Transcriptome profiling of histone acetylation and methylation writers and erasers gene families during mouse spermatogenesis

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Keywords: **spermatogenesis, epigenetic enzymes, histone post-transcriptional modification**

ABSTRACT

Accumulating evidence points out that sperm carry epigenetic instructions to embryo in the form of retained histones marks and RNA cargo that can transmit metabolic and behavioral traits to offspring. However, the mechanisms behind epigenetic inheritance of paternal environment are still poorly understood. Here, we analyzed the expression profile of all known histone lysine writers and erasers enzymes across spermatogenesis, unraveling the developmental windows at which they are upregulated, and the specific activity related to canonical and non-canonical histone marks deposition and removal. We also characterized the epigenetic enzymes signature in the mature sperm RNA cargo, showing most of them positive translation at pre-cleavage zygote, suggesting that paternally-derived enzymes mRNA cooperate with maternal factors to embryo chromatin assembly. Our study shows important mechanistic aspects behind transgenerational epigenetics, where epigenetic enzymes not only can respond to environmental stressors, but could function as vectors of epigenetic information and participate in chromatin organization during maternal-to-zygote transition.

INTRODUCTION

In the last years, accumulating research has documented that male germ cell maturation presents windows of vulnerability for epigenetic reprogramming by environmental stressors that can affect fertility and even transmit developmental, metabolic and behavioral traits to offspring (Bale, 2015). This type of non-genetic "Lamarckian" transmission has been established for paternal lifestyle and different forms of chronic stress, drug abuse, dietary change, and social defeat, involving changes in non-coding RNAs cargo, DNA methylation, and histone post-translational modifications (PTMs) (Bale, 2105). However, most of the research in epigenetic inheritance has documented the role of non-coding RNAs and DNA methylation patterns, whereas the mechanisms by which the environment can trigger specific changes on histone PTMs are still largely unexplored.

Spermatogenesis is a finely regulated process where unipotent spermatogonia undergo meiosis and, subsequently, spermiogenesis, to become sperm cells. During the developmental stages of spermatogenesis, male germ cells experience dramatic chromatin reorganization, where approximately 90% (human) to 95% (mouse) of histones are evicted and replaced by protamines to compact the paternal genome (Rajender et al., 2011). Importantly, a small fraction of histones in the sperm genome is retained in specific locations and carries several PTMs that play critical roles in epigenetic regulation of spermatogenesis and early embryonic development (Luense et al., 2016). The epigenetic program of histone PTMs that controls spermatogenesis is driven by enzymatic families known as writers and erasers of the so-called "histone code", that modulate chromatin structure and transcription (Kouzarides, 2007). These epigenetic enzymes catalyze the deposition or removal of specific PTMs such as acetylation, methylation, phosphorylation, crotonylation, among others, at specific amino acid residues on the nucleosome's core

histones H2A, H2B, H3, and H4, and also on the linker H1/H5 (Luense et al., 2016). In male germ cells, histone PTMs not only control active vs repressed chromatin states but also a range of processes including DNA replication and repair, chromosome maintenance and histone eviction (Luense et al., 2016). So far, the most studied histone PTMs related to the control of germ cells mitosis, meiosis and spermiogenesis are methylated and acetylated lysines (K) on H2A/B, H3 and H4. Histone K acetylation can be dynamically regulated by the opposing action of acetyltransferases (HATs) and deacetylases (HDACs). Acetylation of K residues neutralizes the positive charge on histones, allowing DNA-binding proteins better access to the DNA and resulting in activation of gene expression as well as histone eviction (Rajender et al., 2011). Unlike acetylation, methylation does not affect histone charge but regulates recognition and interaction with chromatin-binding proteins that control the transcription or respond to DNA damage (Rajender et al., 2011). Histone K methylation is finely regulated by methyltransferases (KMTs) and demethylases (KDMs) that control the mono-, di-, and/or tri-methylation of specific residues, and this can either activate or repress transcription depending on the residue position, the number of methylations and the presence of other methyl or acetyl groups in the vicinity (Mosammaparast and Shi, 2010).

The role of epigenetic enzymes comes to focus, as they are responsible for the histones´ PTMs writing and erasing balance. Moreover, histone-modifying enzymes have been shown to respond to the organism physiology and metabolism, several disease conditions, and environmental stressors (Bale, 2015; Nebbioso et al., 2018). Even though epigenetic enzymes are the bridge linking the environment with epigenetic inheritance through retained histones PTMs, an in-depth analysis of their expression patterns throughout the spermatogenic process is still lacking. Moreover, these enzymes provide

new targets for therapies for numerous diseases (Prachayasittikul et al., 2017) but the potential effect of these compounds on epigenetic inheritance is generally overlooked. To further elucidate the mechanisms driving the histone PTMs during spermatogenesis, a better understanding of the gene expression profiles of epigenetic modifying enzymes in male germ cells is required. Here, we curated transcriptomic data from spermatogonia to mature sperm populations and analyzed the expression profile and dynamic changes of all the known families of histone K acetylation and methylation writers and erasers. We provide a complete picture of the epigenetic enzymes across spermatogenesis in mice and the developmental windows when the transcription of these enzymes may be more susceptible to environmental disruption. Moreover, we analyzed pre-cleavage zygote translatome, confirming that mRNAs of several histone modifying enzymes carried by sperm are associated with ribosomes for protein synthesis in 1-cell embryo. Not only do we expand the knowledge on epigenetic enzymes with recognized roles in spermatogenesis, but provide evidence of many more whose roles in male germ cell development and zygote have not been described yet.

