and *Nut*<sup>-/-</sup> round spermatids
- Figure 13. Transcriptomic analysis of *WT* and *Nut*<sup>-/-</sup> germ cells
- Figure 14. Expression of NUT-activated or NUT-repressed genes in the testes from *WT* and *Brdtd* $\Delta$ *BD1* mice
- Table 1. Summary of detected PTMs from acid-extracted histones of *WT* or *Nut*<sup>-/-</sup> round spermatids by MS analysis.

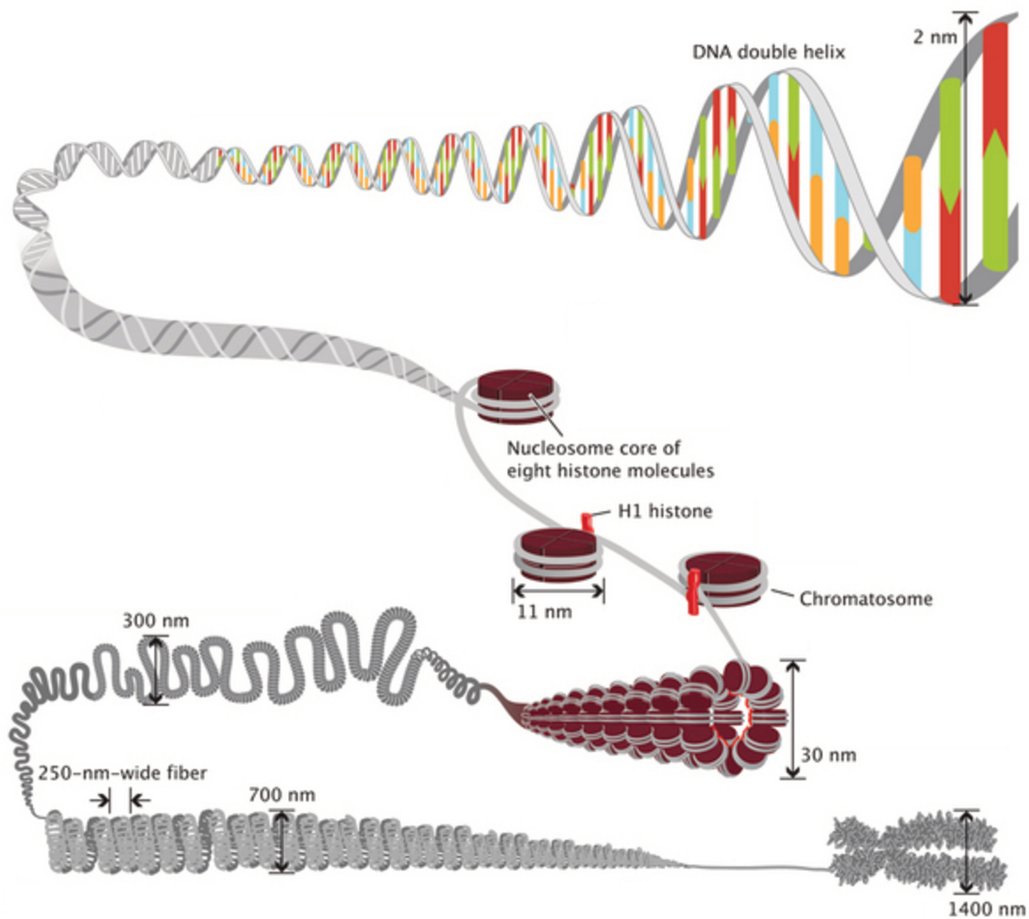
# List of Abbreviations

**DNA:** Deoxyribonucleic acid  
**RNA:** Ribonucleic acid  
**bp:** base pairs  
**PTM:** Post-translational modifications  
**Dnmt:** DNA methyltransferase  
**HAT:** Histone acetyltransferases  
**HDAC:** Histone deacetylase  
**ac:** acetylation  
**me1:** monomethylation  
**me2:** dimethylation  
**me3:** trimethylation  
**bu:** butyrylation  
**bhb:** beta-hydroxyisobutyrylation  
**cr:** crotonylation  
**TSS:** transcription start site  
**TP:** Transition Protein  
**Prm:** Protamine  
**BET:** Bromodomains and extraterminal domain  
**BD:** Bromodomain  
**TSA:** Torichostatin A  
**BRD4:** Bromodomain-containing protein 4  
**BRDT:** Bromodomain testis-specific protein  
**iPSC:** induced pluripotent stem cells  
**ESC:** embryonic stem cells  
**NUT:** Nuclear Protein in Testis  
**NMC:** NUT Midline Carconoma  
**NES:** nuclear export signal  
**NLS:** nuclear localization signal  
**TAD:** topologically associated domain  
**TGC:** total germ cells

# I. Introduction

## I.1. Chromatin and epigenetics

The eukaryotic genome is packaged in a DNA-protein complex called “chromatin”, which allows a meter- long DNA molecule to fit into the cells nucleus with a diameter of micrometers (Figure A). This packaging of the genome is essential



**Figure A:** schematic representation of chromatin fibers composed of nucleosomes, enabling compaction of DNA. (Image from: © 2013 Nature Education Adapted from Pierce, Benjamin. Genetics: A Conceptual Approach, 2nd ed., with some modifications.)

not only for minimizing the space consumption within a nucleus, but also for the establishment of gene expression regulatory circuits. While the functions of the genes directly depend on its sequence, the timing and context under which each gene is expressed is a highly regulated process. A part of these regulations involves epigenetic mechanisms. Epigenetics is the study of such mechanisms, which could induce heritable changes in gene expression profiles without altering the DNA sequence. These mechanisms are also largely relying on chromatin states that take various degrees of compaction under different circumstances to regulate activities of the genome such as gene transcription, DNA replication and repair.

### **I.1.1. Nucleosome: a basic unit of chromatin**

The functional unit of chromatin is the nucleosome (Kornberg, 1977), in which 145-147 base pairs (bp) of DNA wrap around a protein octamer constituted of two copies of each histones H2A, H2B, H3 and H4. The nucleosome is part of a structural unit that covers  $200\pm 40$  bp of DNA. This unit also includes space between nucleosomes which is often bound by linker histones H1. The genome covered by nucleosomes constitutes the chromatin.

#### **I.1.1.1. Core histones**

In 1997, the high-resolution X-ray structure of the nucleosome core particle was revealed (Luger et al., 1997). 146 bp of DNA was shown to create 1.65 turns of a left-handed helix wrapping around the histone octamer (Figure B). Four types of core histones, H2A, H2B, H3 and H4, are constituted of around 100~130 amino acids, which is a relatively small size among all proteins. Core histones are highly conserved proteins among the eukaryotes. Although their sequences are distinct, all core histones are consisted of a central globular domain flanked by amino- and carboxy-terminal (N term and C term) tails. The histone octamer is formed by four histone-folds: two H2A-H2B dimers and two H3-H4 dimers. Two H3-H4 dimers form a tetramer through an interaction between H3 and H3'. The H2A-H2B dimers interact with the tetramer through interactions between H2B and H4 and between H2A and H3. These

histone fold domains are also responsible for histone DNA interaction within a nucleosome.

The N-terminal tails of the histones, which protrude from the nucleosome core particle, are important parts for inter-nucleosome interactions, and affect the higher order folding of the chromatin fiber. The tail region is also subjected to a variety of post-translational modifications (PTMs) characterized by the attachment of small chemical groups to various amino acids, including lysine, arginine, serine (for review see Kouzarides 2007). Recently, modifications in the globular domains of histones were also discovered (Lawrence et al., 2016). Essentially, histone modifications could alter the interaction between nucleosomes, or protein-protein or protein-DNA interaction within nucleosomes, affecting the properties of the chromatin. Various histone modifications have been identified (for review see Zhao & Garcia 2016). Chemical groups involved in these covalent modifications could be attached, removed, or bound by specific histone binding factors which in turn mediate a series of downstream events.

Core histones also have non-allelic variants, which shows different expression profiles, amino acid sequences and enrichment along the chromatin. The



**Figure B:** Crystal structure of the nucleosome core particle. DNA and core histones are labeled with different colors as follows: light green and orange: DNA, blue: H3, green: H4, yellow: H2A, red: H2B (Image from: Luger et al., 1997)

incorporation of the histone variants at specific positions are also part of the regulatory mechanisms affecting chromatin properties and hence its local activities.

#### **I.1.1.2. Linker Histones**

The DNA between nucleosomes, called linker DNA, is bound by the fifth histone, also called linker histone or H1. The role of H1 is essentially to stabilize the higher order chromatin structure, although its dynamic binding to the DNA, as well as the large variety of H1 variants and interacting factors allow H1s to perform diverse functions. The linker histones constitute a large family including a number of variants that are distinct from each other depending on species, cell types and expression profile (Khochbin, 2001). H1 is present in almost all nucleosomes, and its binding is independent from the underlying DNA sequences.

The function of H1 in the stabilization of chromatin structure is observed in studies based on H1 depletion in some model organisms. At the chromatin level, H1 depletion causes multiple effects, such as defective condensation of mitotic chromosomes in *Tetrahymena* (Shen and Gorovsky, 1996), and shorter linker DNA in mice (Fan et al., 2003). However, H1 binding to DNA is quite dynamic and H1 occupancy does not simply mean the silencing of the underlying genes. Depletion of H1 was reported to downregulate some genes in mouse, yeast and *Tetrahymena* (Downs et al., 2003; Lin et al., 2004; Shen and Gorovsky, 1996).

The roles of H1 could also involve its interplay with a network of chromatin binding proteins such as HMG proteins (Catez et al., 2004), MeCP2, UBF, the liver enriched transcription factor HNF-3, and glucocorticoid receptor (Zlatanova et al., 2000), that may also target the linker DNA regions. The binding of these factors could in fact be affected by H1s.

Additionally, not only the accessibility of chromatin-binding proteins could be affected by H1 binding but also the H1-dependent higher-order chromatin structure could be a determinant regulatory event. H1 could also stabilize the position of the nucleosomes, which are the targets of chromatin binding proteins and hence exert a regulatory role.



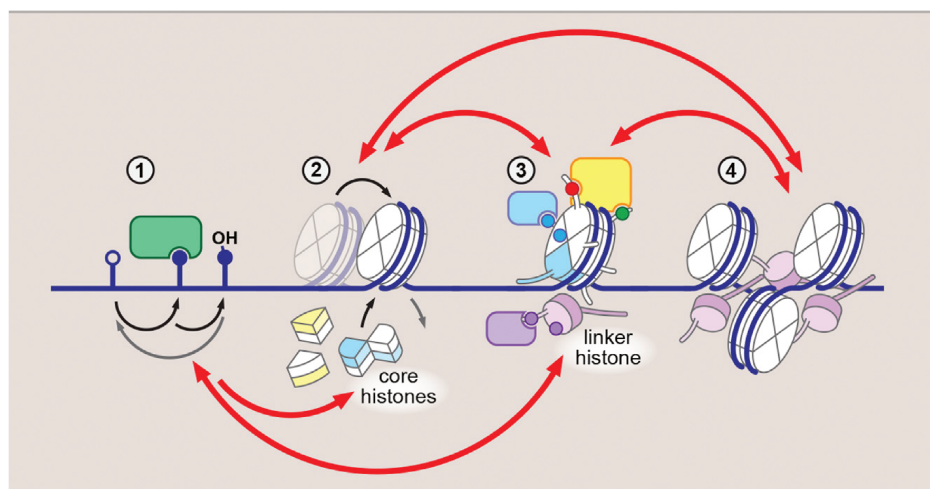
The time during which H1s stay on the chromatin, or “residence time” depends on other chromatin binding proteins as well as on the type of H1 variant. An increase in the HMG protein and specific H1 variants concentration could change the time of residence of H1s (Catez et al., 2004). A change in the time of residence of H1 could ultimately affect the activities of nucleosome remodeling factors such as SWI/SNF (Horn et al., 2002) or of histone modifying factors (Herrera et al., 2000).

### **I.1.2. Epigenetics: What is it?**

In multicellular organisms, the course of embryonic development starts from a single cell, the fertilized egg. After cycles of cell division and differentiation, various organs, made of tissues with various cell types constitute the adult organism. The process of cell division with faithful transmission of the mother cell’s genome is certainly a fundamental process during development. However, at certain point, cells also start differentiating following different and specific gene expression programs, which are established in the course of development. Once established, the cellular identity is transmitted to the next cellular generation, and the cells retain their identity until the production of a fully developed individual. How could an identical genetic content give rise to totally different cell types? How could a differentiating cell remember and keep its identity while going through active cellular replication?

Epigenetics is the study of heritable changes in gene activity which are not based on the genetic code, but mostly relying on the modulation of chromatin properties. In other words, epigenetic phenomena could be defined at least in part as “alterations to chromatin template that establish different patterns of gene expression from the same genome” (Soshnev et al., 2016). Chromatin needs to be a dynamic entity in order to sustain basic cellular tasks as well as complex processes such as cellular differentiation. For example, nucleosome formation antagonizes gene expression, because histone-DNA interactions hinder the access of many transcription factors to the genes. These chromatin-based gene regulation mechanisms play also a critical role in avoiding aberrant activation or repression of certain genes, which are potentially pathogenic, as observed in human diseases, mainly cancer.

In the following sections, fundamental elements of the epigenome constituents (Figure C), such as DNA methylation, histone PTMs, histone variants and their regulation and dynamics are presented and discussed.



**Figure C:** Overview of epigenetic regulations. 1: DNA methylation. 2: histone variant incorporation. 3: histone modifications and PTM readers. 4: higher order chromatin organization. Red arrows represents the possible influences from a regulatory layer to another. (Image from: Soshnev et al., 2016)

#### **I.1.2.1. DNA methylation**

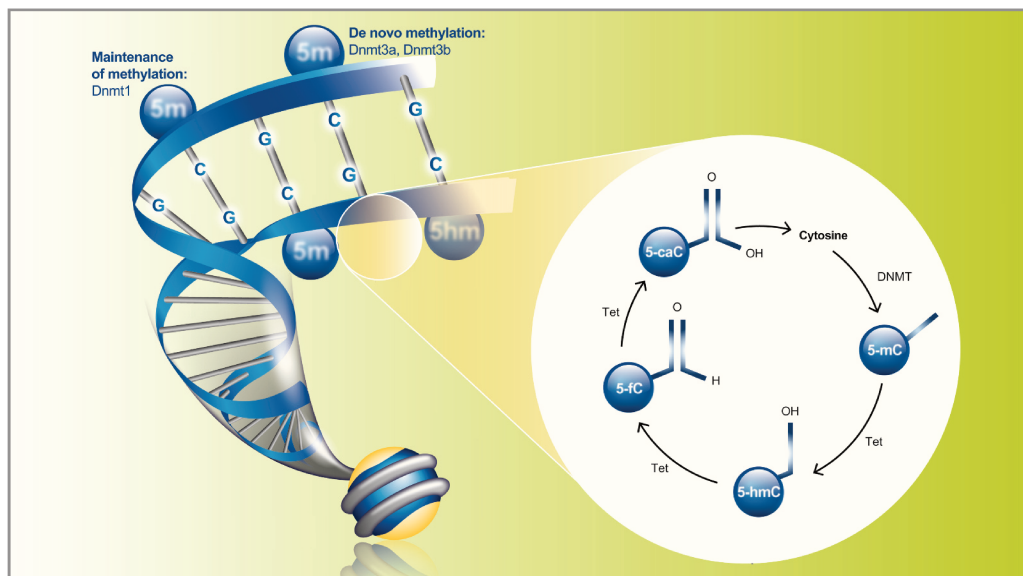
DNA methylation is a modification of genomic DNA, occurring at cytosine base on which a methyl group is added (Jeltsch and Jurkowska, 2014; Rose and Klose, 2014). The role of DNA methylation was first observed in a study comparing chicken beta-globin locus, which led to the finding that DNA methylation is associated with the suppression of gene expression (Mcghee and Ginder, 1979).

DNA can be methylated by two types of DNA methyltransferases, Dnmt1 or Dnmt3. Dnmt1 specifically binds to a DNA bearing the methylation on only one of its strands, a state called hemimethylation (Jurkowska et al., 2011). The other unmethylated strand is then methylated by Dnmt1 to maintain the DNA methylation status. De novo Dnmt, DNMT3a and 3b, bind both unmethylated or hemimethylated

DNA, and methylate previously unmethylated loci (Jurkowska et al., 2011). However, some studies reported the *in vitro* activity of Dnmt1 as a de novo methyltransferase, suggesting that it could actually function in a broader context than initially suggested (Biniszkiwicz et al., 2002; Takagi et al., 1995; Vertino et al., 1996).

Demethylation of DNA could occur passively by replication when there is not sufficient amount of Dnmt1 to maintain DNA methylation. Alternatively, ten eleven translocation (TET) dioxygenases could oxidize methylcytosine into hydroxymethylcytosine, initiating an active demethylation process (Kohli and Zhang, 2013; Pastor et al., 2013; Wu and Zhang, 2014) (Figure D). Active demethylation as well as de novo and replication-dependent DNA methylation underlies the dynamics of DNA methylation which could have critical regulatory roles (Métivier et al., 2008; Stevenson and Prendergast, 2013).

DNA methylation is essential for cell differentiation through the regulation of gene expression. Cancer has been also studied as one of the pathological models to explore the impact of DNA methylation on human diseases. In cancer cells, tumor suppressor genes are often found silenced by local DNA hypermethylation, while the genome is overall hypomethylated at a global level (Shen and Laird, 2013).



**Figure D:** DNA methylation and oxidative demethylation. (Image from: “a guide to epigenetics” by Abcam.)

### **I.1.2.2. Histone modifications**

After their translation, proteins could be modified by the addition of small chemical groups onto certain amino acid residues. Such modifications are called post-translational modifications or PTMs. Like other proteins, histones can be affected by such modifications. Histone PTMs could change the properties of nucleosomes, and hence create a basis for epigenetic regulations. In 2000, the “histone code” hypothesis was introduced, referring to the idea that combinations of histone modifications could serve as a “language” that would be read by various “reader” proteins, regulating down stream events (Strahl and Allis, 2000). Histone PTMs include acetylation, methylation, phosphorylation, ubiquitination, among other modifications. Recent investigations based on mass spectrometry are now indicating that there are many possible histone PTMs (Tan et al., 2011).

Here we will briefly discuss histone acetylation and histone lysine methylation.

#### **Histone Acetylation**

Acetylation (ac) is catalyzed by a large family of enzyme called histone acetyltransferases (HAT), adding an acetyl group to a lysine residue using acetyl-CoA as the acetyl group donor. The added acetyl group neutralizes the basic charge of the lysine with some possible structural effects (Lawrence et al., 2016).

The dynamics of histone acetylation also involves histone deacetylase (HDACs), which deacetylate the histones. Histone acetylation also creates an anchoring point for proteins containing specific domains capable of binding the acetylated histones (Marmorstein and Zhou, 2014). Bromodomain is one of the examples of such domains capable of binding acetylated histones in various cellular processes (see section I. 1. 4. 2. Readers: bromodomain and extraterminal domain family (BETs)).

In general, histone acetylation marks active transcription. After the identification of acetylation of H3 tail at lysine 14, catalyzed by Gcn5 in yeast (Grant

et al., 1999), many acetylation sites were identified. Namely, H3K14 and K18 are acetylated at gene promoters that are actively transcribed (Grant et al., 1999). H4K5, K8, K12 and K16 acetylation marks transcriptionally active regions in decondensed euchromatin (Bannister and Kouzarides, 2011; Dion et al., 2005; Turner, 1991; Wang et al., 2008).

In addition to the transcription coupled histone acetylation, newly synthesized histone H4 and H3 are pre-acetylated before their incorporation into the nucleosome. H4K5ac and H4K12ac are detected in the pre-incorporation complex formed with the histone chaperone (Chang et al., 1997; Sobel et al., 1995), and then are deacetylated after their incorporation to the chromatin (Taddei et al., 1999).

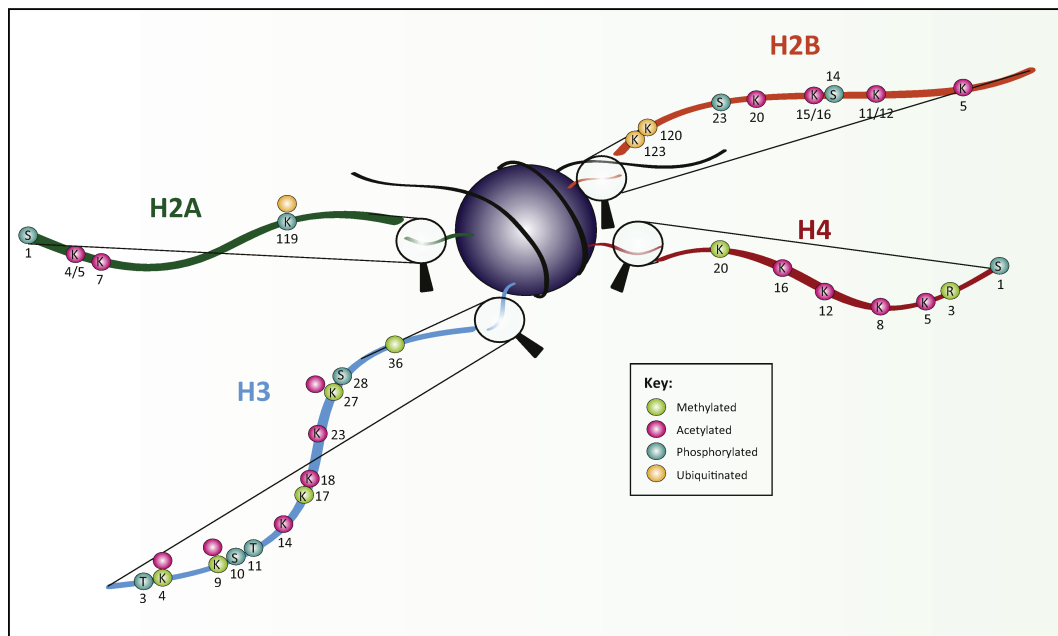
### **Histone lysine Methylation**

Histone can also be modified by the addition of methyl groups, which could take several forms; lysine residue could be mono-, di- or trimethylated (me1, me2 me3). It is of note that arginine residues can also be mono- or dimethylated (Kouzarides, 2007) but here we will focus on lysine methylation. Many histone methyltransferase capable of methylating histone lysines were identified (Zhang and Reinberg, 2001), which have relatively high specificity compared to HATs (Bannister and Kouzarides, 2005). Methylated lysines can be recognized by chromo, Tudor, PHD or MBT domain containing proteins (Kouzarides, 2007). Histone methylation could affect the transcription in either activating or repressing ways. Heterochromatin is associated with low levels of acetylation and high levels of methylation at certain residues, including H3K9 and H4K20, while facultative heterochromatin is characterized by H3K27 methylation (Kouzarides, 2007). Euchromatin regions generally contain various histone marks, and actively transcribed regions typically receive additional marks such as H3K4, K36 and K79 methylation. In some cases, H3K4me3 and H3K27me3, which are active and repressive marks respectively, are found on the same genomic region. Such bivalent marking can be found for example in regulatory elements of developmentally regulated genes in ES cells (Azuara et al., 2006; Bernstein et al., 2005), as well as in the germ cells (Erkek et al., 2013; Sachs et al., 2013).

## Nucleosome core modifications

Like the histone tail regions (Figure E), histones could also be modified in their central globular domains (Di Cerbo et al., 2014; Cosgrove et al., 2004) (Figure F). The lateral surface of the nucleosome, which is in direct contact with DNA, is composed of the globular domains (Di Cerbo et al., 2014; Zhang et al., 2002).

Methylation at H3K79 was first identified from chicken erythrocytes, and one of the first globular modifications studied (Zhang et al., 2002). Mono-, di-, and trimethylated H3K79 were first identified in yeast, and seem to contribute to the silencing of the corresponding genomic regions (Feng et al., 2002; Van Leeuwen et al., 2002). However, in other studies, H3K79 was found to be associated with active transcription (Barry et al., 2009; Guenther et al., 2007; Nakanishi et al., 2009; Ng et al., 2003; Schübeler et al., 2004). For example, H3K79me1 is enriched in the developmental genes harboring the bivalent methylation marks, H3K4me3 and H3K27me3, in ESCs (Bernstein et al., 2006; Steger et al., 2008), while di- and trimethylation marks the TSS of actively transcribed genes (Schübeler et al., 2004;

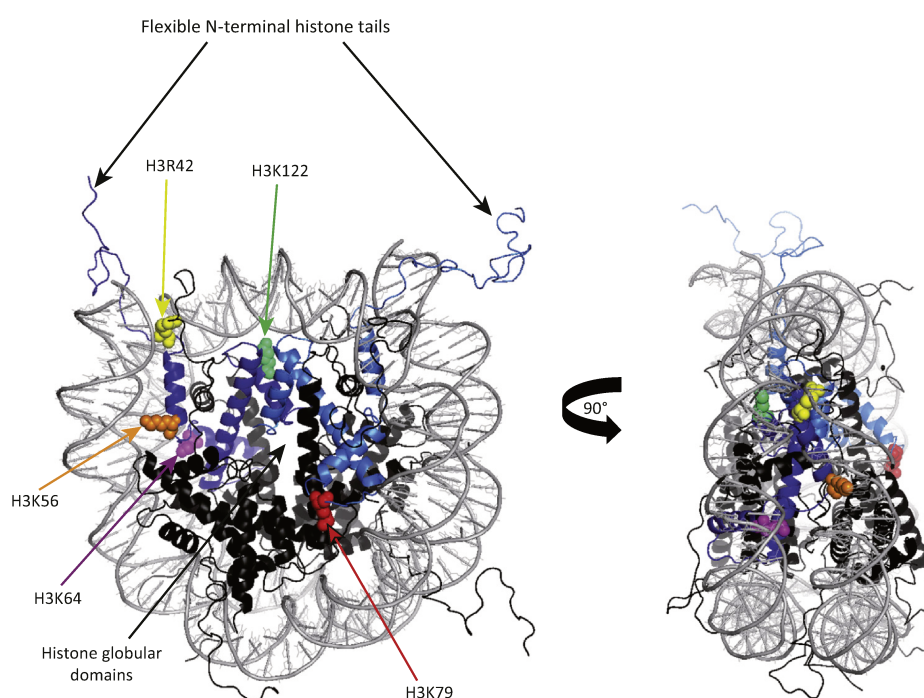


**Figure E:** Examples of histone tail modifications. (Image from: Lawrence et al, 2016)

Steger et al., 2008; Vakoc et al., 2006).

Acetylation at H3K122 is one of the best characterized example (Manohar et al., 2009; Tropberger et al., 2013). H3K122 is located at the point where the strongest interaction between the histone and DNA occurs (Hall et al., 2009). It has been observed that the acetylation of K122 directly induces the transcriptional activity by histone eviction in vitro (Tropberger and Schneider, 2013; Tropberger et al., 2013). Indeed, the mark is enriched in the enhancer regions harboring H3K4me1 and H3K27ac, as well as on active genes marked by H3K4me3, H2A.Z and H3.3 (Tropberger et al., 2013).

Other modifications are readily identified in the globular domains of nucleosomal histones, mainly lysine acetylation and lysine or arginine methylation in all the canonical core histone members (Lawrence et al., 2016). However, the functions of most of those modifications are yet to be characterized. Since no readers for the modifications located on the globular domains have been identified so far, these modifications could have direct effects on the nucleosome integrity, as seen in the H3K122ac. Along with the new types of PTMs that is introduced in next section,



**Figure F:** Examples of histone PTMs occurring in the globular domains and their relative positions within the nucleosome. (Image from: Lawrence et al, 2016)

histone globular PTMs constitute an actively studied domain in the field of epigenetics.

### **Newly identified modifications**

Recent advancements in the mass spectrometry-based proteomic approaches have accelerated the discovery of new types of histone modifications. In Particular, many types of acylations, beside acetylations, have been found, which include propionylation (Chen et al., 2007), formylation (Jiang et al., 2007), butyrylation (Chen et al., 2007), 2-hydroxy isobutyrylation (Dai et al., 2014), beta-hydroxybutyrylation(Xie et al., 2016), crotonylation(Montellier et al., 2011; Tan et al., 2011), malonylation (Xie et al., 2012), succinylation (Xie et al., 2012), and glutarylation (Tan et al., 2014) (Figure G).

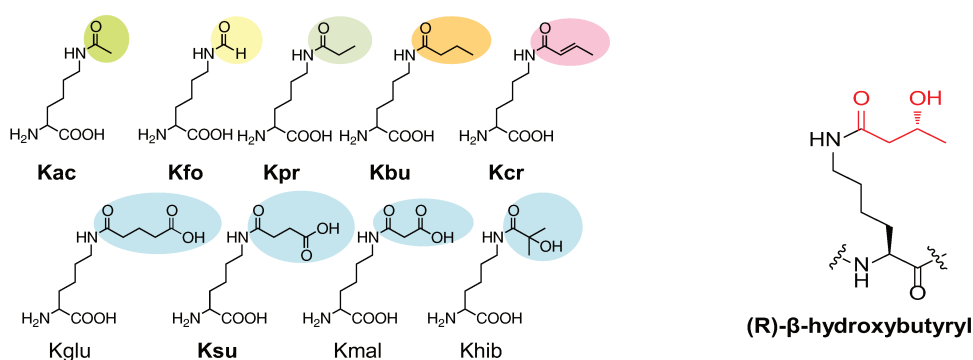
Acetylation occurs by transferring an activated acetyl group from acetyl-CoA to lysine residues. Acetyl-CoA is an intermediate produced in many metabolic pathways including sugars, fatty acids and amino acid metabolism. At the same time, these pathways also produce other intermediates of acyl groups coupled with CoAs. Interestingly, a few studies have shown that some of these acyl-CoA can actually be used by a known HATs. For instance, butyrylation and propioylation are two modifications which could be catalyzed by p300 (Chen et al., 2007). In addition, it was recently reported that p300 is also able to carry out crotonylation, hence contributing to the regulating gene activation (Sabari et al., 2015).

The functions of many of those new modifications are still largely unknown, but they are gradually discovered. One of the critical findings is the role of H4K5 and K8 butyrylation (bu), in association with acetylations on the same sites during mouse spermatogenesis (Goudarzi et al., 2016). Indeed at the late stages of spermatogenesis, in post-meiotic cells (see section I. 2. 2. Mouse spermatogenesis), histones become globally hyperacetylated during spermatids elongation, including positions H4K5 and K8 (see section I. 2. 3. 1. Histone hyperacetylation wave), before they undergo a genome-wide removal. H4K5 and K8 butyrylations are also detected in the same timing together with these hyperacetylations. The hyperacetylated histones are then bound by the bromodomain containing factor BRDT, which is required for their



genome wide removal (see section I. 2. 3. 2. BRDT: a testis specific BET). The experimental data presented in Goudarzi et al. actually suggest that butyrylated histones have a prolonged retention compared to acetylated histones. Strikingly, the *in vitro* experiment demonstrated that the butyrylation mark on H4K5 prevents the binding of BRDT, suggesting that its presence temporarily prevents BRDT binding. Altogether, this study reveals the mechanisms by which the dynamic exchange between acetylation and butyrylation precisely regulates the ordered chromatin remodeling by modulating the binding of BRDT. The same study also shows that the TSS surrounding regions of actively transcribed genes in male germ cells are labeled by the four marks, H4K5ac, K4K8ac, H4K5bu, and K4K8bu and that the absence of any of those marks are linked with decreased levels of expression compared to the genes with the four marks, indicating that H4K5 and K8 are also marking actively transcribed genes.

Beta-hydroxybutyrylation (bhb) is also a recently discovered type of lysine acylation (Xie et al., 2016). In the mice liver cells, 44 modification sites were identified for beta-hydroxyisobutyrylation, which were comparable to known acetylation sites. One of those sites, H3K9, showed clear increase of bhb in response to starvation condition. This increase of the H3K9bhb was correlated with active gene regulation, and the genes associated in metabolic pathway that were known to be activated upon starvation were marked by H3K9bhb, leading to their upregulation.



**Figure G:** Lysine acylations identified in histones. Acetylation (Kac), formylation (Kfo), propionylation (Kpr), butyrylation (Kbu), crotonylation (Kcr), glutarylation (Kglu), succinylation (Ksu), malonylation (Kmal), 2-hydroxyisobutyrylation (Khib), and beta-hydroxybutyrylations are shown. The variable side chain regions are highlighted (images from: Flynn et al., 2015 (left); Xie et al., 2016 (right)).

The protein modules capable of recognizing and binding to histones with these new modifications are also actively studied. In the study of Flynn et al., the binding ability of a large variety of bromodomains to several acylated peptides were screened (Flynn et al., 2015). Among those, human BRD9 bromodomain and a few others showed high affinity for butyrylated lysine containing peptides. YEATS domain in Taf14 was found to interact with crotonylated (cr) H3 at K9 (Andrews et al., 2016). In addition, YEATS domain from another protein, AF9, is also able to bind H3K9cr and H3K18cr (Li et al., 2016).

### **I.1.2.3. Histone variants**

Canonical histone incorporation takes place within S phase following DNA replication and maintaining the correct DNA/histone ratio (Whitfield et al., 2000). However, DNA-templated processes such as transcription are associated with nucleosome remodeling and histone eviction. Therefore, the cells need additional resources of histones that can be incorporated independently from the cell cycle. Currently, variants are known to exist for H2A, H2B, and H3, and many of these variants could be incorporated independently from DNA replication. In addition, histone variants present various degrees of differences in amino acid sequences compared to their canonical counter part. Therefore, compared to canonical nucleosomes, a nucleosome with histone variants could have different properties by exhibiting different affinity to DNA or other histone members, or by introducing additional sites potentially subject to PTMs. The incorporation of replication-independent variants is particularly important in various processes, such as maternal histone incorporation into the paternal pronuclei after fertilization (Loppin et al 2005) or in terminally-differentiated cells in which a large proportion of total H3 content is replaced by the replication independent variant, H3.3 (Piña and Suau, 1987).

### **H3.3**

H3.3 is one of the most abundant replication-independent H3 variant, synthesized throughout the cell cycle (Ahmad and Henikoff, 2002). Compare to the

canonical members H3.1 and H3.2, H3.3 has 5 and 4 amino acid differences, respectively. At position 31, H3.1 and H3.2 have alanine, while H3.3 has a serine, which is phosphorylated during mitosis (Hake et al., 2005). The deposition of H3.3 is carried out by two histone chaperones, HIRA (Tagami et al., 2004) and DAXX/ATRX (Xue et al., 2003). Since the incorporation of H3.3 reflects the nucleosome turn over, H3.3 was thought to be primarily an active gene marker (Ahmad and Henikoff, 2002; Schwartz and Ahmad, 2005). In fact, H3.3 is found wide spread mainly at the transcription factor binding sites, and up to 50% of this deposition is HIRA dependent (Goldberg et al., 2010). H3.3-occupied regions also include promoters of transcriptionally silent genes after abortive transcription (Guenther et al., 2007). Therefore, the replacement of H3.3 is indicative of the locations where nucleosome eviction took place during transcription. It is of note that telomeric repeats also possess H3.3, incorporated by the ATPase chromatin remodeler DAXX/ATRX. H3.3 plays important roles in the silencing of telomeres (Drané et al., 2010; Goldberg et al., 2010).

### **CENP-A**

Centromere is a chromosomal region where kinetochore formation takes place. While the centromeric region of a chromosome is composed of repeat sequences ( $\alpha$  satellite in human and minor satellite in mouse) (Guenatri et al., 2004; Yang et al., 2000), the sequence itself could not explain the identity of the centromere, since ectopic formation of centromere at abnormal genomic region has been reported (Warburton, 2004). Actually the centromere is epigenetically characterized by the presence of a centromere specific H3 variant, CENP-A (for review see Muller and Almouzni, 2014). The incorporation of CENP-A is regulated by the specific histone chaperone HJURP (Dunleavy et al., 2009; Foltz et al., 2009), and the deposition takes place during G1 phase (Hemmerich et al., 2008; Jansen et al., 2007; Shelby et al., 2000). A structural study revealed that CENP-A containing nucleosomes present flexible DNA at both ends due to shorter  $\alpha$ -helix (Tachiwana et al., 2011). This specific organization of the nucleosome induced by CENP-A could provide binding sites for centromere-specific proteins.

## **H2A.Z**

H2A-H2B dimers are also replaced by other dimers containing variant histones, and H2A.Z is one of the replication-independent variant of H2A (review Miller, 2013). H2A.Z differs from its canonical counterparts mainly at its extended acidic patch on the surface of the nucleosome, which is a determinant of chromatin fiber structure (Clarkson et al., 1999; Suto et al., 2000). H2A.Z is particularly enriched at regions flanking TSS (Barski et al., 2007; Luk et al., 2010). H2A.Z is also found in subtelomeric regions, preventing the spreading of a silent chromatin state toward the center of the chromosome (Venkatasubrahmanyam et al., 2007).

## **H2A.X**

H2A.X is a variant particularly important for DNA repair. This variant has a distinct C-terminal region from the canonical H2A, which contains a serine at position 139. H2A.X serine 139 is phosphorylated by ATM kinase upon DNA damage response, and the phosphorylated H2A.X is referred to as  $\gamma$ H2A.X, spreading up to approximately 1 to 2 Mb around the damaged site (Rogaku et al., 1998).  $\gamma$ H2A.X facilitates the assembly of the DNA repairing complex, and the knockdown of  $\gamma$ H2A.X is known to induce hypersensitivity toward irradiation induced DNA damage (Celeste et al., 2002; Morrison et al., 2004). The bidirectional exchange between H2A-H2B dimer and H2A.X-H2B dimer is regulated by the FACT complex (Heo et al., 2008).

## **macroH2A**

MacroH2A is an especially large H2A variant compared to other histones because of its characteristic extended C-terminal globular region of about 25 kDa (Costanzi and Pehrson, 1998). MacroH2A is apparently incorporated in the inactivated X chromosome (Costanzi and Pehrson, 1998; Perche et al., 2000), co-localizing with Xist RNA and H3K27me3 (Chadwick and Willard, 2004). It also has a role in the silencing of pluripotency genes in differentiated cells (Pasque et al., 2012).

The silencing effect of macroH2A was thought to be brought through by its specific interaction with PARP1 (Nusinow et al., 2007; Ouararhni et al., 2006). The interaction with macroH2A suppresses the catalytic activity of PARP1, and the binding of the inactivated PARP to the chromatin silences the underlying gene transcription. However, the inhibitory effect on PARP1 by macroH2A could not be detected in another study (Timinszky et al., 2009). It has been also reported that macroH2A could protect the genome against exonuclease digestion, therefore macroH2A.X could stabilize the nucleosome and blocks the access for the binding of transcription factors to the DNA (Angelov et al., 2003; Chakravarthy et al., 2012).

### **H2A.Bbd and related variants**

H2A.Bbd is a H2A variant primarily expressed in testis and brain, as well as ectopically in in some cancers (Nekrasov et al., 2013). There is family of H2A that are similar to H2ABbd. All members are characterized by a lack of acidic patch, which significantly reduces the folding of the chromatin fiber (Bönisch and Hake, 2012). One member is named H2A.Lap1 (for lack of acidic patch), which like other members significantly reduces the nucleosome stability (Bao et al., 2004; Kelly et al., 2010; Soboleva et al., 2012; Zhou et al., 2007). Indeed, H2A.Lap1 incorporation prevents chromatin compaction, which is linked to the derepression of gene transcription (Soboleva et al., 2012). In mice, 4 types of H2A.Lap variants have been reported, and H2A.Lap2 and Lap3 were in fact discovered earlier and were respectively named H2AL1 and L2 (Govin et al., 2007; Talbert et al., 2012). Despite the sequence similarity between H2A.Lap1 and H2AL1/2, both expressed in testis and lacking the acidic patch, these related variants play distinct roles during spermatogenesis. H2A.Lap1 is expressed in pachytene spermatocytes and round spermatids which are meiotic and post-meiotic cells respectively (Soboleva et al., 2012). H2A.Lap1 is enriched at the TSS of actively transcribed genes, including silenced X chromosome at loci that escapes the repression (Namekawa et al., 2006; Soboleva et al., 2012). H2AL1/2 are exclusively expressed during later spermiogenesis stages when the germ cells undergo nuclear elongation and condensation (Govin et al., 2007). Interestingly, H2AL1/2 is shown to be incorporated at approximately the same timing when the majority of the histones are removed and

replaced by other chromatin organizing proteins such as transition proteins (TPs) and Protamines (Prms). H2AL1/2 is maintained on pericentric regions all through post-meiotic spermatids genome condensation and is present at these regions in mature spermatozoa (Govin et al., 2007).

### **I.1.3. Nucleosome Dynamics**

Nucleosome, by its genome protective role, antagonizes the transcription of underlying gene. A specific genomic locus covered by a nucleosome may not be accessible for the binding by various transcription factors and the transcription machinery. Therefore, in order to express a gene whose regulatory elements are protected by nucleosomes, nucleosome remodeling factors modulate DNA-histone interactions to facilitate the action of regulatory factors.

Nucleosomes are constantly assembled, disassembled and remodeled during cellular processes such as transcription and replication. For these processes, histone chaperones and nucleosome remodelers are playing their roles in the nucleosome dynamics.

#### **I.1.3.1. Histone Chaperones**

In nucleosome core particles, the high basicity of core histones is neutralized by the phosphate backbones of DNA (Luger et al., 1997). However, when histones are free from DNA, the highly basic nature of these proteins could cause their aggregation, as seen in the cases of deregulated histone supplies (Groth et al., 2005; Kim et al., 1988; Meeks-Wagner and Hartwell, 1986; Prado and Aguilera, 2005). Therefore, before their deposition into the nucleosome and after their dissociation from the nucleosome, histones must be handled by factors capable of preventing their aggregation. The term of histone chaperone is used to design a group of proteins regulating non-assembled histones, present as soluble histone pools, regulating nucleosome assembly and disassembly. The activity of histone chaperones is crucial for correct genome functions such as DNA replication, repair and transcription all of

which also require the maintenance and/or the reassembly of proper epigenetic landscape.

The first histone chaperone identified is Nucleoplasmine. The finding that cytoplasmic fractions of oocytes from *X. laevis* and *Drosophila* are capable to assemble nucleosomes on exogenous DNA (Adamson and Woodland, 1974; Laskey et al., 1978; Nelson et al., 1979) lead to identification of Nucleoplasmin, which was isolated from ooplasm extracts of *X. laevis* (Earnshaw et al., 1980). In *X. laevis* oocytes, two types of histone chaperones act on the regulation of soluble histones, which include nucleoplasmin, which pools H2A/H2B dimers, and the protein N1/N2, which were later identified as NASP, which handles H3/H4 dimers (Cook et al., 2011; Kleinschmidt and Franke, 1982; Kleinschmidt et al., 1985). Nucleoplasmin is also a chaperon for linker histones, as shown by a study demonstrating the efficient removal of somatic variant H1 and H1<sup>o</sup> from somatic nuclei incubated in *Xenopus laevis* oocyte extracts (Dimitrov and Wolffe, 1996).

Nucleosome assembly protein (NAP) is a family of histone chaperones involved in the deposition or exchange of H2A-H2B dimers, including human NAP1Ls and yeast Vps75 (Selth and Svejstrup, 2007). Nap1p, a member of NAP in yeast, preferentially binds to H2A-H2B dimers in vivo (Mosammaparast et al., 2002), although it can also bind H3-H4 tetramers in vitro (Andrews et al., 2008). Nap1 regulates H2A-H2B import by shuttling between the nucleus and the cytoplasm (Mosammaparast et al., 2002). Functioning with ACF (ATP-dependent chromatin and remodeling factor), Nap1 is involved in the deposition of H2A-H2B and H3-H4 to form regularly spaced nucleosome (Ito et al., 1997; Torigoe et al., 2011). Nap1 also disrupts unnecessary DNA-histone interactions (Andrews et al., 2010). A recent structural study demonstrated that the elimination of unproductive DNA-histone binding is achieved by oligomerized  $\gamma$ NAP1 binding and masking the DNA interacting region of H2A (Aguilar-Gurrieri et al., 2016). NAP1 is also involved in the nucleosome remodeling which takes place during transcription, allowing the passage of RNA polymerase II during the elongation and maintaining the nucleosome density even after the passage (Kuryan et al., 2012).

FACT is another histone chaperone that could regulate the handling of H2A-H2B, and consists of two subunits: Spt16 and SSRP1 (Belotserkovskaya et al., 2003).

The presence of FACT within chromatin increases its sensitivity against endonuclease *in vitro*, which led to the suggestion that FACT could alter DNA-histone interactions, although without complete removal of histones (Xin et al., 2009). Recently, it has been found that the heterodimer of FACT simultaneously binds to two H2A-H2B dimers through highly conserved acidic regions, which are located at the C-parts of each subunit (Kemble et al., 2015). In addition, a recent structural study showed that FACT invades the nucleosome by displacing H2A-H2B dimers, while keeping the displaced dimers tethered to the proximity of the evicted nucleosome, for later re-assembling (Tsunaka et al., 2016).

CAF-1 (chromatin assembly factor-1) is involved in the *de novo* histone deposition into nucleosome (Smith and Stillman, 1989), initially detected in SV40 DNA replication assay with cytosolic and nuclear extracts (Stillman, 1986; Stillman and Gluzman, 1985). The newly synthesized DNA is assembled into nucleosomes immediately after its synthesis by CAF-1, depositing H3/H4 dimers (Stillman, 1986; Stillman and Gluzman, 1985). CAF-1 is a large complex composed of several subunits; p150, p60 and p48 (Kaufman et al., 1995). p150 subunit interacts with PCNA, an essential factor for the replication, and this association is crucial for the DNA synthesis coupled nucleosome assembly.

Histones can also be deposited into chromatin independently from DNA replication. H3.3 is a DNA replication-independent variant of H3, which is incorporated throughout the interphase by the histone chaperone HIRA (Ray-Gallet et al., 2002, 2011; Tagami et al., 2004). In addition, H3.3 deposition is also carried out by another histone chaperone DAXX (Goldberg et al., 2010). DAXX interacts with a member of the Swi2/snf2 chromatin remodeler family, ATRX, which recruits the histone chaperone to the specific loci (Drané et al., 2010; Goldberg et al., 2010). HIRA dependent and DAXX dependent deposition seem to occur at different positions (Drané et al., 2010; Goldberg et al., 2010). In *Xenopus*, *Drosophila* and mouse, HIRA-dependent H3.3 incorporation into paternal genome is essential for embryogenesis (Inoue and Zhang, 2014; Loppin et al., 2005; Szenker et al., 2012). Paternal genome in mature sperm is initially packaged in protamine-based chromatin (see section I. 2. 1. Mission of the male germ cells and its unique genome compaction). After fertilization the proteins organizing paternal chromatin are rapidly



removed in exchange with maternal ones including H3.3. In the mouse, blocking the H3.3 incorporation by H3.3 or HIRA depletion causes failure of other histone incorporations, resulting in the malformation of paternal pronucleus devoid of nucleosomes (Inoue and Zhang, 2014).

Centromere is epigenetically defined by the nucleosome-containing specific H3 variant CENP-A in human and mice (Black and Bassett, 2008). The incorporation of this variant is also regulated by the unique histone chaperone HJURP (Black and Bassett, 2008). The incorporation of CENP-A by HJURP occurs in late telophase to early G1 phase (Jansen et al., 2007), implying that CENP-A is not immediately assembled after DNA synthesis. It has been suggested that H3.3 could be incorporated and act as a placeholder in those regions until CENP-A is incorporated (Dunleavy et al., 2011).

#### **I.1.3.2. Chromatin remodelers**

Although the nucleosome is a dynamic entity, the cumulative effect of weak interactions between core histones and DNA creates a relatively stable interaction within the nucleosome. Overall the function of nucleosome remodelers is to disrupt, assemble, or slide the nucleosome, using energy from ATP hydrolysis. Nucleosome remodelers could affect both the local (nucleosome level) or global (3D structure of chromatin fiber) properties of the chromatin.

The term “chromatin remodeler” refers to a large protein family. The representative chromatin remodelers could include Swi/Snf, ISWI, CHD, Mi2, INO80 and Swr1. These complexes all contain remodeling ATPase subunits, which often function in large complexes. While ISWI forms a relatively smaller complex with 2 to 4 interacting proteins, INO80 and Swi2 forms much bigger complexes with many more subunits.

Swi/Snf is one of the most studied remodelers (Hargreaves and Crabtree, 2011). Initially identified in *Drosophila*, purified Swi/Snf can disrupt nucleosome and expose the transcription factor binding sites (Vignali et al., 2000).

ISWI is present in several different nucleosome remodeling complexes as an ATPase subunit. Examples of ISWI-containing complexes include NURF, ACF and CHRAC (Ito et al., 1999; Tsukiyama et al., 1995; Varga-Weisz et al., 1997).

Nucleosome remodelers are able to “detach” the DNA from the core histones by mechanisms involving a direct interaction with the DNA double strand and a conformational change of the remodeler itself via its ATPase activity (Flaus and Owen-Hughes, 2011; Gangaraju and Bartholomew, 2007; Racki and Narlikar, 2008; Saha et al., 2006).

While TSS of housekeeping and widely transcribed genes are often depleted of nucleosomes, other genes with a tightly regulated patterns of expression tend to rely on chromatin remodelers for their transcription (Cairns, 2009; Rach et al., 2011). In many cases, sequence specific transcription factors associate with nucleosome remodelers to clear the promoter region of their target genes (Clapier and Cairns, 2009; Fry and Peterson, 2001; Vignali et al., 2000). During gene transcription, the remodelers also assist RNA polymerase progression by evicting the histones (Schwabish and Struhl, 2007). RNA polymerase pausing induced by the nucleosome could be relieved by Swi/Snf remodelers (Vignali et al., 2000).

Since chromatin remodelers could slide the nucleosomes, they could affect the gene transcription by either activating or repressing a gene respectively through exposing or in contrast, covering its regulatory region with nucleosomes (Hargreaves and Crabtree, 2011). The action of Swi/Snf on certain genes could be repressive (Hargreaves and Crabtree, 2011; Martens and Winston, 2003). NuRD complex containing Mi2 plays an important role in the maintenance of heterochromatin. NuRD is recruited by MeCP1, linking chromatin remodeling, histone deacetylation and DNA methylation (Kunert and Brehm, 2009). ISWI involved in nucleosome remodeling could also slide nucleosomes onto a promoter to repress the gene expression of the corresponding gene (Whitehouse and Tsukiyama, 2006).