RESULTS

Epigenetic enzymes and histone K acetylation and methylation marks distribution during spermatogenic stages

Table 1 shows a curated list of writers and erasers families for histone K acetylation and methylation obtained from the Uniprot database, as proteins with confirmed catalytic activity towards histones K residues, and their most representative K targets reported in mammalian cells. Figure 1A shows the canonical histone methylation sites that are found on H3K4/9/27/36/79 and H4K20, with well-established roles in chromatin functions. For instance, H3K4me1/2/3, H3K36me3, H3K9me1, H3K27me1, H3K79me2/3, and H4K20me1 are found in enhancers, promoters, and gene bodies of active genes, whereas H3K9me2/3, H3K27me2/3 and H4K20me3 are found in—heterochromatin, telomeric regions and inactivated X chromosome (Fig. 1A). Open chromatin is characterized by the presence of acetylated histones at several K residues such as H2AK5/9/13/15ac, H2BK5/12/15/20/23/24ac, H3K4/9/14/18/27/36/56/79/122ac, and H4K5/8/12/16/20/79/91ac. Many of these sites are also targets of mutually exclusive methylation and characteristic of active or repressed chromatin. In addition, there are multiple non-canonical histone methylation sites on H2AZK7, H3K23/63 and H4K5/12 with unknown functions. In Figure 1B we summarized the available information on the most important histone K acetylation and methylation marks distribution during spermatogenic stages, obtained from reported immunohistochemical and/or proteomic studies. We observed two main profiles on the marks distribution across spermatogenesis: H3/4ac, H3K4me2/3 and H3K9me2/3 increase with spermatogonia differentiation and early meiosis, decrease at late meiosis, and regain high levels at spermiogenesis, whereas

H3K36me2/3, H3K27me2/3, H3K79me3 and H4K20me3 show sustained increased expression, but H4K20me is erased at RStid stage.

Histone K acetylation and methylation enzymes expression during spermatogenesis

To analyze the expression profiles of writers and erasers families for histone K acetylation and methylation across spermatogenesis, we curated RNA-seq data available in purified male germ cells from adult mice and selected the dataset GSE162740 (Mayorek et al., 2022). This dataset contains the mRNA profiles of 6 populations of germ cells isolated in triplicates: undifferentiated (SGund) and differentiated spermatogonia (SGdiff) (spermatogonial phase), primary spermatocytes, including pre-leptotene (PreL), leptotene/zygotene (LZ) and pachytene/diplotene (PD) stages (meiosis), and round spermatids (RStid) (spermiogenesis phase). We downloaded the FASTQ files and mapped them to MM10 genome using STAR and featureCounts to obtain the count matrix. We corroborated the cell stages and purity of the dataset by analyzing spermatogenic and somatic cell marker genes profiles in each sample (Fig.S1 and Table S1). Figure 2 shows the hierarchical clustering of z-scores for the 99 epigenetic enzymes listed in Table 1. Column clustering shows two main profiles between the spermatogonial and early meiotic vs late meiotic and spermiogenic phases. SGdiff and PreL stages are the most similar, whereas SGund and LZ show large clusters of enzyme upregulation. The late meiotic and spermiogenesis genes show two main clusters: genes specifically upregulated at PD and genes that increase at PD and remain high or even increase expression at RStid stage.

Epigenetic enzymes upregulation across spermatogenesis

To analyze upregulated mRNAs of epigenetic enzymes across male germ cells differentiation, we performed differential gene expression (DEG) analysis using the DESeq2 algorithm and compared consecutive stages of cell development: SGdiff vs SGund, PreL vs SGdiff, LZ vs PreL, PD vs LZ and RStid vs PD. For each analysis, we selected genes with padj < 0.05 and log2FC > 0.5. Figure 3A shows the number of upregulated enzymes detected for each comparison, while Figure 3B shows enzymes that were not found significantly upregulated at any cell stage. These enzymes show a pattern of high expression at the spermatogonial phase that decreases with cell differentiation. To evaluate until which stage these enzymes maintain high expression, we analyzed the downregulated genes at each comparison (padj < 0.05, log2FC < -0.5). The statistical analysis showed that i) *Kat6a*, *Kat6b*, *Mcm3ap* and *Kmt2b* significantly decreased at PreL vs SGdiff, ii) *Hdac2*, *Smyd2* and *Kdm8* significantly decreased at LZ vs PreL, iii) *Naa60*, *Ash1l*, *Kmt2a*, *Kmt5b* and *Kdm3b* significantly decreased at PD vs LZ, and iv) *Setd2* significantly decreased at RStid vs PD.

Expression profile of histone K acetylation and methylation enzymes during spermatogenesis phases

For the analysis of the increased epigenetic enzymes profiles during spermatogonial, meiosis prophase I and spermiogenesis phases, we constructed heatmaps for the upregulated enzymes at each developmental cell stage, and dot plots indicating the mRNA abundance, the writer/eraser nature and target histone mark. During the spermatogonial phase, the SGund population is characterized by a mitotic activity to maintain testicular homeostasis through self-renewal. Some of these SGund initiate extensive chromatin reorganization and their epigenetic landscape shifts dramatically to differentiate to

committed SGdiff (Shirakawa et al., 2013). Figure 4 shows the differential expression of histone K acetylation and methylation writers and erasers in SGund and SGdiff. Several histone acetylation writers decrease with the transition of SGund to SGdiff, including *Kat2a*, *Kat2b*, *Supt7l* and *Ep300*. The SGund also showed high expression of class I *Hdac8*, class II *Hdac4/7/9* and class IV *Hdac11*, whereas SGdiff upregulated class I *Hdac1/3* and class III *Sirt1*. We also found specific expression profiles of enzymes involved in heterochromatin formation, including increased KDM3/4 family of H3K9me erasers at SGund, and increased KMT1 H3K9me and KMT5 H4K20me writers families at SGdiff. Interestingly, the SGund showed high expression of the polycomb H3K27me writer subunit *Ezh1*, and the transition to SGdiff upregulated the H3K27me eraser *Kdm6b*. The transition to SGdiff also decreased the levels of several writers and erasers for H3K4me and H3K36me but specifically upregulated *Prdm9* and *Nsd2* writers. Finally, Figure 2 shows high expression of *Ehmt2* and *Kdm1a* at the spermatogonial phase, coincident with the high protein expression described for these enzymes at this stage.