Chromatin remodelers could interact with histones through certain histone reading domains. For example, CHD chromatin remodelers have tandem chromodomains capable of binding methylated histones (Brehm et al., 2004). The

chromodomains of CHD1 specifically bind methylated H3 at K4, which is an active transcription mark (Hargreaves and Crabtree, 2011).

Chromatin remodelers often cooperate with histone chaperones as well. One of the well-known examples is the H2A.Z variant incorporation by SWR1 type of remodelers. H2A.Z is deposited at the transcription start sites of actively transcribed genes (Altaf et al., 2009; Morrison and Shen, 2009). Human SRCAP and p400/TIP60 complex, could also incorporate H2A.Z at specific loci (Gévry et al., 2007, 2009).

H2A.X is phosphorylated by ATM kinase in response to the DNA damage, and the phosphorylated H2A.X ( $\gamma$ H2A.X) recruits remodelers and double strand break repair machineries (Talbert and Henikoff, 2010).  $\gamma$ H2A.X interacts with Arp4, a subunit participating to various remodeling complexes including NuA4, SWR1 and INO80, contributing to the repair process by recruiting those complexes to the site of DNA damage (Altaf et al., 2009).

#### **I.1.4. Functional aspects of epigenetic marks: examples of histone acetylation**

Among the currently known histone PTMs, histone acetylation is one of the most characterized modifications. In this section, specific players (writers, readers and erasers) of histone acetylation-dependent signaling will be presented and their function discussed. Acetylation is carried out by Histone AcetylTransferases (HATs) (“writers”), and the acetylated histones are bound by the “reader” domain containing proteins. The bromodomain is the best known acetyl lysine recognizing domain. Finally, acetylated histones are deacetylated by Histone DeAcetylases (HDACs) (“erasers”).

##### **I.1.4.1. Writers: histone acetyltransferases (HATs)**

Histone acetylation is one of the most studied histone PTMs. It is the first histone modification that was found correlated with transcriptional activity (Allfrey et al., 1964; Pogo et al., 1966). Acetylated histones were found enriched in DNaseI

sensitive area (Sealy and Chalkley, 1978; Vidali et al., 1978) and in transcriptionally active genes. Indeed using antibody raised against epsilon-N-acetyl lysine to capture acetylated histones from chicken erythrocytes, led to the detection of the actively transcribed gene, alpha D globulin (Hebbes et al., 1988). Regarding the structural aspects, it was observed that acetylation on histone H4 amino tail reduces the affinity of the histone tail to DNA through neutralizing the positive charge of the histone lysines, suggesting that histone acetylation could have some structural roles (Norton et al., 1989), and modulate the accessibility of transcription factors for the transcription of underlying genes (Lee et al., 1993; Vettese-Dadey et al., 1996).

Although histone acetylation was first detected on lysine residues at the N-terminal tails, it is now clear that lysine residues in the core domain could also be subjected to acetylation. The modification is catalyzed by a large group of enzymes called histone acetyltransferases (HATs), which can transfer an acetyl group from acetyl-CoA to substrates. HAT could be categorized into five subfamilies: HAT1 (also called KAT1), GCN5/PCAF, MYST, p300/CBP and Rtt109a. While the first three have human and yeast homologs, p300/CBP is a metazoan specific members, and Rtt109a is a fungal-specific gene, although these two show some similarities in their catalytic core structures (Marmorstein and Zhou, 2014).

## **HAT1**

Yeast HAT1 (yHAT1) is the first HAT whose structure was identified (Dutnall et al. 1998), and became the basis of understanding the structures of other HATs. The core region of the catalytic domain consists of three-stranded beta-sheet and a long helix running in parallel (Neuwald and Landsman, 1997). All HATs share structural identity in their core regions, but the N- and C-side regions flanking the core region are different, creating a variety of acetyl-lysine binding pockets with unique clefts hence providing a diversity required for ligand binding specificity.

Newly synthesized histones are acetylated by Hat1 (Parthun et al., 1996; Sobel et al., 1995). Specifically, H3-H4 dimers are associated with the Hat1- RbAp46 complex and acetylated at H4K5 and K12 (Ejlassi-Lassalette et al., 2011; Parthun et al., 1996). The Hat1- RbAp46-H3-H4 complex interacts with the histone chaperone

Asf1 and importin 4 (Campos et al., 2010). Histone acetylation is important for their interaction with importin 4, hence regulating the nuclear import of newly synthesized histones (Ejlassi-Lassalette et al., 2011).

## **GCN5**

The second HAT, GCN5, was originally identified from *Tetrahymena thermophile*, as an orthologue of yeast transcription regulator Gcn5 (Brownell et al., 1996). Gcn5 is responsible for the acetylation of several lysine residues, including H3K4, K9, K14, K18, K27 and K36. H3K4 acetylation is also induced by a yeast specific HAT, Rtt109a, in budding yeast, and it marks the promoter of actively transcribed genes, just upstream of H3K4me3, a conserved active gene mark in human (Guillemette et al., 2011). H3K9 and K18 acetylations are carried out by a Gcn5-containing 1.8 mDa-complex SAGA, which is made of Ada proteins, Spts, and TBPs. Ada and Gcn5 also make their own complex without the other components (ADA), which further diversifies the substrate specificity (Grant et al., 1999). K14 acetylation is also catalyzed by Gcn5, stimulating gene transcription (Brownell et al., 1996; Kuo et al., 1996). H3K36 is another acetylation site regulated by the Gcn5-SAGA complex; the mark is mutually exclusive with H3K36me, a repressive mark (Morris et al., 2007).

## **PCAF**

PCAF is a paralog of GCN5, which was initially identified through its ability to bind CBP/p300 (Yang et al., 1996). The preferential substrate for PCAF is H3, although it could acetylate all core histones *in vitro* (Yang et al., 1996). PCAF is also one of the first HAT found to acetylate non-histone substrates including p53 (Liu et al., 1999; Sakaguchi et al., 1998). Similar to GCN5, PCAF forms transcription coactivator complexes, SAGA and ATAC. GCN5 or PCAF are mutually exclusive subunits of these complexes (Nagy et al., 2010). While in yeast there is SAGA complex with GCN5, in the human there is a variety of SAGA-like complexes including SAGA with GCN5 or PCAF, and ATAC with GCN5 or PCAF (Spedale et

al., 2012). SAGA is enriched in gene promoter regions, while ATAC is recruited to promoters and enhancer regions, and these two related complexes play different roles in the regulation of gene expression (Krebs et al., 2011).

### **CBP/p300**

p300 and CBP are two related proteins, initially characterized as E1A binding protein and CREB Binding Protein, respectively (Chrivia et al., 1993; Eckner et al., 1994). The two proteins share highly conserved domain organizations (Kalkhoven, 2004), including CH1, KIX (CREB binding domain), CH3 (ZZ-TAZ2), steroid coactivator-1 interaction domain (SID) and NCDB domains. Their catalytic core includes the bromodomain, CH2 domain (containing PHD domain) and HAT domain. The bromodomain in the catalytic core is thought to be essential for the HAT activity of CBP and P300 (Kraus et al., 1999; Manning et al., 2001; Tomita et al., 2000). The PHD domain integrated in the enzymatic core domain cooperates with the bromodomain in binding hyperacetylated histones (Ragvin et al., 2004). CBP and P300 interact with the basal transcription factors such as TATA-binding proteins (TBP) and TFIIB, forming a complex with RNA polymerase II (Cho et al., 1998; Kwok et al., 1994; Nakajima et al., 1997a, 1997b; Neish et al., 1998; Yuan et al., 1996). CBP and p300 acetylate nucleosomal histones at gene promoters, increasing the accessibility of the corresponding DNA for other factors (Bannister and Kouzarides, 1996; Kundu et al., 2000; Ogryzko et al., 1996). In addition, CBP and p300 acetylate other proteins than histones, modulating gene transcription (Chen et al., 1999; Martínez-Balbás et al., 2000; Munshi et al., 1998; Soutoglou et al., 2000; Sterner and Berger, 2000; Yang, 2004). Examples of non-histone proteins interacting with CBP/p300 include PCNA (Hasan et al., 2001a), Fen1 (Hasan et al., 2001b), DNA polymerase beta (Hasan et al., 2002) and thymidine DNA glycosylase (Tini et al., 2002), all involved in DNA repair, in which the latter three could be acetylation substrate. Cdk2 involved in the cell cycle regulation (Ait-Si-Ali et al., 1998, 2000; Perkins et al., 1997), MDM2 catalysing p53 degradation (Grossman, 2003; Grossman et al., 1998), and importin alpha 1 isoform Rch1 and importin 7 are also reported to be interacting proteins with CBP and p300 (Bannister et al., 2000). In the end, CBP and p300 are involved in varieties of different transcriptional coactivator complexes

(Goodman and Smolik, 2000; Vo and Goodman, 2001). Despite their high degree of similarity in the critical domains, and their high functional redundancy in many cases, these two proteins could have different roles. In a mouse model, heterozygous inactivation of p300 results in the abnormal development of heart, lung and small intestine (Shang et al., 2000; Soutoglou and Talianidis, 2002). In comparison, heterozygous inactivation of CBP could lead to growth retardation and craniofocal abnormalities (Cosma et al., 1999). There are also human clinical cases characterized by mutations in these proteins. Human CBP mutation is a cause of Rubinstein-Taybi syndrome (Arany et al., 1994; Jason et al., 2002), while p300 mutation was found in many cancers exhibiting aberrant level of acetylation (Kwok et al., 1994; Partanen et al., 1999; Yuan et al., 1996). Therefore, these two HATs have similar properties but proper dosage of each is essential for the normal cellular activity.

## **MYST**

The largest subgroup of HATs is the MYST family, named after the initial factors identified within this category, MOZ, Ybf2/Sas3, Sas2, and TIP60. According to functional considerations and domain sequence similarities, mammalian MYSTs could be briefly categorized into three groups: TIP60/MOF, MOZ/MORF, and HBO1. In other organisms, zebra fish lacks Hbo1-like members, and yeast has only a MOF/TIP60 type, which is Esa1. All members of the MYST family share a conserved catalytic MYST domain, which by itself is capable to acetylate histones *in vitro* (Champagne et al., 1999, 2001; Kitabayashi et al., 2002; Thomas et al., 2000; Yamamoto and Horikoshi, 1997). MYST members are involved in many cellular processes through histone acetylation such as transcription, DNA replication and repair. In addition, many of the MYST members are capable of acetylating non-histone proteins similar to other HATs. For example, p53, ATM and Myc can be acetylated by TIP60 (Patel et al., 2004; Sun et al., 2005; Sykes et al., 2006; Tang et al., 2006), and MOF can acetylate p53 (Sykes et al., 2006) regulating its interaction with ATM in the regulation of cellular proliferation and apoptosis (Gupta et al., 2005). This wide range of substrates shows that MYST members are involved in diverse cellular functions.

MYST functions in large complexes formed by other proteins. Yeast Sas3 and Esa1 form NuA3 and NuA4 complexes, respectively (Doyon et al., 2004; John et al., 2000). TIP60 is a human protein equivalent to the yeast Esa1, and the complex equivalent to NuA4 is also found in human (Doyon et al., 2004). MOZ and MORF participate to the ING5 complex, and HBO1 is a part of either ING4 or ING5 (Doyon et al., 2006; Ullah et al., 2008). All MYST members are ubiquitously expressed at moderate levels in all cell types with some expression level variations according to the types of organs. For example, TIP60 and MOF were reported to be highly expressed during spermatogenesis (Thomas et al., 2007).

## **MOF**

MOF is one of the first MYST member reported for its loss-of-function effect in a study screening essential genes for X-chromosome dosage compensation in drosophila (Hilfiker et al., 1997). In Drosophila, males up-regulate the expression of X-linked genes in order to compensate for gene dosage, in contrast to Mammals where the X-chromosome is inactivated in female individuals. *Male absent on first* (MOF) null mutant flies fail to induce the up-regulation of X-chromosome gene expression, due to the unsuccessful recruitment of the dosage compensation complex MLE (*Maleless*) as well as to a lack of H4K16 acetylation. Mof is indeed able to directly acetylate H4K16 (Akhtar and Becker, 2000), and this mark is essential for relieving repression and stimulating the expression of the X chromosome (Akhtar and Becker, 2000). In contrast, H4K16ac is not present on the mammalian inactive X chromosome.

Mof is also essential for embryonic development. In mice, Mof depletion causes embryonic lethality at the blastocyst stage with a lack of H4K16ac (Thomas et al., 2008). It is worth mentioning that acetylation at other residues such as H3K9, K14, H4K5, K8 and K12 are unaffected, suggesting that Mof is highly specific for acetylating H4K16. Mof depletion eventually leads to apoptosis after exhibiting severe chromatin condensation anomalies, suggesting the importance of H4K16 acetylation catalyzed by Mof for the maintenance of open chromatin states during embryonic development.



Esa1 is a Mof-related MYST in *S. cerevisiae*. Esa1 mutants exhibit cell cycle arrest, which is rescued by depletion of a DNA damage checkpoint protein rad9 (Clarke et al., 1999; Smith et al., 1998), indicating that Esa1 could be important for maintaining genome integrity. In addition, another yeast MYST protein, Sas2, was found to be a HAT also acetylating H4K16. Several studies showed that Sas2 acetylates H4K16 at the euchromatin and heterochromatin boundaries, counteracting the action of the HDAC Sir2p, which deacetylates this residue (Kimura et al., 2002; Suka et al., 2002). The interplay between Sas2 and Sir2p is essential in order to maintain the interphase of transcriptionally active and inactive chromatin regions.

### **TIP60**

TIP 60 was initially identified as an interactor of the HIV-1 Tat protein, “Tat interactive protein, 60 kDa” (Kamine et al., 1996). TIP60 forms NuA4 complex in mammals, a complex initially discovered as Esa1 containing complex in yeast (Bird et al., 2002). DNA double strand break repair is one of the processes in which TIP60 plays an essential role (Ikura et al., 2000; Kusch et al., 2004), similar to the role of Esa1 (Bird et al., 2002).

TIP60 is an essential factor for embryonic development, since TIP60 depletion causes embryonic lethality before the implantation (Gorrini et al., 2007). In *C. elegans*, TIP60 mutation causes premature cell cycle exit and differentiation (Ceol and Horvitz, 2004), thus this member of MYST is important for regulating cellular proliferation and differentiation. Indeed, in a study of an RNAi screening revealed that TIP60 is essential for the establishment of pluripotency in mouse ESCs (Fazzio et al., 2008).

The TIP60 complex is also involved in acetylation-coupled nucleosome remodeling. One of the subunits in the complex P400 complex is an ATPase-containing chromatin remodeling protein that is essential for the incorporation of the histone H2A variant H2A.Z at chromatin regions undergoing a dynamic turnover of nucleosomes, such as nucleosomes on the TSS of transcriptionally active genes (Gévry et al., 2007). Prior to the incorporation of H2A.Z, the TIP60 complex acetylates H4 and H2A in order to loosen chromatin and “prepare” the nucleosome for the histone

exchange (Altaf et al., 2010). Other H2A variants H2A.X and H2A.V in *Drosophila* are also substrates of TIP60, playing important roles in DNA damage response (Ikura et al., 2007; Kusch et al., 2004). Recently, in contrast with the general view of the acetylation as a transcription stimulating signal, a role of TIP60 in the maintenance of pericentric heterochromatin has been reported. In a mouse model, when Suv39h1 is absent, TIP60 acetylates H4K12 at the heterochromatin of pericentromere region, and this acetylation is essential for the recruitment of the bromodomain containing protein Brd2 (Grézy et al., 2016). The heterochromatin compaction is normally induced and maintained by Suv39h1 that methylates H3K9 and the heterochromatin protein HP1 $\alpha$  recruited to the methylated histone (Peters et al., 2001). Therefore, this TIP60 and Brd2 dependent pericentric heterochromatin maintenance could be thought as a backup system, in case the canonical Suv39h1 pathway is absent, which is seen in some human cancers (Dialynas et al., 2008; Khanal et al., 2013). Pericentromere regions consist of major satellite repeats whose expression is strictly regulated. Brd2 recruitment through TIP60 catalyzed H4K12 acetylation could be important for maintaining the repressive state of the satellite region in absence of Suv39h1 (Grézy et al., 2016).

#### **I.1.4.2. Readers: bromodomains and extraterminal domain family (BETs)**

Bromodomains (BDs) are the first protein module identified to be able to recognize and bind histones modified by lysine acetylation (Dhalluin et al., 1999; Sanchez and Zhou, 2009). In 1999, the bromodomain of P/CAF was reported to have specific binding affinity towards acetylated histones H3 and H4 (Dhalluin et al., 1999). Bromodomains are found in HATs, chromatin remodelers, transcriptional coactivators and many other nuclear proteins (Marmorstein and Zhou, 2014). In human, it is known that there are 46 BD containing proteins with 61 different BDs (Filippakopoulos et al., 2012) (Figure H), suggesting their diverse functions based on the signalization of acetyl-lysine recognition.

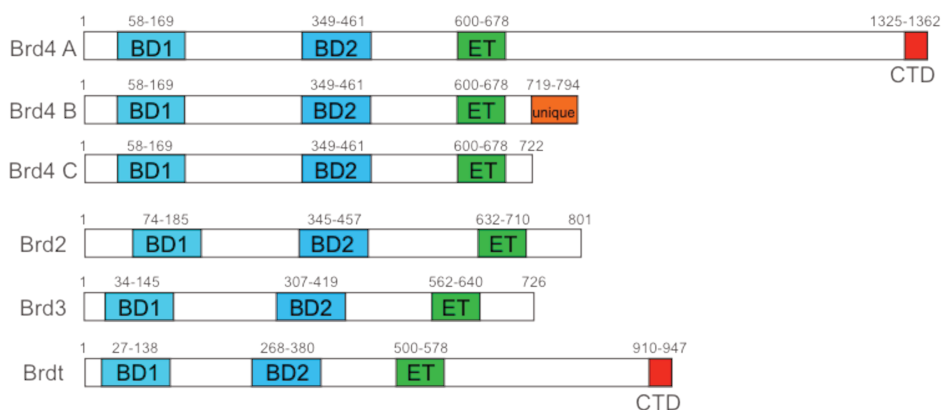
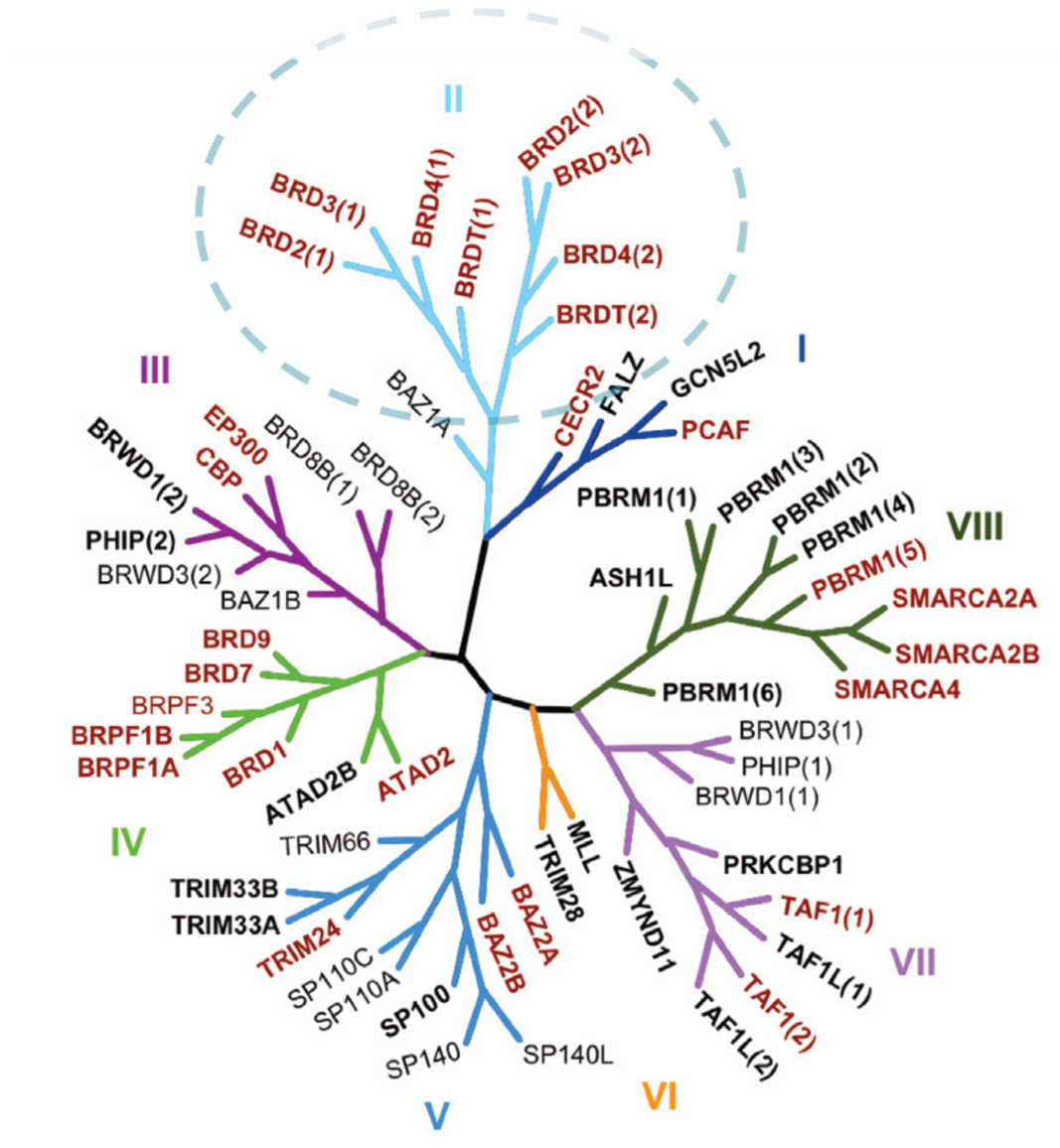
#### **I.1.4.2.1. BET family members**

Among these, the members of the Bromodomain and extraterminal domain (BET) family are presently the most intensely studied BD containing proteins. The BET family is characterized by the two consecutive bromodomains (BD1 and BD2) normally located at the N-part of the protein, and another conserved region named the extra terminal domain (ET) located at either the central region or the C-terminus, which is involved in crucial protein-protein interactions in many cases (Figure H). The other domains that are shared by most but not all BET members include motifs A and B, Ser/Glu/Asp rich SEES domain, and the carboxy terminal motif (CTM). Representative members of the BET family are human BRD2, BRD3, BRD4 and testis specific BRDT, as well as yeast Bdf1/2 and drosophila Fsh. One of the notable characteristics of these BET proteins is their consistent association with chromatin both during the interphase and mitosis (Chua and Roeder, 1995; Dey et al., 2003; Kanno et al., 2004; Pivot-pajot et al., 2003), which might play a role in ensuring immediate gene expression control after cell division (Dey et al., 2009; Mochizuki et al., 2008; Yang et al., 2008).

#### **BRD4**

BRD4 is known for its transcriptional function through its binding to H4K5ac, activating transcription by decondensing chromatin (Zhao et al., 2011). After mitosis, previously activated genes are labeled by H4K5ac, and become re-activated by the recruitment of BRD4 immediately after mitosis. BRD4 is also involved in the viral gene regulation (Zhou et al., 2009) and presents an anti-proteosomal degradation effect on human papillomavirus E2 protein (Gagnon et al., 2009).

BRD4 encodes 2 short isoforms (isoform B and C). Compared to the full-length isoform A, both short isoforms lack the CTM which is important for its interaction with P-TEFb (Floyd et al., 2013). Interestingly, the isoform B, possessing a stretch of 75 amino acids at its C-terminal divergent from other isoforms, has a critical effect on the chromatin compaction upon the DNA damage, attenuating DNA damage response pathway (Floyd et al., 2013).

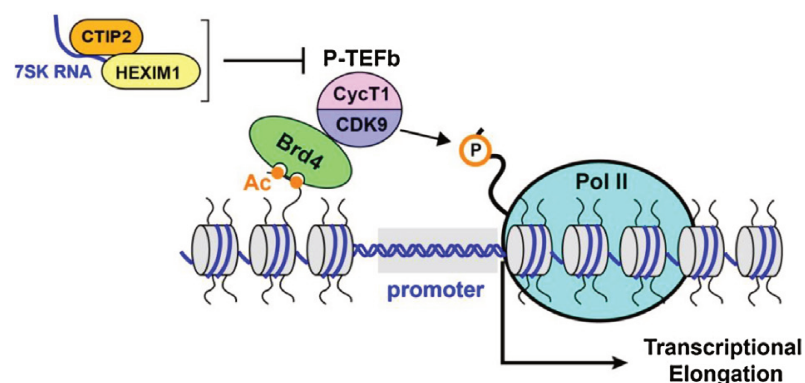


**Figure H:** Members of the human BET family. (top) Phylogenetic tree of human bromodomain (image from: Ferri et al., 2016). Major BET members are marked by blue circle. (bottom) Domain architecture of BET factors (image from: Shi and Vakoc, 2014).

BRD4 is also known for its association with general transcriptional cofactors such as Mediator complex (Thomas and Chiang, 2006) and P-TEFb (Jang et al., 2005; Yang et al., 2005).

The bromodomains of BRD4 binds to H3K14ac, or dual-acetylated H4 at K5 and K12 (Dey et al., 2003). In addition to histones, the substrates of BRD4 include non-histone proteins such as a subunit of the RelA complex NFkappaBeta which interacts with BRD4 in an acetylation dependent manner (Huang et al., 2009). Although the bromodomain itself is able to bind to acetylated histones, the recruitment of BET protein is often associated with other proteins that mediate additional protein-protein or protein-DNA interaction. The RelA-BRD4 interaction is important for the binding of the complex to acetylated H3, representing the commitment step of BRD4-regulated gene transcription (Wu and Chiang, 2007).

BRD4 mediated P-TEFb recruitment to the target genes such as JunB, C-Myc, Cdk7 and cyclin D1 occurs throughout anaphase to early G1 phase (Yang et al., 2008). As shown in BRD4 knock down studies, many of the genes expressed during the G1 phase are activated in a BRD4-dependent manner (Mochizuki et al., 2008). The recruitment of P-TEFb by BRD4 is regulated by the acetyl-histone recognition by its bromodomains (Figure I). So far, it has been reported that BRD4's recruitment of P-TEFb occurs with two patterns of histone PTM combinations; H4K5/K8/K12ac (Hargreaves et al., 2009), or H3K9ac/H3S10ph inducing H4K16ac (Zippo et al., 2009). The existence of different histone marks for the BRD4-P-TEFb recruitment



**Figure I:** BRD4's function during transcription via P-TEF recruitment. (image from: Ferri et al., 2016).

could be attributed to the fact that i/ these residues could be acetylated by several HATs; for example, CBP/P300 for H3K14 and H4K5/K8ac, P/CAF for H3K9/K14ac, TIP60 for H3K14, and MOF for H4K16 (Kouzarides, 2007; Rea et al., 2007), and ii/ the types of HATs that are available could be different depending on the cellular context. Therefore, the use of several pathways to recruit BRD4-P-TEFb could assist maintaining consistent transcriptional activity.

## **BRDT**

BRDT is the testis-specific member of the BET family, possessing similar domain organization and characteristics as BRD4. BD1 of the BRDT specifically recognizes histone H4 acetylated at K5 and K8 which is itself a signature of hyperacetylated H4 (Morinière et al., 2009). Indeed, this particular bromodomain of BRDT enables it to specifically bind and act on hyperacetylated chromatin, as demonstrated in a study revealing chromatin remodeling activity of ectopically expressed murine Brdt in somatic cells after HDAC inhibitor TSA treatment (Pivot-pajot et al., 2003). In later studies, Brdt was shown to be an essential factor involved in i/ regulating specific gene expression during the meiotic and post-meiotic stages of spermatogenesis, as well as in ii/ driving hyperacetylation-dependent histone to protamine replacement (Gaucher et al., 2012). More details about the function of BRDT is discussed in the section I.2.3.2 “BRDT: a testis specific BET”.

## **Other proteins containing bromodomains**

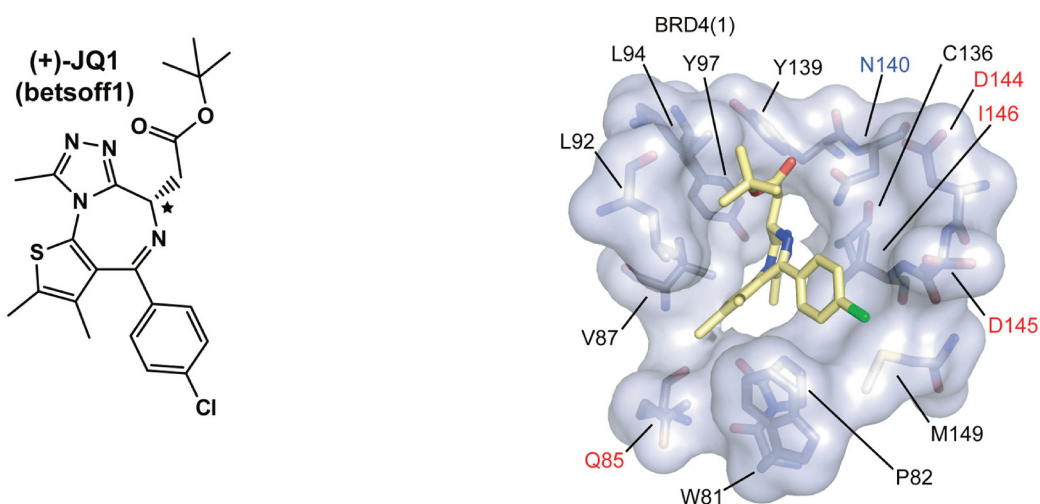
The other members of the BET family also play critical functions in controlling various cellular activities. BRD3 interacts with the hematopoietic transcription factor GATA1 in an acetylation specific manner, to regulate transcription of essential genes for erythroid maturation (Lamonica et al., 2011). GATA1-regulated gene expression indeed requires its association with BRD3. Another BET protein, BRD2, has recently been reported to have an important role in the maintenance of pericentric heterochromatin in the absence of Suv39h1. TIP60 catalyzed histone H4 acetylation at K12 is bound by the bromodomain of BRD2, inducing the chromatin compaction to silence the satellite transcription (Grézy et al., 2016).

Along with the rapid discovery of new types of histone acyl-modifications occurring on lysine residues similar to acetylation, Flynn et al. investigated the binding capacities of diverse human bromodomains to a variety of acylated lysines. The study revealed great diversity of bromodomains binding to acyl-lysines. Notably, BRD9, CER2 and the second BD of TAF1 showed significant binding affinity toward butyryl-lysine (Flynn et al., 2015). In parallel to the newly-identified histone PTMs, the diversity of PTM readers is also expanding. A recent study reported that YEATS domain of AF9 is an acetylated histone binder, specifically interacting with acetylated histone H3 at K9 (Li et al., 2014), and more interestingly with crotonyl H3K9 (Li et al., 2016), whose binding could be linked with transcriptional stimulation (Sabari et al., 2015). Bromodomains could also read a combination of histone acylations as it has been recently shown for Brdt's BD1, which is capable of recognizing H4K5acK8bu (Goudarzi et al., 2016).

#### **I.1.4.2.2. BET inhibitors**

The diverse functions of the BD containing proteins and BETs, as well as their potential to interact with acetylated- (and acylated-) histone or non-histone proteins, make these factors attractive therapeutic target candidates. Indeed, the inhibition of the bromodomains is becoming an actively studied approach for the cure of various diseases. Several members of the BET family are known to be deregulated in many pathological conditions (For review see Belkina & Denis 2012). The first potent BET inhibitors, JQ1 (Figure J) and iBET, were discovered in 2010 (Filippakopoulos et al., 2010; Nicodeme et al., 2010). The efficiency of JQ1 was demonstrated in a rare squamous tumor known as NUT-Midline Carcinoma (NMC). NMC is characterized by the chromosomal translocation fusing BRD4 or BRD3 with Nuclear protein in Testis (NUT) (French, 2012). The fusion protein BRD4-NUT blocks cell differentiation and maintain the proliferative status of NMC through the generation of hyperacetylated nuclear foci by sequestration of CBP/P300 (Reynoird et al., 2010). The blocking of the bromodomain of BRD4 moiety by JQ1 successfully induced squamous differentiation and G1 cell cycle arrest (Filippakopoulos et al., 2010). One of the oncogenic mechanisms driving NMC is the up-regulation of oncogenic genes such as MYC. Indeed, BRD4-NUT is present at the MYC promoter, activating MYC

expression (Grayson et al., 2013). It has been shown that the JQ1 treatment could inhibit the binding of BRD4-NUT to the MYC promoter, and down-regulates MYC expression (Grayson et al., 2013). It is important to note that natural form of BRD4 is also present in the *MYC* promoter region, thus the down regulation of *MYC* by JQ1 treatment could be the effect achieved either through BRD4-NUT, BRD4 or both. However, it is also worth to mention that BRD4-NUT knock down by siRNA specific to *NUT* could down regulate *MYC* expression, suggesting that BRD4-NUT indeed participates to the regulation of *MYC* expression. MYC up-regulation is one of the fundamental mechanisms driving cellular transformation, and the effectiveness of JQ1 in down-regulating MYC has been observed in many other non-BRD4-NUT types of cancers (Asangani et al., 2014; Bandopadhyay et al., 2014; Baratta et al., 2015; Bihani et al., 2015; Chaidos et al., 2014; Cho et al., 2014; Da Costa et al., 2013; Dawson et al., 2011; Delmore et al., 2011; Emadali et al., 2013; Fiskus et al., 2014; Gao et al., 2013; Henssen et al., 2013; Herrmann et al., 2012; Mertz et al., 2011; Ott et al., 2012; Picaud et al., 2013; Qiu et al., 2015; Rajagopalan et al., 2014; Roderick et al., 2014; Segura et al., 2013; Shao et al., 2014; Shimamura et al., 2013; Tan et al., 2015; Tsai et al., 2015; Venkataraman et al., 2014; Wang and Filippakopoulos, 2015; Zuber et al., 2011).



**Figure J:** Structure of JQ1 and acetyl lysine binding pocket of BD1 from BRD4, bound by JQ1 (images from: Filippakopoulos et al., 2010).



#### **I.1.4.3. Erasers: histone deacetylases (HDACs)**

The removal of acetyl groups from lysine residues is catalyzed by a group of enzymes known as histone deacetylases (HDACs). HDACs are sub classified into three groups: class I, class II and NAD<sup>+</sup>-dependent class III.

The first HDAC identified was a mammalian homologue of yeast transcription repressor Rpd3 (Taunton et al., 1996). There are three proteins in mammals related to yeast Rpd3: HDAC1, HDAC2 and HDAC3. With additional members identified later, including HDAC8, these HDACs constitute the class I HDACs. All the members in class I have conserved catalytic domains, and HDAC1, 2 and 3 have quite similar sequence identity. HDAC1 and 2 act in the protein complexes Sin3 and NuRD, respectively (Knoepfler and Eisenman, 1999). HDAC3 is part of nuclear receptor corepressor complexes, SMRT and N-CoR (Guenther et al., 2000; Li et al., 2000; Underhill et al., 2000; Wen et al., 2000).

In yeast, other proteins that are related but distinct from Rpd3, include Hda1 (Rundlett et al., 1996). Since Hda1-related proteins were first identified in Grenoble (Verdel and Khochbin, 1999) as HDA1 and HDA2, they were later re-named respectively HDAC5 and HDAC6 (Grozinger et al., 1999). Later, other members were also identified in human and named HDAC4, 7 and 9 (Fischle et al., 2001; Grozinger et al., 1999; Kao et al., 2000). Although their catalytic domains have some similarities with HDAC1, their size and domain organization are distinct from the class I HDACs (Khochbin et al., 2001). Class II HDACs are not found in canonical complexes. HDAC6 is a unique member in this group (Boyault et al., 2007). Unlike other HDACs, HDAC6 has tandem catalytic domains (Grozinger et al., 1999; Verdel and Khochbin, 1999). While other class II member shuttles between nucleus and cytoplasm, HDAC6 essentially remains in the cytoplasm (Verdel et al., 2000) playing a role distinct from the other members (Boyault et al., 2007).

Class III HDACs, or Sirtuins, are the only members of HDACs using NAD<sup>+</sup> to deacetylate their substrates. It has been shown that SIRT1 is nuclear, SIRT2 localizes in cytoplasm (North et al., 2003), while SIRT3, 4 and 5 are present in mitochondria (Onyango et al., 2002; Schwer et al., 2002). SIRT6 and SIRT7 are also nuclear with important regulatory functions (Chalkiadaki and Guarente, 2012).

Examples of SIRT substrates are the enzyme acetyl Co-A Synthetases (AceCSs). While SIRT2 deacetylates cytoplasmic AceCS1, SIRT3 deacetylates AceCS2 in mitochondria (Hallows et al., 2006; Schwer et al., 2006), which both are controlling the activity of these enzymes.

The discovery of specific inhibitors certainly accelerated the understanding of histone acetylation. Trichostatin A and trapoxine are microbial metabolites that reversibly or irreversibly inhibit the HDAC activity (Kijima et al., 1993; Yoshida et al., 1995). The finding was not only used in wide applications for basic research, but also stimulated the study of drugs that targets epigenetic factors. Vorinostat, also known as SAHA, was the first FDA-approved HDAC inhibitor drug for T cell lymphoma treatment (Richon et al., 1998). Currently, more HDAC inhibitors are approved by FDA, and their applications are going beyond cancer treatment.

## **I.2. Epigenetics of male germ cells**

So far, this introduction has covered the principal components of the epigenetic information: DNA methylation, nucleosomes, histones/variants and PTMs. These elements could also play a role in the inheritance of a non-genetic information transmitted by germ cells. Here we will cover briefly our knowledge on the male germ cell epigenetic characteristics.

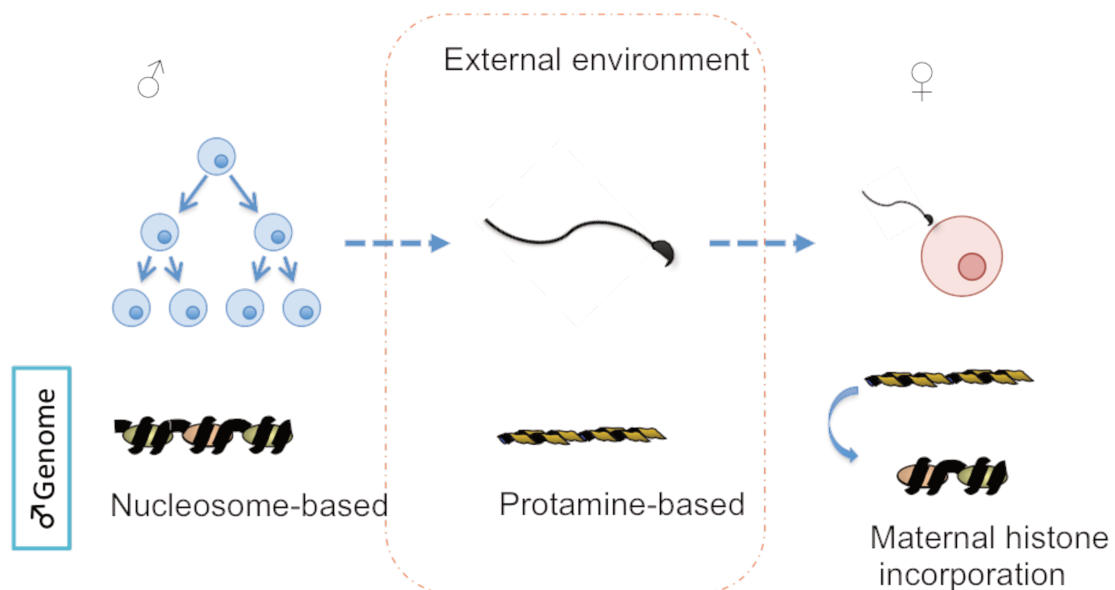
### **I.2.1. Mission of the male germ cells and its unique genome compaction**

The function of the male germ cell is to deliver the paternal genome to the oocytes. In addition to the genetic information, the sperm is also thought to deliver an important epigenetic information. For example, it has long been known that proper gene epigenetic “imprinting”, differentially established during the male and female germ cell differentiation, is essential for embryogenesis (Hammoud et al., 2010; McGrath and Solter, 1984; Trasler, 2009). There are also RNAs that are delivered by the sperm, which is suspected to be essential for the embryonic development (Jodar et al., 2013; Liu et al., 2012; Ostermeier et al., 2004).

Although the mature sperm’s genome mainly consists of DNA associated with highly basic proteins named protamines (see below), about 1%~5% of nucleosomal histones are retained in the mature sperm chromatin (Bench et al., 1996; Brykczynska et al., 2010; Gatewood et al., 1987; Hammoud et al., 2010). The nature of the genomic regions retaining nucleosomes is controversial (for review see Saitou & Kurimoto 2014). Some studies propose that these regions are regulatory elements (Arpanahi et al., 2009; Brykczynska et al., 2010; Erkek et al., 2013; Hammoud et al., 2009). Specifically, regulatory sequences of developmental genes were found enriched in retained nucleosomes (Arpanahi et al., 2009; Hammoud et al., 2009). Other studies reported that a majority of the nucleosome-retained regions were gene poor region termed “gene desert” (Carone et al., 2014), or suggested that these nucleosomal regions could include repetitive sequence area such as SINE and LINE (Samans et al., 2014). This is in agreement with data of our laboratory showing that

the mouse testis specific H2A variant, H2AL2, is found specifically at the pericentromeres of the developing spermatids and retained throughout spermatogenesis and mature sperm (Govin et al., 2007). One of the difficulties of studying the sperm chromatin is that the experimental procedure could have a large impact on the result (Saitou and Kurimoto, 2014). Therefore, where exactly on the male genome nucleosomes are truly retained, and the functional consequences of this retention, are still actively discussed scientific questions.

In addition, in order to achieve highly condensed sperm nucleus, protamines have to replace the majority of the histones. A proper assembly of sperm chromatin is essential for the function of male germ cells, fertilization, and epigenetic inheritance (Figure K). However, the highly conserved and essential process of histone-to-protamine replacement during spermatogenesis has remained one of the most obscure fundamental phenomena in reproductive biology.



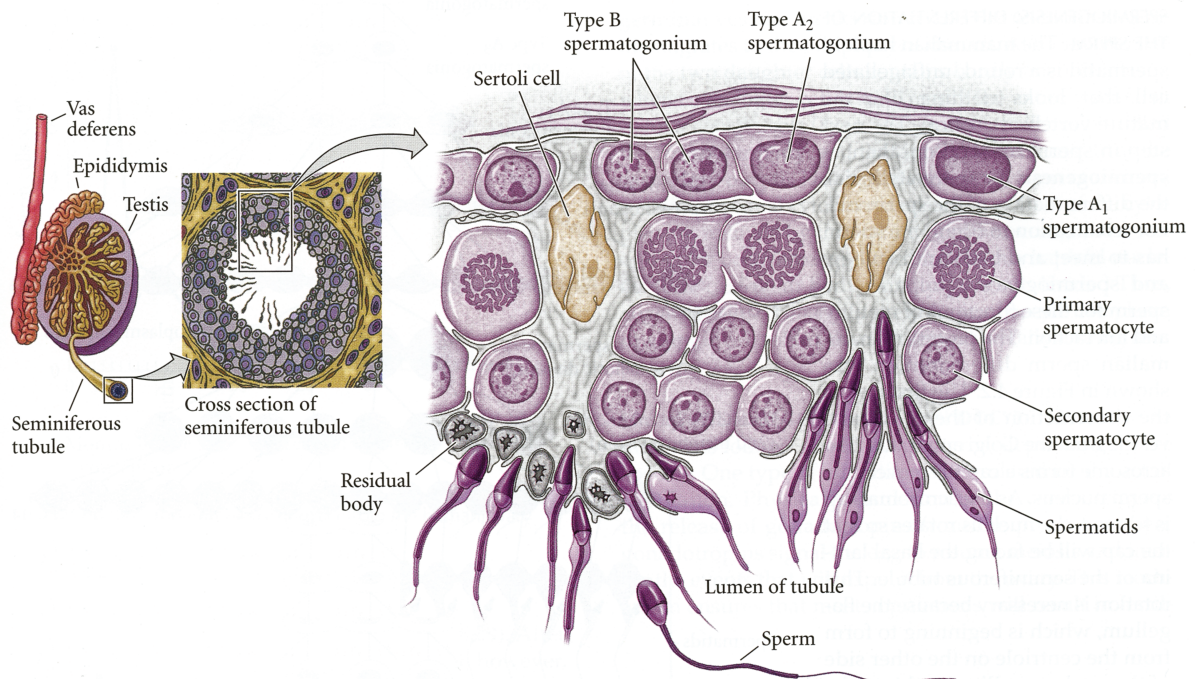
**Figure K:** inheritance of male genome through multiple chromatin reorganization events. Male germ cell epigenome must be precisely maintained throughout the constant remodeling of the chromatin during spermatogenesis and early embryonic development.

## I.2.2. Mouse spermatogenesis

### Anatomy of the mammalian testis

Spermatogenesis, the process of male germ cell differentiation, takes place in the testis (Figure L). The testis consists of seminiferous tubules delimited by a fibrous capsule, the Tunica albuginea. The amount of connective tissues within the capsule varies depending on the species. The testis contains two major compartments: interstitium, and seminiferous tubules.

The interstitium is the space between the seminiferous tubules and the capsule. It contains blood vessels and lymphatic system, as well as a few types of somatic cells. Leydig cells are the major source of androgen, mainly testosterone, which is crucial for the regulation of spermatogenesis. Macrophages are also present in interstitium. They represent about 25% of all the cells in the interstitium.



**Figure L:** Organization of spermatogenic cells differentiating in seminiferous tubules. (image from: Scott F. Gilbert. *Developmental Biology*, 9th Edition. Sinauer Associates, Inc. 2010. P609. Figure 16.27)

Seminiferous tubules are the compartment where spermatogenic development takes place. It appears as convoluted loops with two ends connected into the rete testis, a duct exiting the testis and entering the tubuli recti, which itself is a straight tube connected to the male reproductive tract. The seminiferous tubules are highly convoluted and occupy most of the testis volume. A transverse section through the long axis of the testis can produce cross-sections of the seminiferous tubule, which enables histological analysis and staging of seminiferous tubules (Figure M). The boundary of seminiferous tubule consists of the basal lamina and the peritubulomyoid cells (PTMCs).

### **Spermatogenic differentiation**

The whole process of spermatogenesis could be divided into three phases: mitotic, meiotic and post-meiotic. The spermatogenic differentiation initiates from the most primitive precursor, the spermatogonia, which are mitotically dividing diploid cells. They will generate spermatocytes which are the cells undergoing the two consecutive meiotic divisions, resulting in the production of haploid non-dividing post-meiotic cells. Post-meiotic cells will then undergo a maturation process called spermiogenesis, during which the paternal haploid genome will undergo drastic reorganization events, including histone to protamine exchange.

#### **I.2.2.1. Pre-meiosis**

Mammalian spermatogenesis could produce millions of sperm per day, and these cells are all originated from a large population of spermatogonia. There are three types of spermatogonia: Spermatogonial stem cells (SSCs), proliferative spermatogonia, and differentiated spermatogonia. SSCs and proliferative spermatogonia are considered as undifferentiated cells.

Spermatogonial stem cells (SSC), or type A-isolated spermatogonia ( $A_{is}$ ) are the starting cells of spermatogenesis. SSCs have relatively low frequency of division. Thanks to the slow growth, the SSCs are relatively resistant to a variety of insults, as

since this type of spermatogonia often survives when other spermatogonia or spermatogenic cells are depleted (Russell et al., 1990a). SSCs are the source of the whole population of spermatogenic cells, and the loss of the stem cell could lead to irreversible loss of spermatogenic capacity in the animal.

Division of SSCs produces proliferative spermatogonia. There are two forms: Type A paired ( $A_{pr}$ ) and Type A aligned ( $A_{al}$ ). During this phase, spermatogonia pair up and form intercellular connection with other spermatogonia through areas of open cytoplasmic continuity. This cytoplasmic bridges enable synchronized development in a group of spermatogenic cells.

Differentiating spermatogonia refers to the type  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ , Intermediate (In), and type B spermatogonia. Transformation from  $A_{al}$  to  $A_1$  does not involve cellular division, but a slight change in the morphology. Throughout the differentiation of spermatogonia, the cells remain attached to the basal lamina.

#### **I.2.2.2. Meiosis**

Meiosis consists of two successive divisions following one DNA replication resulting in the generation of four haploid cells from each diploid spermatocyte entering meiosis. During the prophase of the first meiotic division the chromosomes of paternal and maternal origin pair and undergo the process of meiotic recombination, a process which is essential for successful achievement of spermatogenesis.

The most mature spermatogonia (type B spermatogonia) undergo the last mitosis to generate primary spermatocytes. The first cells entering meiotic phase are preleptotene spermatocytes (Pl). These cells are the last spermatogenic cells undergoing DNA synthesis (S phase). The cells appear slightly smaller than type B spermatogonia, with less chromatin along the nuclear envelope.

The preleptotene spermatocytes differentiate into leptotene spermatocytes (L). This cell represents the initiation of a long lasting first meiotic prophase. L spermatocytes forms fine threads of chromatin, which reflect the beginning of

chromosome condensation. Recombination initiates with the formation of programmed DNA double strand breaks. This cell has a round shape, and it starts separating itself from the basal lamina and migrating toward the lumen through intermediate compartment formed by Sertoli cells.

Next, the spermatocytes enter the zygotene stage (Z). In zygotene spermatocytes, homologous chromosomes start pairing and recombining, exhibiting thick highly condensed chromosome filaments. The chromatin also shows the formation of synaptonemal complex, a chromosome bundling complex created by SYCP1, SYCP2 and SYCP3 (Moses, 1968).