Meiotic entry occurs concomitantly with the pre-meiotic S phase and is followed by the meiotic prophase that initiates with PreL stage. Here, we found some enzymes that continue to increase expression from SGdiff to LZ stage, including *Prdm9*, *Setdb2* and *Kmt5a/c*, and enzymes that seem to be specific to each meiotic stage, like *Setd6* at PreL stage, that deposits H2AZK7me1 mark, and *N6amt1* at LZ stage, that deposits H4K12me mark (Fig. 5). Histone acetylation writers increased from PreL to PD stage, with upregulated expression of almost all members of GNATs, NATs, SRC, MYST, and p300/CBP families, and other HATs like *Supt7l*, *Msl3*, *Clock*, *Gtf3c4* and *Brca2*, showing most of them high RPKM values. During meiotic prophase I, several class I, II and III HDACs were increased, including *Hdac1/8*, *Hdac4/5/9* and *Sirt2/3/5/6/7*. Additionally,

note that the major enzymatic regulation of active H3K4me and H3K36me marks occurs during chromosomal synapsis at LZ stage, allowing the crossing over of homologous chromosomes at hotspots and then at PD stage. Moreover, H3K36me writers *Nsd1/2/3*, *Setd5* and *Prdm9* peak expression from PreL to LZ stage, while PD stage does not show peak expression of neither H3K36me writers nor erasers. Also, prophase I stages showed increased expression of KDM5 family and *Kdm1a*, consistent with the decreased H3K4me2/3 global expression reported at this stage (Fig. 1B). We also detected increased expression and high RPKM values of H3K27me writers and erasers at PreL and LZ stages, including polycomb writers *Ezh1/2*, and *Kdm6a*, which form the COMPASS complex with *Kmt2b/c/d* and *Ashl2* H3K4me writers increased at these stages. Meiotic prophase I also showed upregulation of KDM7 family, *Kdm7a* and *Phf2/8*, that mediate gene activation programs by removal of all histone repressive marks. In addition, there is considerable enzymatic regulation on H3K9me mark, evidenced in the number and peak expression of H3K9me writers and erasers during all stages of prophase I, including KDM3/4 families that would mediate the decreased global H3K9me3 levels detected at this stage (Fig. 1B).

Spermiogenesis is the final phase of spermatogenesis, where the haploid RStids engage in chromatin remodeling programs involving specific histone variants incorporation, H3/4 hyperacetylation and subsequent replacement of 90-99% histones by protamines (PRMs) to compact the nucleus. Figures 6 shows that the RStid stage presents expression of histone K acetylation writers and erasers that continued to increase expression from PD stage, including *Kdm4a*, *Msl3*, *Ehmt2*, *Rsbn1* and *Dot1l*, and others that seemed to reactivate expression from previous spermatogenic and early meiotic stages. We found several HATs specifically upregulated at RStid, and also *Kat8* that maintained high levels from PD stage (Fig. 2) consistent with the histone hyperacetylation that takes

place at this stage (Fig. 1B). Also, RStid stage showed high RPKM values for the H3K79me writer *Dot1l*, the H3K27me writer *Ezh2* and the H3K9me2 writer *Ehmt2*, consistent with the high global expression of these marks at spermiogenesis stage (Fig. 1B). Additionally, RStid population showed peak expression of *Rsbn1*, a known specific eraser involved in decreasing H4K20me levels at spermiogenesis (Fig. 1B).

Epigenetic enzymes detected at mature sperm and 1 cell embryo translatome

To characterize the epigenetic enzymes mRNAs present in the mature sperm, we analyzed data from three datasets that performed long non-coding and mRNA RNAseq: GSE81216 (total and head sperm, Schuster et al., 2016), GSE88732 (Zhang et al., 2017), and E-MTAB-5834 (Gapp et al., 2020). For each study, we obtained the mean RPKM for each gene and converted it to percent rank to transform values into a 0-1 scale, where 0 means no detection and 1 is the highest detected gene in each study (see Supplemental Table1). Then, we calculated the mean percent rank for the three studies. Figure 7A shows a positive correlation between sperm and RStid mean percent rank, which favors the idea that the sperm enzymes mRNA comes from the spermiogenesis phase. In addition, with the aim of elucidating if the epigenetic enzymes detected in mature sperm may participate in embryo chromatin assembly, we analyzed recently reported data that profiled the mRNA translation landscape in mouse pre-implantation embryos by Ribo-seq (Zhang et al., 2022). We analyzed the translation efficiency (TE), obtained as mean RPKM detected at ribosomes/mean RPKM detected at whole RNAseq (RPKM>0.5) (see Supplemental Table S3). Figure 7B shows a word cloud plot where word size indicates enzyme abundance (mean percent rank) in mature sperm, and red color indicates positive translation detected at 1 cell embryo. Figure 7C shows that several writers and erasers of all histone acetylation

and methylation marks are detected in the translatome of the 1-cell embryo, where many of these mRNAs are highly detected in the sperm RNA pool.

DISCUSSION

Accumulating evidence has put on focus the existence of windows of vulnerability during spermatogenesis, at which environmental stressors can induce epigenetic reprogramming and the transmission of developmental, metabolic, and behavioral traits to offspring. To further elucidate the mechanisms driving histone PTMs changes during the spermatogenic process, we analyzed the epigenetic enzyme expression programs across germ cell differentiation to mature sperm. We show that cell transition across spermatogenesis is characterized by the upregulation of specific histone K methylation and acetylation writers and erasers driving the epigenome changes necessary for spermatogonia differentiation, meiosis entry and spermiogenesis. Moreover, most of these enzymes are detected in the mature sperm mRNA pool, and some are also detected in the early zygote translatome, suggesting they can be delivered and participate in the 1-cell embryo chromatin assembly. Our study shows important mechanistic aspects behind transgenerational epigenetics, where epigenetic enzymes not only can respond to environmental stressors and alter the germ cell epigenome but could function as vectors of epigenetic information transmission themselves, by participating in chromatin organization at the maternal to zygote transition.

The spermatogonia stages seem to be vulnerable for stress-induced epigenetic reprogramming since they reside outside the blood-testis barrier (González et al., 2020). Furthermore, current data support a dynamic stem cell model in which the fate of SGund population is context-dependent and plastic, and several epigenetic mechanisms are

implicated in the regulation of spermatogonia maintenance and cell fate (Mäkelä et al. 2019). Here, we found that the transition to the SGdiff population is characterized by increased expression of writers of activation marks H3K4me and H3K36me and repression marks H3K9me and H4K20me. At the spermatogonial phase, we found high expression of H3K4me epigenetic enzymes, including *Kmt2a/b/c* writers and *Kdm1a/b* erasers. In this context, at spermatogonial stage occurs the deposition of monovalent and bivalent H3K4me marking at promoters by *Kmt2b* (*Mll2*), preparing germ cells for gene activation at late spermatogenesis and embryonic development (Tomizawa et al., 2018). Accumulating evidence also points to H3K4me2 in the maintenance of transcriptional states during cell development and, its removal by erasers expressed at spermatogonia such as KDM1A/LSD1 is a key step for epigenetic reprogramming and cell fate (Martinez-Gamero et al., 2021). KDM1A/LSD1 was found to mediate spermatogonia commitment and differentiation, as testicular deletion of *Kdm1a* leads to progressive germ cell loss, downregulation of self-renewal factors and abnormal accumulation of meiotic spermatocytes (Lambrot et al., 2015; Myrick et al., 2017). Interestingly, overexpression of *Kdm1a* altered the H3K4me2/3 levels and the RNA profile of sperm and embryos transgenerationally (Siklenka et al., 2015; Lismer et al., 2020). Hence, the spermatogonial phase seems to be especially sensitive to alterations in H3K4me balance linked to epigenetic inheritance. SGdiff showed a specific increase of *Prdm9*, responsible for H3K4me3 and H3K36me3 that mediate the double-strand breaks (DSBs) formation at meiotic hot spots (Sun et al., 2020; Getun et al., 2017). In line with this, it has been shown that meiosis entry of SGdiff is driven by increased levels of H3K36me3 (Godmann et al., 2007; Song et al., 2011; Zuo et al., 2018). Here, we found *Setd2* expression until PD stage, and *Setd5* specifically upregulated at SGund, suggesting their involvement in the specific

H3K36me3 landscape of spermatogonia differentiation. The SGund population also expressed H3K4me/H3K36me eraser *Kdm2b*, a subunit of non-canonical PRC1.1 complex that was found to protect the polycomb-silenced promoters against ectopic de novo H3K36me methylation (Blackledge and Klose, 2021). Furthermore, KDM2B was recently found to erase H3K79me2/3 and to induce transcriptional repression via SIRT1 chromatin silencing (Kang et al., 2018).

It is established that SGund typically lacks heterochromatin, which forms as they differentiate to the spermatogonia population committed to meiosis (Chiarini-Garcia et al., 2002). The SGund showed increased expression of H3K9me erasers *Kdm3a/b* and *Jmjd1c* (*Kdm3c*), that were found to counteract the enzymatic activity of EHMT2 (Tachibana et al., 2007), which is highly expressed in the spermatogonia population. Methylation of H3K9 by EHMT2 blocks gene expression of the stem cell factors OCT4 and NANOG, therefore, demethylation of H3K9 by KDM3 family enzymes may be a key step in the maintenance of self-renewal of spermatogonial stem cells (Kuroki et al., 2020; Chioccarelli et al., 2020). In agreement, we found increased *Ehmt1*, *Suv39h2* and *Setdb2* expression at SGdiff stage, consistent with H3K9me2/3 deposition during spermatogonia differentiation. In particular, EHMT1 was found in a complex that binds E2F- and Myc-Responsive genes to repress the mitotic program (Ogawa et al., 2002). Moreover, SGdiff population showed decreased *Ezh1* and increased *Kdm6b* compared to SGund, suggesting that spermatogonia differentiation involves H3K27me removal. We also observed that the transition to the SGdiff population is characterized by specific HDACs upregulation. In this sense, it was shown that class IIa HDACs enzymatic activity depends on their recruitment into a complex containing class I HDAC3 and retinoid acid (RA) receptor (RAR), suggesting that these HDACs could mediate chromatin silencing induced by RA, essential for the transition

of SGund into SGdiff (Parra, 2015). Moreover, SGdiff population showed increased *Sirt1*, described as a key regulator of spermatogonia differentiation since SIRT1-deficient mice show a delay of pre-meiotic differentiation, aberrant expression of spermatogenic genes, abnormal spermatozoa with elevated DNA damage, and reduced fertility (Coussens et al., 2008; Bell et al., 2014). Among histone acetylation writers, *Kat2b* expressed in SGund functions as a co-activator for RAR and can interact with NCOA1 to promote transcription (Spencer et al., 1997), which is upregulated from SGdiff to LZ stage. Also, we observed peak expression of *Kat2a* in SGund, its germ-cell-specific knockout results in abnormal chromatin dynamics, leading to increased sperm histone retention and severe reproductive phenotype (Luense et al., 2019). Therefore, the action of certain epigenetic enzymes in pre-meiotic stages seems to enable the post-meiotic transcriptome and chromatin remodeling processes.

Initiation of male meiosis is one of the most important events that coincide with spermatocyte differentiation, and meiotic prophase is accompanied by several alterations of epigenetic and gene expression programs for post-meiotic spermiogenesis (Maezawa et al., 2020). Histone acetylation writers increase during prophase I progression, and H3K9/18/23ac and H4K5/8/12/16/91ac marks have been associated with open chromatin and hot spot cores (Getun et al., 2017; Chioccarelli et al., 2020). Accordingly, MYST enzymes were previously found strongly related to meiosis control, increasing their expression from early pachytene through diplotene stages (Getun et al., 2017). Interestingly, p300/CBP family and KAT2B were found to regulate histone eviction by acetylation of transition nuclear protein (TNP) 2, affecting DNA condensation properties and interaction with histone chaperones (Pradeepa et al., 2009). Among other acetylation writers upregulated during prophase I, MSL3 binds to H3K36me3 marked sites and

deposits H4K16ac, controlling meiosis entry and STRA8 (stimulated by retinoic acid 8) functions (McCarthy et al., 2022). On the other hand, we detected several SIRT enzymes throughout prophase I. Deacetylation of H3K9 by SIRT6 modulates telomeric chromatin function (Michishita et al., 2008) while SIRT7 is highly selective toward H3K18ac, and might play an upstream role in DNA repair and telomere maintenance (Wu et al., 2018). We also detected peak expression of *Hdac1* at the PD stage that was shown to form a complex with DNA methyltransferase DNMT3L to regulate X chromosome compaction (Deplus et al., 2002; Zamudio et al., 2011).