When the cells become Pachytene spermatocytes (P), the chromosomes complete the full pairing. The pachytene stage lasts a relatively long time (two weeks in the rat), which helps easier detection of this particular stage in the microscopy. The last stage of chromosome recombination and meiotic cross-overs take place during this phase. Meiotic recombinations between the paternal and maternal allelic versions of each chromosome generates a combination of genes that is unique to each germ cell and not found in the somatic cells of the same animals. Towards the middle of pachytene stage, the cell actively synthesizes RNA and proteins (Monesi, 1965) and their size rapidly increase (Russel and Frank, 1978). The size of nucleus also increases, and the sex chromosomes form a specific body called sex vesicle or XY body. Nuclear materials become more widely distributed, making large area appearing empty under microscope.

The cell differentiates into diplotene spermatocytes (D), which are the largest primary spermatocytes, and the largest spermatogenic cells. The synaptonemal complex disappears from the nucleus. The chromosomes start separating, except the area which correspond to the cross-over sites (chiasma). The diplotene stage is the last stage of the long 1st meiotic prophase, which ends with the chiasma of the condensed chromosomes aligned on the first metaphasic plate. The metaphase /anaphase / telophase of meiosis I is rapid. The division produces secondary spermatocytes. Secondary spermatocytes do not last for a long time, since there is no prophase and the cells quickly undergo the second meiotic metaphase and anaphase, both stages which resemble corresponding mitotic stages. This second division produces cells smaller than meiosis I cells. The resulting haploid cells are called spermatids.



### **I.2.2.3. Post-meiosis**

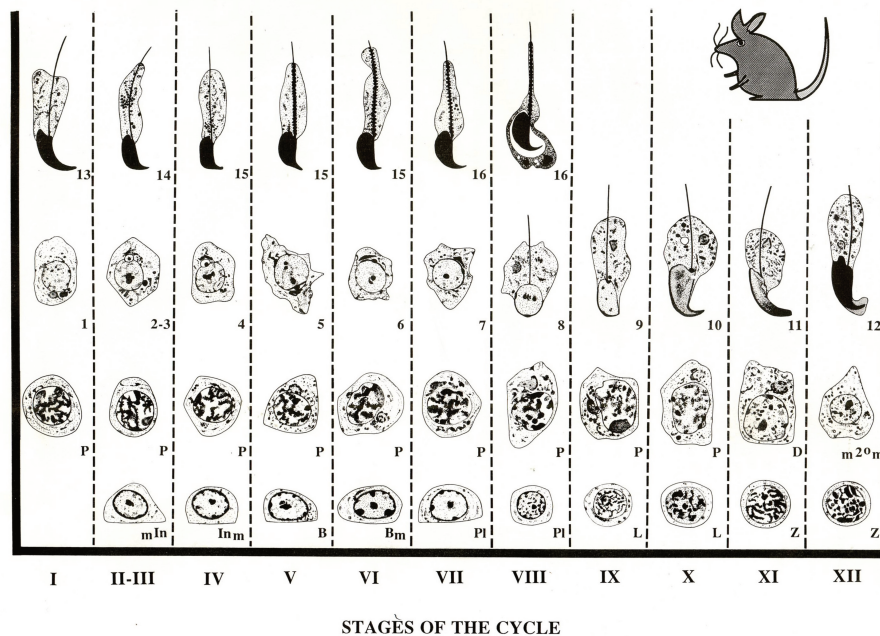
The post-meiotic phase of spermatogenesis, also known as spermiogenesis, involves one of the most dramatic cellular transformation in the body. Indeed, the cells undergoing spermiogenesis acquire a variety of cellular features and functions, including flagellum development, acrosome development, and nuclear elongation and condensation.

Flagellum grows from a pair of centrioles, which migrate to the cell surface in the round spermatid. One of the centriole pair forms axoneme, which consists of microtubules and creates a protrusion. The centriole pair migrates and makes contact with the surface of the nucleus. The nucleus then shows an indent at its side making contacts with centriole, called implantation fossa. This position is opposite to the site of acrosome formation.

The acrosome is one of the crucial structures found in the spermatozoa. It is a compartment containing enzymes that will enable the penetration of the zonapellucida of the oocyte during fertilization. In addition, the experimenter, by following the acrosome development, while checking the types of coinciding cells, can identify precisely each step of spermatid differentiation and perform staging of the seminiferous tubules during their development. The acrosome originates from the proacrosomal vesicle produced by the Golgi apparatus in early round spermatids. The vesicle is full of dense proacrosomal granules. Proacrosomal granules enclosed by membrane are produced from Golgi apparatus, and these small vesicles fuse with each other to form a large proacrosomal vesicle. The vesicle makes contact with nucleus. The Golgi apparatus is also lying near by the proacrosomal vesicle, assisting the maintenance of the vesicle. The nucleus makes indents in response to the contact, and the vesicle becomes flatten on the side making these contacts with the nucleus. The nucleus migrates to the cell surface with the proacrosomal vesicle-side facing the plasma membrane. This configuration of the nucleus and proacrosomal vesicle creates cell polarity, where the nucleus region, more precisely its acrosomal side, is considered as the head (anterior part) and the future flagellum as the tail (posterior or caudal part) are connected by the neck region. The cell then starts its elongation of the cytoplasm along the anterior-caudal axis, while the Golgi apparatus moves to the caudal area of the spermatid. All of these processes are slow and continuously

progress until the late stages of spermiogenesis (Lalli and Clermont, 1981; Leblond and Clermont, 1952). The shape of the elongating spermatids, as well as the shape of later condensing stages and mature sperm, are significantly different between species (Fawcett, 1958, 1975; Fawcett et al., 1971). Rat spermatozoa have a sickle-like shape, mouse spermatozoa have a similar morphology as the rats sperm with slightly rounder shape, whereas dog spermatozoa have a spatulate shape. This dramatic change in the nucleus morphology and its condensation are associated with the replacement of histones by protamines.

Chromatin condensation is an intrinsic phenomena observed in other species. In mature sperm cells from many organisms, the majority of the male genome is packaged by non-histone proteins, such as protamines in mammals. In addition, at this stage of development, the cells lose significant part of their cytoplasm (Sprando and Russell, 1987). At later stages of spermiogenesis, right before the release of matured germ cell into the lumen or spermiation, the spermatid forms tubulobulbar complexes by protrusion of a part of cytoplasm (Russell, 1979; Russell and Clermont, 1976; Russell and Malone, 1980). This complex maintains the connection of spermatids and Sertoli cells until spermiation. At the spermiation, a large part of the cytoplasm is pinched off and released as residual bodies, which contain a variety of materials such as RNAs and organelles in spermatids that are considered unnecessary in the further process. After spermiation, the small portion of cytoplasm remains around the neck of the spermatids, and the release of the residual bodies reduce the cell volume as low as one fourth.



**Figure M:** developmental stages of seminiferous tubules. The developmental stages of seminiferous tubules are indicated in Roman numerals. The letters and numbers indicate types of spermatogenic cells:

$m^{In}$ ,  $In^m$ , **B**: type A<sub>4</sub>-Intermediate, Intermediate- type B, type B spermatogonia, respectively.

**B<sub>m</sub>**: type B spermatogonia – Preleptotene spermatocytes,

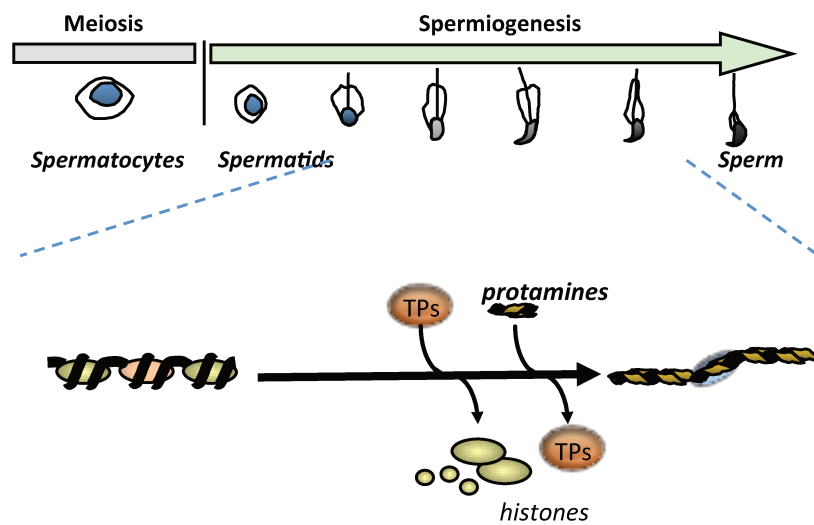
**Pl**, **L**, **Z**, **P**, **D**,  $m^{2o}$ : Preleptotene, Leptotene, Zygotene, Pachytene, Diplotene, and secondary spermatocytes, respectively.

**1-16**: round (1-8) and elongating/condensing (9-16) spermatids, respectively.

(Image from: Russell et al., 1990b)

### I.2.3. Histone-to-protamine replacement during spermiogenesis

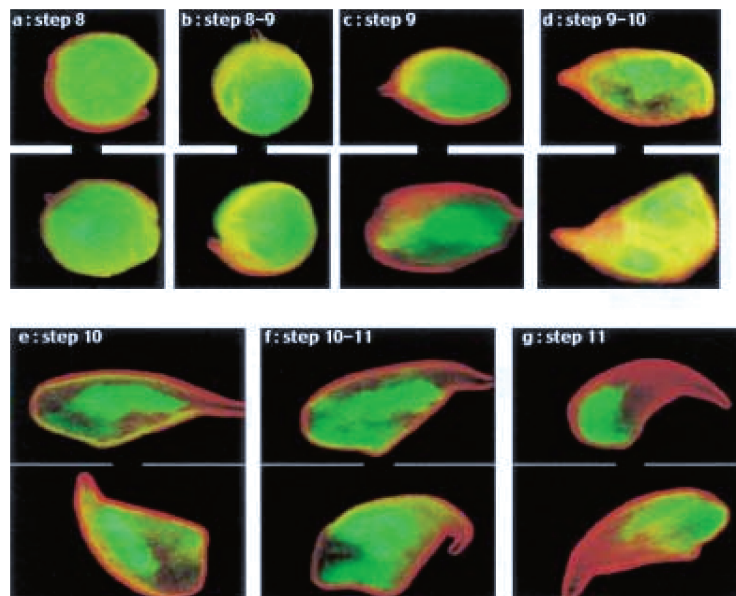
After completion of meiosis, male germ cells undergo one of the most drastic and complex chromatin remodeling events. As spermatids mature, the majority of nucleosomal histones are replaced by transition proteins (TPs) and protamines (Prms) (Figure N). Because protamines are major DNA packaging components in the mature sperm, proper assembly of the protamine-based DNA-packaging is essential to produce functional and viable spermatozoa. Histone replacement is thought to be carried out in a stepwise manner by the act of histone modifications and remodeling factors, including testis specific histone variants. In the following sections, the mechanisms of histone replacement that are understood today are described.



**Figure N:** histone replacement by TPs and Prms during post-meiotic phase of spermatogenesis.

### 1.2.3.1. Histone hyperacetylation

It is now well known that before their replacement histones become hyperacetylated in elongating spermatids (Boussouar et al., 2008; Goudarzi et al., 2014; Govin et al., 2004) (Figure O). Based on the temporal relationship between hyperacetylation and histone replacement, these two events were thought to be functionally linked. In mouse elongating spermatids at step 8 to 11, a number of lysine residues on all core histones become simultaneously acetylated, including the N-terminal lysine at position 5, 8, 12, and 16 of H4 (Hazzouri et al., 2000). The timing of acetylation of the other core histones, as studied by immunohistochemistry, are as follows: H3ac in step 10 to 11, H2Aac in step 9 to 11, H2Bac in step 10 to 11 spermatids. Several studies aiming to uncover the impact of hyperacetylation on histone replacement demonstrated that indeed the hyperacetylation of nucleosomal histones assists protamine incorporation-associated histone evictions *in vitro* (Marushige et al., 1976; Oliva and Mezquita, 1986; Oliva et al., 1987). In spermatids, the underlying molecular mechanism is suspected to be more complex than *in vitro* systems, since chromatin remodeling and histone replacement is normally performed by various factors such as histone chaperones and chromatin remodelers (Burgess and Zhang, 2014; Ray-Gallet and Almouzni, 2010). Therefore, histone hyperacetylation



**Figure O:** hyperacetylation wave in late round and elongating spermatids. H4 acetylation is labeled in green and nucleus in red (images from: Hazzouri et al., 2000).

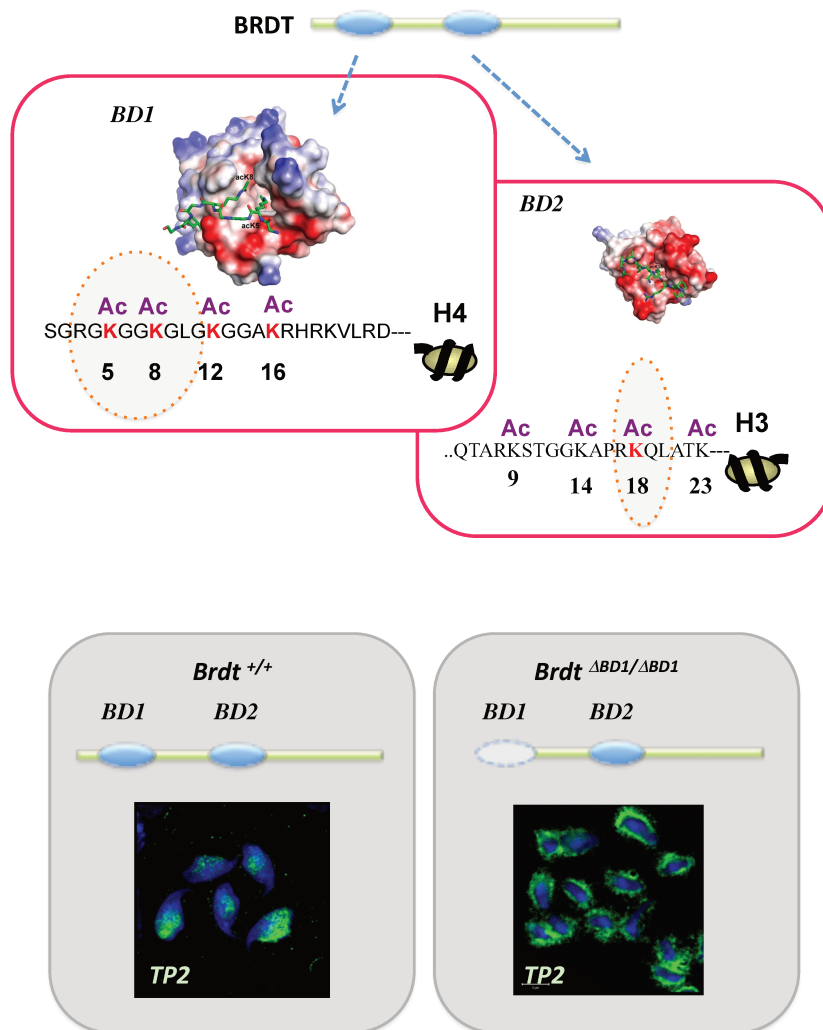
by itself is not sufficient to explain the histone-to-protamine replacement.

### **1.2.3.2. BRDT: a testis specific BET**

In general, post-translationally modified histones are often targeted by “readers” in order to trigger the downstream cellular response. In the case of histone acetylation, bromodomains were identified as acetyl lysine readers. The bromodomain consists of four-helix bundle ( $\alpha_z$ ,  $\alpha_a$ ,  $\alpha_b$ ,  $\alpha_c$ ) with left-handed twists and ZA loop spanning between  $\alpha_z$  and  $\alpha_a$  (Dhalluin et al., 1999). A structural study of the bromodomain of P/CAF (P300/CBP-associated factor) demonstrated that the bromodomain binds to the histone tails in an acetylation dependent manner (Dhalluin et al., 1999). Based on the finding of bromodomains as acetyl lysine readers, and the possibility that such factors could also act on hyperacetylated histones in the spermatid, an *in silico* analysis looking for testis specific proteins with bromodomain(s) led to the identification of BRDT (Pivot-pajot et al., 2003). BRDT belongs to the protein family of Bromodomain and extra terminal domain (BET), whose members all contain two adjacent bromodomains at their N-terminal part and extended domains in their C-terminal part. BRDT’s capability to act on hyperacetylated histones was demonstrated by forced expression of BRDT in somatic cells treated with histone deacetylase inhibitor, TSA. Under these conditions, BRDT was shown to bind and remodel hyperacetylated chromatin (Pivot-pajot et al., 2003). The structural studies of the two bromodomains in BRDT revealed the basis of how BRDT could act on hyperacetylated chromatins. The first bromodomain (BD1) of BRDT preferentially binds to histone H4 harboring simultaneous acetylation at K5 and K8, while the second bromodomain (BD2) is capable of binding H3K18ac (Morinière et al., 2009) (Figure P). Hence, the BD1 of BRDT was the first domain discovered having specificity for hyperacetylated histone H4. This unique feature of BRDT’s BD1 was used in designing a molecular probe for real-time tracking of histone hyperacetylation activity (Sasaki et al., 2009). A probe was genetically engineered in which the double bromodomain region of BRDT, as acetylated histone recognition modules, was connected with histone H4 as a substrate. The probe is then flanked by Venus and CFP which respectively act as the donor and acceptor by fluorescence resonance energy transfer (FRET). A FRET signal is emitted in response

to the binding of BD1 to acetylated histones. This study did not only show the ability of this probe for hyperacetylated histone detection, but also mutational analysis further confirmed that the binding of BRDT entirely depends on the integrity of BD1 and simultaneous acetylation of H4 at both positions at H4K5 and K8.

Studies utilizing transgenic mice revealed the critical function of BRDT during the spermatogenesis. First, Shang et al. demonstrated that the mice expressing *Brd*t lacking its first bromodomain (*Brd*t  $\Delta$ BD1) exhibit male infertility, due to spermatogenic arrest at the elongating stage of the spermatid differentiation (Shang et al., 2007). The epididymal sperm of the *Brd*t  $\Delta$ BD1 male showed decrease sperm numbers and increases of spermatozoa with misshapen head and defective motility. Finally, a comprehensive study of the function of BRDT during spermatogenesis revealed the striking importance of this protein. The expression of BRDT initiates when the male germ cell enters meiotic phase. Combining all possible approaches and several mouse models, this extensive study revealed that BRDT, as soon as it begins to be expressed in early meiotic cells, acts in a stage specific and domain dependent manner as a major regulator of spermatogenesis. It activates meiotic and post-meiotic gene expression programs and its BD1 is indispensable for histone removal and replacement. *Brd*t knockout mouse (*Brd*tKO) exhibits meiotic arrest in spermatogenesis, due to failure of meiotic gene activation (Gaucher et al., 2012). In addition, detailed analysis of *Brd*t  $\Delta$ BD1 revealed BRDT's critical role in post-meiotic cells during histone to protamine replacements. Unlike *Brd*tKO, *Brd*t  $\Delta$ BD1 spermatids can develop up to the elongating spermatids stage without apparent defect, but the later cells exhibit defective histone-TPs-Prms replacement (Gaucher et al., 2012) (Figure P). This phenotype indicates that BD1 is indeed essential for the recognition and replacement of the hyperacetylated histones. Therefore, *Brd*t is the key factor triggering the acetylation-dependent histone replacement at the later stages of spermiogenesis.



**Figure P:** Top: preferential binding of BRDT's BD1 to diacetylated histone H4 at K5 and K8 (image from: Moriniere et al., 2009). Bottom: spermatogenic arrest with defective histone replacement caused by the lack of BRDT's BD1 in *Brdt* $\Delta$ BD1 mice (images from: Gaucher et al., 2012).



### **I.2.3.3. Histone variants in male genome reorganization**

Mammals have variant versions of all the histone members, except H4 whose variant has not been discovered. Within these identified variants, some of them are exclusively or highly expressed during spermatogenesis (Figure Q). Although many of them have not been fully characterized, some studies revealed their critical functions during spermatogenesis.

#### **H2AL1/H2AL2 (H2AL1/2)**

H2AL1/2 are mouse testis specific H2A variant showing unique behavior among all the other histones and their variants. Unlike most histones that are present up to round spermatids and evicted at the histone replacement, H2AL1/2 are expressed in elongating spermatids, at the timing when TPs start occupying the spermatids' chromatin, and persists in condensed spermatids and mature spermatozoa (Govin et al., 2007). In addition, they are enriched in the chromocenter, which consists of major satellite repetitive sequences of pericentromeric region of the mouse genome (Govin et al., 2007). H2AL1/2 are mostly found in unconventional sub-nucleosome structures which are not found in somatic cells, are devoid H3 and H4 and contain approximately 60 bp of DNA (Govin et al., 2007). These features of H2AL1/2 suggest that the pericentromeres of elongating/condensing spermatids undergo specific reorganization, and potentially have a crucial role in the formation of sperm.

#### **H2A.Lap1 (H2A.Bbd)**

H2A.Lap1 is a mouse homologue of human H2A.Bbd, highly expressed from spermatocytes to round spermatids (Ishibashi et al., 2010). In spermatocytes and early round spermatids, H2A.Lap1 is localized at the TSS of actively transcribed genes, and in later round spermatids, TSSs of the genes on inactivated X chromosome that escapes the suppression are enriched by H2A.Lap1 (Soboleva et al., 2012). Therefore,

H2A.Lap1 is suspected to regulate the specific gene expression program in each steps of spermatogenesis.

## **TH2B**

TH2B, or TSH2B in human, is the first H2B variant characterized as germ cell specific variant (Branson et al., 1975; Shires et al., 1975). During mouse spermatogenesis, TH2B initiates its expression in preleptotene spermatocytes and the level increases as the differentiation progresses, while canonical H2B expression gradually decreases (Montellier et al., 2013). In round spermatids, almost all H2B are replaced by TH2B. The genome wide incorporation of TH2B indicates its importance in chromatin organization during spermatogenic differentiation. However, surprisingly, the complete knockout of TH2B did not exhibit abnormality in the sperm production (Montellier et al., 2013). Unexpectedly, in the absence of TH2B, canonical H2B is up regulated with additional histone PTMs, including methylation at H2BR72, H4R35, R55 and R77, crotonylation (Montellier et al., 2011; Tan et al., 2011) at H3K122 and H4K77, which all are strategic in destabilizing nucleosomes. This unexpected compensation using heavily modified core histones suggests a role of TH2B as an inducer of nucleosome instability potentiating chromatin for histone replacement. The nucleosome destabilizing function of TH2B is further supported by a structural study using human TSH2B. Histone octamer containing TSH2B shows decreased stability (Li et al., 2005) compared to octamers possessing canonical H2B. In addition, the hydrogen bond between H2BN84 and H4R78 that is found in the canonical nucleosome is absent in a TH2B incorporated nucleosome (Urahama et al., 2014).

Although TH2B is initially characterized as a testis specific H2B variant, it is also highly expressed in oocytes. (Montellier et al., 2013; Shinagawa et al., 2014). Since maternal TH2B is rapidly incorporated into male genome upon fertilization, it is also considered to play important role during embryonic reprogramming. Along with a germ cell specific H2A variant TH2A (Trostle-Weige et al., 1982), the incorporation of these variants is important for the activation of the paternal genome upon fertilization (Shinagawa et al., 2014). In addition, it has been reported that the

expression of TH2A/TH2B also improves the induced pluripotent stem cell (iPSC) generation, suggesting that these variants are also potent factors for nuclear reprogramming (Shinagawa et al., 2014).

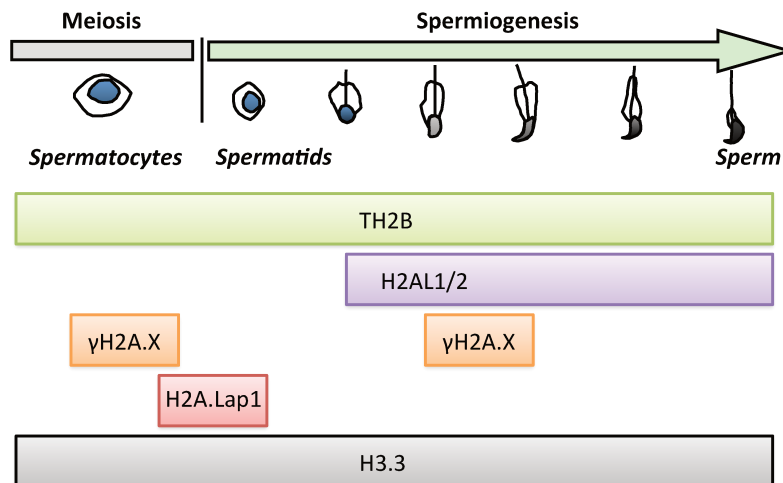
### **H3 variants**

H3T (H3.4) is a testis specific histone H3 variant (Witt et al., 1996). It has been reported that incorporation of H3T induces nucleosome instability in vitro (Tachiwana et al., 2010). Although the structural details on a nucleosome containing this variant has not been unveiled yet, H3T is shown to induce nucleosome instability with potentially important role in male genome reorganization.

As a result of chromatin reorganization during spermiogenesis, most nucleosomal histones are replaced by protamines in mouse mature sperm. Only about 1% of nucleosomal histones are maintained in mature mouse spermatozoa (Brykczynska et al., 2010). Within the small part of nucleosomal regions in the mature sperm, some are found to be unmethylated CpG islands enriched with ubiquitously expressed H3 variant H3.3 (Erkek et al., 2013). These H3.3 containing regions are also found to be labeled with trimethylation H3K4, which is a mark of actively transcribed genes. In contrast, the other nucleosomal regions in the CpG islands bearing H3.1 and H3.2 are enriched by repressive H3K27 trimethylation. Differential marking of CpG islands by H3.3 or H3.1/3.2 may play important roles in paternal epigenome inheritance.

The importance of H3.3 during spermatogenesis has been studied through the use of transgenic mice and drosophila models (Bush et al., 2013; Couldrey et al., 1999; Hödl and Basler, 2009; Sakai et al., 2009). H3.3 is encoded by two genes, H3f3a and H3f3b. Both genes encode identical proteins, but their expression timings are slightly different. H3f3a is expressed throughout spermatogenesis in a constant level, whereas H3f3b is highly expressed in spermatocytes (Bramlage et al., 1997). Mutational studies revealed impact of each gene during spermatogenesis. H3f3a mutant is viable but male mice exhibit subfertility with morphological defects in their sperm (Tang et al., 2015). H3f3b mutant gives more severe phenotypes where the homozygous shows growth defect and birth lethality, and the heterozygous shows

growth deficiency with male sterility due to spermatogenic arrest at round spermatids (Tang et al., 2015; Yuen et al., 2014). H3.3 has been shown to be incorporated into the silenced sex chromosomes (van der Heijden et al., 2007). However, these H3f3b mutant spermatids did not show apparent defect in the sex body formation (Tang et al., 2015). Therefore, although both genes of H3.3 encode identical proteins, the above mentioned studies indicate they have different impacts in the cellular functions including their roles during spermatogenesis.



**Figure Q:** Relative expression timing of histone variants during spermatogenesis.

### **I.3. Nuclear protein in Testis (NUT)**

The mechanisms involved in the regulation of histone hyperacetylation during male genome reorganization are still largely unknown. A study of a specific human cancer, Nut Midline Carcinoma, discovered an intriguing action of the aberrantly expressed testis specific factor, NUClear protein in Testis (NUT). It suggested a relationship between this protein and members of the bromodomain containing factors of the BET family and that it could play an important role in the control of histone acetylation.

In this section, along with a general introduction on NMC, the role of NUT as a critical epigenetic factor in the germ cell development is discussed.

#### **I.3.1. NUT Midline Carcinoma (NMC)**

NUT Midline Carcinoma (NMC) is a rare, aggressive human cancer occurring in men and women at any ages (for review see French 2012). NMC is characterized by a reciprocal chromosomal translocation involving *NUT* (15q14) fused to another gene. In about two thirds of NMC cases, *NUT* is fused with the *BRD4* gene on chromosome 19p13.1 through a t(15;19)(q14;p13.1) translocation creating *BRD4-NUT* oncogene under the control of the *BRD4* promoter (Figure R). Some other variations of NMC were also reported, such as BRD3-NUT and NSD3-NUT.

The oncogenic activity of NMC is considered to be caused by the fusion protein. BRD4 is a ubiquitously expressed factor belonging to the BET family, characterized by its tandem bromodomains with extraterminal domain, and involved in various cellular processes through the regulation of gene transcriptions (see section I. 1. 4. 2. Readers: bromodomain and extraterminal domain family (BETs)). NUT is a functionally poorly characterized protein, and its role in testis, in which NUT is normally expressed, is completely unknown. Two acidic domains, AD1 and AD2, are present in the protein, which could be potentially important for protein-protein interactions. AD1 is included in the region that is reported to interact with p300

(Reynoird et al., 2010). BRD4-NUT interaction with p300 mediated by NUT moiety disrupts the terminal differentiation of squamous epithelial cells by deregulating gene expression (French et al., 2008; Reynoird et al., 2010; Schwartz et al., 2011). Nuclear export signal (NES) and nuclear localization signal (NLS) are also present in the C-terminal part of NUT, implying that the protein may shuttle between the nucleus and the cytoplasm. In t(15;19)(q14;p13.1), the breakage of *BRD4* gene occurs at the C-terminal region of the protein. This fuses the central and N-terminal region of BRD4 including bromodomains to the almost entire gene encoding NUT.

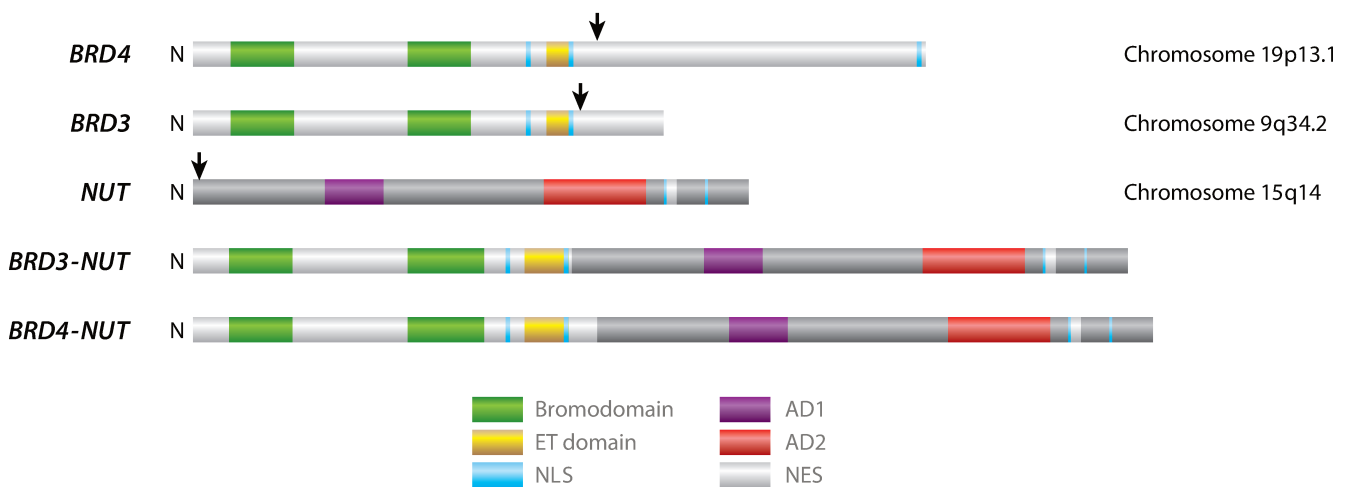
The knockdown of *BRD4-NUT* induces rapid squamous differentiation and proliferation arrest (French et al., 2008; Schwartz et al., 2011), characterized by the up regulation of squamous differentiation genes such as cytoplasmic keratin and involucrin. Morphologically, the differentiation is associated with increase in the size of nucleus and euchromatin, and eventually the cells turn into flat, resting, terminally differentiated squamous cells. Therefore, the main outcome of this gene fusion is to produce an oncogene which blocks cell differentiation. Consequently, perturbation of BRD4-NUT's function has been identified as a promising therapeutic strategy in fighting NMC.

The study from Shwartz et al found that BRD4-NUT decreases the global acetylation level by sequestering HAT activity, hindering the expression of necessary genes for differentiation (Schwartz et al., 2011). HDAC inhibitor treatment successfully induced cellular differentiation and stopped NMC proliferation.

One of the oncogenic mechanisms occurring in NMC is caused by BRD4-dependent upregulation of the oncogene MYC (Grayson et al., 2013). BRD4 protein is often found to be key a regulator of MYC expression in other cancers (Bielas et al., 2006; Bonnet and Dick, 1997; Liebertz et al., 2010). In case of NMC, *BRD4-NUT* knockdown causes down-regulation of MYC, and induces terminal squamous differentiation. Interestingly, MYC overexpression could partially rescue the BRD4-NUT knockdown effect associated with moderate differentiation phenomena, but it did not fully restore the proliferative ability. Therefore, MYC is surely playing a role but is not responsible for the entire oncogenic activity, induced by BRD4-NUT.

Several translocation variants of NMC have been reported, including *BRD3-*

fusion and *NSD3-NUT* fusion (French et al., 2014). While BRD3 is another BET factor similar to BRD4, possessing bromodomains, NSD3 is a methyltransferase that has distinct roles from factors of the BET family. NSD3 translocation is more known in case of leukemia (Rosati et al., 2002; Wang et al., 2007), but the mode of gene fusion is completely different since in NMC involving NSD3 rearrangement does not include many of the domains found in leukemia, including the chromodomain. NSD3 has been previously shown to interact with the ET domain of BRD4 (Rahman et al., 2011). Indeed, NSD3 moiety of the fusion protein interacts with BRD4 mimics the BRD4-NUT type of NMC. In addition, it was shown that wild-type NSD3 was also required for blocking the differentiation in a major type of NMC expressing BRD4-NUT. Therefore, association of ET domain of BRD4 and NSD3 through either direct fusion or protein complex formation is essential for NUT-induced oncogenesis.

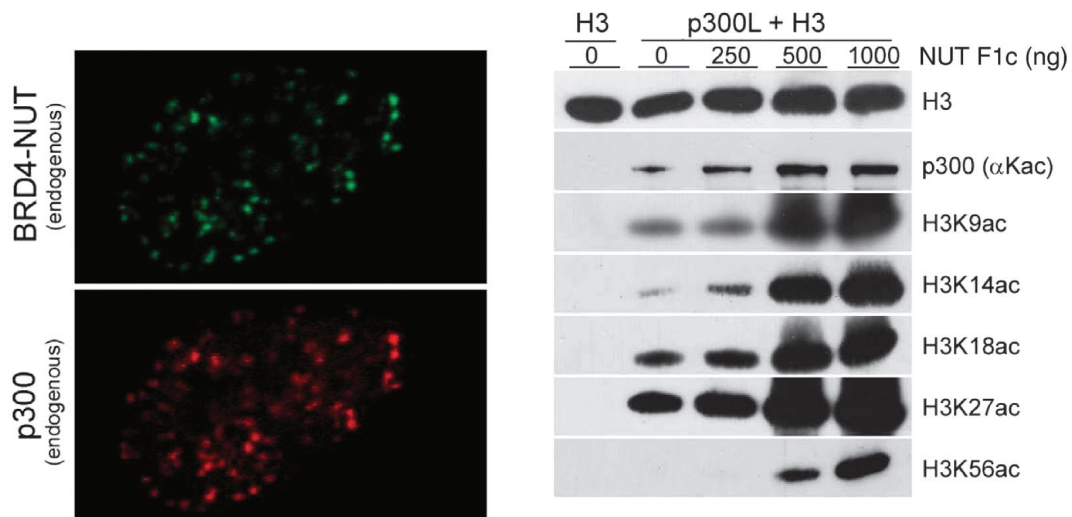


**Figure R:** schematic representation of the position at which BRD4-NUT or BRD3-NUT fusion occurs (image from: French, 2012).

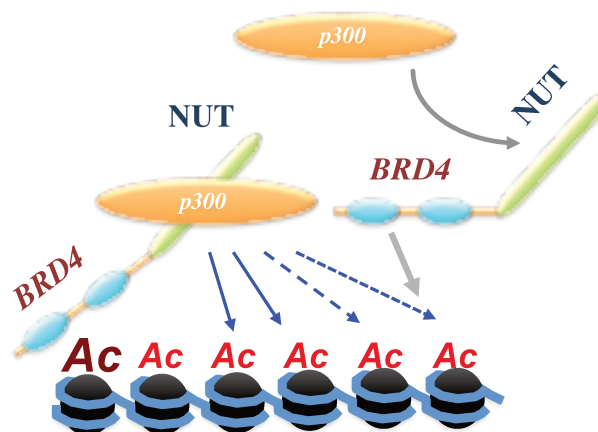
### **I.3.2. BRD4-NUT: a strong enhancer of HAT activity**

A critical study tackling the oncogenic mechanism of NMC was published in 2010 (Reynoird et al., 2010). The bromodomain of BRD4 was known for its specific association with acetylated histones (Dey et al., 2003; Lee and Chiang, 2009). When BRD4-NUT expression is induced in Cos7 cells, it forms characteristic acetylated histone foci that perfectly overlap with BRD4-NUT localization, and shows very similar localization patterns as those found in patient-derived HCC2429 cells (Figure S). p300 and CBP are also found in the same foci. The histones found in BRD4-NUT foci were found to be hyperacetylated at various lysine residues, including H3K14, K18, K27, K56 and H4K8. BRD4-NUT interacts with p300 through its NUT moiety. More precisely, the central region of NUT (amino acid 346 to 593), named F1c, is the key region for its interaction with p300. This is the first characterization of the function of NUT moiety of the fusion protein. Interestingly, the association with NUT F1c enhances the HAT activity of p300 in vitro (Figure S). Increasing amount of purified NUT F1c in the reaction increased H3K9, K14, K18 and K27 acetylation. In addition, H3K56 is acetylated only in presence of NUT F1c. Finally, the study showed that the hyperacetylated foci formation is BRD4-NUT dependent, especially the bromodomain activity and p300 recruitment via NUT moiety. Disruption of the BRD4-NUT and foci formation with p300 recruitment, by inducing global hyperacetylation by HDAC inhibitor TSA treatment, for example, dispersed the BRD4-NUT and p300 foci. Altogether, these studies showed that the oncogenic activity of BRD4-NUT is due to induction of locally hyperacetylated chromatin regions by tethering of itself onto acetylated chromatin, and recruiting p300 and propagating histone hyperacetylation through a feed-forward loop (Figure T).





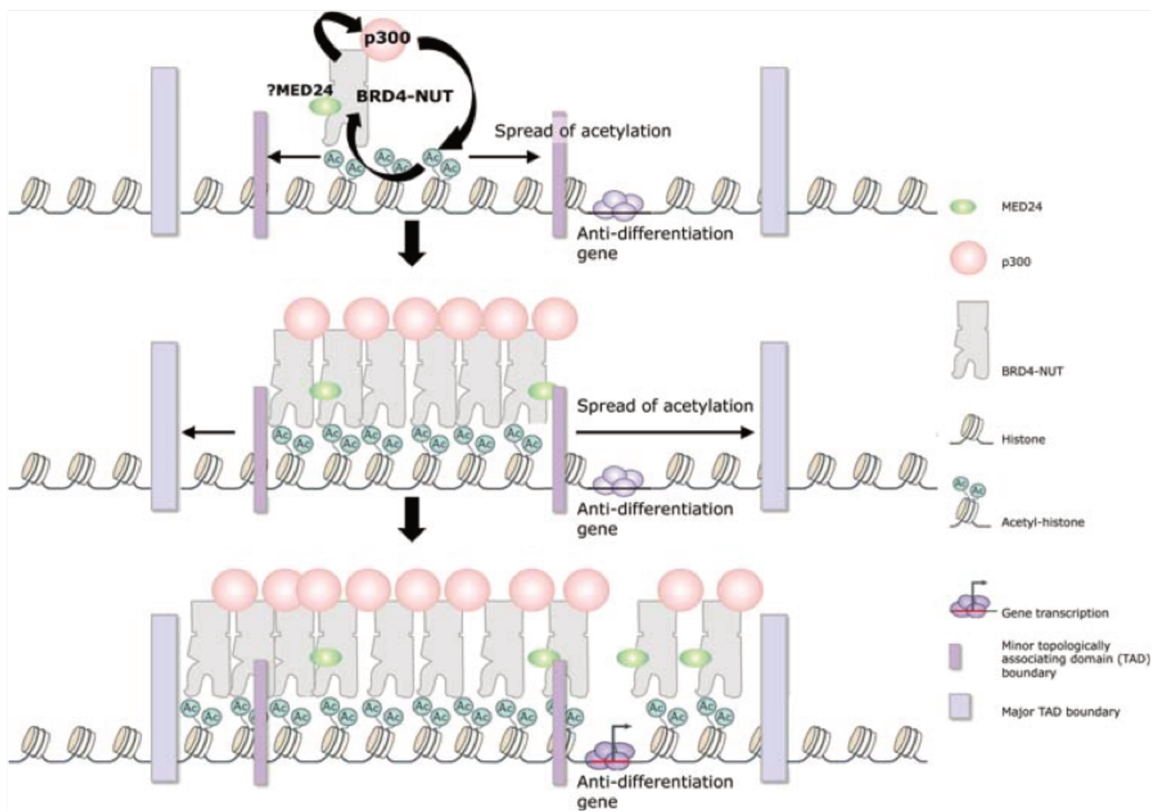
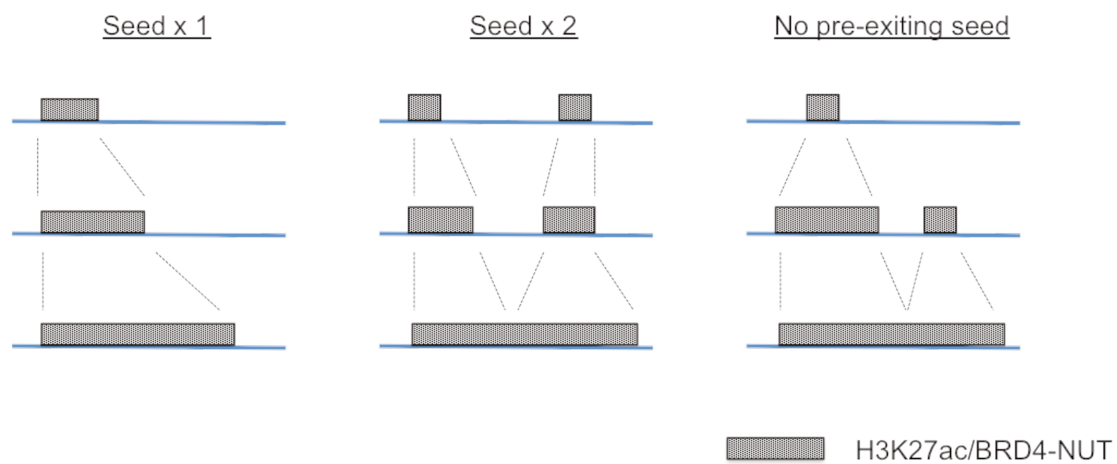
**Figure S:** enhancement of the HAT activity by direct interaction between BRD4-NUT and p300. Left: BRD4-NUT co-localizes with p300 in NMC. Right: p300 interacting region of NUT could enhance HAT activity of p300 toward histone H3 in vitro (images from Reynoird et al., 2010).



**Figure T:** the model of feed-forward hyperacetylation by BRD4-NUT and p300 in NMC. The fusion protein anchors itself to acetylated chromatin via its bromodomain, and NUT moiety recruits p300, which creates the hyperacetylated chromatin region. Newly acetylated chromatin serves as a binding platform for other BRD4-NUT, anchoring of itself, recruiting p300, and acetylating the histones.

### **I.3.3. “Megadomain” formation by BRD4-NUT**

In a recent study, it has been reported that the hyperacetylated chromatin regions induced by BRD4-NUT are organized in wide regions from 95 kb to up to 2 Mb, which the authors called “megadomains” (Alekseyenko et al., 2015). Megadomains are enriched in H3K27ac. This histone PTM generally marks active enhancers or other regulatory regions, and the megadomain is much larger than superenhancers which have been reported to recruit BRD4 (Lovén et al., 2013). 797TRex (NMC origin) (Toretzky et al., 2003) showed similar megadomains defined by H3K27ac and BRD4-NUT enrichment as well as additional marks such as H3K9ac, K14ac and H3K36me3, but in distinct regions from the ones detected in 293TRex cells. The megadomains are responsive to BET inhibitors, and a JQ1 treatment rapidly (4 hr) diminishes the size of the megadomains. Consequently, the genes within megadomains are significantly downregulated upon a JQ1 treatment. BRD4-NUT megadomain formation initiates from “seed” regions, small enhancers or regulatory regions marked by H3K27ac. Three cases were detected: small and single seed domains that grow up to the megadomains compatible size, several regions close to each other which spread and fuse at some point (Figure U). Megadomains could also arise through the formation of enhancer-like regions without pre-existing seed domain. Here again, superenhancers are not overrepresented among seed regions. An analysis of several NMC cell lines and cancer tissues from patients revealed that the regions of megadomain formation depend on the cells of origin. Topologically associated domains (TADs), a chromatin compartment conserved between cell types (Dixon et al., 2012), are filled by megadomains (Figure U). The boundaries of megadomains correspond TAD boundaries, CTCF binding sites, TSS, and active enhancers. The megadomains contain genes that show sensitivity toward JQ1 treatment and potentially critical for the growth of NMC. MYC is one of the genes (Grayson et al., 2013). Other genes detected in the megadomains include TP63 (Ramsey et al., 2013; Stransky et al., 2011; Venkatanarayan et al., 2015), SOX5 (Ma et al., 2009; Zafarana et al., 2002), MED24 (Clark et al., 2002; Hasegawa et al., 2012; Luoh, 2002), as well as 13 long non-coding RNA (lncRNA). Among those, MED24, TP63 and lncRNA PVT1 are newly identified genes that are critical for either proliferation/blocking differentiation or viability of NMCs.



**Figure U:** hyperacetylated megadomain formation by BRD4-NUT. Top: three different origins of the megadomains. Bottom: megadomain fills a wide region of chromatin according to TAD boundaries (image from: Alekseyenko et al., 2015)

## II. Objective

**The aim of my Ph.D project is to characterize the physiological functions of Nut.**

### **Main hypotheses and questions**

Since NUT exhibits a potent effect on chromatin acetylation in the context of NMC where it is expressed in fusion with BRD4, the original function of NUT in its physiological context, which is spermatogenesis, could be also be involved in the establishment of male germ cell epigenome. Until today, the physiological function of the original form of NUT and its potential impact on male germ cell differentiation has not been studied.

Exploring the potential function of NUT would aim at answering the following questions:

***When, or in which types of cells, is Nut expressed in the course of spermatogenic development?*** The expression pattern of Nut is crucial information to determine the function of NUT. The expression profile could be strongly associated with its function.

***Is NUT necessary for spermatogenesis?*** Although no functional study was performed on Nut and its potential impact on other tissue is completely unknown, its expression profile, which is restricted to testis, suggested a potential specific role during spermatogenesis.

***Does NUT affect histone acetylation during spermatogenesis?*** Enhancement of HAT activity is the critical oncogenic function of BRD4-NUT, and this function is achieved by the direct interaction between NUT moiety of BRD4-NUT and p300 (Reynoird et al, 2010). HAT enhancement may also occur when Nut is normally functioning in the spermatogenic cells.

**Does NUT associate with BRDT or other BETs during spermatogenesis?**

Although BRD4-NUT is a gene fusion and therefore its functional data is not directly applicable to the physiological context in which NUT is expressed, the HAT enhancement of NUT in cancer cells is the result of its functional cooperation with BRD4. Nut may associate with BET protein(s) during spermatogenesis, and the testis specific factor Brdt is the major BET member in the male germ cell.

**Which are the interacting proteins with NUT? Does the (potential) complex include HATs?** Which HAT is responsible for the histone hyperacetylation during histone replacement is one of the long persisting questions in the field. The interaction of NUT with HAT in NMC suggests that the physiological function of Nut could also involve interaction with one or several HATs.

Above-questions could be summarized into two hypotheses:

- **NUT could be an essential factor during spermatogenesis.**
- **The hyperacetylation wave in the post-meiotic stage of spermatogenesis could be regulated by NUT.**

**\*The data presented in this manuscript will be published once the preliminary data is completed.**

## III. Materials and Methods

### III.1. Mouse model

Designing the knockout construct and generation of the embryonic stem cell (ESC) are performed by Thierry Buchou and Fayçal Boussouar from our team. *Nut*<sup>-/-</sup> mice were generated in collaboration with Mathieu Gerard (CEA, Saclay).

The knockout of *Nut* gene was performed following the recombineering technique described in Liu et al., 2003. The *Nut null* allele was created by replacing the exon 2 of the endogenous *Nut* gene by *Neo* cassette, which disrupts the *Nut* expression. The targeting vector harbors *Neo* flanked by sequences homologous to *Nut* exon 2 surrounding region. At 3' side of the homologous regions, the targeting vector also contains *TK* gene which induces apoptosis in response to Ganciclovir. The targeting vector was electroporated into mouse ESCs (AT1), and selected for Neomycin and Ganciclovir. The selected ESC clones were confirmed by Southern blot after *EcoR1* digestion and probes with a 3' hybridizing to the region that is shared in *WT* and *null* allele. The genotype is also confirmed by polymerase chain reaction (PCR) using forward primers specific to each allele combined with common reverse primers. The selected ESCs were injected into blastocysts in order to generate the chimeric mice with germ line transimission of the *null* allele. The chimeras were back-crossed with wild type mice to obtain heterozygous pups. The F1 animals were crossed to produce *Nut* null homozygous offspring.

### III.2. RNA purification and expression analysis

The expression of *Nut* during the first wave of spermatogenesis was analyzed from RNA samples extracted from WT male mice of 6, 8, 10, 12, 14, 16, 18, 20, 25 and 25 days postpartum, using TRIzol reagent (Invitrogen). The extracted RNA was reverse transcribed into cDNA using AffinityScript Multiple Temperature cDNA synthesis Kit (Agilent). Using appropriate primer sets, expression of *Nut* was

monitored using Brilliant III Ultra Fast SYBR<sup>®</sup> Green QPCR Master Mix (Agilent) and normalized by Actin expression.