During prophase I, recombination hotspots are mainly marked by H3K4me3 catalyzed by KMT2 family and PRDM9 methyltransferases (Sollier et al., 2004; Buard et al., 2009). Moreover, the levels of H3K4me3 and H3K36me3 are highly correlated at hotspots, but mutually exclusive elsewhere, and PRDM9 is capable of placing both marks on the same nucleosomes in vivo (Powers et al., 2016). It was shown that *Prdm9* knockout changes the distribution of DSBs across the genome inducing defective synapses and male infertility (Paigen and Petkov, 2018; Bhattacharyya et al., 2019). We also detected peak expression of H3K79me writer *Dot1L* at PD stage, consistent with previous reports showing increased levels of DOT1L, and H3K79me2/3 from pachytene onwards (Ontoso et al., 2014). The heterochromatic centromeric regions and the sex body are enriched for H3K79me3, while H3K79me2 is present all over the chromatin, but is largely excluded from the sex body despite the accumulation of DOT1L (Ontoso et al., 2014). Repressive histone methylation marks H3K27me3, H3K9me3 and H4K20me3 are present in chromatin regions with reduced recombination or involved in heterochromatin formation. *In vitro* and *in vivo* models disrupting the action of H3K9me writers *Suv39h1, Setdb1/2 and Ehmt2* detected during prophase I, showed an abnormal distribution of H3K9me3 at pericentromeric

heterochromatin, compromised meiotic silencing of unsynapsed chromatin (MSUC), misregulation of meiotic and somatic genes, anomalous synapsis and misssegregation of chromosomes and apoptosis at pachytene stage (Peters et al., 2001;, Tachibana et al., 2007; Takada et al., 2011). Interestingly, low protein diet in the father was linked to altered levels of H3K9me2 through EHMT2, that changed tRNAs in sperm and transmitted metabolic phenotypes to the offspring (Yoshida et al., 2020). Also, SETDB1 haploinsufficiency in mice was shown to induce changes in DNA methylation in transposable elements, and to influence coat color in the offspring, further linking epigenetic inheritance with altered epigenetic enzymes levels during spermatogenesis (Daxinger et al., 2016). A recent work by Barral et al., 2022 reported dual regions in mouse embryonic stem cells that rely on the SETDB1 and NSD proteins to generate H3K9me3 and H3K36me3, respectively. They found that SETDB1 removal induces loss of both marks in dual regions, gains signatures of active enhancers, and comes into contact with upregulated genes, providing a mechanistic insight by which genes are controlled by heterochromatin (Barral et al., 2022). Moreover, NSD1-mediated H3K36me2 was found to prevent H3K27me3 deposition by PRC2, modulating PRC2-mediated H3K27me domains demarcation (Blackledge and Klose, 2021). In line with this, we detected the expression of H3K27me writers *Ezh1/2* at PreL and LZ stages that were found involved in mammalian X chromosome inactivation (Schuettengruber et al., 2007). Furthermore, *Kdm6a* is part of the COMPASS complex with *Kmt2b/c/d* and *Ashl2* that activate genes in response to RA by H3K4me deposition and H3K27me removal (Lavery et al., 2020). On the contrary, the loss of H3K27me3 from bivalent signature in the promoter of endonuclease SPO11 induces chromatin activation that favors meiotic entry (Hammoud et al., 2014). At PreL stage, we detected greater expression of H3K27me erasers than writers, including the expression of *Kdm6a*, which

alteration in its expression induce defects that persisted transgenerationally (Siklenka et al., 2015, Lesch et al., 2019). We also detected high expression of H2AZK7me1 writer *Setd6* at PreL stage, which in ESC was found with H3K27me3 close to differentiation marker genes and removed upon RA signal (Binda et al., 2013), suggesting that SETD6 may have a role at early male meiosis. Finally, we observed expression of the H4K12me writer *N6amt1* at LZ stage, a recently described epigenetic mark found at promoters of genes encoding cell cycle regulators (Metzger et al., 2019).

Chromatin organization dramatically changes during mid- to late-spermiogenesis, due to histone eviction and replacing by PRMs to facilitate the condensation and packaging of the paternal genome. In mice, 1%-10% of histones are retained in sperm chromatin and form a heterogeneous mixture of nucleo-histones and nucleo-protamines (Rajender et al., 2011; Luense et al., 2016). At the spermiogenesis phase, histone acetylation is essential in destabilization and remodeling of nucleosomes. Here, the RStid population showed high transcriptional levels of H4K16ac writers *Msl3* and *Kat8*, a histone PTM essential for histone eviction which does not occur in the absence of this specific mark (Lu et al., 2010). Also, *p300/CBP* and *Kat2b* keep their expression from spermatocyte stage, due to their crucial role to regulate histone eviction (Pradeepa et al., 2009). Moreover, KAT2B was found in spermatids and involved in H3K9ac, an epigenetic mark detected at unmethylated active genes / enhancers (Hammoud et al., 2014) that could influence gene expression directly after fertilization (Steilmann et al., 2011). Additionally, *Hat1* expression was found related to the incorporation of H4K5/12ac and H3.3 variant at DSBs sites and the promotion of DNA repair (Yang et al., 2013). H4K5/8/12 marks have been also observed just before eviction of histones during spermiogenesis (Meistrich et al, 1992; Hecht et al., 2009) and moreover, H4K8/12ac was detected prior to full decondensation of the sperm

nucleus, suggesting that these marks are transmitted to the zygote (van der Heijden et al., 2006).

Multiple histone methylation have been also identified in spermatids, pointing to a balance of “opened” and “closed” chromatin regions during the histone-to-protamine transition (Wang et al., 2019). Here, we detected upregulated expression of H3K79me writer *Dotl1* and H3K9me writer *Ehtm2* at RStid stage. It was found that H3K79me writer DOTL1 is enriched in post-meiotic stages of mouse germ cells and precedes the histone-to-protamine transition (Dottermusch-Heidel et al., 2014). During spermiogenesis, there is a reactivation of transcription from MSUC and MSCI sites that is enabled by the deposition of histone crotonylation (Kcr). It was proposed that CDYL, an HKcr eraser, prevents post-meiotic chromatin reactivation by binding to H3K9me3 and H3K27me2/3 marked sites and facilitating H3K9me2 deposition by EHMT2 (Mulligan et al., 2008). Moreover, there is specific H3K27me deposition to establish bivalency at developmental genes (Maezawa et al., 2018), and proteomic studies found substantial H3K27me3/H3K36me2 double marking in RStid and sperm (Luense et al., 2016).