### **III.3. Purification of male germ cells**

Our method of the germ cell purification was developed from the technique introduced by Bellevé et al., 1993. Testes were isolated from euthanized mice and the albuginea were removed. Seminiferous tubules were treated with collagenase (1 mg/ml in phosphate-buffered saline [PBS]) for 10 min at 37°C, with occasional vortex. The cells were centrifuged and resuspended in DMEM/F12 medium containing 0.5% bovine serum albumin (BSA) and pipetted with Pasteur pipette for 10 min in order to release germ cells from the seminiferous tubule. The suspension was filtered through 100 µm mesh filter. At this point, the prepared cells are called total germ cells (TGCs), which could be directly used for protein preparation or RNA isolation for further analysis. Stage specific germ cells could be purified from the TGC through a BSA gradient by sedimentation.

The airtight sedimentation chamber was used to generate a BSA gradient in which the TGC would be fractionated into pachytene spermatocytes (P), round spermatids (R), and elongating/condensed spermatids (E/C). The chamber was first filled with Fluorinert FC77 to create air-free environment in the chamber. Then, from the top of the chamber, a 2% and 4% BSA gradient in DMEM/F12 medium was slowly loaded (1 ml/10 sec). Prepared TGC were loaded on top of the 2-4% BSA layer, and additional PBS layer was added on top of the TGC layer. The germ cells were allowed to sediment in 4°C for 70 min. After removing the PBS layer, the germ cell fractions were collected in 10 ml fractions. The collected cells were examined under a phase-contrast microscopy, and corresponding fractions were pooled. The cells were washed with PBS and used for further applications.

#### **III.4. Spermatogenic cell preparation for immunofluorescence**

Slides of staged seminiferous tubule were prepared for immunofluorescence by tubule squash method (Kotaja et al., 2004). Testis were isolated from euthanized mice, washed in PBS and placed in pre-chilled DMEM with 100 mM sucrose. Albugenea was carefully removed and seminiferous tubules were dissected under light microscope. Segments of seminiferous tubules about 2 mm length were placed on the slides, and topped by a coverslip. The slide was rapidly frozen and prefixed by dipping into liquid nitrogen for about 20 s, 10 min in 90% ethanol and air-dried.

As an alternative method, testis “imprints”, was adopted. The isolated testes were frozen in liquid nitrogen. The frozen testis was then cut in two pieces. After brief melting of the cut surface, the germ cells were directly applied on slides by apposition. The slides were fixed by 90% ethanol and air-dried.

Immunofluorescence analysis was performed on the spermatogenic cells prepared through tubule squash or testis imprint methods. The air-dried slides were washed with PBS for 5 min, and permeabilized with 0.5% saponine and 0.2% TritonX-100 in PBS for 5 min 3X. The slides were then washed in PBS and blocked in 5% milk dissolved in PBS containing 0.2% Tween 20 (PBS-T) for 30 min at room temperature (RT). Primary antibody dilution prepared in 50  $\mu$ l per slide with 0.5% milk in PBS-T was applied. The slide was then covered by a coverslip, and incubated in a humid chamber at 4°C overnight. For acrosome labeling, Alexa Fluor® 647-conjugated Lectin PNA (Thermo Fisher Scientific) was added to the antibody solution at 1:100 ratio. The slides were washed with 0.5% milk in PBS-T, 5 min 3X and incubated with 1/500 diluted secondary antibodies conjugated with Alexa Fluor® 488 or 568 in 5% milk in PBS-T at 37°C for 30 min. The slides were washed with 5% milk in PBS-T for 5 min 3X. Cells were stained with Hoechst (2  $\mu$ g/ml) to visualize the nuclei. The slides were washed with PBS, then mounted with either one of the Dako Fluorescence Mounting Medium (Dako) or the Vectashield (Vector Laboratories) and a coverslip.



### **III.5. Histological and immunohistochemical analysis**

Testis and cauda epididymis were fixed in an AAF solution containing eosine (1/500). The testes were then transferred to a PFA solution containing eosine (1/500) and incubated at 4°C overnight. The samples were transferred to 70% ethanol, and dehydrated in the following program: 80% ethanol 90 min/ 90% ethanol 90 min/ 96% ethanol 90 min/ 100% ethanol 90 min/ Toluene overnight/ paraffin (60°C). The samples were embedded in a paraffin block, and sections were cut (7 µm). The cut sections were placed on slides and incubated at 37°C for 16 hr.

Paraffin removal, endogenous peroxidase inhibition and sample rehydration were carried out in the following procedure: Toluene 10 min x 3 times/ 100 ethanol 2 min/ 0.3% H<sub>2</sub>O<sub>2</sub> in methanol 30 min/ 100% ethanol for 2 min/ 90% ethanol for 2 min/ 70% ethanol for 2 min/ H<sub>2</sub>O wash for 5 min. The antigen was exposed through the treatment with 10 mM sodium citrate (made from Citrate Buffer pH 6.0 conc. 10x (Diapath)) for 20 min at 99.5°C, followed by cooling down at RT for 20 min. Blocking was done in 5% milk in PBS for 30 min, as well as blocking of endogenous avidin and biotin by Abidin/Biotin Blocking Kit (Vector Laboratories), following the manufacture's protocol. The primary antibody incubation was done in the same way as in immunofluorescence, except no use of Tween20 in the buffer. After washing the slides in 0.5% milk in PBS for 5 min 3 times, secondary antibody incubation and abiding-biotin complex formation for the antibody detection was performed using VECTASTAIN Elite ABC Kit (Vector Laboratories) following the manufacture's protocol. The signal was developed by 3,3'-DiAminoBensidine (DAB) at RT. The slides were then stained with PAS (periodic acid incubation for 5 min and Schiff reagent for 20 min, both at RT in humid chamber) for acrosome labeling. The nucleus was stained by brief dipping into hematoxyline, for approximately 10 to 20 sec. the slides were washed with running water for 2 min after both staining. The stained samples were dehydrated by incubations with 100% ethanol for 2 min 3 times and Toluene for 2 min 3times, and the slides were mounted using Eukitt<sup>®</sup> quick-hardening mounting medium (Sigma Aldrich).

### **III.6. Histone preparation for PTM analysis**

Round spermatids were purified via the gradient sedimentation method in section III.3. Nuclei were isolated from the purified round spermatids by adding the extraction buffer (10 mM Hepes pH 7.0, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 340 mM Sucrose, 0.5% NP-40, 2 mM sodium butyrate, 5 mM nicotinamide and Complete protease inhibitor (Roche)). Nuclei were washed with the extraction buffer without NP-40. 10 X nuclei volume of 0.2 M H<sub>2</sub>SO<sub>4</sub> were added to the pellet and vortexed until the pellet was dissolved. Nuclei were rotated overnight at 4°C. The samples were centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant was collected. Proteins in the supernatant were precipitated with a trichloroacetic acid (TCA) solution that was added to a 20 % final concentration, incubated for 30 min at 4°C. The samples were centrifuged at 14000 rpm for 10 min, and the pellet containing histones were washed by cold acetone with 0.1 % HCl once, and twice with cold acetone. Pellets were air dried, and submitted for proteomic studies or re-dissolved in 8 M Urea for Western blot.

### **III.7. Protein sample preparation and Western blot**

Proteins were prepared for Western blot from various samples depending on the needs of each experiments. For the whole testis extract (WTE), testes isolated from euthanized mice were decapsulated, and the tubules were placed in 8 M urea. The tissue was homogenized, and sonicated at 250 J. The samples were then centrifuged at 14000 rpm at 10 min, and the supernatants were collected.

Whole cell extract were prepared from either TGC or purified round spermatids. The cells were lysed in LSDB500 (50 mM Hepes pH 7.0, 3 mM MgCl<sub>2</sub>, 20% glycerol, 500 mM NaCl, 0.25% NP-40, 1 mM DTT, Complete protease inhibitor), rotated for 1 hr at 4°C. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4°C, and the supernatant was diluted twice by LSDB0, in order to obtain a final concentration of 250 mM NaCl.

Nuclear extracts were prepared from TGC or round spermatids. The nuclei were isolated by lysing the cells in the hypotonic buffer (10 mM Hepes NaOH 7.0, 15

mM MgCl<sub>2</sub>, 10 mM KCl, 340 mM sucrose, 0.25% NP-40, 1 mM DTT, Complete protease inhibitor) on ice for 5 min, and centrifuged at 2000 rpm for 5 min at 4°C. The nuclear proteins were extracted as described for the whole cell extract.

Western blot were performed after running the protein samples on a SDS sample buffer (62.5 mM Tris HCl pH 6.8, 10% glycerol, 3% SDS, 350 mM beta-mercaptoethanol) on SDS-PAGE gels (6 to 15% polyacrylamide) or NuPAGE 4-12% Tris-glycine gradient gel (Life Technologies). The resolved proteins on a gel were transferred onto nitrocellulose membrane. The membrane was blocked with 5% milk in PBS-T. The primary antibody was diluted in 1% milk in PBS-T and incubated for 3 hr or overnight. After three washing steps with PBS-T, the membrane was incubated with corresponding secondary antibodies conjugated with HRP diluted in 1% milk in PBS-T for 30 min at RT. The membrane was washed three times in PBS-T, and the signal was revealed by treating the membrane with Clarity™ Western ECL Substrate (Biorad), exposing to the X-ray film.

### **III.8. Immunoprecipitation, pull-down experiments**

Anti-p300 and anti-Nut immunoprecipitations were performed on WT TGC total cell extract in the LSDB250 (LSDB containing 250 mM NaCl.). 2 µg of the primary antibody was coupled with 30 µl of Dynabeads® Protein G (Life Technologies), and incubated with the protein extract for 4 hr at 4°C. Non-specific rabbit IgG was used as control. The beads were washed with LSDB250 3X, and dissolved in 30 µl of SDS sample buffer.

Peptide pull-down was carried out using nonacetylated or acetylated histone H4 tail peptide conjugated with biotin (Eurogentec). 6 nmol per reaction of the peptide was bound by 40 µl of Streptavidin Sepharose™ High Performance (GE Healthcare Life Sciences). Protein extracts of Cos cells transfected with HA-mNUT only or co-transfected with GFP-Brdt SP6, GFP-Brdt CP or GFP-Brdt FLS were prepared with LSDB500 extraction. The peptide bound beads were added to the extracts, and the pull-down reaction was done in LSDB250 incubated for 2 hr at 4°C. The beads were washed with LSDB250 three times, and 40 µl of the SDS sample buffer was added.

JQ1 conjugated beads used for JQ1 pull-down and corresponding naked agarose beads (to be used for the control) were provided by Dr. Carlo Petosa (IBS, Grenoble). Testis extracts were prepared with the LSDB500 and then diluted to 250 mM NaCl. 75  $\mu$ l of the beads were mixed with the extract and incubated at 4°C overnight. Beads were washed with LSDB250 3X, and mixed with 30  $\mu$ l of the SDS sample buffer.

### **III.9. Antibody generation and purification**

Antibodies against mouse NUT (mNUT) were generated by immunizing rabbits (Covalab) with five different NUT fragments. The NUT fragments were provided by Dr. Panagis Filippakopoulos (Oxford).

Generated anti-mNUT antibodies were further purified from the sera by affinity purification using GST-conjugated human NUT F1c fragment (GST-F1c). GST-F1c was purified through bacterial expression (BL21). After transformation and induction of GST-F1c in 2400 ml BL21 culture, cells were lysed in 40 ml of lysis buffer (40 mM Tris HCl pH 8.0, 0.3% TritonX-100, 750 mM NaCl, 2 mM DTT and complete protease inhibitor). The cells were sonicated at 250 J 5X, and centrifuged at 9000 rpm for 30 min. 4 ml of Glutathione Sepharose<sup>™</sup> 4 Fast Flow (GE Healthcare Life Sciences) was added to the lysate and incubated for 1 hr at 4°C. The beads were washed by the buffer CB (100 mM carbonate buffer pH8.3, 500 mM NaCl) 3X, and the bound GST protein was eluted by the buffer CB containing 30 mM glutathione. Fractions enriched with GST-F1c were pooled and used as baits for the affinity purification of the antibody.

For the affinity purification of the antibody, CNBr-activated Sepharose<sup>™</sup> 4B (GE Healthcare Life Sciences) was used. 285 mg of the CNBr beads were swelled in 1 mM HCl, and washed with buffer CB 3X. The beads were mixed with pooled fraction of GST-F1c (1 mg) and incubated at 4°C overnight. The beads were washed with buffer CB twice and blocked by 0.1 M Tris-NaCl pH 8.0 for 1 hr at RT. The beads were then washed 3x in the following buffers: 0.1 M Tris-NaCl pH 8.0, then acetate buffer pH 4.0 (100 mM sodium acetate, 500 mM NaCl). The beads were further washed with PBS 3x, and the antibody was captured by incubating the GST-F1c

beads with 3 ml of antiserum mixed with 3 ml of PBS for 16hr at 4°C. Finally, the beads were washed 3x with PBS and the bound antibodies were eluted by 100 mM glycine pH 2.2. The eluted fractions were neutralized by 2 M Tris HCl pH 8.0, and the fractions enriched in the antibodies were pooled. Amicon® Ultra-0.5 Centrifugal Filter Devices 10K (Merck Millipore) were used to concentrate the purified antibody. The specificity of the antibody was confirmed by Western blot with the *WT* and *Nut*<sup>-/-</sup> TGC nuclear extracts.

### **III.10. *In vitro* HAT assay**

The *in vitro* HAT assay with purified NUT F1c, p300 short (320-3094) and histone dimers, tetramers and octamers were performed as described in Reynoird et al., 2010. The experiment was performed in collaboration with Dr. Daniel Panne (EMBL, Grenoble).

Briefly, GST-fused NUT F1c fragment was produced in IPTG-induced BL21 bacterial expression with Pet28 plasmids. GST-F1c was purified by Glutathione sepharose beads (GE healthcare). GST-F1c bound to the beads were eluted with 25 mM Glutathione in 50 mM Tris pH 8.0, and the eluted fractions were concentrated by using Amicon Ultra 10,000 NMWL (Millipore).

p300 short (324-2094) was produced by vaculovirus expression system. FLAG-p300 short was cloned in the pFASTBAC1 (Invitrogen) which were used for infecting Hi5 insect cells. The cells were collected after 72 hr from the infection and lysed by freezing and thawing in equilibration buffer (20 mM Tris pH 8.0, 300 mM NaCl, Complete Protease Inhibitors (Roche)). FLAG-p300 short was purified by FLAG M2-agarose affinity gel (Sigma). The bound protein was eluted with 0.1 mg/ml triple FLAG peptide (MDYKDHDADYKDHDIDYKDDDDK) dissolved in equilibration buffer. Eluted fractions were concentrated using an Amicon-ultra 30 unit (Amicon) and further purified on a Hi-Load 16/60 Superdex 200 gel filtration column (GE healthcare) equilibrated in 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM dithiothreitol (DTT).

Purified core histones were obtained by IPTG-Induced BL21 bacterial expression, followed by histone acid extraction protocol from Abcam.

For HAT assays, all the purified components (GST-F1c, p300 short and core histones) were incubated 2h at 30 °C with 25 µl of HAT buffer (10 % Glycerol, 25 mM Tris HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, Complete EDTA free (Roche), 0.05 µg/ml TSA, Acetyl CoA 14C 1 µl at 20nCurie/µl (MPbio)). The result was evaluated by Western blot with the acetyl-lysine recognizing antibody.

### **III.11. Proteomic-based analysis of histone PTMs**

Histones extracted from *WT* and *Nut*<sup>-/-</sup> round spermatids were subjected for PTM analysis through HPLC/MS/MS, as described in Montellier et al., 2013, in collaboration with Dr. Yingming Zhao (University of Chicago) and Dr. Minjia Tan (SIMM, Shanghai).

Histone bands were in-gel digested as described in Chen et al., 2005, and in-solution tryptic digestion and in vitro propinylation were performed as described in Garcia et al., 2007 and Tan et al., 2011. Treated peptides from *WT* and *Nut*<sup>-/-</sup> were labeled with light (<sup>12</sup>C<sub>6</sub>) and heavy (<sup>13</sup>C<sub>6</sub>) propionic anhydride, respectively, and then both labeled peptides were mixed in equal amount. The peptides were fractionated by Agilent 3100 OFFGEL fractionator (Agilent) and desalted by µ-C18 Ziptip before HPLC/MS/MS analysis.

The peptides were dissolved in 10 µL of HPLC buffer A (Formic acid 0.1% (v/v) in water), and 2 µL was injected into a Nano-HPLC system (Eksigent Technologies, Dublin, CA). The peptides were separated using capillary HPLC column (100-mm length x 75-µm inner diameter) containing Jupiter C12 resin (4-µm particle size, 90-Å pore diameter, Phenomenex, St. Torrance, CA) with a 120 min HPLC gradient from 5 to 90% HPLC buffer B (formic acid 0.1% in acetonitrile) at a flow rate of 200 nL/min.

The eluted materials were directly electrosprayed into an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) by a nanospray source. The operation of the mass spectrometer was in a data-dependent mode with resolution 6000 at  $m/z$  400.

Peak lists were generated by the `extract_msn.exe` software (v5.0, Thermo Scientific). All MS/MS spectra were searched against the NCBI IPI\_mouse\_v3\_74 protein sequence database using the Mascot search engine (version 2.1.0, Matrix Science, London). Trypsin was specified as digesting enzyme. A maximum of 5 missing cleavages were allowed. Mass tolerances for precursor ions were set at  $\pm 10$  ppm for precursor ions and  $\pm 0.5$  Da for MS/MS. Lysine acetylation, lysine mono and di-methylation, lysine crotonylation, and arginine methylation were included as variable modifications. All the identified peptides with Mascot score above 20 and E-value below 0.05 were manually verified as described previously (Chen et al., 2005). Quantification of PTMs was based on the precursor ion intensities of peptides labeled by light and heavy stable isotopes. Briefly, after identification of all PTM site and modification type, the highest intensity of the peptide in the mass spectra was selected manually. The intensity ratio of the light and heavy isotope peaks was used for quantifying the relative abundance of the PTM between the two samples.

### **III.12. Transcriptomic analysis**

Analysis of purified RNA was carried out in collaboration with Dr. Philippe Guardiola (Université d'Angers), and Dr. Sophie Rousseaux and Florent Chuffart from our team performed the treatment of the data.

RNA from pachytene spermatocytes (P) and round spermatids (R) were extracted from *WT* (WT) and *Nut*<sup>-/-</sup> (KO) using the Qiagen RNeasy minikit, and analyzed on the Illumina whole-genome chip as described in Montellier et al., 2013 and Gaucher et al. (2012). For each of the 4 conditions (WT P, WT R, KO P, KO R), 6 replicates were analyzed.

The Illumina Total Prep RNA Amplification Kit (Applied Biosystems / Ambion, Austin, USA) was used to generate biotinylated, amplified cRNA according to the manufacturer recommendations. Hybridization, staining and detection of

cRNAs on Illumina Mouse WG-6 v2 Expression BeadChips were performed according to the manufacturer's protocol. The Illumina I-Scan system was used to scan all Expression BeadChips, according to Illumina recommendations.

Raw signals of probes across all samples were filtered according to their levels. Only probes with the associated p-values lower than 0.05 across all 6 replicates were kept. From the initial 45281 probes, we retained 17851 relevant probes. The Quantile normalization method was applied to these relevant probes using the function *normalized.quantiles* from the R package *preprocessCore*. Filtered and normalized data were then log<sub>2</sub>-transformed.

To define a list of genes affected by the knockout (KO) of *Nut*, two analyses of variance (ANOVA) were performed. The first analysis concerns WT R vs. KO R. For each gene, we consider the log<sub>2</sub> of its expression according to genotype. The effect of the KO on the expression level ("KO effect") was computed as the sign of the *beta* multiplied by the Benjamini-Hochberg adjusted to the level of 5% FDR –  $\log_{10}(p\text{-value})$  of the ANOVA test. The second ANOVA concerns 4 conditions: WT R, WT P, KO R, and KO P. For each gene, we consider the log<sub>2</sub> of its expression according to the genotype, the cell type and the combination of these two conditions. Since our interest was in the differentially expressed genes between WT R and KO R, we pay a particular attention on genes that are differentially expressed in R and not in P. We calculated "R:KO effect" which define the combined effect of R (cell type condition) and KO (genotype condition) as following: a sign of the *beta* interaction term multiplied by the Benjamini-Hochberg adjusted to the level of 5% FDR –  $\log_{10}(p\text{-value})$  of the ANOVA test corresponding to this interaction term. Then we consider the genes that had KO effects and a R:KO effects lower than  $-(-\log_{10}(0.05))$ , greater than  $-\log_{10}(-0.05)$  and between  $-(-\log_{10}(-0.05))$  and  $-\log_{10}(-0.05)$  were considered as "downregulated in R KO samples", "upregulated in R KO samples", or "not affected by the KO", respectively. The expression patterns of the NUT-affected genes ("downregulated in R KO samples" or "upregulated in R KO samples") were represented in heatmaps using the R software.

In order to test if the genes affected by the absence of NUT were also those whose expression levels would be affected by the absence of BRDT's first bromodomain BD1, the expression profile of NUT-affected genes were searched



against the transcriptomic data of *Brdt* $\Delta$ *BD1* mice's testes at age of 20 d.p.p. (our data published in Gaucher et al., 2012). The expressions of all genes that were downregulated in *Nut* $^{-/-}$ , all genes that were up regulated in *Nut* $^{-/-}$ , and 1000 genes not affected by the knockout of *Nut* were compared between *WT* and *Brdt* $\Delta$ *BD1*. The result was represented in box plots. In each category of genes, the expression levels between *WT* and *Brdt* $\Delta$ *BD1* were compared by Mann-Whitney test under 5% thresholds.

### III.13. Cell culture and transfection

Cos7 cells were cultured in DMEM supplemented with 1% Penicillin-Streptomycin, 10% fetal bovine serum and 2% L-glutamine, in the humidified incubator maintaining 5% CO<sub>2</sub>. Cells were grown to 60 to 80 % confluence in 10 cm dishes by the day of transfection. Transfection was done using Lipofectamine 2000 (Invitrogen) in the ratio of 1 ul per 1  $\mu$ g of transfected plasmid. The plasmids were transfected in the following amount: HA-NUT 8  $\mu$ g, GFP-BRDT SP6 2  $\mu$ g, GFP-BRDT CP 10  $\mu$ g, GFP-BRDT FLS 4  $\mu$ g. The cells were collected 24 hr after the transfection.

### III.14. Antibodies

Experiments	Names	Manufacture	Reference	Dilution/amount	
Western blot	NUT	Homemade		1/1000	
	BRDT	Homemade		1/1000	
	p300 C-20	Santa Cruz	sc-585		
	Kac	Cell Signaling Technology	9441S	1/1000	
	H4K5ac	Active Motif	61523	1/1000	
	H4K8ac	Active Motif	61525	1/1000	
	H4K12ac	Active Motif	39165	1/2500	
	H3	Abcam	ab1791	1/1000	
	$\beta$ -Actin	Sigma Aldrich	A5441		
	HA	Covance	MMS-101R	1/1000	
	GFP	Covance	MMS-118R	1/5000	
	Immunofluorescence	NUT	Homemade		1/25
		H4K5ac	Active Motif	61523	1/500

	TH2B	Abcam	Ab23919	1/500
	TP2 (TNP2 K-18)	Santa Cruz	21706	1/200
	PRM1 (M-51)	Santa Cruz	30176	1/200
	$\gamma$ H2A.X	Epitomics	2212-1	1/500
	Lectin PNA-Alexa 647	Thermo Fisher Scientific	L32467	1/50
Immunohistochemistry	NUT	Homemade		1/25
	H4K5ac	Epitomics	1808-1	1/500
Immunoprecipitation	p300 C-20	Santa Cruz	sc-585	2 $\mu$ g
	NUT	Homemade		2 $\mu$ g

### III.15. PCR primers

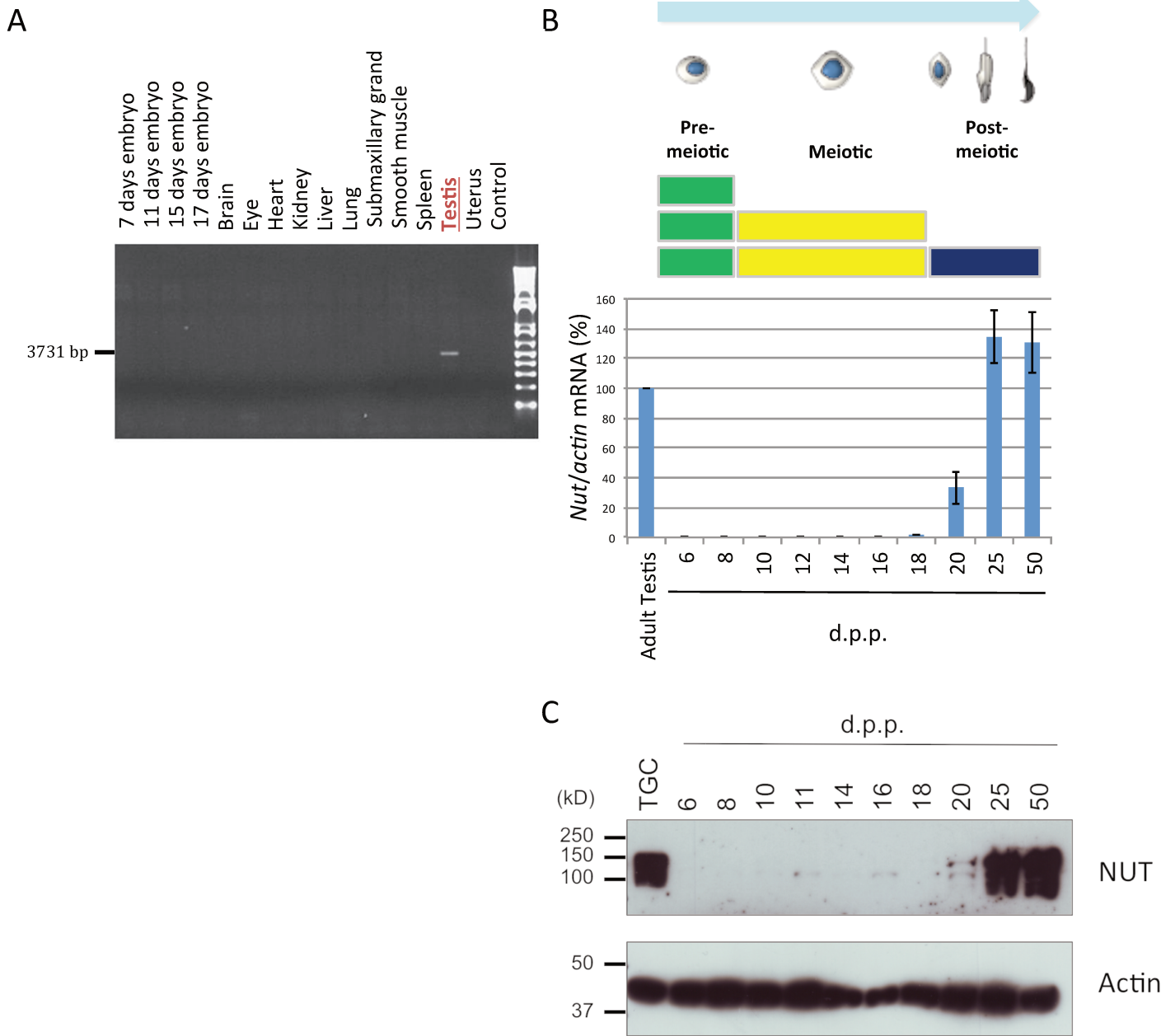
Experiment	gene	Forward	Reverse
RT-PCR	<i>Nut</i>	CAGGACTTCCTCTGCAGGTC	CCCTGGAGGATCAAGTTTCA
RT-qPCR	<i>Nut</i>	ACCTCGGATTATGGCATCAG	GCCAGTGCCAGGTTTCAT
Genotyping	<i>Nut WT</i>	ACACAAGCAACAACCATGGA	TCTGGATTCATCGACTGTGG
	<i>Nut null</i>	AGAGGAAGTCTCCAGCCTT	
Southern blot	<i>Nut</i>	AAATACATGGTGGCAGGAA	ACAGTCTGGGCTGAGAGAA

## IV. Results

### IV.1. Expression of *Nut* starts after completion of meiosis

As an initial step to investigate the function of NUT, its expression profile in mice was determined. RT-PCR revealed that *Nut* transcript was specifically detected in the RNA extracted from testes, but not the other mouse tissues (**Figure 1A**). These data agree with the previous finding from the search in human cDNA database that *NUT* is specifically expressed in testis (French et al., 2003). Therefore, the expression pattern of *Nut* is conserved between human and mouse, and NUT is indeed a testis specific factor.

Next, the expression pattern of *Nut* during spermatogenic cells differentiation was determined using the cells from the first wave of spermatogenesis. In mice, the first cycle of spermatogenesis completes within the first 50 days postpartum (d.p.p.). From 6 to 8 d.p.p., spermatogonia multiply via mitosis. Between 10 and 18 d.p.p., the spermatocytes appear and undergo two consecutive meiotic divisions to produce spermatids. After 20 d.p.p., the first post-meiotic cells (round spermatids) appear and differentiate. RNAs were collected from the developing testes of postnatal and adult mice at ages of 6, 8, 10, 12, 14, 16, 18, 20, 25 and 50 d.p.p., and analyzed by RT-qPCR. *Nut* mRNA became detectable first at 20 d.p.p., and highly accumulated up to levels comparable to the adult testis levels at 25 d.p.p (**Figure 1B**). This timing corresponds to the specific period when round spermatids appear in the testis. Western blot using extracts of testes during the first wave of spermatogenesis confirmed that the protein levels follow the same pattern as the mRNA levels; the protein started being detected at 20 d.p.p., and the levels increase until 25 d.p.p. (**Figure 1C**). Therefore, *Nut* is specifically expressed in post-meiotic cells undergoing spermiogenesis.

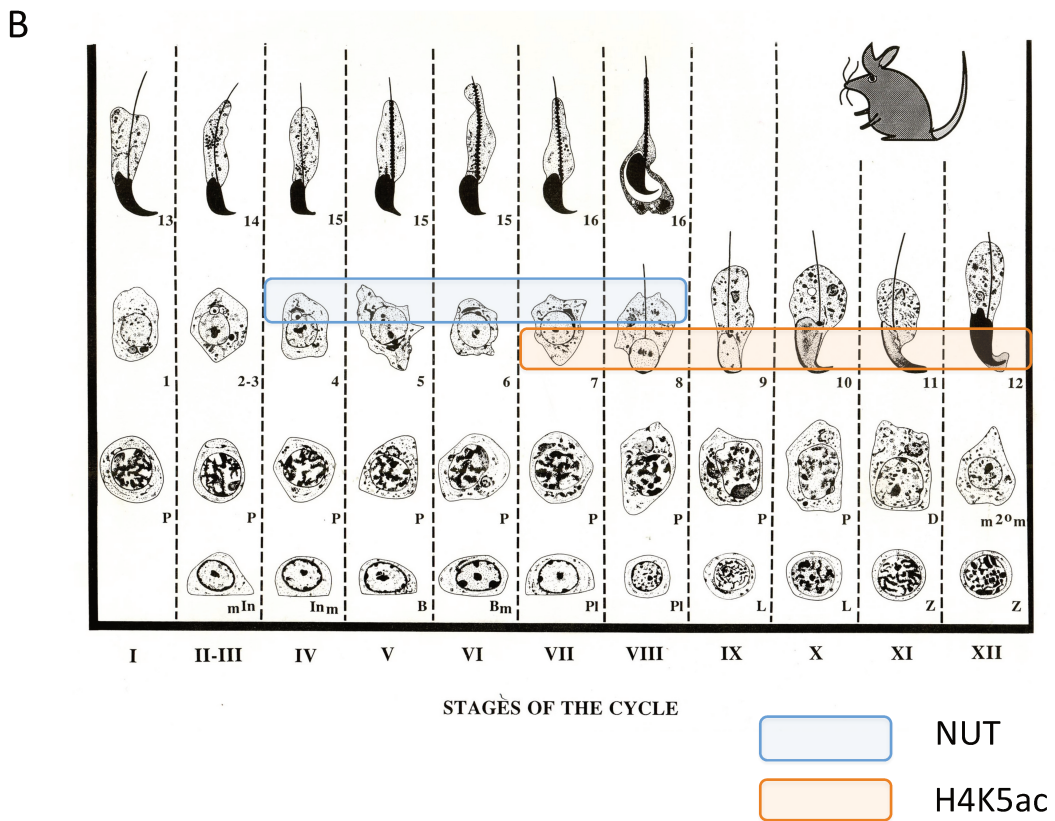
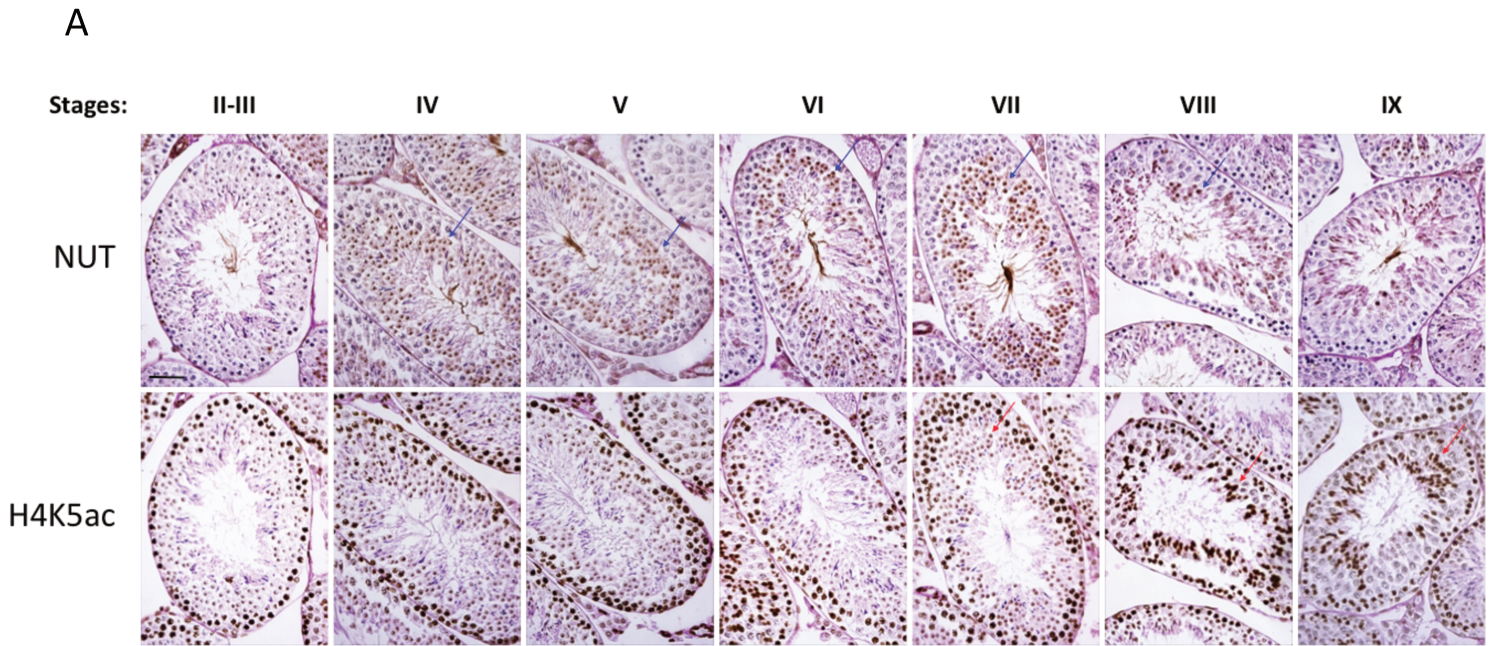


**Figure 1:** Expression profile of *Nut* gene. **(A)** *Nut* transcript was detected by RT-PCR with RNAs extracted from different tissues of mouse, followed by electrophoresis on 1 % agarose gel after PCR amplification. **(B)** Expression timing of *Nut* mRNA during the first wave of spermatogenesis was evaluated in RNAs extracted from the 6 to 50 days postpartum (p.p.t) and adult testes by RT-qPCR. The signal was normalized to *Actb*. Bars represent standard deviation from four experiments. **(C)** Accumulation of NUT protein in testis extracts from 6 to 50 d.p.p. mice and total germ cell (TGC) extracts from adult mice was revealed by Western blot. Actin was used as a loading control.

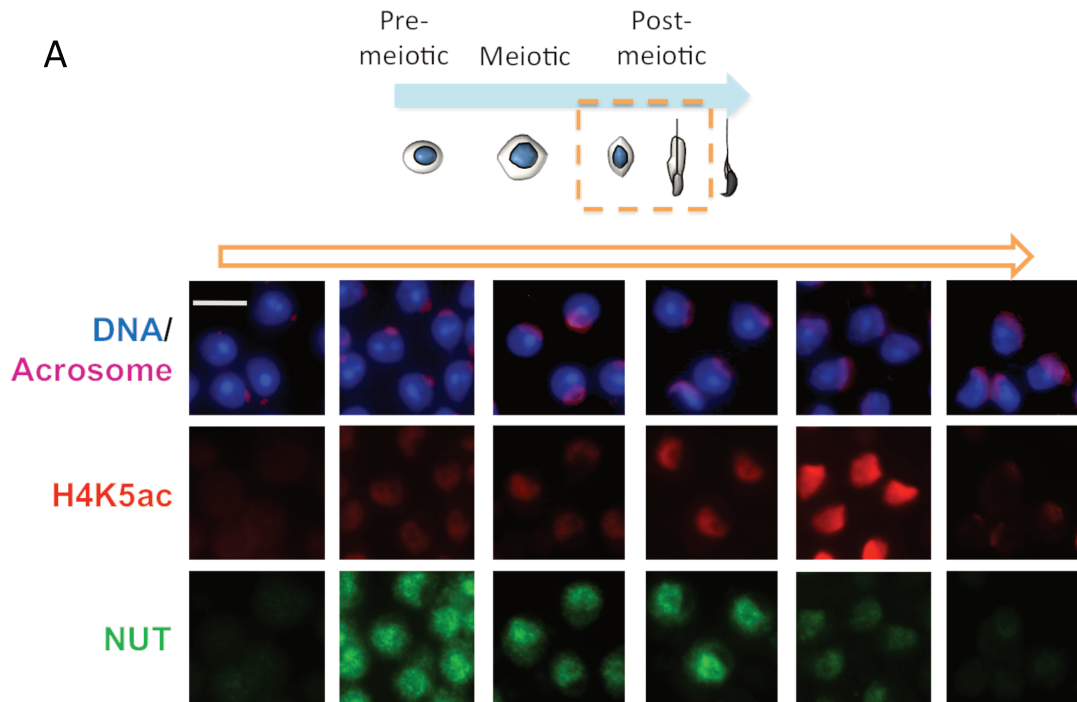
To confirm the expression pattern of NUT during the development of seminiferous tubule in adult mouse testis, sections of paraffin embedded mouse testis were analyzed by immunohistochemistry. Developmental stages of seminiferous tubules were defined based on the types of cells present in each tubule, using staging according to acrosomal development in the spermatids visualized by PAS staining. The localization of H4K5ac was also tracked. H4K5 is one of the residue become acetylated in the course of histone hyperacetylation wave. Two adjacent sections were obtained to capture the same tubules for NUT and H4K5ac detection. Within the development of male germ cells, the expression of NUT was detected in particular populations of round spermatids in stage IV to IX seminiferous tubules (**Figure 2A**). The expression starts in stage IV. The expression level increases in VI to VIII, and becomes almost undetectable after stage IX. (**Figure 2A**). These stages contain round spermatids at step 4 to 8 (**Figure 2B**). H4K5ac started appearing in step 7 round spermatids in stage VII tubules. The level of the H4K5ac strongly increases in step 8 round spermatids in the tubules at stage VIII and persists through the maturation of the cells until the histone are removed, representing the pattern of hyperacetylation wave. Overall, NUT is highly expressed when the hyperacetylation wave initiates, and decreases as the cells undergo further differentiation.

To further characterize the expression pattern of NUT, its subcellular localization was determined by immunofluorescence on squashed tubule preparation. The relative steps of the round spermatids maturation were defined by the acrosome development, visualized by Alexa 647-conjugated lectin. Here again, Nut is highly expressed when H4K5ac initiates, agreeing with the result from immunohistochemistry. In all the round spermatids expressing Nut, the protein is present in entire area of nucleus (**Figure 3A**). Therefore, NUT could act on the entire region of the chromatin rather than on specific or limited areas of nucleus.

Altogether, these results indicate that NUT is specifically activated in early round spermatids when the cells initiate the hyperacetylation wave, and that it could act on wide area of the round spermatid's nucleus.



**Figure 2:** (A) Expression of NUT in relative to a wave of histone hyperacetylation during seminiferous tubule development revealed by immunohistochemistry. In each stage, two successive sections were obtained to follow the hyperacetylation wave indicated by H4K5ac. Cells showing NUT or H4K5ac signals are indicated by blue and red arrows, respectively. Scale bar = 50  $\mu$ m. (B) Schematic representation of the steps of round spermatids in which NUT is present (blue box) and H4K5 is acetylated (orange box). The stages of seminiferous tubules are indicated by Roman numerals at the bottom. Numbers at the right bottom of each picture of spermatids represent steps of differentiation (1-16). The original table is from Lonnie D. Russell et al 1990.



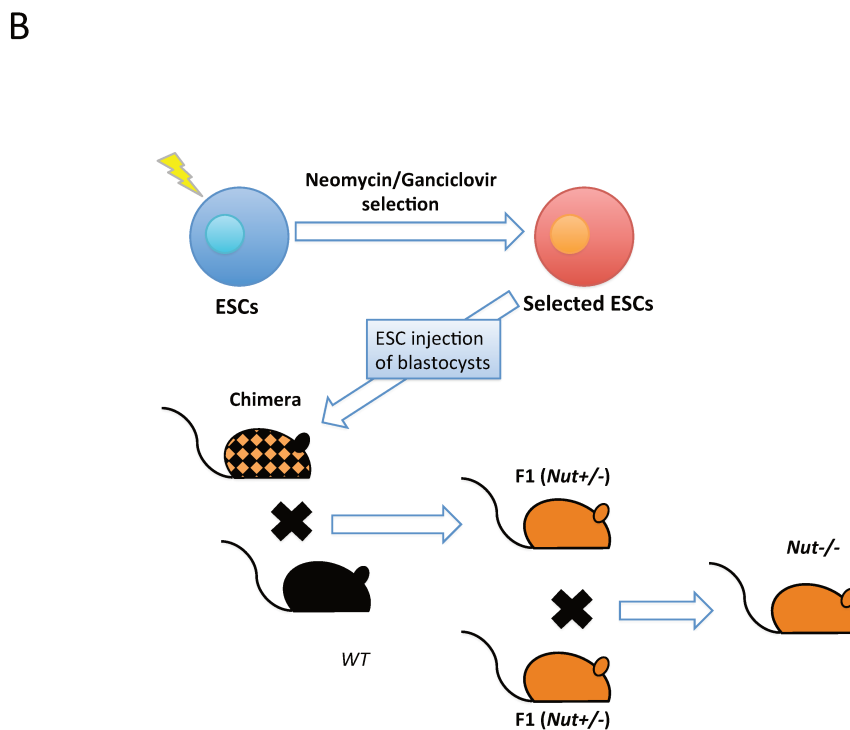
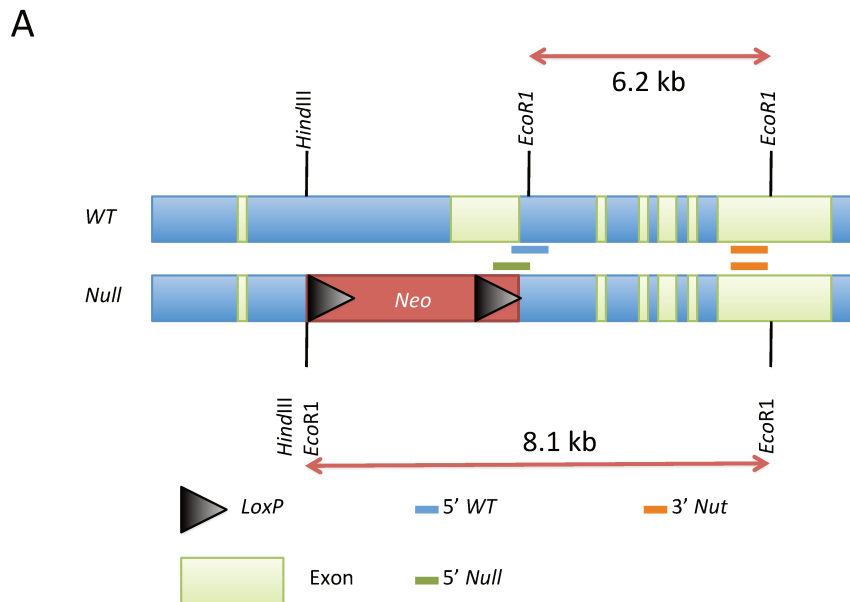
**Figure 3: (A)** Expression timing of NUT relative to the onset of the histone hyperacetylation and its intracellular localization revealed by immunofluorescence. The pictures represent the cells at the early to middle post-meiotic phase of spermatogenesis, as indicated in the schematic representation with the orange dotted box. The orange block-arrow indicates the maturation of spermatids (less mature to more mature). The relative developmental steps of the spermatids were identified based on the shapes of acrosome, visualized by alexa 647-lectin. H4K5ac was used as an indicator of the hyperacetylation wave. Scale bar = 10  $\mu$ m.

## IV.2. Generation of *Nut* knockout mice

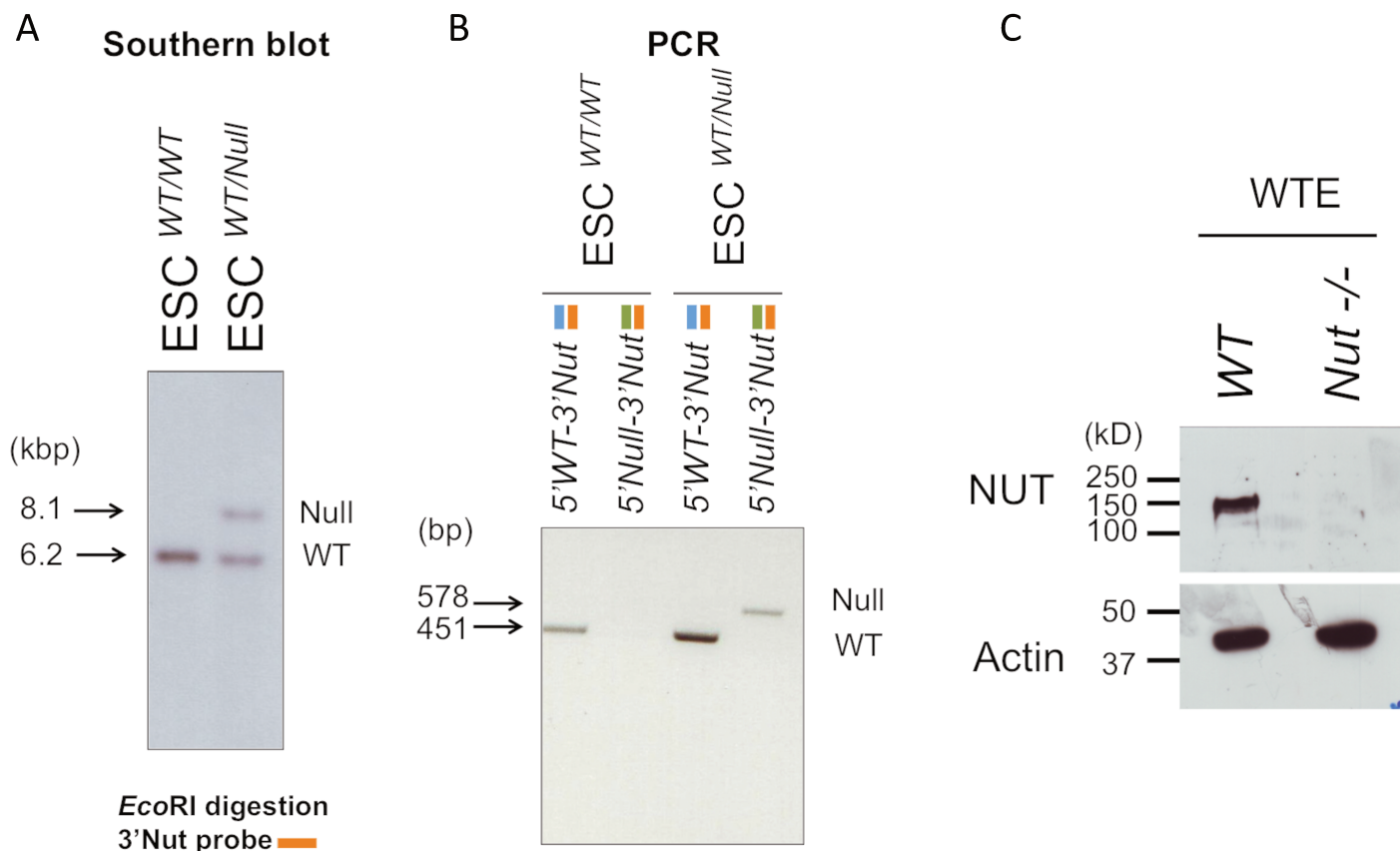
\**Nut*<sup>-/-</sup> mice were produced in collaboration with Dr. Matthieu Gérard (CEA, Saclay), based on the ES cells containing the knockout construct generated by Thierry Buchou and Fayçal Boussouar from our group.

In order to examine the specific effect of NUT, a knockout mouse model lacking the expression of *Nut* was generated. The *Nut null* allele was achieved by replacing the second exon of *Nut* gene by a *neo* cassette (**Figure 4A**). The targeting vector containing the *Nut null* allele was electroporated into mouse embryonic stem cells (ESCs) and integrated by homologous recombination. Beside the *neo* cassette flanked by the homologous sequences, the targeting vector also contained *TK* gene outside of the region of recombination. *TK* encodes thymidine kinase that induces cell death in response to Ganciclovir treatment. Utilizing the *TK* and *neo*, the electroporated ESCs were screened by Ganciclovir and neomycin in order to select a colony of ESCs harboring properly recombined allele (**Figure 4B**). The successful recombination of the *Nut null* allele was confirmed by Southern blot (**Figure 5A**) and PCR (**Figure 5B**). The selected ESCs were injected into blastocysts to generate chimeric mice, which were then crossed with wild-type (*WT*) mice to produce heterozygous F1s. Breeding between F1s successfully produced homozygous males for *Nut null* allele (*Nut*<sup>-/-</sup>). Western blot of testis extracts from a *WT* and *Nut*<sup>-/-</sup> confirmed the absence of Nut in the *Nut*<sup>-/-</sup> testis (**Figure 5C**).