It is established that mature sperm transport a cargo of miRNAs, tsRNAs, lncRNAs, circRNAs, and protein-coding mRNAs, that carry an epigenetic blueprint involved in early embryo development (Guo et al., 2017). Some of these RNAs remain from the last stages of elongated spermatids, and others are acquired along the passage through the epididymis, as sperm absorb epididymosomes released by somatic cells (Trigg et al., 2019). Here, we found that the mature sperm transport a signature of epigenetic enzymes mRNAs similar to the one detected at spermiogenesis phase, and most of these enzymes show positive translation at 1 cell embryo. After fertilization, the zygote genome is transcriptionally silent, and cellular processes are carried on with inherited RNAs and proteins until the

onset of zygotic genome activation (ZGA) around the 2-4 cells stage (Schulz and Harrison, 2019). Also, at 1 cell stage there is significant removal of some maternal RNAs and specific translation of others, including RNAs specifically delivered by the sperm like the egg-activating factor PLC-zeta (Sone et al., 2005; Yao et al., 2010). The parental pronuclei have asymmetric reprogramming capacities and the reprogramming factors reside predominantly in the male pronucleus. Here, we detected high sperm values and embryo translation of several enzymes involved in histone acetylation and methylation, including the H4K20me2/3 writer *Kmt5c*, the H3K9me2 writers *Ehmt1/2*, and the H3K9me3 writers *Suv39h2* and *Setdb1/2*, with established roles in chromatin structure organization. In this context, it was found that EHMT2 activates soon after fertilization and deposit H3K9me2 patterns in the paternal genome (Ma et al., 2015). Also, previous work suggested that KMT5C deposits H4K20me3 to allow the timely and coordinated progression of replication after fertilization (Eid et al., 2016).

In summary, the analysis performed here shows important windows during spermatogenesis, where interference with epigenetic enzymes gene expression may have phenotypic consequences in the offspring, even when they are not inherited. Moreover, we show that epigenetic enzymes mRNA could become functional in pre-cleavage zygotes and contribute to early chromatin organization with deep implications in future embryo development. The epigenetic enzyme's paternal contribution could be another mechanism for epigenetic inheritance that deserves further consideration.

METHODS

Data retrieval

FASTQ files were downloaded from NCBI GEO and ArrayExpress databases. Germ cells data transcriptomic was obtained from GSE162740, that used crossed Stra8-cre mice with CAG-LSLtdTomato mice to generate "Stra8-Tom" mice. The gradient expression of the transgene under Stra8 expression profile, combined with immunostaining for cKit to further discriminate the SGund and SGdiff spermatogonia, was used to FACS sort 6 populations of germ cells in triplicates, and sequence in an Illumina HiSeq 2500 platform. Sperm cells data were obtained from: i) GSE81216, that sampled 2 total sperm and 2 sperm heads from C57BL/6J (JAX) mice using Ion Torrent PGM platform, ii) GSE, that sampled 4 mature sperm from adult C57BL/6J mice using Illumina HiSeq 4000 platform, and iii) E-MTAB-5834, that sampled 4 control mature sperm from adult C57BL/6J mice using Illumina HiSeq 2500 platform. One cell embryo, 2 total and 2 ribosome-bound RNA RPKMs were obtained from GSE169632.

Data processing

FASTQ files were processed with the following pipeline: quality control with FASTQC, trimming with fastp, mapping with STAR and GRCm39 (MM10 gencode.vM29.annotation), and counting with featureCounts. Processed data was manipulated using Rstudio (v1.2.1033) and Tidyverse packages Dplyr and ggplot2 (Wickham et al., 2019). Counts were converted to RPKM using mean gene length extracted from the gencode.vM29 file. The count matrix was converted to z score by gene and performed hierarchical clustering with heatmap package from R base. The sperm RPKMs obtained for the three selected datasets were converted to percentile rank, and the sperm mean percentile rank calculated. Word cloud plot was performed with ggwordcloud package. The embryo traslational eficiency (TE) was calculated as Ribo-seq RPKM / total

RPKM for each gene, with RPKM > 0.5. All the plots shown in this study can be reproduced by downloading the counts tables, enzymes metadata table and R scripts available at https://github.com/Gonzalez-Lab/Gonzalez-2022-germ-cells.

Author contributions

B.G and C.R.G conceived the project, analyzed the data and wrote the manuscript. G.B, M.S.F and C.P.L contributed to data mining and analysis. A.D.V contributed to the critical reading and editing of the manuscript. All authors read and approved the submitted version.

Declaration of interests

The authors declare no competing interests.

Table 1. Writers and erasers families for histone K acetylation and methylation and the targets described.

Acetylation					
WRITERS			ERASERS		
FAMILY	NAME	TARGET	FAMILY	NAME	TARGET
GNAT	KAT2A - GCN5	H3ac	Class I	HDAC1	Hac
	KAT2B - PCAF	H3ac		HDAC2	Hac
	HAT1	H4ac		HDAC3	Hac
	ATF2	H4/2Bac		HDAC8	Hac
NAT	NAA40 - NAT11	H4ac	Class IIa	HDAC4	Hac
	NAA50 - NAT13	H4ac		HDAC5	Hac
	NAA60 - NAT15	H4K79/91ac		HDAC7	Hac
MYST	KAT5 - TIP60	H4/ H2AZac		HDAC9	Hac
	KAT8 - MYST1 - MOF	H4K16ac	Class III	SIRT1	H4K16ac
	KAT7 - MYST2 - HBO1	H3/4ac		SIRT2	H4K16ac
	KAT6A - MYST3 - MOZ	H3/4ac		SIRT3	H4K16ac
	KAT6B - MYST4 - MORF	H3ac		SIRT5	Hac
p300/CBP	KAT3A - CREBBP	H3ac		SIRT6	H3K9ac
	KAT3B - EP300	H3ac		SIRT7	H3K18ac
SRC	NCOA1	H3/4ac	Class IV	HDAC11	Hac
	NCOA3	H3ac			
Other	CLOCK	H3ac			
	MSL3	H4K16ac			
	GTF3C4	H3ac			
	BRCA2	H3ac			
	MCM3AP	H3ac			
	SUPT7L	H3ac			