**Figure 4:** Schematic representations of the knockout strategy to generate *Nut*<sup>-/-</sup>. **(A)** The second exon of *Nut* gene is replaced by neo cassette in the *Null* allele. *Eco*R1 sites and the indicated probes were used for recognition of *WT* or *Null* allele. **(B)** Workflow of generation of homozygous mice for *Nut* *Null* allele (*Nut*<sup>-/-</sup>). The targeting vector containing the construct shown in (A) was electroporated into mouse ESCs. The cells with the recombined allele were selected by Neomycin and Ganciclovir. The selected ESCs are injected to blastocysts to produce chimeric mice. The chimera was crossed with a WT to produce heterozygous F1s (*Nut*<sup>+/-</sup>). F1s were bred to produce *Nut*<sup>-/-</sup> mice.



**Figure 5:** (A) Southern blot from the selected ESCs with *EcoRI* digestion and 3' *Nut* probe (refer to the figure IV 4A) detection. 0.8% agarose gel was used for the electrophoresis. Detection of specific lengths of DNA fragments (6.2 kbp for *WT* allele and 8.1 kbp for *Null* allele) indicates the proper recombination of the *Null* allele. (B) PCR with the indicated primers that are shown in figure IV 4A. The 1.1% agarose gel was used for the electrophoresis. Successful amplification of specific fragments (451 bp for *WT* allele and 578 bp for *Null* allele) indicates the proper recombination of the *Null* allele. (C) Protein levels of NUT in *WT* or *Nut*<sup>-/-</sup> whole testis extract (WTE) revealed by Western blot. Actin was used as a loading control.

### IV.3. NUT is essential for sperm production

It is important to note that the *Nut*<sup>-/-</sup> mice did not show any deleterious defect in their viability. *Nut*<sup>-/-</sup> mice were generated from the crossing between heterozygous parents, and there was no apparent defect in heterozygous mice in male or female germ cell production or in fertility. *Nut*<sup>-/-</sup> mice did not show any embryonic lethality, and the pups fully grew to adult animals without any detectable defects. Their appearance is indistinguishable from that of *WT* mice. The female mice were completely fertile.

In order to evaluate the effect of absence of NUT in the adult mice, fertility and the spermatogenic ability was examined in *Nut*<sup>-/-</sup> males. Strikingly, crossing *Nut*<sup>-/-</sup> males with *WT* females did not produce any pups, demonstrating that *Nut*<sup>-/-</sup> exhibits complete male sterility (**Figure 6A**). In addition, *Nut*<sup>-/-</sup> male exhibited reduced testes weight compared to *WT* (**Figure 6B**), suggesting spermatogenic defects leading to infertility in *Nut*<sup>-/-</sup> males. In order to examine sperm production, cauda epididymides were collected from *WT* and *Nut*<sup>-/-</sup> mice, fixed in paraffin and stained with hematoxylin. Cauda epididymis normally contain maturing sperm exited from the testis. Strikingly, *Nut*<sup>-/-</sup> cauda epididymis were completely devoid of mature spermatozoa (**Figure 6C**), indicating a strong perturbation in spermatogenesis.

Overall, this is the first case reporting the knockout of *Nut* causes a complete sterility specifically in male mice. It is important to note that *Nut*<sup>-/-</sup> females were completely fertile, and that the knockout specifically affects male germ cell production. All together, NUT is an essential factor for production of spermatozoa and male fertility.

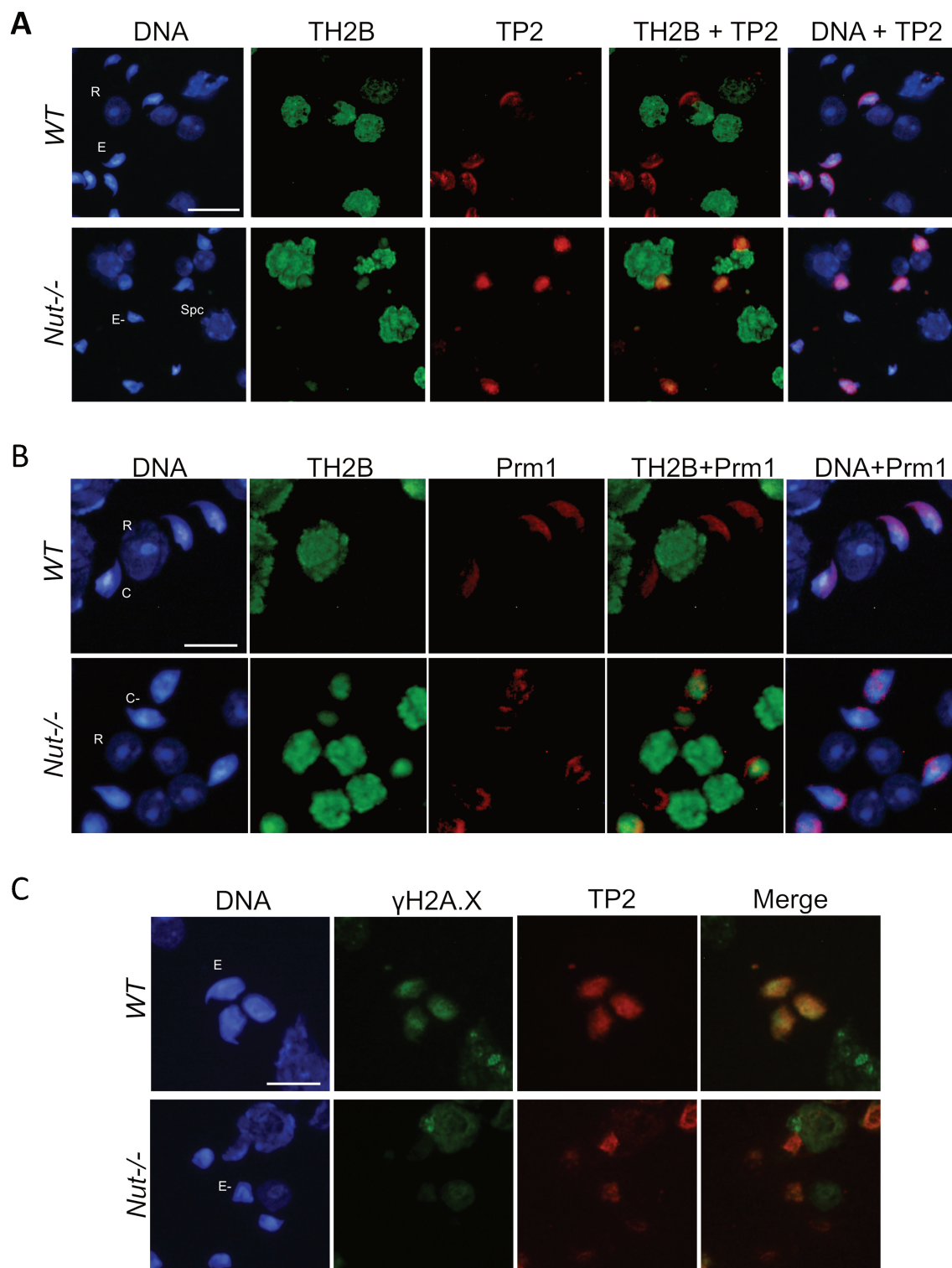


#### IV.4. NUT is essential for histone-to-protamine replacement

NUT being specifically expressed in round spermatids (**Figure 1B, C**) and essential for sperm production, suggested that the role of NUT could be important for the post-meiotic phase of spermatogenesis, the development of spermatids during spermiogenesis, including the histone-to-protamine replacement. Therefore, the effect of the absence of Nut in post-meiotic maturation was evaluated.

In order to investigate the cause of spermatogenic arrest, testis imprints and squashed seminiferous tubules were prepared with *WT* and *Nut*<sup>-/-</sup> testis for the immunofluorescence detection of TH2B, TP2 and Prm1 were tested. TH2B is a germ cell specific histone H2B variant that replaces almost all the H2Bs in the male germ cells (Montellier et al., 2013). Here, TH2B was used as an indicator for nucleosome retention, and TP2 and Prm1 for labeling cells during and after completion of histone replacement. Strikingly, TP2 and TH2B were detected in the same *Nut*<sup>-/-</sup> elongating spermatids, unlike *WT* cells in which TP2 and TH2B do not overlap within the same cell (**Figure 7A**). Moreover, in *Nut*<sup>-/-</sup> spermatids at later stage, although Prm1 is synthesized, it accumulates at the periphery of the nucleus in cells that also show prolonged retention of TH2B (**Figure 7B**).

During chromatin remodeling in spermiogenesis, it has been suggested that DNA double strand breaks are associated with histone-to-protamine replacement that could be detected by the presence of H2A.X phosphorylated at serine 139, or  $\gamma$ H2A.X (Leduc et al., 2008). TP2 and  $\gamma$ H2A.X normally show overlap in elongating spermatids as these cells are undergoing histone replacement. TP2 positive *Nut*<sup>-/-</sup> spermatids, however, lacked signal of  $\gamma$ H2A.X (**Figure 7C**). This absence of  $\gamma$ H2A.X in TP2 expressing spermatids is an additional evidence that histone replacement does not take place in the mutant. These results indicate that, although TP2 and PRM1 are both synthesized, they cannot be incorporated because histones are not removed. The morphology of *Nut*<sup>-/-</sup> spermatids that undergo nuclear elongation and condensation appeared abnormal, with defective shapes which could be related to the defective chromatin remodeling. Therefore, NUT is essential for histone replacement and proper assembly of the chromatin in late spermatids.



**Figure 7:** spermatogenic cells collected from WT or *Nut*<sup>-/-</sup> mice for evaluation of histone replacement. Cells shown in the pictures include: spermatocytes (Spc), Round spermatids (R), TP2-positive elongating spermatids (E) or corresponding misshapen cells in *Nut*<sup>-/-</sup> (E-), and Prm1-positive condensing spermatids (C) or corresponding cells in *Nut*<sup>-/-</sup> (C-) as indicated. **(A)** Co-detection of TH2B (green) and TP2 (red). Scale bar = 10  $\mu$ m. **(B)** Co-detection of TH2B (green) and Prm1 (red). Scale bar = 5  $\mu$ m. **(C)** Codetection of  $\gamma$ H2A.X (green) and TP2 (red). Scale bar = 5  $\mu$ m.

The incomplete histone replacement observed in *Nut*<sup>-/-</sup> is strikingly similar to the phenotype of *Brdt* $\Delta$ *BD1* (Gaucher et al., 2012.), other mouse model that expresses a mutated form of BRDT lacking its first bromodomain (BD1). The binding of its first bromodomain to hyperacetylated histones is essential to trigger the histone replacement, and the lack of BD1 makes the binding of BRDT less efficient in *Brdt* $\Delta$ *BD1*. However, in *Nut*<sup>-/-</sup>, histone replacement do not take place despite fact that BRDT is intact. This suggests that BRDT by itself is not sufficient to drive the histone replacement, and that NUT could be a crucial factor for the BRDT-dependent chromatin reorganization. Since the histone replacement is closely linked with the global histone hyperacetylation, one hypothesis was that NUT could be involved in the regulation of histone hyperacetylation in spermatids before the histone replacement. This hypothesis is also supported by investigations of the molecular basis of BRD4-NUT oncogenic activity. Indeed, these investigations showed that NUT recruits p300 and modulates its HAT activity (Reynoird et al., 2010) suggesting that NUT could act as a modulator of histone acetylation. Additionally, the fusion of NUT with BRD4 observed in NMC, indicated that NUT, in its physiological setting, could also cooperate with a member of BET family. We therefore tried to test these two hypotheses in spermatogenic cells.

## IV.5. Potential interaction between NUT and BET factors in spermatids (preliminary results)

\*The *in vitro* study in this section and was obtained through collaboration with the group of Dr. Daniel Panne (EMBL, Grenoble). The JQ1 beads that I used in one of the experiments in this section was produced from Dr. Carlo Petosa (IBS, Grenoble).

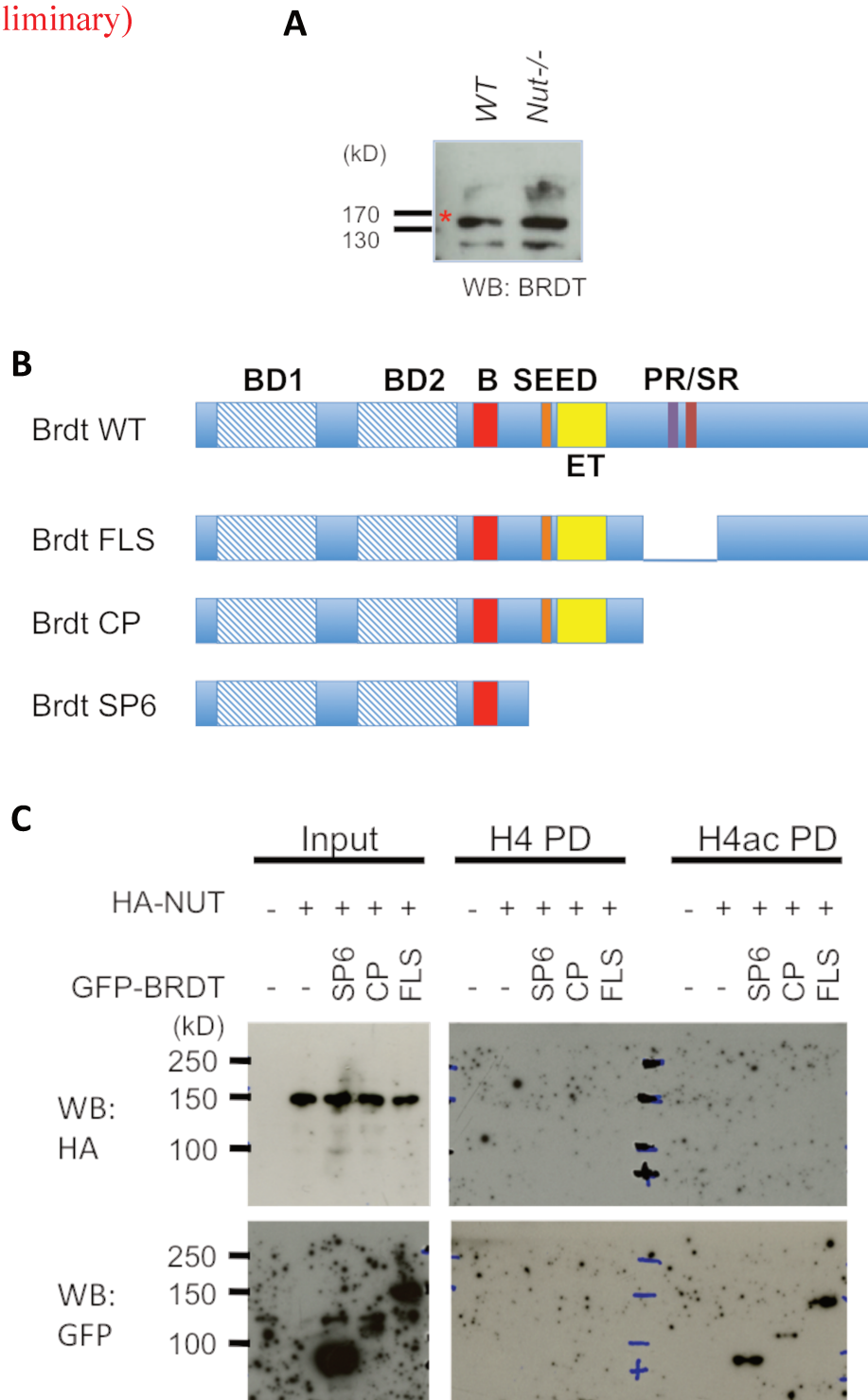
The overlapping expression timing of NUT and BRDT in post –meiotic cells, and the similarity between *Nut*<sup>-/-</sup> and *BrdtdABD1* phenotypes in terms of histone replacement defect, suggested that NUT and BRDT could be acting on the chromatin through the same pathway. In order to test their potential interaction, immunoprecipitation and pull-down based experiments were performed.

First, the levels of BRDT were compared between *WT* and *Nut*<sup>-/-</sup> testis by Western blot (**Figure 8A**). The protein levels of BRDT in *Nut*<sup>-/-</sup> appeared to be comparable to the *WT*, therefore the absence of *Nut* does not seem to affect BRDT expression.

Next, we evaluated the possibility of an interaction between NUT and BRDT. Three different BRDT fragments conjugated with GFP (GFP-BRDT) were co-transfected with HA conjugated mouse NUT (HA-NUT). Full-length fragment (FLS) includes entire BRDT protein except PR/SR region. DNA sequence of these parts of *Brdtd* gene is rich in CG content and did not allow PCR amplification, thus these parts were removed to ease the cloning. The SP6 fragment represents N terminal half of BRDT and includes the BD1, BD2 and motif B. A slightly larger fragment, CP, includes the N terminal half of BRDT including SEED and extraterminal domain in addition to all the regions found in SP6 (**Figure 8B**). BRDT as well as other bromodomain-containing proteins interacting factors were purified via acetylated H4 tail peptide (H4ac) pull down, and the result was checked by Western blot (**Figure 8C**). H4ac successfully captured all the BRDT fragments, while non-acetylated H4 peptide did not precipitate any BRDT. Unfortunately, none of the precipitated materials showed the presence of NUT.



(Preliminary)

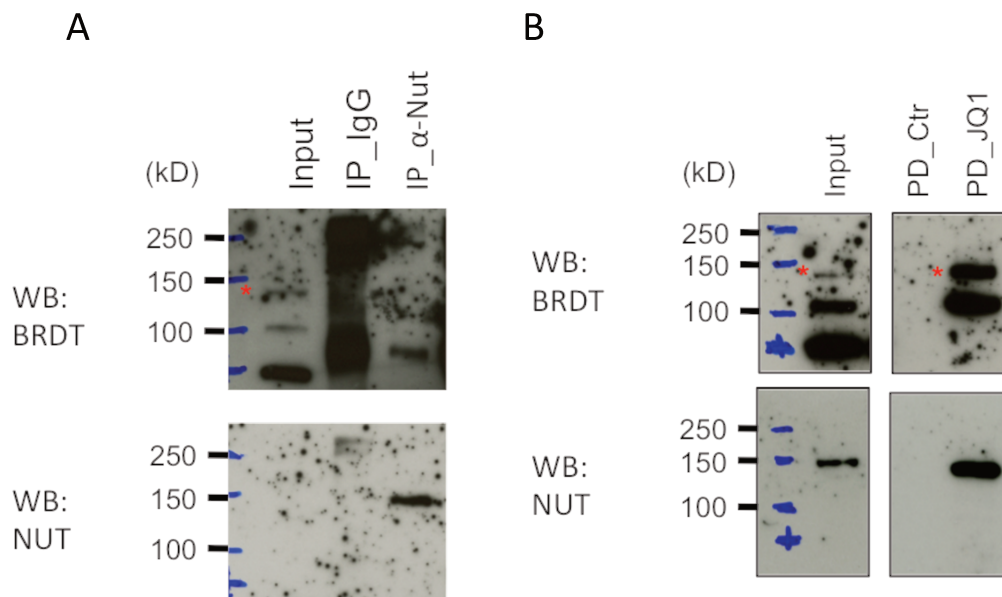


**Figure 8:** Evaluation of the potential interaction between NUT and BRDT. **(A)** The protein levels of BRDT in *WT* or *Nut*<sup>-/-</sup> testis extracts. BRDT is indicated by red asterisk. Other bands are non-specific bands or degraded product. **(B)** Three different BRDT constructs (FLS, CP and SP6) shown in the schematic representation were used. The native protein is shown as BRDT WT for the comparison. FLS differs from the native form in absence of PR/SR region. **(C)** The constructs shown in B fused with GFP were co-transfected with HA-NUT into Cos7 cells, and pull-down experiments were performed with either acetylated or non-acetylated H4 tail peptides, shown as H4acPD and H4PD, respectively. The result was revealed by Western blot with HA antibody or GFP antibody, detecting HA-NUT or GFP-BRDT fragments, respectively.

To test the potential interaction of BRDT and NUT in the germ cells, we attempted to precipitate NUT and its interacting proteins from *WT* male total germ cells (TGC) by using NUT antibody. The precipitation of NUT was confirmed by Western blot, however, BRDT did not seem to be co-precipitated in this experimental condition (**Figure 9A**).

We obtained a BET bromodomain inhibitor JQ1 immobilized on agarose beads, to purify BET proteins from testis extracts. Western blot confirmed BRDT was successfully captured in this experiment. Strikingly, the precipitated material also contained NUT (**Figure 9B**). Since JQ1 is not specific to BRDT, but has preferential binding ability to all bromodomains of BET family, the result does not directly represents the interaction between BRDT and NUT. However, this result suggests the interaction of NUT with at least one or more members of the BET family. In order to define the BET protein associating with Nut, further experiment is needed.

(Preliminary)



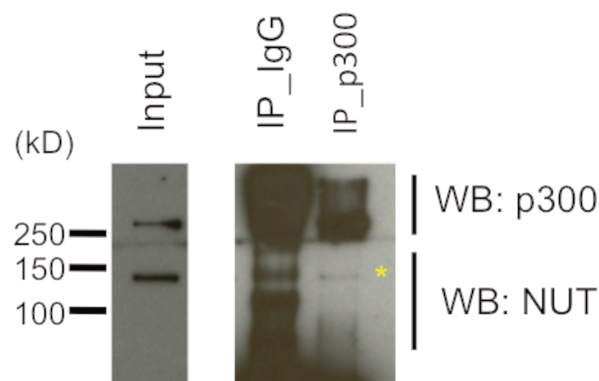
**Figure 9:** Potential interaction of BRDT or other members of BET in male germ cells. The bands of BRDT are indicated by red asterisks, and other bands in BRDT Western blots are backgrounds. **(A)** *WT* TGC were subjected for anti-NUT immunoprecipitation. IgG was used as the control. The final product was tested for NUT and BRDT by Western blot. **(B)** *WT* testis extract was used for the pull-down experiment using JQ1 fixed on the agarose beads. Empty agarose beads were used as the control. The presence of BRDT and NUT was checked by Western blot.

Specifically, more potent antibodies that could be used for NUT and BRDT immunoprecipitations than the conditions that is already tested, and specific antibodies against other BET factors are essential for further analysis.

#### IV.6. Potential interaction between NUT and p300 in spermatids (Preliminary results)

Our previous investigations characterized p300 expression in spermatogenic cells (Boussouar et al., 2014). Therefore p300 association with NUT is a possibility that was tested by immunoprecipitation by using antibodies against p300 in *WT* male TGC (**Figure 10**). The preliminary result shows that p300 and NUT co-precipitated, although the interaction is very weak. The result suggests the possibility for NUT to interact with p300 also in male germ cells, but further analyses with optimized IP conditions will be essential to definitely conclude.

(Preliminary)



**Figure 10:** Potential interaction between NUT and p300 in the spermatogenic cells. TGC from *WT* male were subjected for immunoprecipitation with p300 antibody. Nonspecific IgG was used as the control. The result was analyzed by Western blot with NUT or p300 antibodies. The position of NUT is indicated by a yellow asterisk.

## IV.7. Round spermatid- specific pattern of histone PTM is regulated by NUT

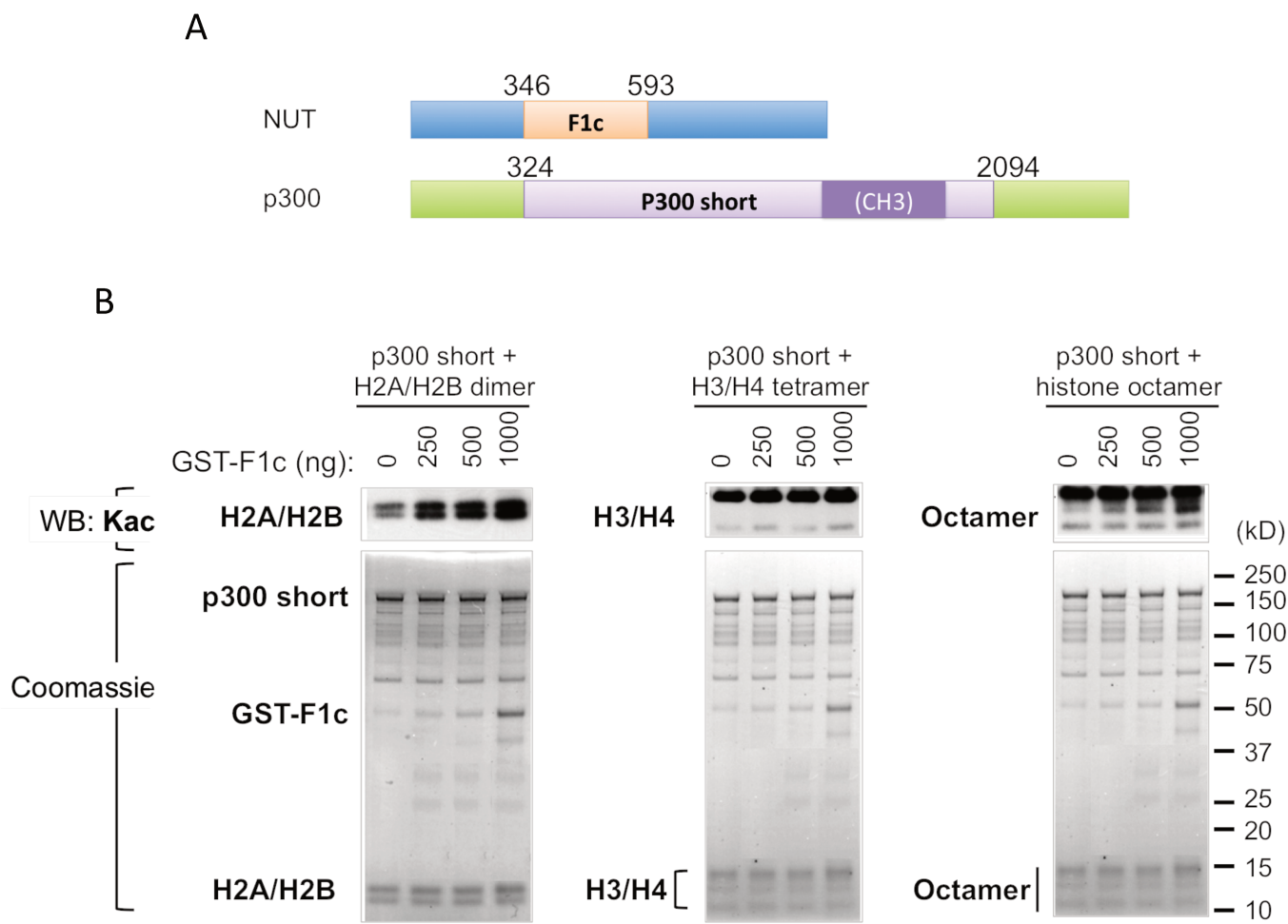
\*The result of this section was obtained in collaboration with Dr. Yingming Zhao (University of Chicago) and Dr. Minjia Tan (SIMM, Shanghai) who performed proteomic analysis on histones that Thierry Buchou from our team and I purified.

In the previous study of NMC, NUT was identified as the key moiety in BRD4-NUT responsible for the enhancement of HAT activity, acting by direct interaction with p300 (Reynoird, et al. 2010). In the case of spermatogenesis, we hypothesized that NUT could also be involved in histone hyperacetylation, setting up the binding platform for the BRDT to act on histone replacement. Based on the similarities in the phenotypes with defective histone replacement between *Nut*<sup>-/-</sup> and *Brd4ABD1*, as well as the HAT activity enhancement effect observed in NMC, we suspected that histone acetylation could be altered in *Nut*<sup>-/-</sup> round spermatids.

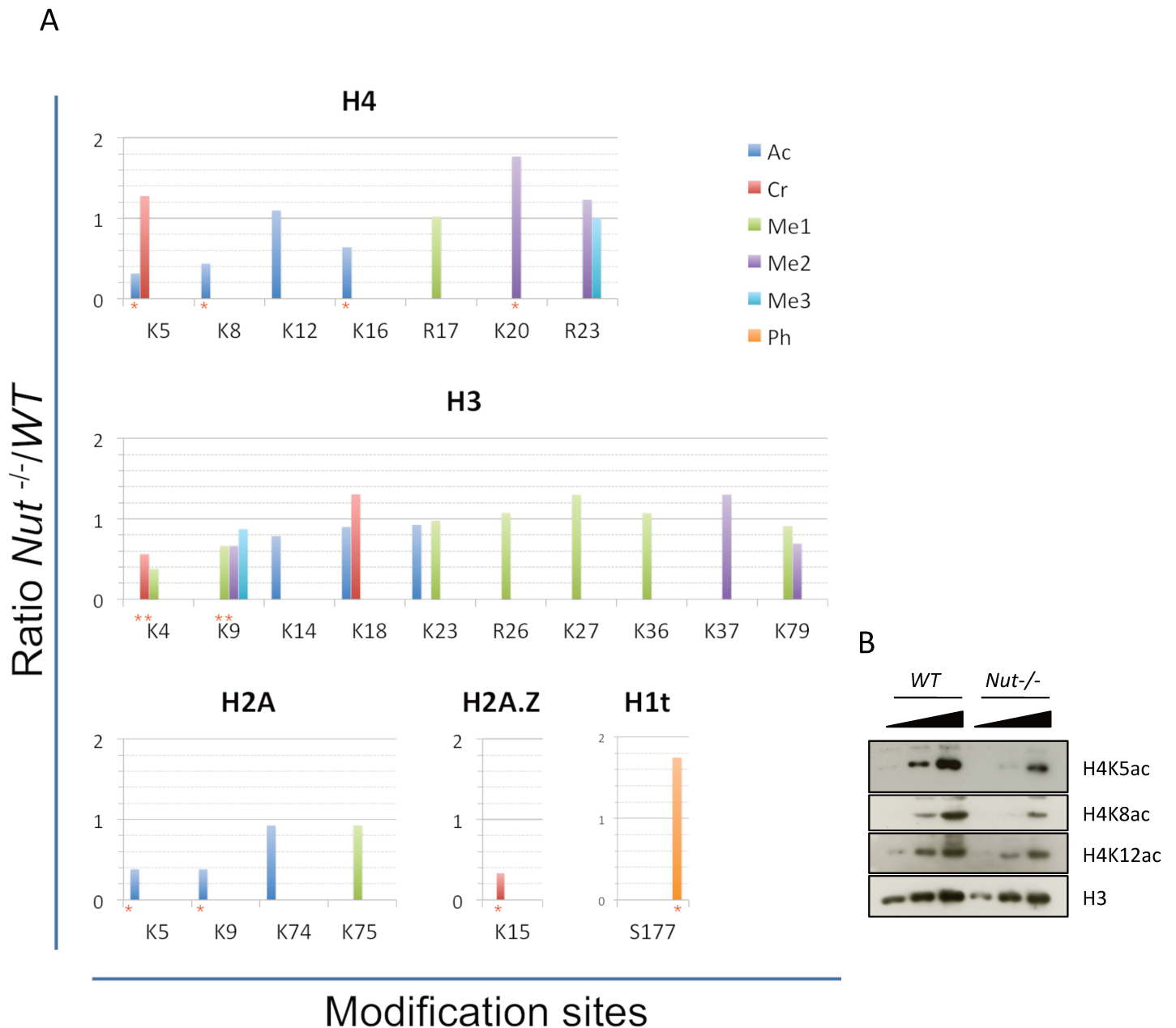
In order to investigate the ability of Nut to enhance histone acetylation on chromatin, *in vitro* HAT assay was performed using bacterially expressed fragments of NUT and recombinant p300 (expressed in baculovirus). It was demonstrated previously that NUT is able to enhance HAT activity on soluble non-nucleosomal histones (Reynoird et al., 2010.). The NUT fragment corresponds to the region of 346 to 593 amino acids which can interact with p300, and the p300 fragment which corresponds to 324 to 2094 amino acids containing NUT interacting domain (CH3) as well as catalytic domain; these fragments are referred respectively as F1c and p300 short, respectively (**Figure 11A**). As substrates, H2A/H2B dimers, H3/H4 tetramers or histone octamers were incubated with GST-F1c and p300 short to detect histone acetylation activity. The result revealed that H2A/H2B dimer acetylation is enhanced by the presence of F1c in a dose dependent manner (**Figure 11B**, left). H3 acetylation also showed slight increase in its level of acetylation with 250 ng of F1c, although it was not possible to accurately interpret the result due to the signal saturation (**Figure 11B**, middle). The effect in H4 acetylation was not conclusive in these data because of the preferential recognition of acetylated H3 over H4 by the Kac antibody used for the detection. Finally, the assay with histone octamer again clearly showed positive correlation between the dose of F1c and the level of acetylation in certain histones,

H2A and H2B based on the migration (**Figure 11B**, right). These results suggest that Nut could enhance overall histone acetylation *in vitro*, particularly on H2A/H2B when these are forming octamers. This result led us to carryout more detailed analysis of histone PTMs in the male germ cells.

In order to analyze the precise effect of NUT on histone PTMs, round spermatids were purified from *WT* and *Nut*<sup>-/-</sup> mice, and acid extraction was performed to obtain histones from those cells for mass spectrometry analysis. Briefly, the extracted histones from *WT* and *Nut*<sup>-/-</sup> round spermatids were digested by trypsin and labeled by light (<sup>12</sup>C<sub>6</sub>) and heavy (<sup>13</sup>C<sub>6</sub>) propionic anhydride, respectively. The propionylated peptides were mixed and analyzed by HPLC/MS/MS. The result of the MS analysis is summarized in **Table 1**. Through this approach, we identified ten Lys acetylation sites, five Lys crotonylation sites, seven Lys and two Arg monomethylation sites, five Lys and one Arg dimethylation sites, one Lys and one Arg trimethylation sites, and one Ser phosphorylation site from *WT* and *Nut*<sup>-/-</sup> round spermatids (**Figure 12A**). Within the PTMs commonly found in both cells, the peak intensities from *Nut*<sup>-/-</sup> were normalized to those from *WT* to determine the relative abundance of each histone mark in *Nut*<sup>-/-</sup> round spermatids. In the absence of NUT, several histone modifications were down regulated. Most of them were located at the N-terminal tails. In *Nut*<sup>-/-</sup> cells, five lysine residues (H2AK5, K9, H4K5, K8 and K16) displayed reduced acetylation at more than 1.5 times in ratio to *WT*. These reductions of H2A acetylation are in agreement with the results of the *in vitro* HAT assays, which showed that H2A acetylation is generally enhanced in the presence of Nut F1c fragment. The reductions of acetylation at H4K5 and K8 in *Nut*<sup>-/-</sup> cells are the critical alteration in the context of spermiogenesis. These two lysine residues are the binding sites of BRDT's BD1 when both become acetylated. The down regulation of H4K5ac and K8ac in *Nut*<sup>-/-</sup> cells was further confirmed by Western blots (**Figure 12B**), while acetylation at H4K12 shown to be unaffected, agreeing with the result from the mass spectrometry. Altogether, the absence of NUT caused critical epigenetic alteration which could affect the binding of BRDT.



**Figure 11:** Ability of NUT to enhance HAT activity *in vitro*. **(A)** Schematic representation of Nut F1c fragment (p300 interacting region) and p300 short fragment (containing Nut-interacting domain, CH3), which were used for *in vitro* HAT assay. These fragments were previously used for *in vitro* HAT assay with purified H3 (Reynold et al., 2010). **(B)** *In vitro* HAT activity of purified p300 short in absence (0 ng) or presence of GST-F1c (250, 500 or 1000 ng) with H2A/H2B dimers (left), H3/H4 tetramers (center) or histone octamers (right) as substrates. Western blot with acetyl lysine (Kac) detection shows acetylation status in each condition, and SDS-PAGE with Coomassie staining indicates the components of each reaction.



**Figure 12: (A)** Histone PTMs that were detected in *WT* and *Nut*<sup>-/-</sup> round spermatids by Mass spectrometry. Each modification's relative abundance was represented as *Nut*<sup>-/-</sup> / *WT* ratio. Vertical axes represent the relative abundance of detected histone PTMs expressed in the ratio of *Nut*<sup>-/-</sup> / *WT*, and the horizontal axes represent modification sites in each histone. Relative abundances of Acetylation (Ac), crotonylation (Cr), monomethylation (Me1), dimethylation (Me2), trimethylation (Me3), phosphorylation (Ph) are shown in different colors as indicated. PTMs found with more than 1.5 times differences in their abundances are marked with red asterisks. **(B)** Western blot with acid-extracted histones from nuclei of *WT* or *Nut*<sup>-/-</sup> round spermatids, confirming H4K5, K8 and K12 acetylation levels detected in (A). H3 was used as a loading control.

No	Modifications	Average Ratio <i>Nut</i> <sup>-/-</sup> /WT (Normalized)
H3	H3K4me1	0,38
	H3K4cr	0,56
	H3K9me1	0,67
	H3K9me2	0,67
	H3K9me3	0,88
	H3K14ac	0,79
	H3K18ac	0,90
	H3K18cr	1,31
	H3K23ac	0,93
	H3K23me1	0,98
	H3R26me1	1,08
	H3K27me1	1,30
	H3K36me1	1,08
	H3K37me2	1,30
	H3K79me1	0,91
	H3K79me2	0,70
H4	H4K5ac	0,32
	H4K8ac	0,44
	H4K12ac	1,10
	H4K16ac	0,64
	H4K5cr	1,28
	H4R17me1	1,02
	H4K20me2	1,77
	H4R23me2	1,23
H4R23me3	1,00	
H2A	H2AK5ac (Isoform, Uniprot No.A0AUV1)	0,39
	H2AK9ac (Isoform, Uniprot No.A0AUV1)	0,39
	H2AK13cr (Isoform, Uniprot No.BRRWH3)	0,69
	H2AK15me2 (Isoform, Uniprot No.BRRWH3)	1,49
	H2AK74ac (Isoform, Uniprot No.A0AUV1)	0,93
	H2AK75me1 (Isoform, Uniprot No.A0AUV1)	0,93
H2A.Z	H2AK15cr (Isoform, Uniprot No.B2RVP5)	0,33
H1t	H1tS177ph (Isoform, Uniprot No.I7HGT9)	1,75
H4	H4K79me1	<i>Nut</i> <sup>-/-</sup> only
H2A	H2AK4me1 (Isoform, Uniprot No.P0C0S6)	
	H2AK5me1 (Isoform, Uniprot No.A0AUV1)	
H2B	H2BK16me1 (Isoform, Uniprot No.A0JNS9)	
H1t	H1tK27me1 (Isoform, Uniprot No.I7HGT9)	
	H1tK166me1 (Isoform, Uniprot No.I7HGT9)	

**Table 1:** Summary of detected PTMs from acid-extracted histones of *WT* or *Nut*<sup>-/-</sup> round spermatids by MS analysis. PTMs shown in red were found with more than 1.5 times differences in their abundances (ratio of *Nut*<sup>-/-</sup> / *WT*).

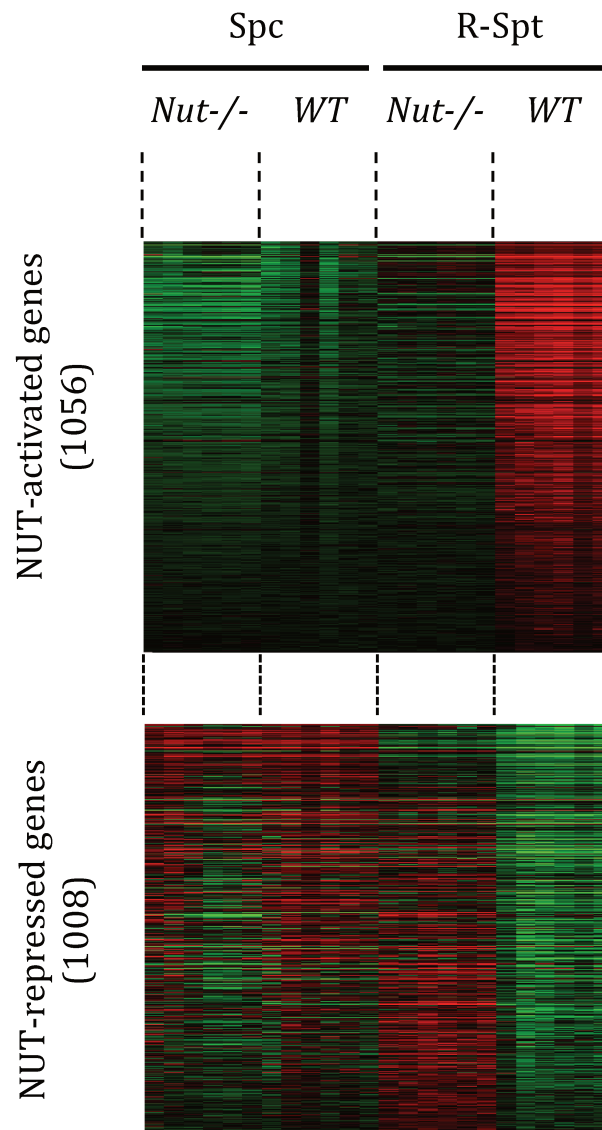


#### **IV.8. Altered transcriptome in *Nut*<sup>-/-</sup> round spermatids**

\*The results of following two sections were obtained through collaboration with the group of Dr. Phillippe Guardiola (Université d'Ange) who performed the microarray experiment on the RNA samples that I purified. and Dr. Sophie Rousseaux and Florent Chuffart from our team who performed the analysis of obtained data from microarray.

Since the absence of NUT is associated with alterations of histone PTMs including the ones that are critical for histone replacement, we hypothesized that NUT could also be involved in controlling gene expression programs through histone modifications. Indeed, histone acetylation, more specifically H4K5 K8 acetylation is also associated with the promoter of highly active genes and their transcriptional activity.

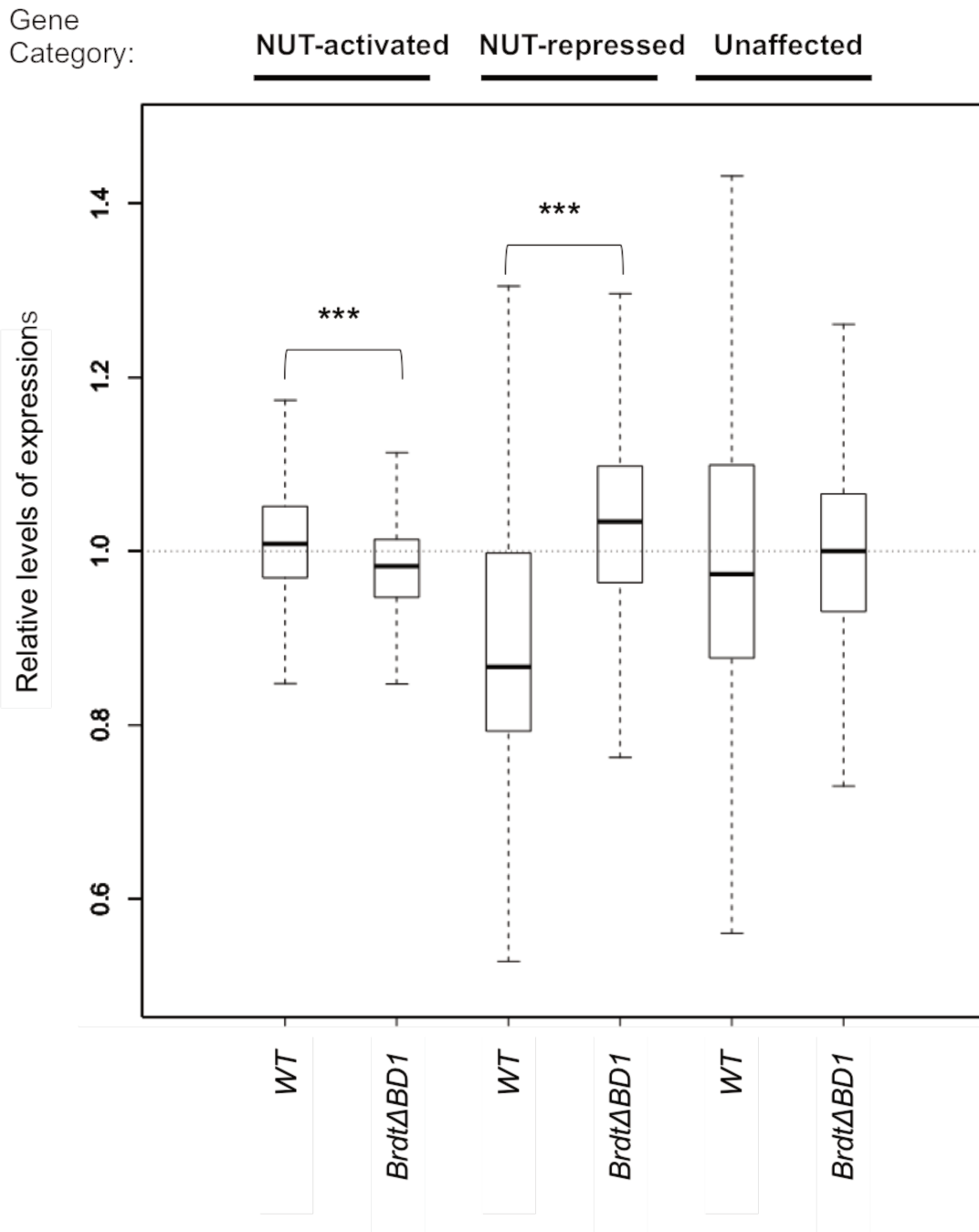
In order to assess the effect of NUT in gene transcription, a transcriptomic analysis was performed using RNA purified from *WT* or *Nut*<sup>-/-</sup> spermatocytes and round spermatids. Transcriptomic profile of these cells were presented as a heatmap (**Figure 13**). Strikingly, a number of genes showed an expression specifically affected in round spermatids: by either up or down regulated in *Nut*<sup>-/-</sup> cells. Among the 13289 detected genes, 1056 were down regulated, and 1008 were up regulated in the absence of NUT in round spermatids. All the rest were not affected by absence of NUT. Those genes that showed reduced level of expression in the *Nut*<sup>-/-</sup> cells were categorized as “NUT-activated” genes. The ones that showed up regulation were categorized as “NUT-repressed”, since these genes are possibly repressed in presence of NUT. Other genes that did not show any significant difference in their transcriptional activity between *WT* and *Nut*<sup>-/-</sup> were identified as “unaffected”.



**Figure 13:** Transcriptomic analysis of *WT* and *Nut*<sup>-/-</sup> spermatocyte (Spc) and dpsermatids (R-Spt) represented in heatmap. In each genotype and the cell type, 6 samples were analyzed (24 samples total). The genes are categorized by the pattern of the expression in round spermatids either down- or up-regulated by the absence of NUT, as “NUT-activated” or “NUT-repressed”, respectively. Numbers in the parentheses indicates the number of genes in each category.

## IV.9. NUT-BRDT-regulated genes

BRDT is one of the major transcription-regulatory factors controlling gene expression during spermatogenesis (Gaucher et al., 2012). The first bromodomain of BRDT has particular specificity toward diacetylated histone H4 at K5 and K8. In a previous work from our team, transcriptomic analysis was performed on the testis from 20 d.p.p. males of *BrdtdABD1* compared with *WT* testes. Using this transcriptomic dataset, we assessed whether the Nut-affected genes were regulated through BRDT-controlled transcriptional regulation. Expression of each category of genes, NUT-activated, NUT-repressed or unaffected in *BrdtdABD1* or *WT* were analyzed. Strikingly, a significant proportion of NUT-activated genes were also downregulated in *BrdtdABD1* and NUT-repressed genes were also up regulated in *BrdtdABD1* (**Figure 14**). In addition, those genes categorized as “unaffected” showed no clear difference in their expression level between *WT* and *BrdtdABD1*. Taken together, these results suggest that the target genes of NUT and genes controlled by BRDT’s BD1 are partly shared (if not totally overlapping), and that these genes are possibly regulated by acetylation on H4K5 and K8.



**Figure 14:** Expression of “NUT-activated”, “NUT-repressed”, or neither categories of the genes (“unaffected”) found in the transcriptomic analysis in the testes from 20 d.p.p. *WT* and *BrdtABD1*. Comparison of all “NUT activated” genes (1056), all “NUT-repressed” genes, and 1000 genes that were least affected by knocking out Nut (“Unaffected”). The transcriptomic data of *BrdtABD1* was obtained from Gaucher et al., 2012. According to Mann-Whitney test under 5% threshold, “Nut-activated” (\*\*\*,  $p = 1.81 \times 10^{-23}$ ) and “Nut-repressed” (\*\*\*,  $p = 2.96 \times 10^{-81}$ ) genes showed significant down or up regulation in *BrdtABD1*, while the expression of “Unaffected” genes ( $p = 0.076$ ) did not show significant alteration.

## V. Discussion

### V.1. Precise assembly of male epigenome

Protamine based genome compaction in mature sperm is essential for its survival (Gaucher et al., 2012; Montellier et al., 2013; Shirley et al., 2004; Zhao et al., 2004). Although nucleo-protamine assembly is carried out in a stepwise manner in mammals, some organisms, such as the cephalopod *Sepia officinalis*, undergo direct replacement of histones by protamines, or non-histone proteins (Martínez-Soler et al., 2007). The highly basic nature of protamines could explain their ability to disassemble nucleosomes (Oliva and Mezquita, 1986; Oliva et al., 1987) and to generate highly compacted aggregates with DNA *in vitro* (Balhorn et al., 2000; Brewer, 1999). Also the induced expression of protamines in somatic cell generates spermatid-like elongated shape cells (Iuso et al., 2015). Despite such a potent chromatin compacting effect of protamines, execution of direct replacement of histones by protamines in mammals could lead to malformation of spermatozoa and infertility of the affected animals, as shown in mouse models depleted of TPs (Shirley et al., 2004; Zhao et al., 2004). In addition, high basicity of protamine itself is not sufficient to displace and replace the histones, as evidenced in mouse models defective for histone replacement as exemplified by *BrdtABD1* and *TH2Btag* mice (Gaucher et al., 2012; Montellier et al., 2013). The recent finding from our group on the dynamic turnover of acetylation and butyrylation at the histone H4K5 and K8 highlighted the existence of an additional layer in the mechanism of BRDT-regulated histone replacement (Goudarzi et al., 2016). Indeed, a precise cooperative action of each of the factors involved in male genome reorganization is essential for proper assembly of the mature sperm epigenome.