Methylation					
WRITERS			ERASERS		
FAMILY	NAME	TARGET	FAMILY	NAME	TARGET
KMT1	KMT1A - SUV39H1	H3K9me3	KDM1	KDM1A - LSD1	H3K4me1/2
	KMT1B - SUV39H2	H3K9me3			H3K9me1/2
	KMT1C - EHMT2 - G9a	H3K9me1/2		KDM1B - LSD2	H3K4me1/2
	KMT1D - EHMT1 - GLP	H3K9me1/2	KDM2	KDM2A - JHDM1A	H3K36me2
	KMT1E - SETDB1	H3K9me3		KDM2B - JHDM1B	H3K4me3
	KMT1F - SETDB2	H3K9me3			H3K36me2
KMT2	KMT2A - MLL1	H3K4me1/2/3			H3K79me2/3
	KMT2B - MLL2	H3K4me1/2/3	KDM3	KDM3A - JMJD1A - JHDM2A	H3K9me1/2
	KMT2C - MLL3	H3K4me1		KDM3B - JMJD1B	H3K9me1/2
	KMT2D - MLL4	H3K4me1		KDM3C - JMJD1C	H3K9me1/2
	KMT2F - SETD1A	H3K4me1/2/3	KDM4	KDM4A - JMJD2A - JHDM3A	H3K9me2/3
	KMT2G - SETD1B	H3K4me1/2/3			H3K36me2/3
	KMT2H - ASH1L	H3K36me2		KDM4B - JMJD2B - JHDM3B	H3K9me3
KMT2 core	ASH2L	H3K4me1/2/3			H3K36me3
KMT3	KMT3A - SETD2	H3K36me3		KDM4C - JMJD2C - JHDM3C	H3K9me3
	KMT3B - NSD1	H3K36me2			H3K36me3
	KMT3C - SMYD2	H3K4me1/2/3		KDM4D - JMJD2D - JHDM3E	H3K9me2/3
		H3K36me2	KDM5	KDM5A - JARID1A	H3K4me2/3
	KMT3D - SMYD1	H3K4me1/2/3		KDM5B - JARID1B	H3K4me1/2/3
	KMT3E - SMYD3	H3K4me2/3		KDM5C - JARID1C	H3K4me2/3
	KMT3G - NSD2 - WHSC1	H3K36me2		KDM5D - JARID1D	H3K4me2/3
	KMT3F - NSD3 - WHSC1L1	H3K36me2	KDM6	KDM6A - UTX	H3K27me2/3
KMT4	DOT1L	H3K79me1/2/3		KDM6B - JMJD3	H3K27me2/3
KMT5	KMT5A - SETD8	H4K20me1	KDM7	KDM7A - JHDM1D	H3K9me2
	KMT5B - SUV420H1	H4K20me1/2/3			H3K27me2
	KMT5C - SUV420H2	H4K20me1/2/3			H3K9me1/2
KMT6	KMT6A - EZH2	H3K27me1/2/3		KDM7B - JHDM1F - PHF8	H3K27me2
	KMT6B - EZH1	H3K27me1/2/3			H4K20me1
KMT7	KMT7 - SETD7	H3K4me1		KDM7C- JHDM1E - PHF2	H3K9me2
KMT8	KMT8B - PRDM9	H3K4me1/2/3			H4K20me3
		H3K36me1/2/3	KDM8	KDM8 - JMJD5	H3K9me1
	KMT8A - PRDM2	H3K9me1			H3K36me1/2
	KMT8D - PRDM8	H3K9me2	KDM9	KDM9 - RSBN1	H4K20me2/3
	KMT8C - PRDM6	H4K20me			
KMT9	KMT9 - N6AMT1- HEMK2	H4K12me1			
SMYD	SMYD4	H3K4me1/2/3			
	SMYD5	H4K20me3			
SETD	SETD4	H3K4me1/2			
		H4K20me3			
	SETD5	H3K36me3			
	SETD6	H2AZK7me			
	SETMAR	H3K36me2			
PRDM	PRDM15	H3K9me1			

The HATs can be divided into six major families: 1) the GNATs (GCN5-related N-acetyltransferases), 2) the NATs (N-terminal acetylases, which also possess a GNAT domain), 3) the MYST, 4) the p300/CBP, 5) the SRC steroid receptors coactivators, and 6) other HATs (Wapenaar and Dekker, 2016). HATs actions are counteracted by the HDACs, which include zinc-dependent class I, class IIa, and class IV HDACs, and NAD-dependent class III Sirtuins (Wapenaar and Dekker, 2016). On the other hand, the KMTs present two domains with annotated lysine methyltransferase activity: 1) SET domains, that catalyze all the canonical methylation sites except for H3K79, and 2) 7βS domains (DOT1L and N6AMT1) (Mosammaparast and Shi, 2010). The KDMs can be divided according to their catalytic activity in: 1) amine oxidases (KDM1), and 2) Jumonji C demethylases (KDM2/3/4/5/6/7/8) (Mosammaparast and Shi, 2010).