In this study, I have shown that NUT is an essential protein for the formation of the sperm, precisely functioning at the timing of histone-to-protamine replacement. Although its critical function when it is expressed as a fusion protein with BRD4 in NMC was characterized (Alekseyenko et al., 2015; Reynoird et al., 2010), the function of NUT when it is normally expressed in testis was completely unknown. This study could bring valuable information for two major reasons: 1) It would bring further understanding of the molecular mechanisms of male genome reorganization,

and 2) It would help the development of the effective therapies targetting the fusion oncoproteins in NMC.

## **V.2. *Nut* expression is specific in post-meiosis**

The first series of experiments not only confirm the previously reported testis specific expression of NUT, but also revealed the restricted expression only in late spermatogenesis. Further analysis by immunofluorescence and immunohistochemistry, performed on spermatogenic cells and testis sections respectively, revealed that *Nut* expression occurs even in more limited steps of spermatogenesis, which are from round spermatids to elongating spermatids. This timing overlaps with the initiation of the hyperacetylation wave. Therefore, these results imply that NUT could also play a role at the initiation of the hyperacetylation wave.

## **V.3. Knockout of *Nut* specifically affects male fertility**

Since the knockout of *Nut* had been never reported before, the effect of its absence was not predictable, although we hypothesized that it could affect spermatogenesis based on the expression profile defined in the earlier experiments. The knockout did not show any apparent defects in the mice's health and growth. The female homozygous mutant mice were completely fertile, therefore the oocyte formation is not relying on NUT expression or function. Strikingly, in agreement with its post-meiotic expression pattern, its absence causes spermatogenic arrest specifically at the spermatid development. Therefore, the development of primordial germ cells, spermatogonia, as well as spermatocytes are not affected by the absence of NUT. The complete absence of maturing spermatozoa in the cauda epididymis of *Nut*<sup>-/-</sup> mice is due to the post-meiotic arrest of spermatogenesis, leading to sterility and reduced testis size. Therefore, this study is the first to show that NUT is necessary for proper progression of spermatogenesis.

#### **V.4. Post-meiotic arrest in *Nut*<sup>-/-</sup> males due to defective histone replacement**

The clear defect of *Nut*<sup>-/-</sup> spermatogenic cells become apparent in elongating spermatids. In these mice during the histone replacement, the signal of TH2B and TP2 as well as TH2B and Prm were detected within the same cells, which does not normally occur in *WT* mice. TPs and Prms accumulated around the peri-nuclear region and failed to be assembled on the genome. Since TH2B was detected even in the condensed spermatids expressing Prm, we conclude that histones are abnormally retained in *Nut*<sup>-/-</sup>. In other words, in the absence of NUT, histones cannot be removed and thus TP and Prm incorporation remains defective.

Similar defective histone replacement was also observed in *Brdtd $\Delta$ BD1* and *TH2Btag* mice (Gaucher et al., 2012; Montellier et al., 2013), which were both used in previous studies from our team. Although the latter two models showed similar phenotypes, our mechanistic investigations show that the causes of defective histone replacement are different; *Brdtd $\Delta$ BD1* could not replace the histones because of the lack of BD1 in BRDT, a domain which specifically binds acetylated histones, while in the case of *TH2Btag*, the C-terminal tag somehow hinders the full assembly of Prms (Gaucher et al., 2012; Montellier et al., 2013). In the case of *TH2Btag*, the exogenously added tag could interfere with the interaction of the nucleosomes with potential histone removal machineries. In the case of *Nut*<sup>-/-</sup>, the cause of defective histone replacement could be closer to the situation observed in *Brdtd $\Delta$ BD1* male germ cells. In the case of *Brdtd $\Delta$ BD1*, BRDT do not bind to the histones because of its lack of BD1. In the case of *Nut*<sup>-/-</sup>, although Brdt is fully intact, the absence of NUT could somehow cause insufficient binding of Brdt. The possible explanation for this could be the downregulation of histone hyperacetylation. This is also in agreement with our previous study in NMC, in which NUT was shown to enhance the HAT activity of p300 (Reynoird et al., 2010). The last series of investigations confirm this hypothesis since they show that NUT is in fact involved in the control of histone acetylation and hence controls all the events that are subsequent to this hyperacetylation.

## V.5. Regulation of histone PTMs by NUT

The HPLC/MS/MS analysis of purified histones from *WT* and *Nut*<sup>-/-</sup> round spermatids revealed the alteration in the levels of varieties of PTMs at different sites. Strikingly, these included the down regulation of histone acetylation at H4K5 and K8, two critical sites bound by Brdt upon simultaneous acetylation. The down regulation of H4K5ac and K8ac, preventing the binding of BRDT, could indeed be the explanation for the defective histone replacement. In the context of lysine acetylation and BRDT binding, a recent study from our team revealed the dynamic exchange of competitive lysine modifications at H4K5 and K8 and its involvement in the recognition of the corresponding chromatin regions by BRDT (Goudarzi et al., 2016). In the study, it was shown that acetylation and butyrylation (bu) show an overall similar enrichment pattern through the genome, and show similar upregulation in the elongating spermatids. Interestingly, in addition to the specific recognition of H4K5acK8ac, the BD1 of BRDT was shown to bind also H4K5acK8bu. Other combinations, K5buK8ac and K5buK8bu, do not bind BRDT. All of those modifications show similar distribution throughout the genome yet having different affinity to BRDT, suggesting that the precise timing of histone replacement is regulated by rapid turnover of those histone PTMs, altering the binding of BRDT. It is also important to note that a relatively prolonged retention of butyrylated histones is observed compared to acetylated histones, again suggesting that the butyrylated histones tend to escape from the binding of BRDT and histone removal. It would be interesting to see the effect of the absence of NUT in the butyrylation levels. One possibility is that since acetylation was reduced in *Nut*<sup>-/-</sup>, butyrylation would possibly occupy the histones that failed to become acetylated. Or, according to another study, p300 is shown to be able to catalyze butyrylation of histones (Chen et al., 2007). If NUT is directly influencing the HAT, its absence may possibly alter the substrate specificity. This may lead to either up- or downregulation of butyrylation, or even change the nature of PTMs at a given site.

Although at this point the ideas discussed are all speculations, and butyrylation was not detected in the mass spectrometry due to technical difficulties, further analysis of *Nut*<sup>-/-</sup> spermatids could be informative in studying possible new HAT pathways regulating different acylation.



## **V.6. NUT-dependent Transcriptome**

Transcriptomic analysis on round spermatids revealed that 1056 genes were downregulated and 1008 genes were upregulated in absence of NUT. Although the former set of genes was categorized as “NUT-activated” and the latter as “NUT-repressed”, the effect of NUT on those genes could be direct or indirect. However, deregulations in histone PTMs in *Nut*<sup>-/-</sup> imply that the absence of NUT could influence the expression of a number of underlying genes. In addition, the analysis of the expression of these genes in *Brd1ABD1* revealed the similar tendency of the expression levels of these genes in the absence of NUT and BD1 mutant. These data therefore suggest that the regulation of some of these genes are also BRDT’s BD1 dependent, and thus possibly H4K5 and K8 acetylation dependent.

## **V.7. Enhancement of cellular HAT activity by NUT**

How histone PTM alteration was induced in the absence of NUT, is one of the critical questions that remain to be solved. This phenomenon could be discussed from mainly two perspectives: NUT-HAT interaction, and NUT-BET interaction.

In a previous study concerning the function of BRD4-NUT in NMC, NUT was shown to interact with a ubiquitous HAT, p300, enhancing its catalytic activity. The HAT interacting domain of NUT, named F1c, was also identified in this study (Reynoird et al., 2010). In this work, the enhancement of p300 HAT activity was tested on soluble non-nucleosomal histone H3 *in vitro*. Here we newly tested the effect of NUT (F1c) on p300 HAT activity on H2A/H2B dimers, H3/H4 tetramers as well as on histone octamers. The enhancement was again seen in F1c dose dependent manner, however the complete conclusion is yet to be made since the detection of acetylation was only done using pan-Kac antibody (presenting a biased detection of acetylated lysine sites). Additional experiments with detection by site-specific antibodies are essential in order to evaluate the enhancement of p300 HAT activity on nucleosomal histones.

In NMC cells, p300 was shown to interact with BRD4-NUT through F1c region of the NUT moiety. This region is also present in NUT expressed in mouse spermatogenic cells, therefore the interaction with p300 is likely to be conserved in germ cells. As a preliminary result, we showed here that p300 could interact with NUT in spermatogenic cells. If this result is confirmed, it agrees with the observation in NMC, in which NUT mediates the spread of hyperacetylation in the wide area of chromatin. In either case, the data presented here need to be completed, and further refinement is required to conclude about NUT-HAT interaction in the male germ cells. I should note that ectopic expression of NUT alone without BRD4-fusion does not induce apparent hyperacetylation effect, thus the activity of NUT in the male germ cell may require additional testis specific factor.

### **V.8. NUT interaction with the proteins of BET family**

In the case of BRD4-NUT in NMC, the association of NUT with a bromodomain-containing protein is essential for the establishment of the feed-forward acetylation loop. I should note that the expression of NUT alone in somatic cells is not sufficient to enhance the HAT activity like observed in NMC. Based on these facts, the interaction between NUT and BRDT, a major BET factor expressed in the germ cell, was suspected. Although the fusion protein and protein-protein interaction are different situations, the resemblance of the phenotypes of *Nut*<sup>-/-</sup> and *Brd4ABD1* mice in terms of defective histone replacement also supports the idea that NUT and BRDT could be acting on the same pathway during histone replacement. In a series of preliminary investigations testing a potential physical interaction between NUT and BRDT did not allow us to draw definitive conclusions. However, interestingly, the pull-down experiments using the BET bromodomain specific inhibitor JQ1 led to a strong enrichment in NUT. NUT does not possess any bromodomain-like sequence, therefore this result suggests that NUT could interact with a BET factor that could be bound by JQ1. It is important to note that JQ1 could bind to the bromodomain of any of BET members, thus, the potential interacting partner of NUT could include any of the BETs and not necessarily BRDT.

Up to today, no interacting proteins have been reported for NUT, except p300. At this stage it is therefore required to purify NUT and associating proteins, in order to draw a full picture of the NUT-containing protein complexes and thus obtain important hints on various functions of NUT. The immunoprecipitation of NUT from the male germ cells has been attempted several times, but the purified products were not abundant enough to conduct proteomic study. The improvement in the experimental conditions is urgently needed.

## **V.9. Comparison of the function of NUT in spermatids and NMC**

With this study, the effect of NUT during spermatogenesis and in NMC cancer cells could be compared.

The common point in these two contexts is that NUT has been shown to be involved in the regulation of histone acetylation in both systems (Reynoird et al., 2010). In NMC, the bromodomain of BRD4-NUT anchors itself to the acetylated histones, and the NUT moiety recruits p300 via direct interaction to induce local hyperacetylation. The cycles of anchoring and HAT recruitment create a feed forward loop that induces the characteristic hyperacetylation foci occupying distinct areas of the chromatin.

In the hyperacetylation wave during spermiogenesis, the acetylated regions also need to spread. Therefore, the involvement of NUT could be important for such a rapid expansion of hyperacetylated chromatin domain. In the case of *Nut*<sup>-/-</sup> spermatids, histone acetylation was not completely abolished by the absence of NUT, although it showed significant decrease. This suggests that NUT may not be necessary for the initiation of the histone acetylation, but for the spreading of the acetylated regions. Megadomain formation by BRD4-NUT (Alekseyenko et al., 2015) provides another aspect to look into during spermatogenesis. Indeed, a recent study beautifully showed that large domains of chromatin spanning up to 2Mb were filled by BRD4-NUT, p300 and hyperacetylated histones. The megadomain corresponds to active genes enhancers including that of *MYC* gene (Alekseyenko et al., 2015). It would be

interesting to see the effect of NUT on the establishment of histone marks according to the topologically associated domains. It is important to note that the direct interaction of NUT and p300 increases the HAT activity on non-nucleosomal histones *in vitro* (Reynoird et al., 2010). In addition, the same effect was observed on histone dimers, tetramers or octamers *in vitro*. Therefore, the role of NUT is not only the expansion of the acetylated chromatin but also the enhancement of the HAT activity itself. Since no structural information is available for NUT, the exact mechanism of the enhancement of the HAT activity of p300 is yet to be understood. However, the p300 interacting region of NUT, F1c, is sufficient to observe the enhancement of HAT activity *in vitro*.

Also, there seems to be a difference in the function of NUT in NMC and spermatogenesis. The hyperacetylation in NMC induced by BRD4-NUT affects various acetylation sites, such as H3K27ac and H3K9ac. In contrast, although those sites are acetylated also during spermiogenesis, the effect of the absence of NUT on histone acetylation during spermatogenesis seems to be relatively restricted. In general, the same factor could deliver different effects depending on the cellular context of its action (cancer cells or germ cells for example) therefore this difference is not surprising. However, if NUT and p300 are in action in both cases, in the germ cells at this point their cooperation is less clear and is still hypothetical. The complex involving NUT in NMC and in germ cells could be different. Also, the combination of the BET factor and NUT could be different in these two different settings, therefore the histone modifications recognized by the bromodomain could be different. This second hypothesis could be interesting to discuss. In NMC, the association of NUT and BRD4 is tied by a chromosomal translocation, since they are expressed as a fusion protein. In the germ cells, our preliminary JQ1 pull-down experiment showed the possibility of interaction between NUT and yet to be identified BETs. If the bromodomain proteins associated with NUT are different from BRD4, the expansion of acetylation may occur differently. The purification of the NUT complex from germ cells should provide some insights into the underlying mechanism.

## **V.10. From cancer to sperm and from sperm to cancer**

This study could open a door to new potential therapeutic strategies for the treatment of NMC. The knockout mice generated in this study showed specific effect in the development of post-meiotic male germ cells. Since NUT is a spermatid specific factor, it makes sense that there was no detectable defect other than spermiogenesis and virtually no defect was observed in the female mice by the absence of NUT.

Importantly, this high specificity of the effect of NUT makes it an attractive potential therapeutic target for NMC treatment. The BET inhibitor JQ1 was originally tested in NMC (Filippakopoulos et al., 2010), and the inhibition of the BET's bromodomain has become a main strategy of the drug development attempting to design a drug that could reduce the tumor (for review see Wang & Filippakopoulos 2015).

Bromodomains could be indeed druggable targets in different pathological situations, but this domain is also present in many essential proteins (see the section "Reader: bromodomain" in the introduction). BET members are particularly abundant factors, such as BRD4, which acts as a general transcriptional coactivator (Jang et al., 2005; Thomas and Chiang, 2006; Yang et al., 2005). Therefore, although significant effectiveness against stopping tumor growth has been reported in quite a few cases, the risk of currently known and yet to be known side effect of those inhibitors cannot be ignored. A recent report about clinical trial of new bromodomain inhibitor OTX015/MK-8628 showed the efficacy of this new compound in NMC, with mild yet various side effects including thrombocytopenia (Stathis et al., 2016). Presumably, if NUT could be targeted, it may cause much less side effects. The worst side effect might be male infertility. Within the full length of NUT protein, the already identified F1c region could be suitable for the inhibition since this is the direct binding site for p300. A compound that could specifically interfere with the interaction between F1c and p300, would be an effective and safe drug. The detailed molecular mechanism of the function and structural information of NUT are largely missing, and determination of these aspects would be the main challenge in creating anti-NUT drugs. However, the phenotypic evidence presented in this study provides a basis to initiate the exploration of possible inhibitory molecules against NUT.

## **V.11. Future works**

### **V.11.1. Identification of NUT interacting proteins**

In order to understand the mechanistic details of the function of NUT, it is indeed essential to identify its interacting partners. As already, discussed, the binding partners could reveal several aspects regarding to the NUT's function.

The first thing would be the identification of the HAT acting with NUT. Although the interaction between NUT and p300 was already defined in NMC cancer cells (Reynoird et al., 2010), the interaction in the male germ cells are not yet fully confirmed in the presented data. Optimization of the experimental condition is currently ongoing.

Another aim would be to verify the relationship between NUT and BET factors. BRDT is still one of the strong candidates based on its expression pattern and on the phenotypic overlap between *Brdt* $\Delta$ *BD1* and *Nut*<sup>-/-</sup> spermatids. However, the possibility also includes the interactions with other BET factors. The result from the JQ1 pull down experiment indeed shows a considerable accumulation of NUT, therefore the link between BET factors and NUT are currently being investigated via additional pull down and immunoprecipitation experiments.

The ideal analysis to be performed would be the proteomic analysis to identify NUT-interacting proteins. Although the current experimental conditions using the anti-NUT antibody generated in our lab has enabled some degrees of successful NUT accumulation, the final product is still not sufficient to carry out mass spectrometry. Therefore, in parallel with the exploration of NUT-p300 or -BET interaction, immunoprecipitation targeting NUT is also the task to be done.

### **V.11.2. Additional analysis of transcriptomic data**

The current result from the transcriptomic study from *Nut*<sup>-/-</sup> spermatids showed the significant effect of the absence of NUT in the gene expression. The potential link between NUT and BRDT in the regulation of those NUT affected genes

was also indicated. To draw the full picture of the consequence of NUT knockout, categorization of the types of affected genes would be informative. For example, previous study from our team regarding p300 conditional knockout mice in which p300 is down regulated specifically in the post-meiotic spermatids revealed its major effect on the genes involved in metabolic pathways (Boussouar et al., 2014). If the transcriptomic profile from the p300 KD spermatids and *Nut*<sup>-/-</sup> spermatids show some overlaps, this would suggest NUT and p300 are influencing the transcription of the same sets of genes, and thus would complete our view of the effect of NUT in the gene transcription.

### **V.11.3. Analysis of H4K5/K8 ac/bu of the genes regulated by NUT**

H4K5 and K8 acetylation status is shown to be the key for the activities regulated by NUT and BRDT. Recent study from our group also showed that these two modification sites undergo a dynamic turnover of the acetylation and butyrylation in spermatids (Goudarzi et al., 2016). Therefore, NUT could be involved in the regulation of specific gene expression programs driven by H4K5 and K8 acetylation and butyrylation. Genes regulated by NUT in *Nut*<sup>-/-</sup> could be marked by particular combinations of these PTMs. Thus, we will evaluate the H4K5 K8 ac/bu status of those genes that are shown to be affected by the absence of NUT, by searching the affected genes against the ChIP-seq data obtained from *WT*.

## VI. Annex

During my PhD period, I participated to several studies which are now published. In the annex, I will present two research articles, one review article and two short research highlight article to which I contributed.

### **“Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B” (Montellier et al., 2013)**

**Summary:** The germ cell specific H2B variant TH2B plays critical role in nucleosome destabilization prior to the histone replacement by TPs and Prms at later stages of spermatogenesis. TH2B replaces canonical H2B at genome wide scale by the completion of meiosis. The mouse model expressing TH2B with molecular tag fused at its C-terminal exhibit post-meiotic arrest due to defective histone-to-protamine replacement. Strikingly, the knockout of TH2B completely rescued the phenotype, by up-regulation of canonical H2B and a number of additional histone PTMs compensating the nucleosome destabilization, potentiating the chromatin for the histone replacement. In addition, TH2B is also found in oocytes, and the maternal TH2B rapidly replaces the male chromatin organizers during the reassembly of nucleosome-based chromatin. Altogether, TH2B is a critical factor involved the genome reprogramming both during spermatogenesis and after fertilization.

**My contribution to the work:** I performed the analysis of the TH2B expression in the mouse zygote. Immunofluorescence analysis nicely showed the reincorporation of maternal TH2B into the paternal chromatin right after fertilization, whereas no canonical H2B was observed. The presence of TH2B was confirmed during the early embryonic development, but this variant was lost in the developed embryo (confirmed at 13.5 d.p.c. in this study). The conclusion of this part of the work is that TH2B is a histone variant that acts specifically during genome reprogramming in early embryonic development, in addition to its role during spermatogenesis.





## Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B

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# Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B

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**The conversion of male germ cell chromatin to a nucleoprotamine structure is fundamental to the life cycle, yet the underlying molecular details remain obscure. Here we show that an essential step is the genome-wide incorporation of TH2B, a histone H2B variant of hitherto unknown function. Using mouse models in which TH2B is depleted or C-terminally modified, we show that TH2B directs the final transformation of dissociating nucleosomes into protamine-packed structures. Depletion of TH2B induces compensatory mechanisms that permit histone removal by up-regulating H2B and programming nucleosome instability through targeted histone modifications, including lysine crotonylation and arginine methylation. Furthermore, after fertilization, TH2B reassembles onto the male genome during protamine-to-histone exchange. Thus, TH2B is a unique histone variant that plays a key role in the histone-to-protamine packing of the male genome and guides genome-wide chromatin transitions that both precede and follow transmission of the male genome to the egg.**

[*Keywords:* H2AZ; BRDT; male contraception; reprogramming; male infertility; sex chromosome inactivation; histone eviction]

Supplemental material is available for this article.

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An essential feature of spermatogenesis is the generation of a transportable genome placed in the nucleus of highly specialized cells, spermatozoa, capable of leaving and surviving the parent organism. After meiosis, young spermatids (known as round spermatids) inherit a chromatin-containing transcriptionally active genome that, during subsequent stages in elongating spermatids, undergoes a genome-wide histone hyperacetylation followed

by histone removal and the assembly of transition proteins (TPs) and protamines in condensing spermatids. These chromatin transitions constitute a unique feature among eukaryotes, since the universal nucleosome-based organization of the genome undergoes a metamorphosis into new and unique genome-packaging structures based on nonhistone proteins. Although essential to the life cycle, the molecular basis of these dramatic changes remains one of the most obscure issues in modern biology (Boussouar et al. 2008; Gaucher et al. 2010). Following the commitment of self-renewing proliferative spermatogonia to meiotic divisions, all of the specific drivers of meiotic and post-meiotic events are expressed through

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Montellier et al.

successive waves of gene activation in spermatocytes (meiotic cells) and spermatids (Gaucher et al. 2012). Actually, the gradual male genome programming has, by this stage of development, already undergone large-scale exchanges of histones in spermatocytes. De Boer's group (van der Heijden et al. 2007) discovered that the transcriptional inactivation of meiotic sex chromosomes, which occurs at the time of autosomal chromosome pairing (Montellier et al. 2012), is associated with the chromosome-wide replacement of H3.1/2 by H3.3. In spermatids, histone removal is preceded by a large-scale incorporation of new H2A variants, H2AL1/2, which are synthesized almost at the same time as TPs (Govin et al. 2007). Other studies have also shown more targeted incorporations of H2A variants such as H2A.Z and H2A.Lap1 occurring at earlier stages (Greaves et al. 2006; Soboleva et al. 2012). These observations raise two fundamental issues. First, what are the molecular mechanisms underlying such large-scale chromatin dynamics? More specifically, which molecular determinants are responsible for the partial or total disassembly of nucleosomes and for the subsequent incorporation of new histones, TPs, and protamines into chromatin? Second, what are the functions of the histone variants synthesized and incorporated into chromatin at the different stages of spermatogenic cell differentiation?

The testis-specific histone variant TH2B, or TSH2B.1 in the new unified nomenclature (Talbert et al. 2012), was discovered in mammalian testes histone extracts in 1975 and is one of the earliest histone variants to have been identified (Branson et al. 1975; Shires et al. 1975). Later studies showed that its synthesis occurs in a replication-independent manner, beginning in early spermatocytes (Brock et al. 1980). The generation of TH2B-recognizing antibodies allowed a better characterization of its expression in spermatogenic cells (for review, see Govin et al. 2004), while *in vitro* studies revealed some of its biochemical properties (Li et al. 2005). However, the role of TH2B in the physiological context of spermatogenic cell differentiation still remains obscure. In particular, no gene invalidation approach has yet been undertaken to probe TH2B function. This is probably due to the complex structure of the gene, whose regulatory elements are shared with a neighboring *Th2a* gene (Supplemental Fig. S1; Huh et al. 1991; Choi and Chae 1993), making attempts to specifically abolish *Th2b* gene expression without deregulating *Th2a* challenging.

Here, we describe the genome-wide removal of H2B, which begins in early spermatocytes, and its replacement by TH2B, demonstrating that TH2B acts on a much larger scale than any of the other known histone variants. In addition, we investigate the functions of this histone variant in spermatogenic cells. Using a knock-in approach, we added three consecutive affinity tags to the C terminus of TH2B and generated a mouse strain expressing the tagged variant in spermatogenic cells. We also generated mice where the expression of TH2B was abrogated. Overall, the results obtained by using these mouse models strongly suggest that TH2B sets nucleosome stability parameters ensuring a genome-wide transition of nucleosomes into intermediate structural entities, which in

turn are required for the assembly of TPs and protamines. This study also highlights a role for TH2B in coordinating somatic-type H2B gene expression and fine-tuning histone post-translational modifications (PTMs). Additionally, we show that TH2B is also maternally expressed and replaces protamines at fertilization. Taken together, these data considerably increase our understanding of the molecular basis of male genome-wide chromatin disassembly and reassembly while assigning a function to TH2B nearly four decades after its discovery.

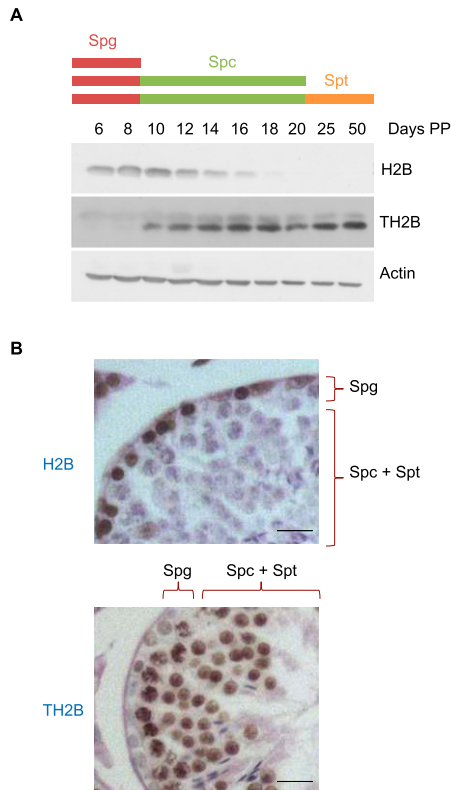
## Results

### *Genome-wide replacement of H2B by TH2B*

To investigate the function of TH2B, we examined the timing of TH2B protein expression in the developing testis of postnatal mice during the first wave of spermatogenesis. Samples were analyzed at time points corresponding to the appearance of successive spermatogenic cell types. Commitment to meiotic divisions occurs at 10 d post-partum (dpp) when spermatogonial divisions give rise to preleptotene spermatocytes. Spermatogenesis continues with spermatocytes undergoing meiotic divisions followed by the generation of post-meiotic haploid cells, which first appear at 20 dpp. The 50-dpp testis encompasses all spermatogenic cell types, including mature spermatozoa. Western blot analysis shows that TH2B starts to accumulate at ~10 dpp, when pre-leptotene/leptotene spermatocytes first appear (Fig. 1A). Monitoring H2B expression in the same experiment revealed that a drastic decrease of H2B mirrors the accumulation of TH2B, with the latter largely replacing H2B by 18 dpp in spermatocytes. These findings were corroborated by immunohistochemistry of adult testis sections, confirming that H2B is expressed in spermatogonia and nearly depleted from spermatocytes and spermatids, which instead stain strongly for the presence of TH2B (Fig. 1B).

### *A C-terminal affinity tag on TH2B causes spermatid differentiation to abort*

To further explore TH2B function, the *Th2b* gene was tagged in embryonic stem (ES) cells following a knock-in strategy based on homologous recombination, and the corresponding mouse strain was generated. Heterozygous *Th2b*<sup>+/tag</sup> mice expressed the *Th2b* tagged allele (TH2B C-terminally fused to three consecutive affinity tags: His, Flag, and Ha) (Supplemental Fig. S1) in the same spermatogenic cells as *Th2b*<sup>+/+</sup> mice and at the expected timing, showing a pattern of genome association very similar to that of wild-type TH2B (Fig. 2). In meiotic cells, both TH2B and TH2B-tag associated homogeneously with the genome, and meiotic events occurred normally. These included chromosome pairing, visualized by immunodetection of synaptonemal complexes (Sycp3) (Fig. 2B); sex body formation, visualized by the accumulation of  $\gamma$ H2A.X on the X and Y chromosomes (Fig. 2C); and the sex chromosome-specific H3-to-H3.3 histone exchange (van der Heijden et al. 2007), as judged by the accumulation of H3.3 on the sex body (Fig. 2D). In spermatids, wild-



**Figure 1.** A major H2B-to-TH2B transition occurs in early spermatocytes. (A) TH2B and H2B accumulation was analyzed in testes extracts at the indicated times (in days post-partum [Days PP]) by Western blots. (B) The expression of TH2B and H2B was analyzed by immunohistochemistry. Testis sections at stage II/III are represented. Spermatogonia (Spg) and spermatocytes and spermatids (Spc + Spt) are indicated. Bars, 10  $\mu$ m.

type TH2B and the tagged protein also exhibited a highly similar appearance, with an enhanced polar localization in round spermatids (Fig. 2A).

These data show that the C-terminally tagged TH2B does not interfere with any of the finely tuned events requiring dynamic chromatin, including meiotic recombination, sex chromosome inactivation and associated H2A.X phosphorylation, and the chromosome-wide H3-to-H3.3 exchange on the sex chromosome of meiotic cells. In striking contrast, at subsequent post-meiotic stages, TH2B-tag expression resulted in abnormalities occurring in elongating spermatids and a total absence of cells at later stages (Figs. 3A, 5B,C [below]; Supplemental Fig. S4). These defects lead to a total absence of epididymal spermatozoa (Fig. 3B) and complete male sterility (Supplemental Fig. S6B). Thus, C-terminally tagged TH2B causes no obvious phenotypical defects at the early stages of spermatogenesis but leads to the arrest of sperm cell development in condensing spermatids.

#### *TH2B-tag is efficiently assembled into nucleosomes in meiotic and post-meiotic cells*

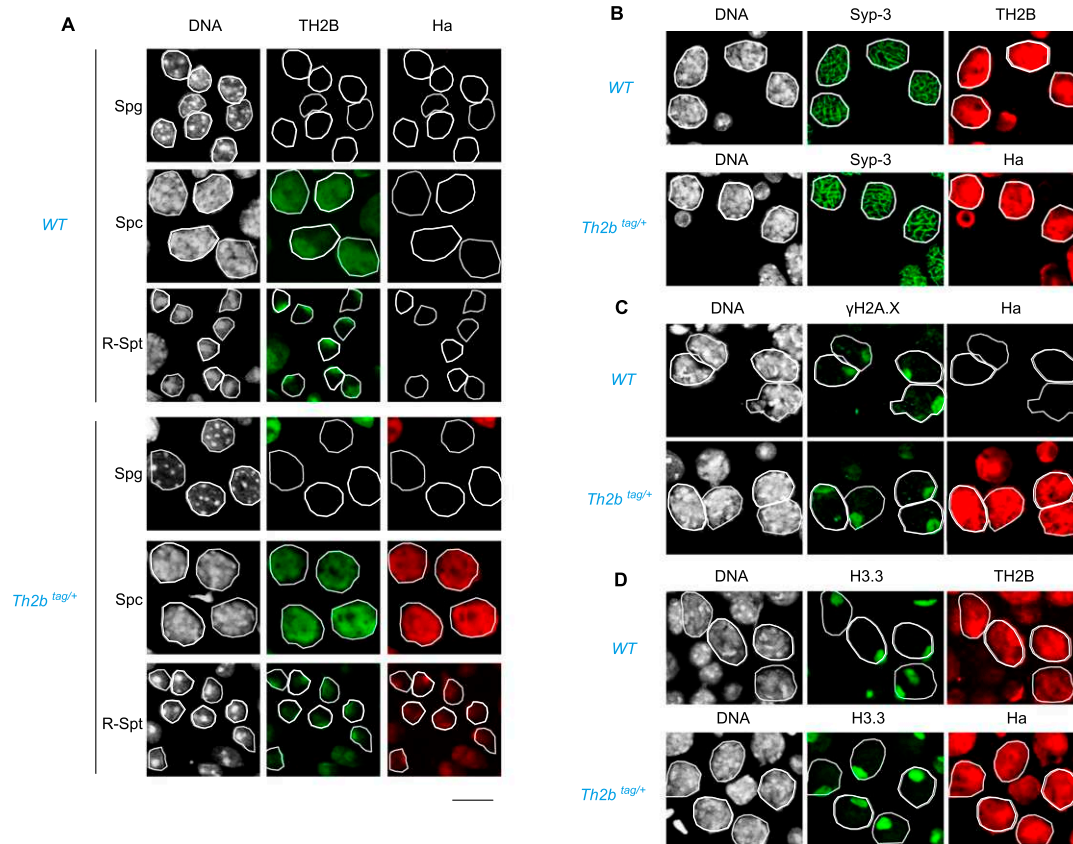
The data described above suggest that the C-terminal tag interferes with a critical TH2B function in elongating

spermatids. Since there is almost no transcription in these cells (Zhao et al. 2004), we hypothesized that the tag may perturb a distinct chromatin-related event such as the genome-wide histone replacement that occurs in elongating/condensing spermatids. To check whether the C-terminal tag could interfere with earlier events such as the assembly of TH2B or of other histones into nucleosomes, we generated mononucleosome-enriched chromatin from spermatocytes and round spermatids and used an anti-Ha antibody to immunopurify TH2B-tag-containing nucleosomes. Individual histones were then resolved by SDS-PAGE (Fig. 4A) and subjected to tryptic digestion and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis. Using this approach, we confirmed that all core histones (H2A, H2B, H3, and H4) as well as linker histones were associated with TH2B-tag-containing nucleosomes. The H2B detected here probably originates from early spermatocytes during the course of H2B-to-TH2B exchange at a stage where “mixed” nucleosomes are present. Histone variants normally expressed in spermatogenic cells—including H3.3, TH3, H2A.Z, H2A.X, and macroH2A—were also identified (Fig. 4B; summarized in Supplemental Tables S1, S2; see also Supplemental Fig. S2). Thus, the C-terminal tag on TH2B does not obviously interfere with proper nucleosome assembly.

#### *TH2B-tag is assembled genome-wide*

Next, we asked whether a biased TH2B-tag chromatin assembly and a subsequent defective gene expression could account for the defective histone-to-protamine replacement. To address this, we isolated mononucleosomes from TH2B-tag-expressing spermatocytes and round spermatids as described above and performed chromatin immunoprecipitation sequencing (ChIP-seq) analyses. In parallel, whole-genome transcriptome profiling was obtained from the same cell fractions comparing wild-type or TH2B-tag-expressing cells. The recent mapping of H2A.Z from spermatogenic cells (Soboleva et al. 2012) provided a useful reference for this analysis. In contrast to H2A.Z, TH2B showed no bias toward gene regulatory elements (Fig. 4C), which is consistent with a genome-wide action of TH2B in replacing H2B (Fig. 1), as opposed to gene regulatory functions. Moreover, TH2B-tag was depleted from the transcriptional start sites (TSSs) of a large group of genes. Specifically, this depletion was observed from the TSSs of genes showing H2A.Z enrichment (Fig. 4D). This anti-correlation between TH2B and H2A.Z at gene TSSs was observed in independent experiments performed in two different laboratories (in France and Australia), confirming the robustness of our mapping approach. Interestingly, Tremethick and colleagues (Soboleva et al. 2012) recently found that H2A histone variants H2A.Z and H2A.Lap1 form two adjacent nucleosomes located 200 base pairs (bp) upstream of and at the TSSs, respectively. We observed that these same regions were depleted in TH2B (Fig. 4E). Remarkably, TH2B was not depleted from TSS regions not enriched in H2A.Z (hereafter named “H2A.Z-less”) (Fig. 4D). Finally,





**Figure 2.** TH2B and TH2B-tag show similar intracellular distribution in wild-type and TH2B-tag-expressing spermatogenic cells. (A) Codetection of TH2B and TH2B-tag was performed by immunofluorescence on seminiferous tubule preparations from testes from the indicated genotypes using anti-TH2B or anti-Ha antibodies. The stages considered are indicated at the left of each panel: spermatogonia (Spg), pachytene spermatocytes (Spc) and round spermatids (R-Spt). (B) Syp3 was codetected with TH2B (*top* panel) or TH2B-tag (anti-Ha, *bottom* panel) in spermatocytes of the indicated genotypes. (C) Codetection of TH2B-tag (anti-Ha panels) and  $\gamma$ H2A.X in spermatocytes with the indicated genotypes is shown. (D) H3.3 was codetected with TH2B or TH2B-tag in spermatocytes of the indicated genotypes. Please note that the immunodetection with anti-Ha antibody is more sensitive than the detection based on the anti-TH2B antibody.

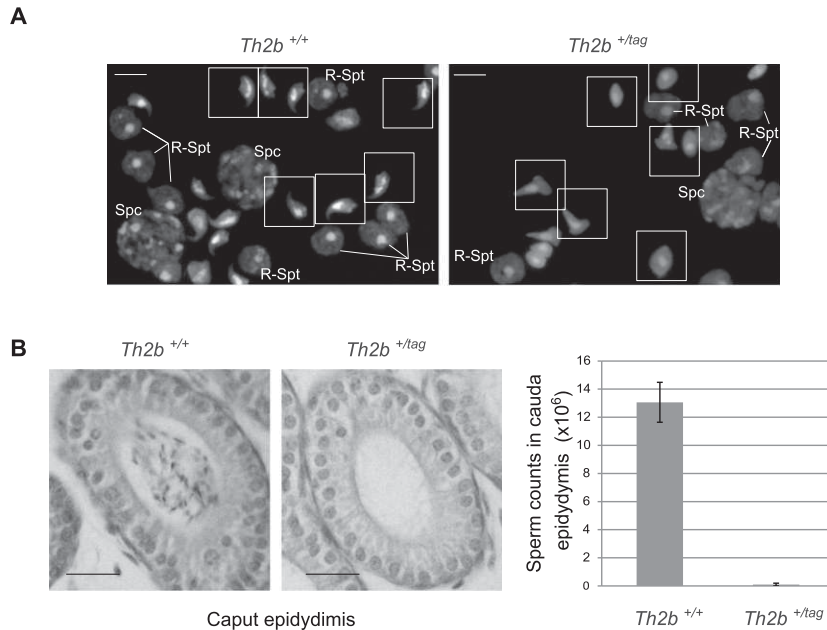
we also performed ChIP-seq using wild-type spermatogenic cells and an anti-TH2B antibody. Comparison of the mapping of TH2B-tag with that of TH2B in wild-type cells showed a large overlap as well as a similar depletion of TH2B from H2A.Z-enriched TSSs (Fig. 4C,D). These data therefore confirm that the presence of the tag does not affect either the assembly of TH2B or its functional dynamics in chromatin. It is noteworthy that, although TH2B is excluded from H2A.Z-containing TSSs, the two variants should be able to coexist in other genomic regions, since our proteomic approach revealed the presence of H2A.Z in TH2B-containing nucleosomes (Fig. 4B).

Whole-transcriptome analysis of spermatocytes and round spermatids from wild-type or TH2B-tag-expressing mice demonstrated that the presence of TH2B-tag had no significant effect on gene expression (Fig. 4F). We also observed that, overall, genes bearing H2A.Z at their TSSs had higher transcriptional activity than H2A.Z-less genes in both spermatocytes and round spermatids (Supplemental Fig. S3A). Taken together, these results show that first,

TH2B is a histone variant with genome-wide rather than locus-specific activity, and second, the C-terminal tag has no obvious effect on chromatin-dependent regulatory events in spermatocytes or round spermatids. The effect of the tag on TH2B turnover was also measured, reinforcing this conclusion. Somatic cells stably expressing GFP-TH2B or GFP-TH2B-tag were generated and used in a FRAP (fluorescence recovery after photobleaching) experiment to compare the dynamics of both fluorescent histones. This approach did not reveal any significant effect of the tag on the turnover of TH2B (Supplemental Fig. S3B).

#### *TH2B directs a stepwise nucleosome-to-nucleoprotamine transition*

The above data suggest that the C-terminal TH2B tag does not affect nucleosome dynamics per se, since events involving nucleosome disassembly and reassembly, such as the H3-to-H3.3 transition in meiotic cells, occur normally. In contrast, the presence of the tag might disrupt



**Figure 3.** TH2B-tag induces late arrest of spermatogenesis. (A) Different cell types stained with DAPI from the indicated genotypes are shown. Bars, 5  $\mu$ m. (B) Cauda epididymis sections stained with hematoxylin are shown in the *left* panel, and spermatozoa counts from isolated cauda epididymis from mice with the indicated genotypes are presented as histograms in the *right* panel. Bars, 20  $\mu$ m. Bars represent standard deviations of sperm counts from cauda epididymis of five mice of each genotype.

certain chromatin states specific to elongated/condensing spermatids; i.e., transitional states linking the dissociating nucleosomes to the assembly of TPs and protamines. Accordingly, we investigated the DNA-packaging transitional states known to occur in condensing spermatids (Govin et al. 2007). In agreement with previous findings, extensive micrococcal nuclease (MNase) treatment of nuclei purified from elongated/condensing spermatids resulted in the isolation of MNase-resistant nucleosomal DNA as well as more sensitive subnucleosomal DNA fragments (Fig. 5A). The subnucleosome particles, previously shown to be devoid of H3 and H4 and to contain TH2B and the late-expressing H2A variant H2AL2, are thought to be transitional states preceding the complete replacement of histones by TPs (Govin et al. 2007). We monitored the time course of MNase-induced release of nucleosomal and subnucleosomal fragments in wild-type and TH2B-tag-expressing elongated/condensing spermatids. While in both wild-type and TH2B-tag-expressing cells extensive MNase digestion released the two expected nucleosomal and subnucleosomal fragments, we observed that first, slightly higher amounts of mononucleosomes were consistently obtained in TH2B-tag-expressing cells compared with wild-type cells, and second, the subnucleosomal particles from these cells were more resistant to MNase digestion than those from wild-type cells, where these particles were rapidly digested (Fig. 5A). In contrast, when the MNase sensitivity of chromatin-containing total spermatogenic cell nuclei was compared between wild-type and TH2B-tag-expressing cells, no significant difference was found (Fig. 5A, bottom panel). Altogether, these experiments suggest that the tag-dependent defects specifically occur at a time when TP and protamines should fully displace TH2B.

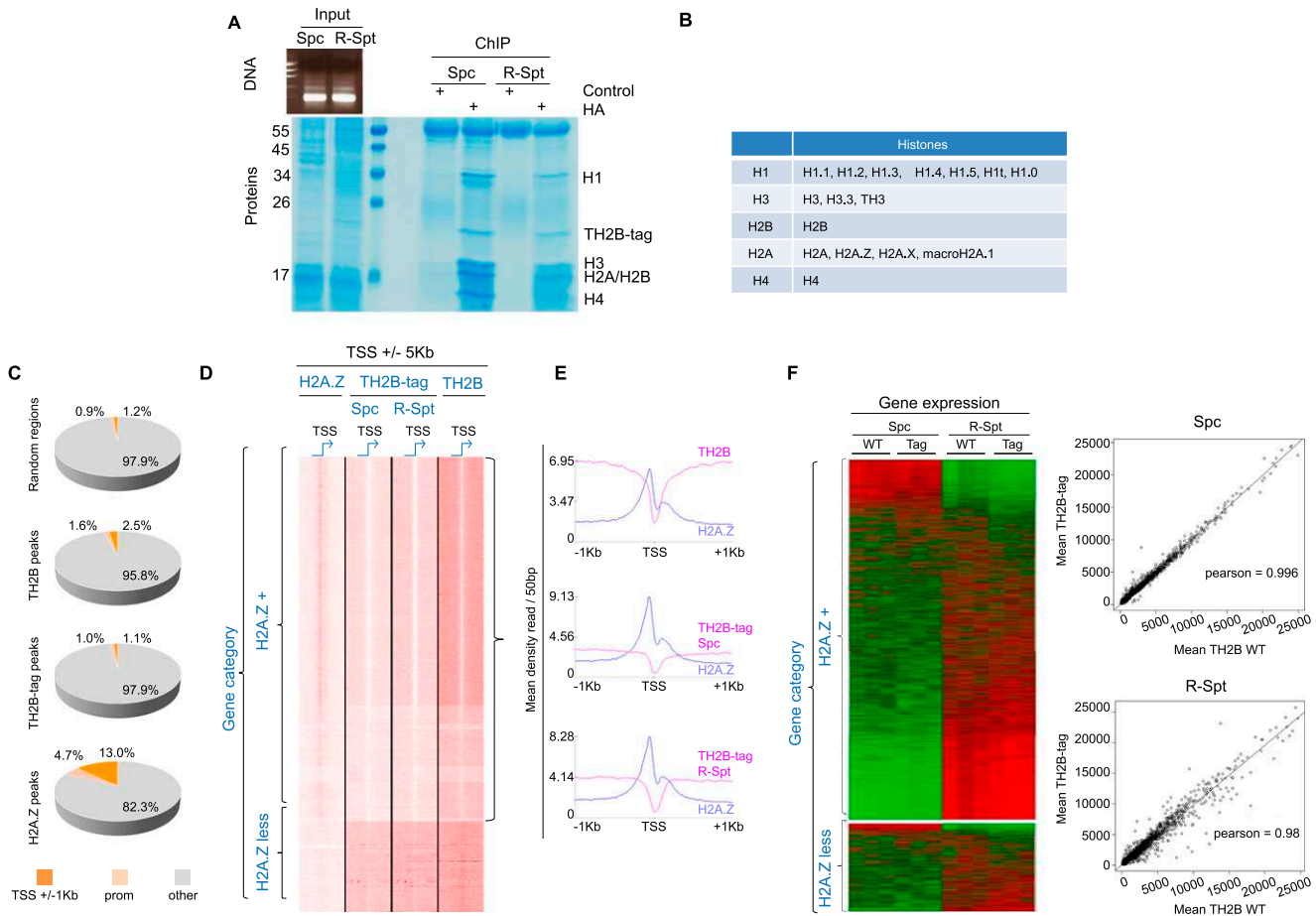
In support of this hypothesis, TPs and protamines are coimmunodetected with TH2B in elongating/condensing

spermatids, showing that TH2B is not replaced by either TP or protamines in TH2B-tag-expressing mice. Indeed, although both nonhistone proteins are synthesized and imported into the nucleus of TH2B-tag-expressing spermatids, TH2B remains present in the same nucleus (Fig. 5B; Supplemental Fig. S4). In striking contrast, in wild-type spermatids, histones disappear when TPs and protamines accumulate (Fig. 5B; Supplemental Fig. S4, *Th2b*<sup>+/+</sup> cells). Furthermore, electron microscopy shows that chromatin compaction is impaired in TH2B-tag-expressing spermatids, suggesting that TPs and protamines are unable to condense chromatin in these cells (Fig. 5C). These data suggest that the presence of the tag affects the properties of TH2B that are specifically required at the time of histone-to-TP transition.

#### *The complete depletion of TH2B induces enhanced H2B expression and restores normal sperm cell development*

The presence of the *neo* cassette downstream from the *Th2b-tag* gene (Supplemental Fig. S1) severely interferes with *Th2b* gene expression and generates a phenotypically null *Th2b* allele. Triton acid urea (TAU) gel separation of H2B and TH2B followed by immunodetection (Fig. 6A) and highly sensitive LC-MS analysis of histones extracted from testes (Fig. 6B; Contrepois et al. 2010) demonstrated a total absence of TH2B. Furthermore, both approaches showed that H2B expression is enhanced in the absence of TH2B (Fig. 6A,B). The profiling of histones from wild-type and TH2B-less testes by ultrahigh-performance LC-MS (UHPLC-MS) allowed us to define which isoforms of H2B were up-regulated (Supplemental Fig. S5A). Six H2B isoforms were readily identified (Supplemental Fig. S5A; Supplemental Table S3), whereas others, such as 1-H/1-K/2-B, could not be distinguished

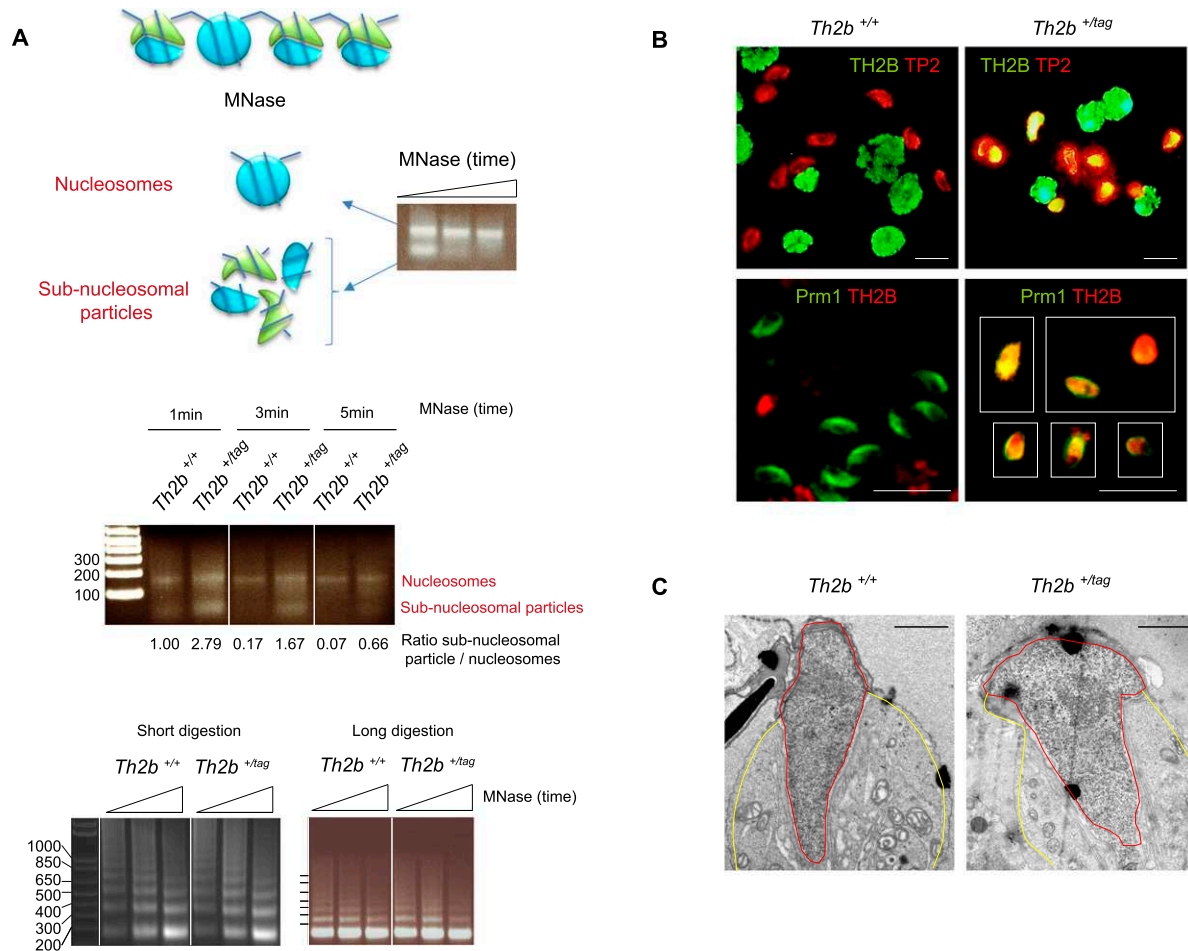
Montellier et al.



**Figure 4.** TH2B-tag is assembled into nucleosomes in spermatocytes and round spermatids and does not affect any of the fine-tuned chromatin activities. (A) Chromatin from spermatocytes (Spc) and round spermatids (R-Spt) was extensively digested with MNase (DNA gel) and subjected to immunoprecipitation using an anti-Ha antibody. The materials obtained from ChIP with anti-Ha or an irrelevant antibody were visualized after SDS-PAGE followed by Coomassie staining. The position of histones identified by MS is indicated. (B) The different variants found associated with TH2B nucleosomes are indicated (see also Supplemental Table S1). All of the unique peptides identified corresponding to each of the linker histone types are shown in Supplemental Table S2. (C) Mononucleosomes from cells isolated from *Th2b*<sup>+/tag</sup> (spermatocytes and round spermatids) or *Th2b*<sup>+/+</sup> (total spermatogenic cell suspension) mouse testes were subjected to ChIP with anti-Ha and anti-TH2B antibodies, respectively, followed by DNA sequencing (ChIP-seq). The respective proportions of TH2B-tag and TH2B peaks (this experiment) distributed on gene regulatory regions (color-coded), including TSSs (regions covering TSSs  $\pm 1$  kb) and promoter regions (from TSSs to 5 kb upstream), were compared with that of H2A.Z peaks (from spermatogenic cells, GSE29913) as well as with “random” genomic localizations (using a set of 75,000 200-bp segment regions randomly selected from the input raw read files). (D) SeqMiner software (Ye et al. 2011) was used to compare TH2B-tag’s, TH2B’s, and H2A.Z’s respective distribution around the TSSs ( $\pm 1$  kb) of the same genes in the indicated spermatogenic cell populations ([Spc] spermatocytes; [R-Spt] spermatids) for the TH2B-tag and total spermatogenic cell population for TH2B. The genes were classified as either H2A.Z-positive (H2A.Z<sup>+</sup>) or H2A.Z-negative (H2A.Z-less). (E) Metagenesis analysis of TH2B-tag (in spermatocytes [Spc] and spermatids [R-Spt]) and TH2B (in total wild-type spermatogenic cells) together with H2A.Z distribution centered around the TSSs of the H2A.Z<sup>+</sup> gene group. (F, left panel) Whole-genome gene expression pattern of *Th2b*<sup>+/+</sup> (WT) and *Th2b*<sup>+/tag</sup> (Tag) spermatocytes (Spc) and round spermatids (R-Spt) are shown as heat maps for the H2A.Z<sup>+</sup> and H2A.Z-less gene classes. (Right panel) Pearson correlation plots between individual gene expression in spermatocytes (Spc) and round spermatids (R-Spc), wild-type or expressing TH2B-tag, are shown.

due to their high sequence identity. As shown in Figure 6B and Supplemental Figure S5A, the peak corresponding to TH2B is the most intense peak in the spectrum obtained from wild-type cells, while it completely disappears from TH2B-less cells. To better visualize the cross-talk between TH2B and H2B isoforms, the peak heights corresponding to these isoforms from Supplemental Figure S5A were normalized relative to the peak heights of the major H4 forms (unacetylated H4 + monoacetylated H4)

whose total abundance is expected to remain constant (Supplemental Fig. S5B). As a control, we determined the ratio of histone H2A isoforms 2-A to histone H4, which remained unchanged between the two samples. Most histone H2B isoforms exhibited a twofold to fourfold increase in TH2B-less spermatogenic cells compared with wild-type cells, except for histone H2B isoforms 1-P, which showed an approximately sevenfold increase (Supplemental Fig. S5B).



**Figure 5.** TH2B-tag affects subnucleosomal transitional states in elongating spermatids during histone replacement. (*A*, top panel) Nuclei isolated from elongated/condensing wild-type spermatids were digested with MNase for increasing lengths of time to release both nucleosomal MNase-resistant regions and MNase-sensitive subnucleosomal particles (histone and nonhistone proteins are represented in blue and green, respectively). (*Middle panel*) Nuclei from wild-type elongated/condensing spermatids or the corresponding cells isolated from TH2B-tag-expressing spermatogenic cells were extensively digested by MNase as above. The agarose gel shows nucleosomal and subnucleosomal DNA fragments released from the two cell types by MNase digestion during the indicated times. The normalized ratios of intensity of subnucleosomal particles to nucleosomes obtained with ImageJ software are indicated below each lane. This ratio was set to 1 for *Th2b*<sup>+/+</sup> after 1 min of MNase digestion and was used to normalize the other values. The bottom panels show chromatin digestion by MNase of suspensions of total spermatogenic cells from wild-type and TH2B-tag mouse testes. (*B*) Codetection of TH2B (green) and TP2 (red) (*top panel*) and of Th2B (red) and protamine 1 (Prm1, green) (*bottom panel*) were performed on spermatogenic cell preparations from wild-type or *Th2b*<sup>+/tag</sup> testes by immunofluorescence using the corresponding antibodies. Bars, 10  $\mu$ m. (*C*) Electron micrographs show representative spermatids of both genotypes. Nucleus and cytoplasm are delimited by red and yellow lines, respectively. Bars, 1  $\mu$ m.

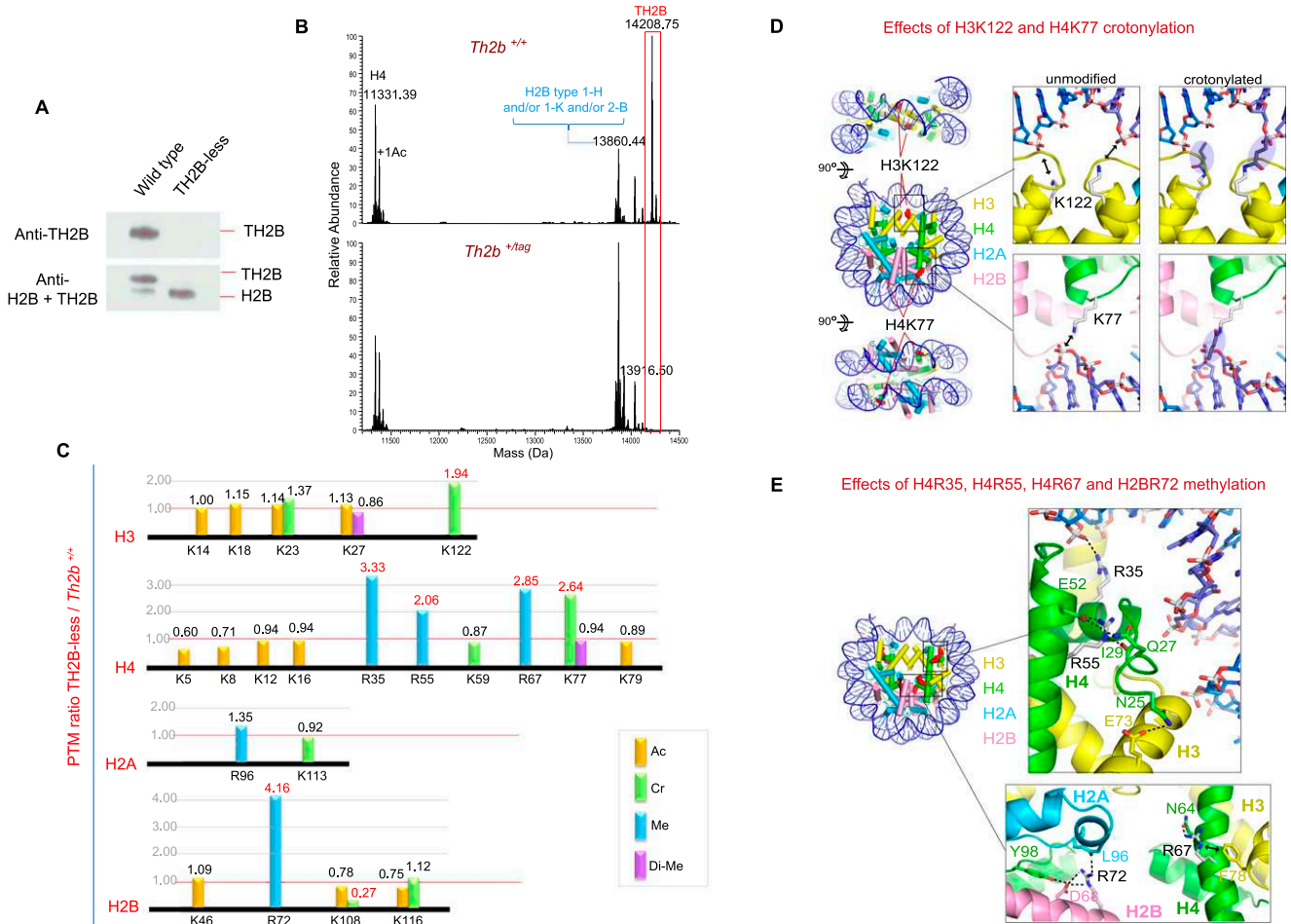
Unexpectedly, the absence of TH2B led to the total rescue of the defects observed in TH2B-tag-expressing elongating spermatids (data not shown) and to the restoration of normal mature sperm production and male fertility (Supplemental Fig. S6A). Indeed, breeding experiments showed that wild-type and TH2B-less male mice were equally fertile, in striking contrast to *Th2b*<sup>+/tag</sup> male mice, which failed to produce any offspring (Supplemental Fig. S6A,B). Thus, whereas modifying TH2B with a C-terminal affinity tag specifically causes sperm cell development to abort, the complete ablation of TH2B appears to have no phenotypic consequences, suggesting the existence of mechanisms that compensate for the loss of TH2B function.

#### *TH2B* depletion induces the epigenetic reprogramming of nucleosomes in spermatogenic cells

The notion that TH2B depletion might be rescued by simply up-regulating H2B expression seems improbable, as this would argue against TH2B having any specialized function in spermatogenesis. We therefore conjectured that H2B up-regulation was only part of the rescue mechanism and that additional chromatin alterations were needed to recapitulate the functions ordinarily sustained by TH2B—specifically, nucleosome destabilization (Govin et al. 2007). Since certain histone PTMs are known to modulate nucleosome stability (Tropberger et al. 2013), we wondered whether altered histone PTMs, by increas-



Montellier et al.



**Figure 6.** A highly specific compensation mechanism is activated in the absence of TH2B. (A) Histone extracts from wild-type and TH2B-less spermatogenic cells were subjected to electrophoresis on TAU gels to allow separation of TH2B and H2B and were subsequently used for the immunodetection of the two histones by Western blotting with a specific anti-TH2B antibody (*top* panel) and an antibody recognizing both TH2B and H2B (*bottom* panel). (B) Profiling of histones from wild-type and TH2B-less testes was performed by UHPLC-MS. The figure shows the deconvoluted electrospray mass spectra of H4, TH2B, and H2B (see Supplemental Fig. S5A,B for additional information). (C) Histones from wild-type and TH2B-less testes were subjected to *in vitro* isotopic labeling followed by HPLC/MS/MS analysis of histone peptides. The relative abundance of the identified histone PTMs is expressed as TH2B-less/wild-type ratio (for details see Supplemental Table S4; Supplemental Fig. S5D). Lysine acetylation (Ac), crotonylation (Cr), and dimethylation (Di-Me) and arginine monomethylation (Me) are color-coded as indicated. (D) Lysine residues exhibiting enhanced crotonylation. (*Left*) Nucleosome core particle (Protein Data Bank [PDB] code 3AZH). Histones H2A, H2B, H3, and H4 are in cyan, pink, yellow, and green, respectively; DNA is in blue. Modified lysines are shown as red spheres. The dyad axis is vertical in the *middle* view. (*Insets*) Close-up of H3K122 (*top*) and H4K77 (*bottom*). Hypothetical crotonyl groups are also shown (blue ovals). (E) Arginine residues displaying enhanced methylation. (*Left*) Nucleosome showing modified arginines as red spheres. (*Top inset*) Close-up of H4R35 and H4R55. H4R35 forms a direct hydrogen (H) bond to the DNA backbone by adopting an orientation that is itself stabilized by an H bond with H4 residue Tyr51 (not shown). Disruption of either H bond by methylation would weaken DNA binding. H4R55 is in an intricate H bond network with H4 residues Gln27 and Glu52 and the backbone carbonyl of Ile29. This network orients a stretch of N-terminal H4 residues (residues 25–29) such that residue Asn25 is ideally placed to H-bond with H3 residue Glu73. R55 methylation would likely destabilize this network and perturb the precise orientation of the H4 N-terminal stretch, thereby weakening the H3–H4 interface. (*Bottom inset*) Close-up of H4R67 and H2BR72. Residue H4R67 is stabilized by an H bond with Asn64 in a conformation allowing it to form a cation– $\pi$  interaction with H3 residue Phe78. Methylation would destabilize the latter interaction by disrupting the H bond and/or delocalizing the positive charge on the R67 guanidino group due to the electron-releasing inductive effect of the added methyl group. H2BR72 is in an H bond network with H2B residue Asp68, H4 residue Tyr98, and the backbone carbonyl of H2A residue Leu96. Methylation would disrupt this network and thus destabilize the H2B–H2A and/or H2B–H4 interfaces.

ing nucleosome instability, could restore an efficient histone-to-protamine exchange in the absence of TH2B. To verify this, we used *in vitro* isotopic labeling followed by HPLC/MS/MS analysis to quantify histone PTMs in

wild-type and TH2B-less cells. Briefly, histone extracts from wild-type and TH2B-less testes were digested with trypsin and propionylated using light ( $^{12}\text{C}_6$ ) and heavy ( $^{13}\text{C}_6$ ) propionic anhydride, respectively. Subsequently,

the light and heavy propionylated tryptic peptides were mixed in equal amounts, resolved into 12 fractions by isoelectric focusing (IEF), and analyzed by HPLC/MS/MS. Using this procedure, we identified 12 Lys acetylation sites, two Lys dimethylation sites, seven Lys crotonylation sites, and five Arg methylation sites in core histones (Fig. 6C). The MS/MS spectra of all PTM-containing propionylated peptides are presented in Supplemental Figure S5D. We then determined the relative abundance of histone marks at every PTM site by normalizing the peak intensities from TH2B-less cells to those from wild-type cells (Fig. 6C; Supplemental Table S4).

In the absence of TH2B, several enhancements of histone PTMs were observed. All of these localized to the histone fold domains as opposed to the flexible N-terminal tails, which, in contrast, showed little or no alteration (Fig. 6C). In TH2B-less cells, four sites (H4R35, H4R55, H4R67, and H2BR72) displayed enhanced arginine methylation, while two (H3K122 and H4K77) displayed enhanced crotonylation. Crotonylation is a newly discovered lysine PTM that adds a bulky four-carbon moiety to the lysine amino group and (like acetylation) neutralizes the positive charge (Tan et al. 2011). The two crotonylated sites, located near (H4K77) or next to (H3K122) the dyad axis on opposite sides of the nucleosome, both mediate electrostatic interactions with the DNA backbone (Fig. 6D; Iwasaki et al. 2011). Crotonylation would abolish these interactions and hence considerably destabilize the nucleosome core. Indeed, the acetylation of H3K122 is known to induce significant nucleosome instability (Manohar et al. 2009; Tropberger et al. 2013). Similarly, the enhanced methylation observed for the four Arg sites is also expected to have a destabilizing effect. These (unmodified) residues either participate in the histone–DNA interface (H4R35) or directly (H2BR72 and H4R67) or indirectly (H4R55) stabilize histone–histone interfaces (Fig. 6E). The specific geometry of all of these residues is such that their methylation is predicted to weaken the corresponding interface (see the legend for Fig. 6E for details). Thus, the absence of TH2B in spermatogenic cells seems to induce the epigenetic reprogramming of nucleosomes whereby strategically positioned histone residues are modified so as to enhance nucleosome instability. We also monitored nucleosome disassembly and the occurrence of transitional states in wild-type and TH2B-less elongating/condensing spermatids following extensive MNase digestion, as described above. In contrast to what was observed in TH2B-tag-expressing cells, the genome of TH2B-less cells displayed exactly the same sensitivity to MNase as the genome of wild-type cells (Supplemental Fig. S5C).

We next examined the genome-scale gene expression of our TH2B-less mouse model to test for changes in gene expression consistent with the up-regulated H2B expression and histone PTM enhancements observed upon TH2B depletion. Spermatocytes and round spermatids were purified from wild-type and TH2B-less testes, and the corresponding whole-genome gene expression measurements were compared. Only a small number (25) of genes were significantly up-regulated in the absence of

TH2B (data not shown). None of these were obvious candidates that might explain the changes in histone PTMs induced by TH2B depletion. In contrast, five were H2B-encoding genes (*h2bm*, *h2bf*, *h2bj*, *h2bk*, and *h2bc*), accounting for the observed increase in H2B expression upon TH2B depletion.

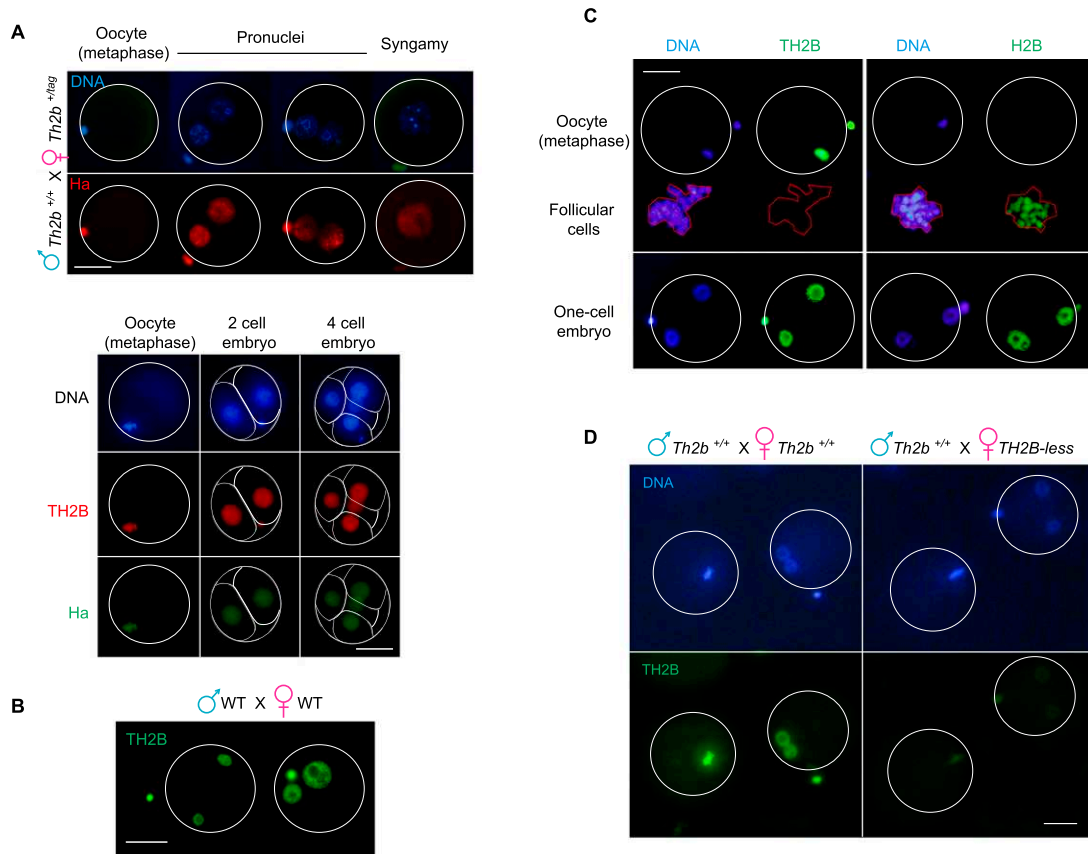
#### *TH2B is assembled into the male pronucleus at fertilization*

Taking advantage of our ability to detect TH2B with enhanced sensitivity using high-affinity anti-tag antibodies, we also investigated TH2B expression in TH2B<sup>tag/+</sup> female mice. We observed that TH2B-tag is present in metaphase oocytes and the female pronucleus at fertilization and is also rapidly incorporated into the male pronucleus (Fig. 7A, top panel). This was confirmed in wild-type cells using an anti-TH2B antibody (Fig. 7B). TH2B remains associated with embryonic cell chromatin (Fig. 7A, bottom panel) until later developmental stages, when it completely disappears in favor of H2B (Supplemental Fig. S7). As in spermatocytes, we once again observed that TH2B is the major H2B species in the mature oocyte, as the metaphase oocyte genome is only stained with an anti-TH2B antibody and not with an anti-H2B antibody, although the latter efficiently detects contaminating somatic nuclei in the field (Fig. 7C). This observation also confirmed the absence of any defective activity of TH2B-tag, further highlighting the highly stage-specific and restricted effects of the tag seen in elongated/condensing spermatids. Also of note, embryos obtained from TH2B-less females and males developed normally and did not show any noticeable defects, as reflected by litter sizes comparable with those of wild-type mice (Fig. 7D; Supplemental Fig. S6A). In summary, TH2B not only replaces H2B during spermatogenesis, but may also play a role following fertilization.

## Discussion

This study not only presents the first thorough characterization of an H2B variant in its physiological context, but also highly increases our understanding of the nucleosome-to-protamine transition that characterizes spermatogenesis. We show that, prior to this transition, the histone variant TH2B almost entirely replaces H2B in male germ cells. This activity differentiates TH2B from other known histone variants, which instead constitute only a fraction of the corresponding histones and display “region-specific” action potentials (Talbert and Henikoff 2010; Bonisch and Hake 2012). TH2B-tag/TH2B ChIP-seq analyses and a comparison of the genomic elements occupied by H2A.Z and TH2B show that H2B replacement by TH2B affects the whole genome. Since the histone-to-protamine transition also affects almost the entire male genome, we hypothesized a role for TH2B in the genome-scale nucleosome disassembly that precedes TP and protamine assembly. Our findings not only confirmed this prediction, but also highlighted the stepwise nature of histone replacement. In a previous study, we

Montellier et al.



**Figure 7.** TH2B is assembled on the male genome at fertilization. (A, top panel) *Th2b*<sup>+/*lag*</sup> females were crossed with wild-type males, and TH2B-tag was detected with an anti-Ha antibody. Metaphase oocytes (indicated) and embryos at different stages are shown in the top and bottom panels. In the bottom panels, a codetection of TH2B-tag (anti-Ha) and TH2B (anti-TH2B) was performed. (B) TH2B was detected in wild-type embryos. (C) Specific antibodies against TH2B and H2B were used to detect the corresponding histones in metaphase oocytes or male and female pronuclei. The contaminating somatic follicular cells are highlighted (red line). (D, right panel) TH2B was detected in embryos from wild-type males and TH2B-less females. (Left panel) As a control, immunodetection was carried out in parallel on wild-type embryos. A specific anti-TH2B antibody was used in both cases. Bars, 40  $\mu$ m.

observed the occurrence of H3/H4-less subnucleosomal particles in elongated/condensing spermatids containing TH2B and H2AL2 (Govin et al. 2007). The present study shows that C-terminally tagged TH2B disrupts the processing of these particles, indicating that these transitional states are specific intermediary structures generated after nucleosome disassembly and are required for full TP/protamine loading and final histone displacement. The disruptive effect of TH2B-tag is highly specific to this particular step among the changes in genome organization that accompany histone removal, since all other chromatin-directed events occur normally. Specifically, profiling the meiotic and post-meiotic transcriptome of TH2B-tag-expressing spermatogenic cells showed no remarkable effect of the tag on gene expression during these stages. Furthermore, genome-wide ChIP-seq analysis demonstrated that TH2B-tag and wild-type TH2B behave similarly and are both excluded from H2A.Z-containing gene TSSs, suggesting normal chromatin dynamics. The chromosome-wide eviction of H3 and incorporation of H3.3 on the sex chromosomes also occur normally in TH2B-tag-expressing pachytene cells. Fi-

nally, the presence of TH2B-tag in male and female pronuclei and early embryonic cell chromatin causes no noticeable defects in embryonic development. These findings suggest that the tag interferes uniquely and very precisely with TH2B function during the processing of subnucleosomal transitional states. However, since the nature and structure of these transitional states are unknown at the present time, one can only speculate on how the TH2B-tag might hamper the final histone displacement. Nevertheless, these data demonstrate a stepwise displacement of histones and the occurrence of a critical TH2B-dependent transitional state required for the full assembly of TP and protamines.

The observation that TH2B depletion induces the activation of several *h2b* genes suggests that H2B replacement by TH2B primarily leads to a shutdown of gene expression in the histone gene cluster domain, with specific effects on *h2b* genes. Remarkably, TH2B depletion also induces dramatic changes in the PTM pattern of core histones, as revealed by the HPLC/MS/MS analysis of histones from TH2B-less mice. Specific sites within the globular domains of histones H3, H4, and H2B exhibit

an enhanced degree of modification, while no particular changes were observed in the accessible N-terminal tails. Two of these sites, H3K122 and H4K77, are lysine residues that directly contact DNA and exhibit enhanced crotonylation. The acetylation of H3K122 was previously shown to induce transcriptional derepression and significant nucleosome instability (Hyland et al. 2005; Manohar et al. 2009; Hainer and Martens 2011; Tropberger et al. 2013). The presence of the bulkier, charge-neutralizing crotonyl group is expected to destabilize nucleosome structure in a manner similar to, if not more severe than, acetylation (Manohar et al. 2009; Tropberger et al. 2013). The fact that TH2B depletion can be compensated by enhanced levels of destabilizing PTMs suggests that the normal function of TH2B is to mediate nucleosome instability. Such a role for TH2B is corroborated by previous biochemical studies, which showed that, upon expression of TH2B and H2AL2 in somatic cells, the nucleosomes containing both variants are dissociated at salt concentrations that do not affect regular somatic chromatin (Govin et al. 2007). Furthermore, *in vitro* reconstituted octamers containing human TH2B have also been found to be particularly unstable (Li et al. 2005). These observations agree with earlier studies of pachytene cell chromatin, which revealed that nucleosomes in these cells were less stable than in somatic chromatin, as shown by their enhanced accessibility to DNase I (Rao et al. 1983). A more detailed mapping revealed that pachytene-specific DNase I-hypersensitive regions mostly correspond to H2B-contacting nucleosomal DNA fragments (Rao and Rao 1987), consistent with a specific role for TH2B in the alteration of chromatin structure. Altogether, these data strongly support the idea that the primary function of TH2B is to create a more dynamic chromatin, thereby facilitating the large-scale exchange of histones, which occurs in meiotic and post-meiotic cells. Our mouse models fully support this hypothesis. Indeed, in TH2B-tag-expressing spermatogenic cells, a decrease in the ability of nucleosomes to dissociate leads to a specific blockade of histone replacement, whereas in the total absence of TH2B, an increase in nucleosome-destabilizing PTMs allows H2B to functionally replace TH2B. Additionally, we found that TH2B is involved in the generation of transitional states, which precede genome-wide nucleosome disassembly in elongating spermatids. Other factors, such as the late-expressing H2AL2 variant (Govin et al. 2007) as well as histone hyperacetylation, which induces the action of the bromodomain-containing factor Brdt (Gaucher et al. 2012), should also be required for full nucleosome disassembly in these cells.

Finally, the assembly of TH2B on the male genome at fertilization while this variant is already present in the female pronucleus indicates a need to cover the whole embryonic cell genome with TH2B during early development. Our models did not allow us to identify a specific role for TH2B in oogenesis or during early development. Neither the TH2B-tag nor TH2B depletion revealed any major or minor defects in oogenesis or embryonic development. However, based on our spermatogenesis data, a chromatin-destabilizing role for TH2B is also conceiv-

able during early embryonic development, when there is a strong requirement for genome plasticity.

## Materials and methods

### *Histone preparation*

Histone extraction was performed on germ cell nuclei using H<sub>2</sub>SO<sub>4</sub> at 4°C for 1 h followed by centrifugation at 16,000g for 10 min at 4°C to isolate solubilized histones (for germ cell nuclei preparation, see “Chromatin Preparation and ChIP”). Histone precipitation was performed with TCA 33% final concentration for 30 min at 4°C, followed by centrifugation at 16,000g for 10 min at 4°C. The histone pellet was washed three times with ice-cold acetone and resuspended in H<sub>2</sub>O.

### *Proteomic-based approaches*

All of the experimental procedures regarding the proteomic approaches are detailed in the Supplemental Material.

### *Chromatin preparation and ChIP*

Mononucleosomes were prepared from fractionated spermatocytes and round spermatids and used for ChIP-seq analyses following the protocols described in Gaucher et al. (2012) and detailed in the Supplemental Material.

### *Step 12–16 condensed spermatid isolation and chromatin preparation*

Step 12–16 spermatid fractionation and chromatin preparation are detailed in the Supplemental Material.

### *Embryo and oocyte collection and immunofluorescence*

Superovulation and collection of oocytes and one-cell embryos were performed as described in Wu et al. (2008). Briefly, collected cells were treated by 0.1% hyaluronidase in M2 medium for 2 min to remove follicular cells and washed in M2 medium. Removal of the zona pellucida was performed by a short incubation in Tyrode solution. After washes in M2 medium, cells were deposited on concavaline A-pretreated slides. Cells were fixed with 4% PFA in PBS overnight at 4°C, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and blocked with 1% BSA in M2 for 30 min at room temperature. Incubation with primary antibody diluted in blocking buffer was performed for 1 h at 37°C for rabbit anti-TH2B antibody (1/700; Abcam, ab23913), for rat anti-HA antibody (1/700; Roche, 3F10), and for rabbit anti-H2B antibody (1/200; Abcam, 61245). The cells were washed with blocking buffer and subjected to secondary antibody incubation for 30 min at 37°C. After the final wash, cells were mounted with VectaShield mounting medium with DAPI.

The two-cell to four-cell embryos were collected and subjected to immunofluorescence analysis as described in Torres-Padilla et al. (2006).

### *Mouse models*

*Th2b* tap-tagging was performed following the recombineering technique as previously described (Liu et al. 2003) to construct the *Th2b* tap tag targeting vector for homologous recombination in ES cells (AT1 line). Animal experiments were approved by ad hoc committees, and all of the investigators directly involved have an official animal-handling authorization obtained after



Montellier et al.

2 wk of intensive training and a final formal evaluation. Knock-in through homologous recombination in ES cells is described in detail in Gaucher et al. (2012). The crossing of *Th2b<sup>neo</sup>* mice with a CMV-driven Cre-expressing mouse line lead to the deletion of the *neo* cassette and the rescue of TH2B-tag expression (see Supplemental Fig. S1 for details).

#### Testis and epididymis histology and immunohistochemistry

Testis and epididymides were AFA-fixed overnight and paraffin-embedded. Slides were either counterstained with eosin hematoxyline or subjected to immunohistochemistry as described in Hazzouri et al. (2000). Polyclonal anti-TH2B antibody (1/500; Millipore, 07-680) and polyclonal anti-H2B antibody (1/500; Abcam, ab61245) were used.

#### Sperm count and purification of germ cells

Cauda epididymides were dilacerated in DMEM medium and incubated for 10 min at room temperature. Spermatozoa were counted on Malassez slides under a microscope.

Fractions enriched in spermatogenic cells at different stages of maturation (spermatocytes and round spermatids) were obtained by sedimentation on a BSA gradient as previously described in Pivot-Pajot et al. (2003). Pure fractions of condensed spermatids at steps 12–16 were obtained by sonication of total mouse spermatogenic cell preparation as described by Marushige and Marushige (1983).

#### Spermatogenic cell preparation, immunofluorescence, and FRAP experiments

Staged seminiferous tubules and testis imprints were prepared as described in Gaucher et al. (2012) and detailed in the Supplemental Material. FRAP experiments were performed on H1299 cell lines stably expressing GFP-TH2B and GFP-TH2B-tag and photobleaching, and the measurement of fluorescence recovery and the calculation of different parameters were carried out essentially as previously described (Moriniere et al. 2009).

#### RNA preparation and transcriptomic and statistical analyses

RNA from fractionated cells (spermatocytes and round spermatids) was extracted using the Qiagen RNeasy minikit and analyzed on the Illumina whole-genome chip as performed in Gaucher et al. (2012). The global gene expression level in spermatocytes and round spermatids was compared between TH2B-tag-expressing and wild-type cells, and Pearson correlation coefficients were calculated.

#### Protein sample preparation and Western blotting

For total protein extracts, whole testes were homogenized in 8 M urea and sonicated at 200 J. Protein dosage was assessed by Bradford. Western blots using SDS-PAGE or TAU gels were carried out using anti-TH2B antibody (1/5000; Millipore, 07-680), anti-actin antibody (1/5000; Sigma, A5441), and anti-H2B C-term antibody (1/2500; Millipore, 07-371).

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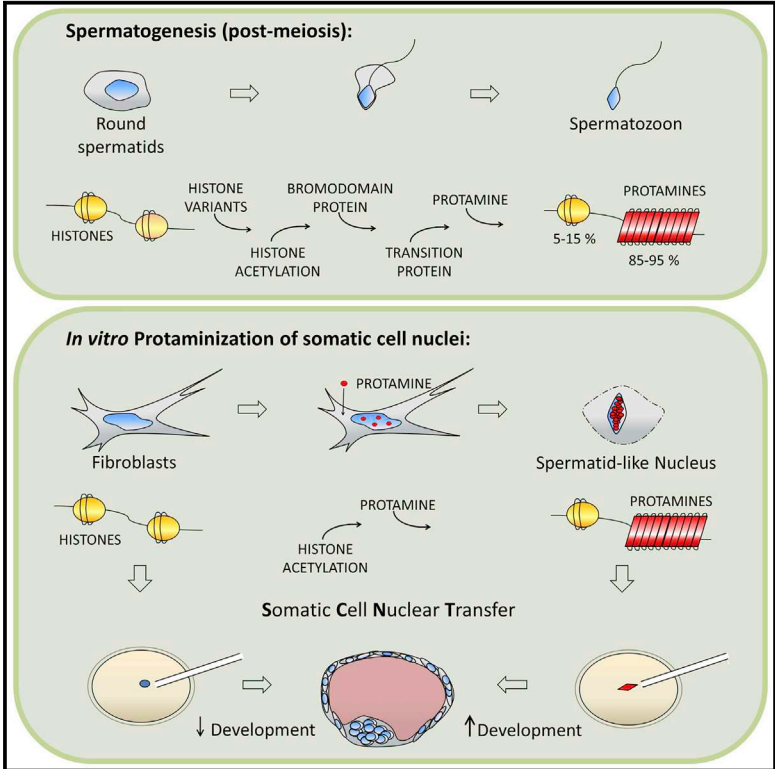
**“Exogenous expression of human protamine (hPrm1) remodels fibroblast nuclei into spermatid-like structures.”**  
**(Iuso et al., 2015)**

**Summary:** Protamine is one of the fundamental components that shapes male germ cell chromatin. Somatic Cell Nuclear Transfer (SCNT) is a method that reprograms the genome by injecting a somatic nucleus into an enucleated oocyte. In this study, induced expression of human protamine 1 (hPrm1) in sheep fibroblasts could induce dramatic remodeling of their nuclei into spermatid-like compacted organization. The reorganized somatic nucleus is reprogrammable upon SNTC, which was evidenced by oocyte’s TH2B incorporation into injected protaminized nucleus.

**My contribution to the work:** I contributed to the immufluorescence analysis and evaluation of TH2B incorporation in the oocyte injected with hPrm-expressing sheep fibroblasts (injected oocytes were sent to our laboratory by the team of Dr. Pasqualino Loi (University of Teramo, Italy)). In this situation of ectopic “protaminization”, TH2B from the oocyte is also incorporated into these injected somatic nuclei, confirming the ability of protaminized nuclei to undergo reprogramming, along with rapid removal of hPrm1.

## Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures

### Graphical Abstract



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### In Brief

Iuso et al. find that exogenous expression of human protamine 1 remodels interphase fibroblast nuclei into spermatid-like structures. The process is reversible upon nuclear transfer into enucleated oocytes. This finding could be exploited as a simplified model for investigating protamine-induced genome compaction and may also boost somatic cell nuclear transfer efficiency.

### Highlights

- In vitro protaminization of somatic cell nuclei
- Conversion of interphase somatic nuclei into “spermatid-like” structures
- Protaminization of somatic nuclei that is reversed upon injection into enucleated oocytes
- A simplified model of nuclear remodeling and reprogramming in vitro



# Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures

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## SUMMARY

Protamines confer a compact structure to the genome of male gametes. Here, we find that somatic cells can be remodeled by transient expression of protamine 1 (Prm1). Ectopically expressed Prm1 forms scattered foci in the nuclei of fibroblasts, which coalesce into spermatid-like structures, concomitant with a loss of histones and a reprogramming barrier, H3 lysine 9 methylation. Protaminized nuclei injected into enucleated oocytes efficiently underwent protamine to maternal histone TH2B exchange and developed into normal blastocyst stage embryos *in vitro*. Altogether, our findings present a model to study male-specific chromatin remodeling, which can be exploited for the improvement of somatic cell nuclear transfer.

## INTRODUCTION

Spermatogenesis is conserved from flies to mammals. Post-meiotic spermatocytes undergo a radical nuclear and cytoplasmic reorganization. Nuclear remodeling relies on a timely translation of stabilized mRNA transcribed at the round spermatid stage (Brock *et al.*, 1980). The first transcripts are testis-specific core and linker histone variants (Gaucher *et al.*, 2010; Kota and Feil 2010). Testis-specific histones have a lower affinity for DNA than somatic ones (Gaucher *et al.*, 2010), and their subsequent post-translational modification (Goudarzi *et al.*, 2014; Rousseaux and Khochbin, 2015; Morinière *et al.*, 2009) further destabilizes the nucleosome. Subsequently, acetylated histones are recognized by bromodomain testis-specific proteins (Brd1) (Pivot-Pajot *et al.*, 2003; Gaucher *et al.*, 2012), proteins that prepare the ground for the incorporation of transition proteins (TPs). TPs cooperate with topoisomerases to relieve torsional stress (Singh and Rao, 1988) and with DNA repairing enzymes (Akama

*et al.*, 1999). The substitution of TPs with protamine (Prm) completes nuclear remodeling, conferring a toroid structure to DNA and the unique shape of male gametes (Miller *et al.*, 2010). Upon fertilization, remodeling is completely reversed. Paternal chromosomes rapidly lose Prm and testis-specific histones (van der Heijden *et al.*, 2005; Wu *et al.*, 2008; Loppin *et al.*, 2005) and regain a nucleosomal organization built upon maternally provided histones.

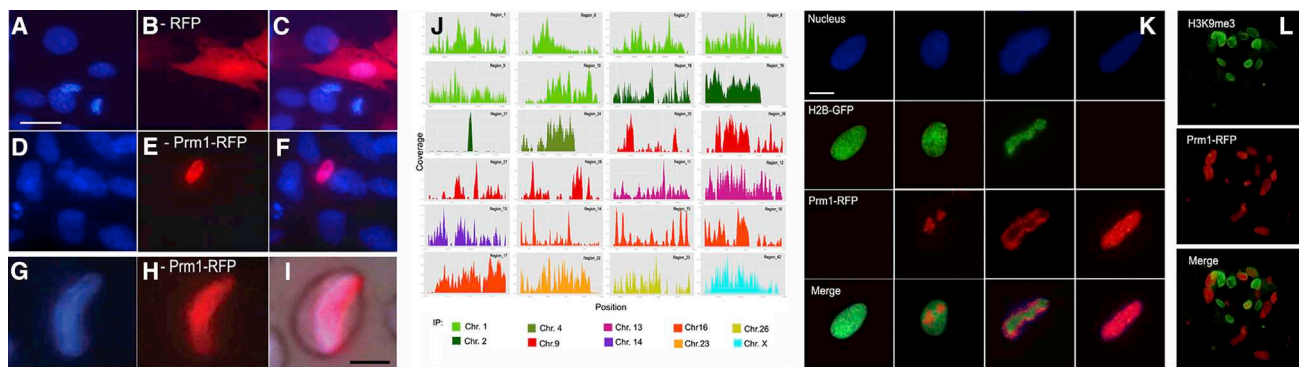
When, instead, a somatic cell is used to “fertilize” an oocyte, as in somatic cell nuclear transfer (SCNT; Wilmut *et al.*, 1997), the nucleosome organization of the chromatin is only occasionally reversed by the oocyte, often leading to developmental failure (Loi *et al.*, 2013).

The current study set out to explore the possibility of conferring a somatic cell nucleus with a Prm-based toroid organization. Here, we demonstrate that the nuclei of adult somatic cells undergo a dramatic chromatin reorganization following the induced expression of the Prm1 gene, transforming the nuclei into “spermatid-like” structures. The Prm-induced nuclear remodeling is reversible upon nuclear transfer (NT) of protaminized cells into enucleated oocytes, and the resulting embryos develop normally *in vitro*.

## RESULTS

### Spermatid-Specific Stepwise Chromatin Remodeling Does Not Function in Somatic Cells

Our first approach to remodel somatic cell nuclei was to induce the co-sequential and sequential expression of four main testis-specific genes in primary cultures of sheep fibroblasts, with the aim to repeat the nuclear remodeling occurring in spermatids. To this end, expression vectors for the bromodomain testis-specific (Brd1-GFP tag), TPs I and II (HA tag), and protamine 1 (Prm1-red variant of GFP tag) were generated and transfected into the fibroblasts. However, our attempts to induce chromatin remodeling of somatic cells following either a co-stepwise or a stepwise transfection of all four expression vectors failed.



### Figure 1. Ectopic Prm1 Binds to Somatic DNA, Replaces the Histones, and Changes Completely Nuclear Morphology

(A–F) Nuclear incorporation of Prm1 in somatic chromatin. This shows tracking the RFP tag in cells transfected with pTag-RFP (A–C) and pPrm1-RFP (D–F); (A and D) Nuclei. (B) RFP. (E) Prm1-RFP localizations. (C and F) Merge. Scale bar represents 10  $\mu$ m.

(G–I) Elongated nucleus in pPrm1-RFP-transfected cells. Scale bar represents 10  $\mu$ m.

(J) ChIP-seq profiles in genomic regions of different chromosomes for pPrm1-RFP in transfected cells (after peak calling the represented genomic regions from pPrm1-RFP sample are enriched than input samples).

(K) Histones/Prm exchange in H2B-GFP fibroblasts: nuclei (blue); H2A.B-GFP (green); Prm1-RFP (red); Merge H2A.B+Prm1. (Left to right) The gradual incorporation (0-, 16-, 24-, 48-hr post-transfection) of Prm1 into somatic nuclei. H3K9me3/Prm exchange (L) was as follows: H3K9me3 (green), Prm1-RFP (red), and Merge H3K9me3+ Prm1.

The nuclear reorganization in spermatids relies on a hierarchic translation of synthesized mRNA, and each of the protein prepares the ground for the next one (Barckmann et al., 2013), a scenario we failed to repeat in cultured somatic cells. There are, however, exceptions to the canonical nucleosome disassembly pathway during spermatogenesis. In the male germline of the cephalopod *Sepia officinalis*, for instance, histones are replaced directly by Prm-like proteins (Martínez-Soler et al., 2007). Hence, we attempted to induce a direct nuclear remodeling in somatic cells through the transient expression of Prm. We selected Prm1 because ram spermatozoa contain Prm1 only and because Prm1 is synthesized as a mature protein with no further processing (de Mateo et al., 2011).

#### Direct Incorporation of Prm1 into Somatic Chromatin

We transfected sheep adult fibroblasts (SAFs) with pPrm1-RFP, WT Prm1, and RFP tag-only vectors using a lipofectamine transfection kit. Transfected cells approached 40% in all three groups. Expression of mRNA and protein Prm1 was confirmed by RT-PCR, by western blotting, and by tracking the RFP-tag (Figures 1, S1A, and S1B). Prm1-RFP co-localized with nuclei stained by Hoechst (Figure 1), whereas in control, i.e., pRFP transfected fibroblasts, the red signal was diffused in the cytoplasm and nucleus (Figure 1). Fibroblasts transfected with WT Prm1 showed nuclear reorganization similar to that detected in pPrm1-RFP transfected ones (Figures 1 and S2). At 40- to 48-hr post-transfection/trichostatin A (TSA) treatment, 93.3% of Prm1-cells did uptake Prm in nuclei, and 83.3% of Prm1-positive cells completely changed nuclear morphology (Table 1).

#### Prm1 Binds to DNA

Prm1 contains numerous cysteine residues that might form intra-Prm/inter-Prm disulfide cross-links (Balhorn et al., 1992). To exclude nuclear Prm1 polymerization, chromatin immunoprecipitation (ChIP)-seq assay was carried on of sorted Prm1-positive

cells. The ChIP-seq assay confirmed the effective binding of Prm1 to DNA at 42 DNA binding sites on 10 of 27 chromosomes, as early as 16-hr post-pPrm1 transfection (Figure 1J; Table S1).

#### Protamination of Somatic Cells' Nuclei Is Not Cell Cycle Dependent

Cell-cycle analysis by cytofluorimetry of transfected cells revealed that Prm1 was incorporated in all cell-cycle stages (72.6% G1, 4.4% S, 23% G2 stage) (Table S2). Moreover, fibroblasts forced to enter G0 (by serum starvation) were transfected with pPrm1-RFP, and even in this case, Prm1 incorporation and chromatin compaction took place (Table 1). Thus, Prm incorporation on DNA is cell cycle independent. A likely mechanism for Prm1 deposition on chromatin might be the conformational changes that nucleosomes undergo as a result of chromatin remodeling enzymes or thermal fluctuations (Chereji and Morozov, 2015). These nucleosomal dynamics make available free stretches of 11 bp between the dyads, enough for Prm1 docking on DNA (Zhang et al., 1996). The low-off rates of Prm1 (Brewer et al., 2003) might facilitate the prolonged condensation of DNA even after transient Prm1 expression. To indirectly test this hypothesis, we transfected GFP-H2B fibroblasts with Prm1-RFP. A histone-to-Prm exchange was observed in GFP-H2B nuclei 48 hr after Prm1 transfection (Figure 1K). Unequivocally, the red fluorescent signal of Prm replaced the green H2B, confirming the over competition of Prm1 on histones (Figure 1K). Furthermore, we observed that the Prm replaced the histone H3 trimethylated on lysine 9 (H3K9me3), a critical epigenetic barrier of SCNT reprogramming in 30% (36 of 120) of Prm-positive cells (Figure 1L). H3K9me3 was no longer detectable in fully condensed nuclei totally devoid of H3K9me3.

#### Dynamics of Prm1 Incorporation

Prm1 assembly on DNA of somatic cells leads to an overlapping of nuclear morphology like the one found in elongating

**Table 1. Incorporation of Prm1 in Somatic Nuclei 16 to 48 hr Post-transfection**

Hours Post-transfection	Group	Prm1 Not in Nucleus	Prm1 Spots in Nucleus	Prm1 in Whole Nucleus
16–20 hr	CTR	15/147 (10.2%)	94/147 (63.9%)	38/147 (25.8%)
	TSA	4/69 (5.8%)	32/69 (46.3%) <sup>a</sup>	33/69 (47.8%) <sup>b</sup>
	G0	4/80 (5%)	52/80 (65%)	24/80 (30%)
	G0 TSA	2/74 (2.7%)	40/74 (54%)	32/74 (43.2%)
40–48 hr	CTR	33/191 (17.3%)	42/191 (21.9%)	116/191 (60.8%)
	TSA	4/60 (6.7%)	6/60 (10%) <sup>c</sup>	50/60 (83.3%) <sup>d</sup>
	G0	6/63 (9.5%)	21/63 (33.3%)	36/63 (57.14%)
	G0 TSA	6/102 (5.9%)	21/102 (20.6%)	75/102 (73.5%) <sup>e</sup>

<sup>a</sup>CTR versus TSA,  $p = 0.0179$ .

<sup>b</sup>CTR versus TSA,  $p = 0.0018$ , Fisher's exact test.

<sup>c</sup>CTR versus TSA  $p = 0.0399$ .

<sup>d</sup>CTR versus TSA,  $p = 0.0010$ .

<sup>e</sup>G0 versus G0 TSA,  $p = 0.0401$ , Fisher's exact test.

spermatids (Figures 1G–1I). Prm1 appears in foci scattered throughout the nucleus 20-hr post-transfection (Figure 2B) and fully coalescences 48 hr later in an elongated structure (Table 1). The final outcome is an elongated and flattened nucleus, very much similar to the spermatozoa's, but larger, due to the diploid DNA content (Figure 2I). The degree of nuclear compaction in protaminized somatic cells approaches that of spermatozoa when observed by transmission electron microscopy (TEM) (Figures 2F–2G).

Histone hyperacetylation opens chromatin structure in spermatids and is a pre-requisite for histone/Prm replacement. Accordingly, transfected cells treated with TSA incorporated more and faster Prm1 than control without TSA (at 16- to 20-hr post-transfection: TSA 47.8% [33 of 69]; without TSA 25.8% [38 of 147],  $p = 0.0018$ ; at 40–48 hr: TSA 83.3% [50 of 60]; without TSA 60.8% [116 of 191],  $p = 0.0010$ ; Table 1).

Physiologically, the massive DNA compaction proper of spermatozoa is achieved through the induction of double strand DNA breaks and repair (Rathke et al., 2014). Therefore, we tested whether the same would apply to our forced conversion of somatic chromatin to a protaminized structure. We analyzed via immunofluorescence the nuclei for the presence of the histone variant  $\gamma$ H2A.X (a marker of double-strand DNA breaks) around the Prm1 condensed foci, but no positive  $\gamma$ H2A.X signal was detected (H2A.X positive: CTR 10.2% [6 of 59]; CTR + [UV irradiated] 63.2% [48 of 76]; Prm1-RFP 10% [6 of 60]; Prm1-RFP/TSA 12.5% [8 of 64]; Figure 2J). A comet assay also excluded the presence of DNA fragmentation in fully protaminized fibroblasts (Figure S3).

Protaminized cells showed signs of degeneration on the third day after transfection, likely a consequence of the global transcription shut down resulting from chromatin compaction (Figure 2C).

### Prm-to-Histone Transition after NT

Finally, we wanted to verify the reversibility of genome protaminization using NT as a biological assay (Figure 3C, a and a1). A large pronucleus was observed in the injected oocytes starting 6 hr after activation and swelling in size ( $16.2 \pm 2.3 \mu\text{m}$ ) by 8 hr. Meanwhile, Prm progressively disappeared in the pronuclei of

77% of injected oocytes (28 of 36; Figure 3A) and replaced by TH2B (Figure 3B), an oocyte-specific histone variant that plays a key role in nuclear reprogramming (Shinagawa et al., 2014; Montellier et al., 2013). Hence, protaminized somatic nuclei decondense and reacquire a nucleosomal organization following a physiological path after artificial activation of the injected oocyte.

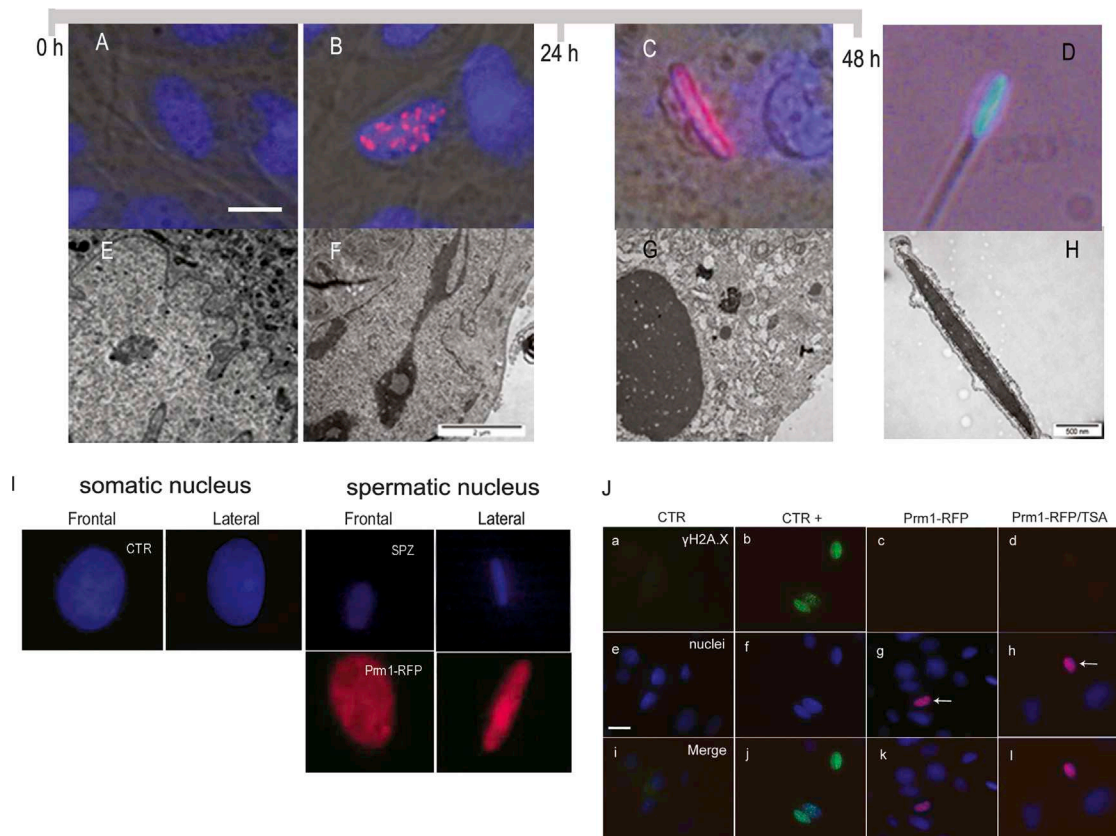
The enucleated oocytes reconstructed with protaminized somatic nuclei were further cultured in vitro in seven separated replicates, indicating their full competence to direct early embryonic cleavages until the blastocyst stage (Figure 3C, a2). The quality of the embryos (total cell count and karyotype) was comparable to that of normal in vitro fertilized embryos (Figure 3D).

## DISCUSSION

Here, we have found that ectopic expression of Prm remodels the interphase chromatin of somatic cells, leading to a nuclear compaction strikingly similar to that of elongated spermatids. The absence of  $\gamma$ H2AX immune localization was surprising, for it is hardly conceivable a genome-wide protaminization without torsional stress alleviation through DNA double-strand breaks (Labege and Boissonneault, 2005). Probably, other DNA repairing enzymes not tested here might be involved; accordingly,  $\gamma$ H2AX has not been detected during chromatin remodeling in human spermatids (De Vries et al., 2012). Another possibility is that the histone variant  $\gamma$ H2AX might have been evicted by Prm binding; however, a comet assay of fully protaminized cells excluded major DNA damage (Figure S3).

The ChIP-seq dataset demonstrated the effective binding of Prm1 to DNA on 42 DNA binding sites on 10 out of 27 chromosomes ( $2n = 54$  in sheep) (Figure 2J; Table S1). Binding occurred in 28 gene-rich domains—14 (50%) genic and 14 (50%) intergenic—while the other 14 were classified as scaffold (Table S1).

Early nuclear remodeling in round human spermatids is marked by the appearance of a single doughnut-like structure. The doughnut is the morphological expression of nucleosome disassembly, for nucleosome-destabilizing histones (H4K8ac and H4K16ac, H3K9me2) co-localize with it (De Vries et al., 2012). These findings suggest that genome remodeling starts



**Figure 2. The Gradual Prm1 Incorporation Leads to Nuclear Compaction Similar to that of Elongated Spermatisms without Causing DNA Brakes**

(A–C; E–G) Timing of incorporation of Prm in somatic nuclei. This is a representation of post-transfection incorporation of Prm in somatic nuclei. pPrm1-RFP-Prm plasmide is tagged with RFP.

(A) Nuclei (Hoechst, blue) of fibroblasts before the transcription of Prm1.

(B) Incorporation of Prm1 in nucleus 16- to 20-hr post-transfection, visible as red spots.

(C) Complete incorporation of Prm1 in nucleus 40- to 48-hr post-transfection. Prm1 is red, and nuclei are blue.

(E–G) TEM analysis of adult sheep fibroblasts transfected with pPrm1-RFP.

(E) Nucleus of fibroblasts before the transcription of Prm1.

(F) Nucleus of fibroblast after 16- to 20-hr post-transfection. Visible partial compaction of chromatin is seen.

(G) Nucleus of fibroblasts after 40- to 48-hr post-transfection. Visible complete chromatin compaction is seen. Bars represent 8  $\mu$ m (A) and 2  $\mu$ m (F).

(D and H) Nucleus of spermatozoa stained with Hoechst (D) and analyzed by TEM (H). Scale bar represents 500 nm.

(I) Representation of nucleus of control (CTR) and Prm-transfected cells (Prm1-RFP) from frontal and lateral view. Nucleus of spermatozoa has been use as a control (SPZ).

(J) DNA double-strand breaks in sheep fibroblasts transfected with pPrm1-RFP visualized by  $\gamma$ H2A.X immunostaining. (a, e, and i) Control adult fibroblasts. (b, f, and j) Positive control: adult fibroblasts irradiated with UV light. (c, g, and k) Adult fibroblasts 20-hr post-transfection with pPrm1-RFP (red). (d, h, l) Adult fibroblasts treated with TSA and transfected with pPrm1-RFP. Stained with antibody anti- $\gamma$ H2A.X. Scale bar represents 10  $\mu$ m.

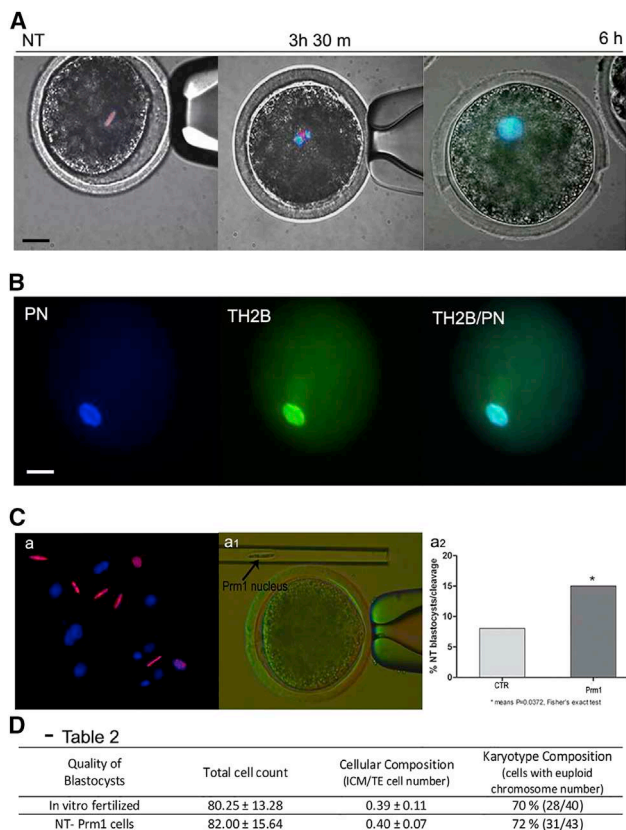
at a defined site in the genome and spreads in a spatially regulated manner, ending up with the nuclear compaction found universally in male gametes. Live imaging of the histone-to-Prm transition could be a powerful tool to test this hypothesis, but it is only available in *Drosophila* (Awe and Renkawitz-Pohl, 2010), not in mice or man. Also, currently unavailable are in vitro models to monitor the whole process of spermatogenesis.

The protaminization of somatic cells might provide unique insights on the early steps of chromatin remodeling, allowing the identification of the very first genome domain(s) binding with Prm. The simplified model for nucleosome-to-Prm exchange reported here might also add to the ongoing debate on nucleo-

somal retention in male gametes (Samans et al., 2014; Weiner et al., 2015). It would be interesting to map on a genome-wide scale, the Prm-histones' footprints on Prm1-expressing somatic cells, to see whether the pattern described in spermatozoa is conserved.

Our findings also impact SCNT (Wilmut et al., 1997). SCNT is a promising technology whose full implementation is on hold because of its low efficiency. Recent successes on human embryonic stem cells isolated from somatic cell-derived cloned embryos have boosted interest in SCNT (Tachibana et al., 2013; Chung et al., 2014). This renewed interest in SCNT goes along with targeted nuclear reprogramming strategies, like





**Figure 3. Protaminized Somatic Nuclei Re-acquire a Nucleosome Organization after Nuclear Transfer**

(A) Displacement Prrm1 during pronucleus formation after nuclear transfer (NT). Scale bar represents 20  $\mu$ m. Prrm1 is red, and nucleus is blue.  
 (B) Incorporation TH2B in pronucleus (PN) after NT of protaminized nuclei: pronucleus (PN, blue), TH2B (green), Merge (TH2B/PN). Scale bar represents 20  $\mu$ m.  
 (C) SCNT of fibroblast transfected with pPrrm1-RFP. (a) Picture represents Prrm1 (red)-positive fibroblasts used as donors for SCNT. (a1) Protaminized nucleus in injected capillary before nuclear transfer into enucleated MII sheep oocyte. (a2) In vitro development of nuclear transfer embryos.  
 (D) Table 2. Quality of blastocysts is as follows: IVF, in vitro fertilized; NT Prrm1 cells. Total cell number is mean  $\pm$  SD. Cellular composition is the number of inner cell mass/trophectoderm cell number. Karyotype composition is the cell with euploid chromosome number divided by total cells (%).

RNAi-mediated downregulation of Xist (Ogura et al., 2013; Matoba et al., 2011) or the depletion of H3K9 methyltransferases in somatic cells before NT (Matoba et al., 2014). In vitro protamination of somatic cells simplifies the nuclear structure of a somatic cell enormously, formatting it in a way that is easily readable by the oocytes and thus holds potential to improve SCNT efficiency.

## EXPERIMENTAL PROCEDURES

### Cell Culture

SAFs were derived from ear biopsy of three female Sarda breed sheep (2 years old). SAFs (between second and eighth passage) were cultured in DMEM (GIBCO) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 3.7 g/L NaHCO<sub>3</sub>, and 0.5% gentamicin.

### Plasmids Construction

Sperm Prrm1 cDNA (GenBank: NM\_002761.2) was amplified from a human testis cDNA library with appropriate primes and cloned into a pTagRFP vector (Evrogen). The identity of the cloned cDNA and its in-frame cloning C-terminal to RFP was verified by sequencing. Mouse TP1 and TP2 cDNAs were amplified by PCR from testis total RNA and cloned in a Ha-tagged pcDNA vector (derived from a Life Technologies pcDNA His-tagged vector). The GFP-Brdt construct is described in Pivot-Pajot et al. (2003) and Gaucher et al. (2012).

### Transfection

SAFs at 80% confluence were transfected with 3  $\mu$ g of pBrdT-GFP, pTP1, pTP2, pPrrm1-RFP, pPrrm1, or pRFP by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 4-hr post-transfection, medium was changed for DMEM containing 5 nM TSA or control (no TSA), and SAFs were cultured for additional 16, 20, 40, 48 hr. For cell-cycle analysis, SAFs before transfection were starved with 0.5% FBS for 5 days.

### Histone Exchange Visualization

To visualize histone-to-Prrm exchange, SAFs were first transfected with construct expressed GFP fused to histone 2B using BacMam 2.0 Technology (C10594, Celllight Histone 2B-GFP, BacMam 2.0, Life Technologies) according to the manufacturer's instructions and then with pPrrm1-RFP, as described above. Histone 2B-GFP and Prrm1-RFP behavior in live cells using fluorescent imaging were observed.

### TEM

Cells were fixed in glutaraldehyde (2.5% in 0.1 M cacodylate buffer [pH 7.4]) for 24 hr. After washing in ddH<sub>2</sub>O, cells were post-fixed in 2% OsO<sub>4</sub> in ddH<sub>2</sub>O for 2 hr. Next, cells were dehydrated through a graded series of ethanol solutions (30%, 10 min; 50%, 15 min; 70%, 24 hr; 80%, 10 min; 96%, 10 min; 100%, 10 min; acetone, twice for 15 min) and were infiltrated with graded concentrations of Epoxy 812 Resin (EPON) resin in 100% acetone (1:3, 20 min; 1:1, 24 hr; 3:1, 2 hr), infused twice for 1 hr in pure EPON resin, and polymerized at 65°C for 24 hr. Next, 60-nm sections were prepared and examined using a LEO 912AB electron microscope. Images were captured using a Slow Scan CCD camera (Proscan) and EsiVision Pro 3.2 software (Soft Imaging Systems GmbH).

### Immunostaining

#### $\gamma$ H2A.X and Trimethyl-HistoneH3

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 or 20 min, and blocked (0.1% BSA/0.05% Tween 20 [v/v] in PBS) for 1 hr at room temperature (RT). Then cells were incubated with the anti-phospho-Histone H2A.X (Ser139) ( $\gamma$ H2A.X) mouse antibody (1:100) (05-636, Millipore) at RT for 2 hr or anti-Trimethyl-HistoneH3 (Lys9) rabbit antibody (1:200) (07-523, Millipore) at 4°C overnight. Subsequently, the secondary anti-mouse IgG-FITC (1:100) or anti-rabbit IgG-FITC (1:200) was added for 2 hr or 50 min at RT. Nuclei were counterstained with 5  $\mu$ g/ml of 4',6-diamidin-2-fenilindolo (DAPI).

### ChIP-seq Assay

#### Chromatin Immunoprecipitation

ChIP assay was performed with the EZ-Magna ChIP kit (Millipore) according to the manufacturer's instructions with minor modification. Protein and DNA complexes from pPrrm1-RFP-transfected cells were cross-linked with 0.5% formaldehyde for 10 min at RT. Sonications were done in nuclear buffer (six 30-s pulses, power setting 10 and six 30-s pulses, power setting 15 in ice with 50-s rest between pulses; Bandelin Sonopuls). Soluble chromatin was immunoprecipitated with anti-RFP antibody (Evrogen) directly conjugated with Magnetic Protein A beads. DNA and protein immune complexes were eluted and reverse cross-linked, and then DNA was extracted using a spin filter column. DNA obtained from pRFP, pPR1-RFP, and Input were subjected to ChIP sequencing.

### ChIP Sequencing

Next-generation sequencing experiments, comprising samples quality control, were performed. Indexed libraries were prepared from 10 ng/each ChIP DNA

with TruSeq ChIP Sample Prep Kit (Illumina) according to the manufacturer's instructions (for details see [Supplemental Experimental Procedures](#)).

## NT

In vitro embryos production was adapted from those previously described (Ptak et al., 2002). Oocytes manipulation was carried out with a piezo-driven enucleation and injection pipette (PiezoXpert), as previously described. Enucleate oocytes were injected with a nucleus, either from CTR or a Prm1-RFP fibroblasts suspended in PBS with 6% polyvinylpyrrolidone (Sigma-Aldrich). Activation and culture of reconstructed oocytes were processed as described (Ptak et al., 2002; Iuso et al., 2013a). Qualities of obtained blastocysts were performed as described by Iuso et al. (2013b).

## TH2B Immunostaining on Pronuclear Stage Embryos

The zona pellucida of embryos at pronuclear stage (10- to 12-hr post-activation) was removed by incubation in 0.5% (w/v) pronase and acid Tyrode's solution for 30 s. Then embryos were fixed in 4% PFA for 15 min and subjected to immunofluorescence analysis as described in Torres-Padilla et al. (2006) with the rabbit anti-TH2B antibody (1:700, ab23913 Abcam). Finally, zygotes were mounted with VectaShield mounting medium with 5 µg/ml of DAPI.

## Statistical Analyses

The Fisher's exact were used to compare quantitative data on nuclear incorporation of Prm1 and in vitro development. Statistical analyses were performed using GraphPad Prism 5.0 software.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.10.066>.

## AUTHOR CONTRIBUTIONS

P.L., D.I., M.C., and S.K. conceived the experiments. D.I. and M.C. performed all NT and characterization work of Prm cells, and M.C. performed the vector expressions and F.Z. did the embryological work. G.E.P. supervised the embryological work and discussed the results. P.T. and A.F. performed the molecular work. S.H. cloned Prm. T.B., S.C., and H.S. performed TH2B immunofluorescence and microscopy of the injected eggs. R.F. designed and interpreted the data. P.L., D.I., M.C., S.K., and R.F. wrote the manuscript.

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## **Review: “Genome-scale acetylation-dependent histone eviction during spermatogenesis” (Goudarzi et al., 2014)**

**Summary:** Genome-wide chromatin reorganization during spermatogenesis has been studied for decades. Increasing amount of evidence suggests tight functional relationship between histone hyperacetylation and histone replacement, as well as the participation of testis-specific factors during the chromatin-remodeling event. Despite the fact that the understanding of this process has been indeed deepened underlying mechanism of such dynamic chromatin remodeling is still largely obscure. This review highlights the findings in this field and yet-to-be-solved questions from four perspectives:

“Does histone hyperacetylation signal histone removal?”

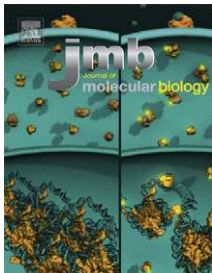
“Mechanism underlying acetylated histone eviction”

“Histone degradation requires facilitated histone removal”

“Burning questions” (“Is histone acetylation active or passive?” “What are the functions of genome-wide histone PTMs in late spermatids?”)

The review conclude with future perspective of this field of study, which solving the questions raised in this review would ultimately help understanding the heterogeneous, yet to be identified complete landscape of mature sperm epigenome and the histone turn over events that could take place in a variety of somatic cells.





# Genome-Scale Acetylation-Dependent Histone Eviction during Spermatogenesis

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## Abstract

A genome-wide histone hyperacetylation is known to occur in the absence of transcription in haploid male germ cells, spermatids, before and during the global histone eviction and their replacement by non-histone DNA-packaging proteins. Although the occurrence of this histone hyperacetylation has been correlated with histone removal for a long time, the underlying mechanisms have remained largely obscure. Important recent discoveries have not only shed light on how histone acetylation could drive a subsequent transformation in genome organization but also revealed that the associated nucleosome dismantlement is a multi-step process, requiring the contribution of histone variants, critical destabilizing histone modifications and chromatin readers, including Brdt, working together to achieve the full packaging of the male genome, indispensable for the propagation of life.

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## Introduction

In both female and male organisms, germ cells become distinct early in life and pursue a specific differentiation process with the aim of generating cells that will survive the parent organisms and perpetuate the species. Within this setting, male germ cells need to develop very unique cellular functions to fulfil their final task, which, in their particular case, includes the transport the genome out of the parent organism. This genome delivery “mission” of the male germ cells imposes specific constraints to their genome that are not seen in any other cells in an organism. Although both male and female germ cells undergo meiosis to end up with a single-copy genome, in the specific case of male germ cells, meiosis also tolls the bells for the start of what can be considered as the most spectacular and large-scale known chromatin remodeling. In mammals, this chromatin remodeling is characterized by an almost genome-wide histone removal and their step-wise replacement by small basic proteins. During this process, histones are first replaced by transition proteins (TPs), which are themselves replaced by protamines (Prms) [1,2]. Prms ensure

the full protection of the genome against the harsh environment encountered by spermatozoa during their journey to reach the female gametes.

These dramatic changes within the essence of genome organization require the action of specialized factors that are themselves expressed exclusively in the male germ cells. They are encoded by groups of genes expressed in successive waves as soon as the progenitor spermatogenic cells, spermatogonia, become committed into spermatogenic differentiation and give rise to spermatocytes entering their first meiotic division [3–6]. During meiosis and following the generation of the haploid post-meiotic cells, named spermatids, a particular gene expression program is turned on, leading to the production of specific factors that will target the genome and drive its transformation into an inert transportable package. Some elements of these new genome organizations have been known for a long time. They include DNA-packaging proteins, histone variants, TPs and Prms that are expressed and that act sequentially following a precise and well-established timing [1]. However, critical information on specific factors managing these elements is still missing. The molecular basis of the precise function

of specific histone variants in haploid cells and the machineries removing histones and assembling TPs and Prms are awaiting to be defined. Additionally, it is also important to understand how, in the context of general histone eviction, some histones escape replacement and are finally retained in the mature spermatozoa [7–9].

The situation has changed in the past few years with the publication of several key investigations that now shed light on how a nucleosome-based genome loses its fundamental organizing structural unit and adopts a new packaging principle, which is apt to be recognized and taken in charge by the maternal genome reprogramming factors in the egg. These new findings will be described and critically commented here, and currently available working models for the molecular basis of histone-to-protamine transition will be presented and discussed.

## Does Histone Hyperacetylation Signal Histone Removal?

In different species, the occurrence of a genome-wide histone hyperacetylation has been observed before and during the disappearance of histones and their replacement by TPs (for review, see Refs. [10] and [11]). These correlative observations and the conserved nature of the association between histone acetylation and removal in different species brought the idea that there could be a causal relationship between the two events. Following this hypothesis, early investigations revealed that histone hyperacetylation could facilitate histone eviction *in vitro* upon the mere incubation of hyperacetylated chromatin with Prms [12–14]. However, although these experiments revealed how histone hyperacetylation could be associated with an enhanced histone exchange, the underlying *in vivo* mechanisms are unlikely to follow such a simple process since, in somatic cells, both the assembly and removal of histones involve specialized machineries [15,16].

Following the discovery of bromodomains as a structural determinant capable of specifically recognizing an acetylated histone tail in 1999 [17], there emerged the hypothesis that putative testis-specific bromodomain-containing factors could in fact recognize hyperacetylated histones and mediate their removal [18]. An analysis of public sequence database for testis-specific genes encoding bromodomain-containing proteins pointed to Brdt, a double-bromodomain-containing factor of BET family, exclusively expressed in spermatogenic cells, as a promising candidate [18]. The early functional investigations of Brdt revealed its capacity to specifically act on hyperacetylated chromatin both *in vitro* and *in vivo* [18,19]. This hypothesis received additional supports from the observation of the phenotype of mice expressing a truncated form of

Brdt deleted for its first bromodomain (BD1). Indeed, *Brdt*<sup>ΔBD1/ΔBD1</sup> male mice were infertile with dramatic defects observed in elongating spermatids [20] at the time when histone hyperacetylation is normally expected to occur. Although the first published analyses of these mice did not consider histone hyperacetylation and their removal in spermatogenic cells [20], the reported blockade of spermatogenesis at the elongation step designated Brdt as a critical factor in managing the biology of elongating spermatids.

The strongest support for the hypothesis presented above, involving Brdt in the removal of hyperacetylated histones, came from the structural characterization of Brdt's two bromodomains. These studies showed that Brdt's BD1 histone binding requires simultaneous acetylation of K5 and K8 of histone H4 tail [21]. Remarkably, the conclusions of these structural studies were confirmed *in vivo* using an original Förster resonance energy transfer probe encompassing Brdt's two bromodomains fused N-terminal to histone H4. This approach demonstrated that the acetylation-dependent interaction of Brdt with histone H4 tail requires the presence of acetylated lysines at both positions 5 and 8 of H4 also in an *in vivo* setting [22].

The abovementioned structural studies, besides describing the first example of a combinatorial reading of histone marks by a single domain, also revealed that Brdt's BD1 is the first identified module specific for the recognition of hyperacetylated histone H4. Indeed, the simultaneous acetylation of H4K5 and K8 is considered as an indicator of the full acetylation of the four acetyl-acceptor lysines of the histone H4 N-terminal tail [23–25].

Taking into account these structural data on the recognition of hyperacetylated histone H4 by Brdt and the fact that a genome-wide histone H4 hyperacetylation occurs only in elongating spermatids, one could therefore easily predict an outstanding action of Brdt on hyperacetylated histones in elongating spermatids, in agreement with the reported impairment of spermatogenesis that precisely occurs in these cells in the absence of Brdt's BD1 [21].

The investigation of histone removal and the assembly of TPs and Prms in elongating spermatids expressing the BD1-less Brdt mutant finally demonstrated the specific involvement of Brdt in the process of histone-to-Prm exchange. Indeed, in these cells, although histone hyperacetylation occurs normally and TP and Prms are synthesized at the expected timing, the removal of hyperacetylated histones does not occur and TPs and Prms are not assembled and remain in the perinuclear regions of the cells [5].

These studies also involving other mouse models for Brdt, including a complete deletion of *Brdt* gene and the use of a dominant-negative non-functional Brdt, also showed that Brdt is a multifunctional factor and that, depending on the spermatogenic cell types,

its activities range from transcriptional regulation in early stages [5,26] to the discussed exchange of histones with TPs in elongating spermatids [5].

## Mechanisms Underlying Acetylated Histone Eviction

All the arguments and data described above strongly support the role of Brdt, mainly its first bromodomain, in the exchange of acetylated histones by TPs in elongating spermatids. However, these data, besides revealing the ability of Brdt to recognize hyperacetylated histone H4 [21,22] and the requirement of its first bromodomain to mediate histone-to-TP exchange [5], did not show how Brdt is actually managing the exchange of nucleosomal histones.

From the early functional data on Brdt, it is possible to propose a working model explaining how Brdt is in fact controlling histone removal and their exchange with TPs. Indeed, a work published in 2003 already showed that Brdt is capable of inducing a dramatic acetylation-dependent chromatin compaction [18] leading to the release and degradation of free histones (in this case, it was shown for linker histone H1<sup>o</sup>) in the nucleoplasm after chromatin compaction [19]. These studies also showed that the chromatin-compacting activity of Brdt requires not only the bromodomains but also the regions flanking the two bromodomains [18]. Based on these data, a working model can be proposed where Brdt and the acetylation-dependent chromatin compaction would constitute a step-wise process initially involving the recognition and binding of hyperacetylated chromatin by Brdt, followed by Brdt–Brdt interactions, finally leading to the cross-link of adjacent nucleosomes and chromatin compaction. In support of this model, the acetylation-dependent Brdt–Brdt interaction was demonstrated using a Förster resonance energy transfer approach [5].

The question underlying this observation is how does this Brdt-mediated chromatin compaction induce histone eviction?

One possibility is that the nucleosomes, already highly destabilized by the incorporation of histone variants and histone post-translational modifications (PTMs), would “squeeze out” their histones through the compacting action of Brdt. The evicted Brdt-bound histones could then be taken in charge by chaperons and a degradation machinery, while TP-associated chaperones would assemble the new DNA-packaging structures.

However, this scheme does not give any indication about the fate of hyperacetylated histones. A probable step following the Brdt-dependent exchange of acetylated histones by TPs could be a direct degradation of these acetylated histones by a specialized 20S testis-specific proteasome, associated with the proteasome activator PA200, named

“spermatoproteasome”. Indeed, a recently published report proposes that the PA200 regulatory module is capable of directly recognizing acetylated histones through a bromodomain-like module and directing their ubiquitin-independent degradation [27].

However, this interpretation is challenged by the observation from another published work clearly indicating that the spermatoproteasome cannot degrade acetylated nucleosomal histones in the elongating spermatid chromatin. Indeed, considering the data discussed above on the role of Brdt's first bromodomain, it clearly appears that, in the absence of Brdt's BD1, despite the occurrence of histone hyperacetylation, histone removal is impaired and cells retain acetylated histones until their death [5]. Additionally, histone removal is also severely disturbed in elongating spermatids expressing a modified form of the testis-specific histone H2B variant, TH2B. In this latter case also, histone hyperacetylation occurs in elongating spermatids in the right timing and in the expected proportion, but again, histone removal does not follow their hyperacetylation [6]. Therefore, a possible explanation for these observations is that acetylated histones should first be removed and then passed onto the spermatoproteasome for degradation.

It is also important to note that the possibility of the ubiquitin-dependent degradation of core histones in spermatids could not be ruled out since previous studies have demonstrated the occurrence of mono-ubiquitination and polyubiquitination of histones H2B, H3 and H3T in the rat elongating spermatids [28]. Along this line, investigators have also identified a testis-specific E2 (UBC4-testis) that may work with an E3 named E3<sup>histone</sup> because of its ability to ubiquitinate core histones, but the involvement of this machinery in histone degradation in elongating spermatids was not demonstrated [29–31].

Additionally, both in mice lacking PA200 and in drosophila lacking a proteasome subunit, Prosa6 T, only a delay in histone removal is observed. Indeed, in the absence of PA200 condensing spermatids devoid of histones could be observed [27], and in the elongating spermatids of Prosa6 T-less drosophila, Prm assembly finally takes place [32], strongly suggesting that a proteasome-independent histone degradation system should also be operational in spermatogenic cells and ensure the degradation of the major part of the removed histones. Altogether, it seems clear that histone acetylation is *a priori* a signal for histone removal and histone degradation could be a consequence of this process.

## Histone Degradation Requires Facilitated Histone Removal

In at least two cases, a normal histone hyperacetylation occurs despite an impairment of histone

removal in elongating spermatids, which strongly suggests that histone hyperacetylation by itself is not enough to mediate the subsequent nucleosome dismantlement. Indeed, as mentioned above, in the absence of Brdt's BD1 [5] or in the presence of a C-terminally modified TH2B [6], histone hyperacetylation occurs without inducing any subsequent histone removal.

These observations strongly suggest that acetylation-dependent histone removal is a multi-step process and involves various and independent factors. Based on the literature, it can be argued that at least two independent events cooperate to mediate the nucleosome disassembly, which is itself also a multi-step process.

One of the first determinants in nucleosome dismantlement in elongating spermatids is a prior drastic destabilization of nucleosomes. Spermatogenic cells express almost all the histone variants encoded by the genome including a variety of testis-specific members exclusively expressed in spermatogenic cells [11,33]. Several reports investigating the biochemical properties of these testis-specific variants encompassing H2A, H2B and H3 variants all support their capacity to create a nucleosome that is less stable than the ones containing canonical histones. Indeed, data on testis-specific histones H3T, TH2B and H2AL2 and H2Abbd show that the presence of these histones in a nucleosome is associated with increased nucleosome instability [33–36]. Since these histones are massively synthesized and incorporated in stages preceding histone removal, one could argue that their synthesis and assembly in chromatin is a requisite for a subsequent nucleosome disassembly. A recent publication reporting the genetic manipulation of the testis-specific H2B variant, TH2B, adding a C-terminal extension to the protein, revealed that, despite the occurrence of histone hyperacetylation and the presence of functional Brdt, histone removal is impaired in elongating spermatids. This report demonstrated that this interference with histone removal is specific to elongating spermatids, since the same TH2B molecule could be normally assembled and later removed in early embryos [6].

The same report also reveals another mechanism that presides at the dismantlement of nucleosomes in spermatogenic cells, involving PTMs at critical positions in histones, which directly affect the stability of nucleosomes. Indeed, in the total absence of TH2B, the canonical H2B can compensate for the lack of TH2B and histone replacement normally occurs, but the nucleosomes undergoing this disassembly contain histones that bear specific PTMs at several critical histone–DNA and histone–histone interaction points. These histone PTMs would confer to the nucleosomes the instability that is normally associated with TH2B recruitment [6].

The uncovering of this mechanism also argues in favor of the existence of other nucleosome-destabilizing mechanisms involving machineries capable of modifying histones within the histone-fold regions [37]. Therefore, it is expected that, in elongating spermatids, in parallel with the well-known acetylations of the histone tails, lysines present in the core regions of histones also show an increased acetylation aiming at inducing increased nucleosome instability. Such situations have already been described in somatic cells, where acetylation of critical histone-fold lysines, mainly H3K56 and H3K122, has clearly been shown to induce nucleosome instability [38]. Although the occurrence of H3K122 and H3K56 acetylation has not been studied in spermatogenic cells, a very recent investigation of a newly discovered histone H3K64 acetylation, also inducing nucleosome instability and facilitating histone eviction, revealed its genome-wide occurrence at the time of histone exchange in elongating spermatids [39].

In summary, taking into account all the data discussed above, it is possible to propose the following temporal scheme for histone removal in elongating spermatids. First, canonical histones are replaced at a very large scale by testis-specific histone variants capable of creating less stable nucleosomes [6,33]. Second, a genome-wide histone hyperacetylation occurs with two distinct objectives: tail acetylation, which provides a surface for the recruitment of Brdt [5], and histone-fold acetylation, to further destabilize the nucleosomes [38]. The recognition of acetylated histone tails by Brdt would then initiate the process of histone removal, which can only occur if it has been previously “prepared” by the combined destabilizing actions of histone variants and acetylation of histone-fold regions. The removed histones could then eventually be degraded following the action of the spermatoproteasome and other yet unknown proteolytic machineries (Fig. 1).

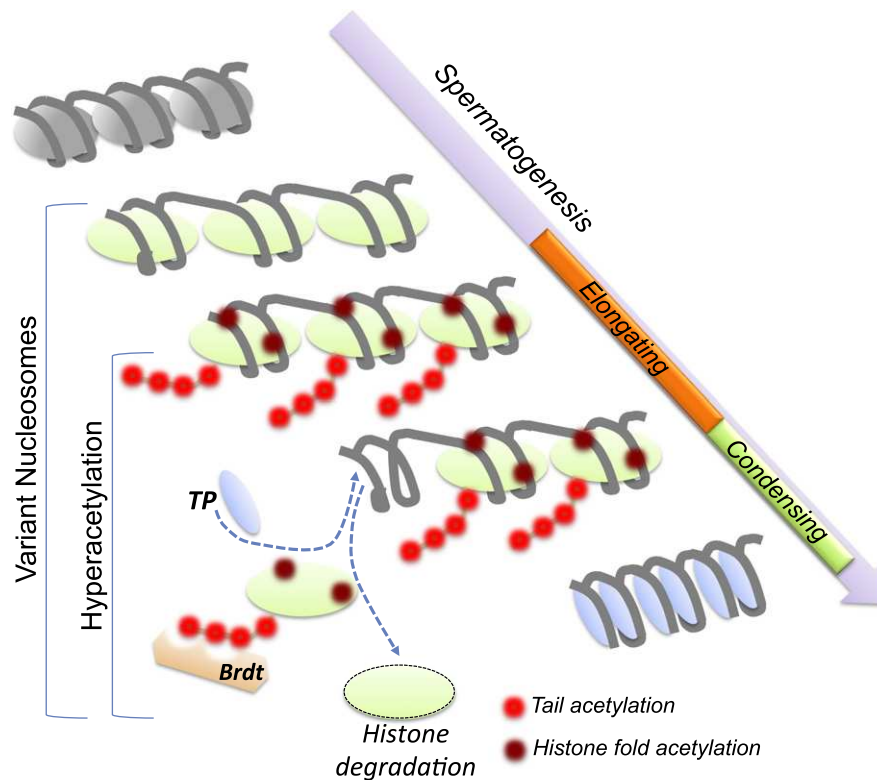
## Burning Questions

In the light of the literature and especially of the data discussed above, histone hyperacetylation in elongating spermatids appears as a determinant event in the process of histone-to-Prm transition. An important unsolved question is whether this histone hyperacetylation is an active process or rather an indirect consequence of prior events.

Although today there is no clear response to this question, some hints from published reports could support the proposition of a working model.

The simplest explanation for the increase in histone acetylation could merely be a decrease in the amount of cellular histone deacetylase (HDAC) at the time of spermatid elongation. This is supported





**Fig. 1.** A working model for the histone-to-transition proteins exchange in elongating spermatids. Global incorporation of various H2A, H2B and H3 histone variants creates highly unstable nucleosomes, which then undergo hyperacetylation both at their accessible N-terminal tails and within their histone-fold regions. Acetylation at critical lysines, such as H3K122 and H3K64, further destabilizes the nucleosomes, while tail acetylation generates a platform for the recruitment of Brdt. Brdt could then start the process of histone eviction and replacement by TPs. Evicted acetylated histones would then be degraded through the action of spermatoproteasome or/and yet unknown proteolytic systems.

by the observation of a dramatic decrease in the amounts of HDAC1 and HDAC2 observed by Western blot in fractionated elongating mouse spermatids [40]. The observed increase in histone acetylation in these cells could therefore be largely explained by the loss of the enzymes removing the acetyl group in these cells. Another argument in favor of a major role for HDACs in this process is the fact that histone hyperacetylation could be induced in round spermatids by a simple treatment of a spermatogenic cell suspension with the HDAC inhibitor TSA. Indeed, this treatment induced a dramatic histone hyperacetylation in round spermatids, which normally have hypoacetylated chromatin, whereas no remarkable change in acetylation could be observed once histone hyperacetylation starts in elongating cells [41], suggesting that there are no more HDACs to inhibit.

In the literature, some data also support the involvement of specific histone acetyltransferases (HATs) in the process of histone hyperacetylation. However, almost all the corresponding data are controversial and do not support the active involvement of a particular HAT in histone hyperacetylation.

First, a work published about a decade ago, identifying Cdy1 as a new HAT, proposed its involvement in the occurrence of histone hyperacetylation in elongating spermatid [42]. However, the HAT activity of Cdy1 specifically tested by other researchers could not be confirmed [40] and, since then, no other report on a HAT activity associated with Cdy1 has been published.

The study of the phenotype associated with the invalidation of *RNF8*, a gene encoding a histone E3 ubiquitin ligase, also led to the observation of a defective histone removal associated with a lack of histone hyperacetylation. The authors then attributed this lack of histone acetylation to a down-regulation of the Mof HAT, a known H4K16 HAT, in the *RNF8*<sup>-/-</sup> spermatogenic cells, and suggested that this HAT could be a major player in mediating histone hyperacetylation [43]. However, here again using the same *RNF8*<sup>-/-</sup> mice strain, another group did not observe any abnormality in histone H4K16 acetylation or in the process of histone-to-Prm exchange [44]. Finally, the analysis of the expression of two ubiquitous and generalist HATs, CBP and p300, in spermatogenic cells showed their important down-regulation in post-

meiotic cells, especially in elongating spermatids, when histone hyperacetylation occurs. Additionally, their further partial knockdown in post-meiotic cells by the targeted deletion of the corresponding genes did not reveal any alterations in the occurrence of histone acetylation in elongating spermatids [3].

All these observations suggest that the occurrence of histone hyperacetylation could only be a consequence of a decrease in the amount of cellular HDACs occurring prior to the exchange of histones by TPs and that cellular HATs present in these cells could ensure this histone hyperacetylation, without a need for additional and specific HATs.

An intriguing observation remains however the fact that, in elongating spermatids, acetylation is not the only PTM that globally affects histones prior to their removal. Indeed, phosphorylation of H2A.X [45], H2A ubiquitination [46] and histone poly-ADP ribosylation [47] are also reported to occur at almost the same timing as histone acetylation. Surprisingly, the discovery of new histone PTMs and the investigation of their occurrence in spermatogenic cells again pointed to elongating spermatids as a particular cell population showing a dramatic enhancement of these new modifications [48]. A question therefore arises: why do nearly all the histone PTMs investigated so far suddenly show an enhanced occurrence on the chromatin in elongating spermatids? As for histone acetylation, which increases as HDACs decrease, it is likely that these other PTMs are increased following prior events, such as the down-regulation of the enzymes involved in their removal from histones. Additionally, the opening of chromatin and histone removal may provide a window of opportunity for all the present histone PTM machineries to efficiently act on histones before their degradation.

Another important question is the functional implication of these genome-wide histone modifications occurring in parallel with acetylation. Some of these reported histone modifications could also be associated with the process of nucleosome dismantlement and histone eviction. For instance, histone poly-ADP ribosylation and H2A.X phosphorylation could be linked to the cell response to naturally occurring double-strand DNA break that accompanies histone eviction [45,49]. One can also question the role of the newly identified histone PTMs but an answer should await more investigations on the functional significance of these new PTMs.

## Concluding Remarks

Histones have been largely studied as major elements in the control of gene expression and support for epigenetic mechanisms. However, de-

spite being very abundant cellular proteins and a focus of intense investigations, their metabolism is not very well understood. Although the synthesis of the canonical forms is coupled to DNA replication and follows well-defined rules, their degradation and the machineries involved have only recently become known [50–52].

A complicated matter in these studies is perhaps that histone turnover must be highly region dependent and variant dependent and that histone half-life in active chromatin and heterochromatin should be very different [53]. The almost genome-scale histone removal in elongating spermatids provides therefore a unique model to tackle mechanisms controlling histone turnover in cells. It could also guide our understanding of histone degradation in somatic cells. Indeed, the definition of the spermatoproteasome in spermatogenic cells, especially the role of the PA200 subunit in the recognition and direct degradation of acetylated histones, allowed the authors to show the occurrence of a similar mechanism in somatic cells degrading histones during DNA repair [27]. However, as discussed above, this particular system does not seem to degrade nucleosomal histones and a collaboration with histone removal machineries is probably necessary.

Finally, although we are advancing in the understanding of the genome-wide histone eviction in spermatogenic cells, it is not clear how histones associated with specific genomic loci could escape removal [7–9]. A recent study established a tight relationship between the histone retention and non-methylated CpG islands and histone H3 variants and their methylation at K4 and K27 [54]. However, the question remains on why factors such as Brdt and the spermatoproteasome do not act on these nucleosomes. One possibility is that histone H4 in these retained nucleosomes does not bear acetylation at K5 or K8 or both, considering that their simultaneous acetylation is absolutely necessary for Brdt binding [21]. A hint from the literature suggests that this may well be the case. Indeed, the analysis of H4 acetylation in the retained human mature sperm histones revealed hypoacetylation of H4K5 [55]. In the light of this observation, it could be proposed that regions escaping full acetylation of nucleosomal histone H4 may become protected against histone removal simply because they do not bear the code necessary for Brdt recognition.

In summary, although recent investigations brought an unprecedented insight into the molecular basis presiding at the dramatic transitions in the male genome packaging and the mechanisms discovered also shed some lights into the mechanisms underlying histone turnover in somatic cells, hundreds of questions still remain unanswered and years of investigations will be necessary to finally unravel the mysteries of the male genome packing.

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### Abbreviations used:

PTM, post-translational modification; HDAC, histone deacetylase; HAT, histone acetyltransferase.

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## **Research highlights**

### **“Transgenerational inheritance of chromatin states”**

**(Shiota et al., 2013)**

(highlighting: Raychaudhuri et al. “Transgenerational propagation and quantitative maintenance of paternal centromeres depends on Cid/Cenp-A presence in *Drosophila* sperm” . PLoS Biol. 10 (12), e1001434 (2012).)

### **“ Shielding Cenp-A removal in post-meiotic male cells”**

**(Shiota et al., 2013)**

(highlighting: Dunleavy et al. “The cell cycle timing of centromeric chromatin assembly in *Drosophila* meiosis is distinct from meiosis yet requires CAL1 and CENP-C”. PLoS Biol. 10 (12), e1001460 (2012).)

**Summary:** These are short articles highlighting two studies about Cid, a *Drosophila* homologue of centromere specific histone H3 variant CENP-A. The discussion concerns about the potential importance of centromere specific histone variants and their association with other neighboring chromatin components (for example, H2AL2 in pericentromeric region).

# Research Highlights

Highlights from the latest articles in epigenomics



## Transgenerational inheritance of chromatin states

**Evaluation of:** Raychaudhvir N, Dubruille R, Orsi GA *et al.* Transgenerational propagation and quantitative maintenance of paternal centromeres depends on Cid/Cenp-A presence in *Drosophila* sperm. *PLoS Biol.* 10(12), e1001434 (2012).

The genome-wide histone-to-protamine transition in the post-meiotic phases of spermatogenesis in many organisms, including *Drosophila*, involves one of the most spectacular genome reorganizations [1], derived by mechanisms that are set in the preceding stages [2]. This transition in genome packaging and, most importantly, the disappearance of histones, raises an essential issue relative to the nature of the final mature male gamete epigenome and the specific associated elements that are transmitted to the embryo.

Early investigations have revealed that in mammals, mature sperm cells partially retain elements of chromatin. For instance, in humans, it is believed that between 5 and 15% of histones survive histone-to-protamine exchange. The recent genome-wide mapping of residual nucleosomes and histone marks in human sperm cells revealed the existence of a possible sperm epigenome organized by the selective association of defined genomic regions – that is, developmental and imprinted genes, with canonical histones [3,4]. These studies also imply that these specific organizational states of the male genome would be inherited by the embryo and could have a functional impact on gene expression during early development. However, up to now, no experiments had been designated to test this hypothesis and no data were available to support this fundamental claim.

Among the somatic chromatin elements that were considered to escape histone

removal was the centromeric histone H3 variant, Cenp-A [5]. However, in this case there was no data explaining the reason behind the retention of this specific histone. Very recently, a breakthrough came out of the study of the male germ cell Cenp-A homolog, Cid, in *Drosophila*, whose male germ cells also undergo a genome-wide histone-to-protamine replacement [6]. These data show not only that the *Drosophila* Cenp-A (Cid) almost completely escapes histone-to-protamine replacement, but also that its maintenance in the sperm is of critical importance. Indeed, the authors found that Cid elimination from sperm results in the loss of paternal chromosomes during the initial syncytial cycles of early embryogenesis, hence demonstrating that the Cid inherited along with the male genome is indispensable for the paternal genome contribution to the embryo, ensuring its propagation. This constitutes the first demonstration of the essential role of a retained histone variant in the male gametes, and may also shed light on the possible importance of other histone variant maintenance in the sperm.

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## Shielding Cenp-A removal in post-meiotic male cells

**Evaluation of:** Dunleavy EM, Beier NL, Gorgescu W *et al.* The cell cycle timing of centromeric chromatin assembly in *Drosophila* meiosis is distinct from mitosis yet requires CAL1 and CENP-C. *PLoS Biol.* 10(12), e1001460 (2012).

The focus of this particular discussion is the discovery by Dunleavy and colleagues of a significant Cid assembly in male post-meiotic cells in addition to that observed during meiosis [1]. It is of note that, as mentioned in the above discussion, in *Drosophila*, like in many mammalian species, the post-meiotic haploid cells, named spermatids, prepare for a genome-wide histone replacement [2]. It is therefore of interest that Cid that escapes histone removal [1,3] is at least partially assembled in haploid cells prior to histone displacement. There is another example of such an incorporation of a histone variant before the process of general histone eviction, observed recently during mouse spermatogenesis, which concerns a H2A variant [4]. Indeed, a testis-specific H2A variant, H2AL2, was found to be incorporated into chromatin just before the accumulation of protamines. Remarkably, with regard to Cenp-A/Cid, this H2AL2 is also retained in the mature

sperm and specifically organizes the pericentric regions [4].

It is therefore possible that the observed assembly of Cid in nonreplicative haploid spermatids is associated with the assembly of a histone-like or a specific DNA-packaging protein on the pericentric regions. Similarly, the assembly of H2AL2 in mouse spermatids could also be associated with that of Cenp-A. This would imply that, in mammals as well as in the fly sperms, centromeric and pericentric regions would both be associated with specific DNA-associated proteins. If this hypothesis is verified, the next question would be whether the repackaging of pericentric regions could have a role in protecting Cid/Cenp-A against histone removal. Since the mouse H2AL2 present in pericentric regions resists histone displacement, one can propose that its deposition around centromeres could 'shield' Cenp-A against the wave of nucleosomes disassembly affecting the rest of the genome.

In the light of these discussions, one could also speculate not only that centromeric histones could be inherited and are indispensable to the male genome propagation [1,3], but also that pericentric regions bear specific chromatin organization of equally important functions. However, in

contrast to Cid, the possible protective role of pericentric chromatin should be critical only in sperms since, after fertilization in the mouse, H2AL2 is rapidly removed and replaced by maternal histones [5].

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