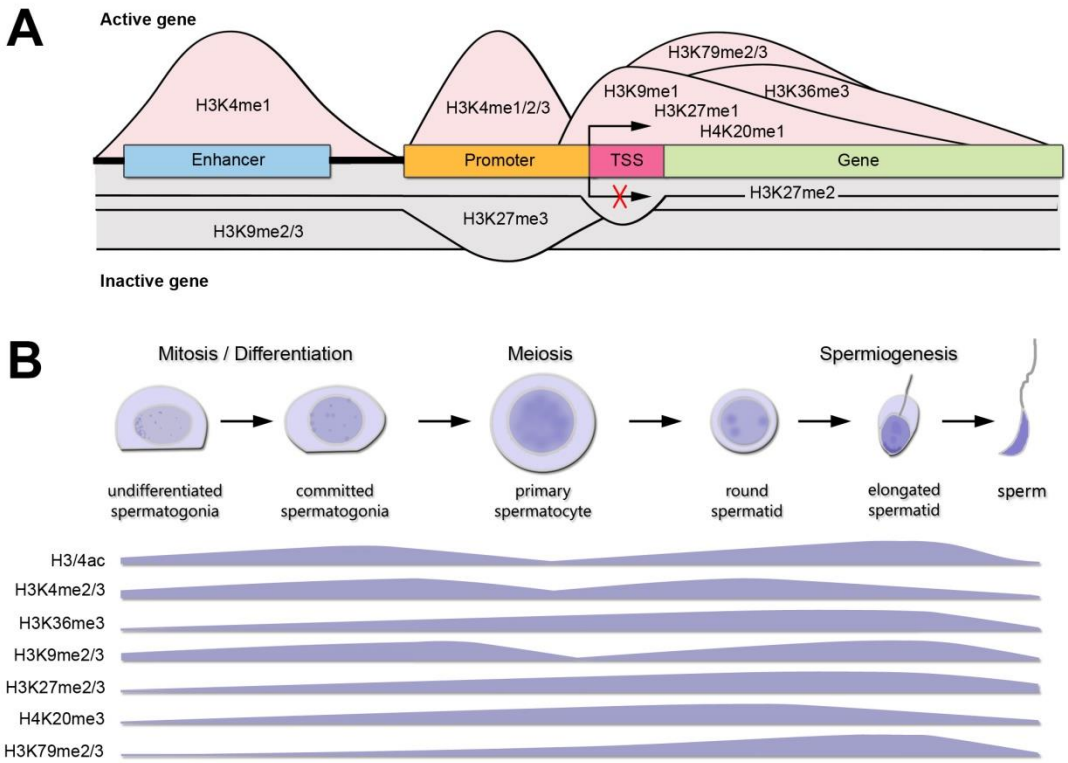
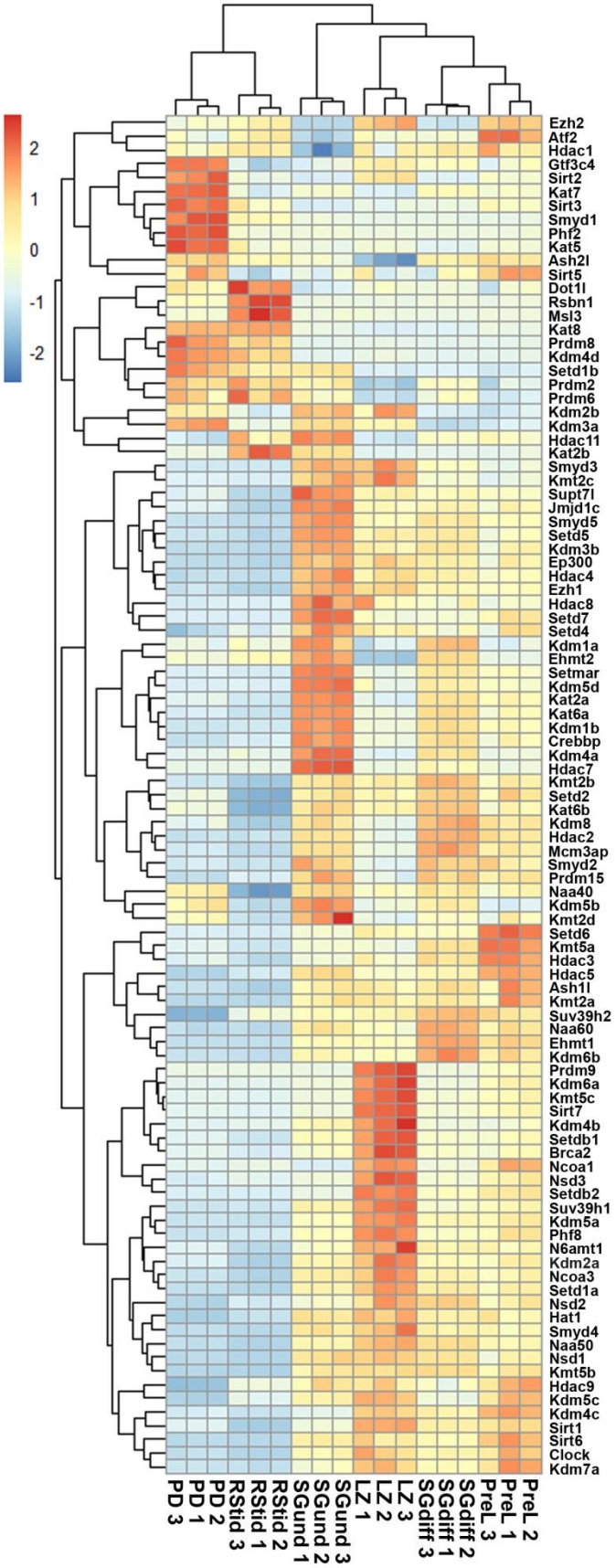


Figure 1. Canonical histone K marks and writers/erasers families distribution during spermatogenesis. A) Histone K methylation PTMs distribution at DNA elements and functional roles in gene expression for an active gene (pink shading) or inactive gene (gray shading). TSS: transcription start site. Only K modifications with sufficient and consensual information about distribution across genes are shown. B) Main histone K PTMs levels involved in chromatin remodeling during spermatogenesis. The data presented is summarized from the following references: Song et al., 2011; Godmann et al., 2007; Zuo et al., 2018; Luense et al., 2016; Wang et al., 2021). SGund: spermatogonia Thy1+/Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD: pachytene/diplotene spermatocytes, RStid: round spermatid.


2
1
0
-1
-2
PD 3
PD 1
PD 2
RStid 3
RStid 1
RStid 2
SGund 1
SGund 2
SGund 3
LZ 1
LZ 2
LZ 3
SGdiff 3
SGdiff 1
SGdiff 2
PreL 3
PreL 1
PreL 2
Ezh2
Atf2
Hdac1
Gtf3c4
Sirt2
Kat7
Sirt3
Smyd1
Phf2
Kat5
Ash2l
Sirt5
Dot1l
Rsbn1
Msl3
Kat8
Prdm8
Kdm4d
Setd1b
Prdm2
Prdm6
Kdm2b
Kdm3a
Hdac11
Kat2b
Smyd3
Kmt2c
Supt7l
Jmjd1c
Smyd5
Setd5
Kdm3b
Ep300
Hdac4
Ezh1
Hdac8
Setd7
Setd4
Kdm1a
Ehmt2
Setmar
Kdm5d
Kat2a
Kat6a
Kdm1b
Crebbp
Kdm4a
Hdac7
Kmt2b
Setd2
Kat6b
Kdm8
Hdac2
Mcm3ap
Smyd2
Prdm15
Naa40
Kdm5b
Kmt2d
Setd6
Kmt5a
Hdac3
Hdac5
Ash1l
Kmt2a
Suv39h2
Naa60
Ehmt1
Kdm6b
Prdm9
Kdm6a
Kmt5c
Sirt7
Kdm4b
Setdb1
Brca2
Ncoa1
Nsd3
Setdb2
Suv39h1
Kdm5a
Phf8
N6amt1
Kdm2a
Ncoa3
Setd1a
Nsd2
Hat1
Smyd4
Naa50
Nsd1
Kmt5b
Hdac9
Kdm5c
Kdm4c
Sirt1
Sirt6
Clock
Kdm7a

515

Figure 2. Unsupervised hierarchical clustering analysis of male germ cells transcriptome. Heatmap showing z-score values of histone K acetylation and methylation writer/eraser enzymes. SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD: pachytene/diplotene spermatocytes, RStid: round spermatid.

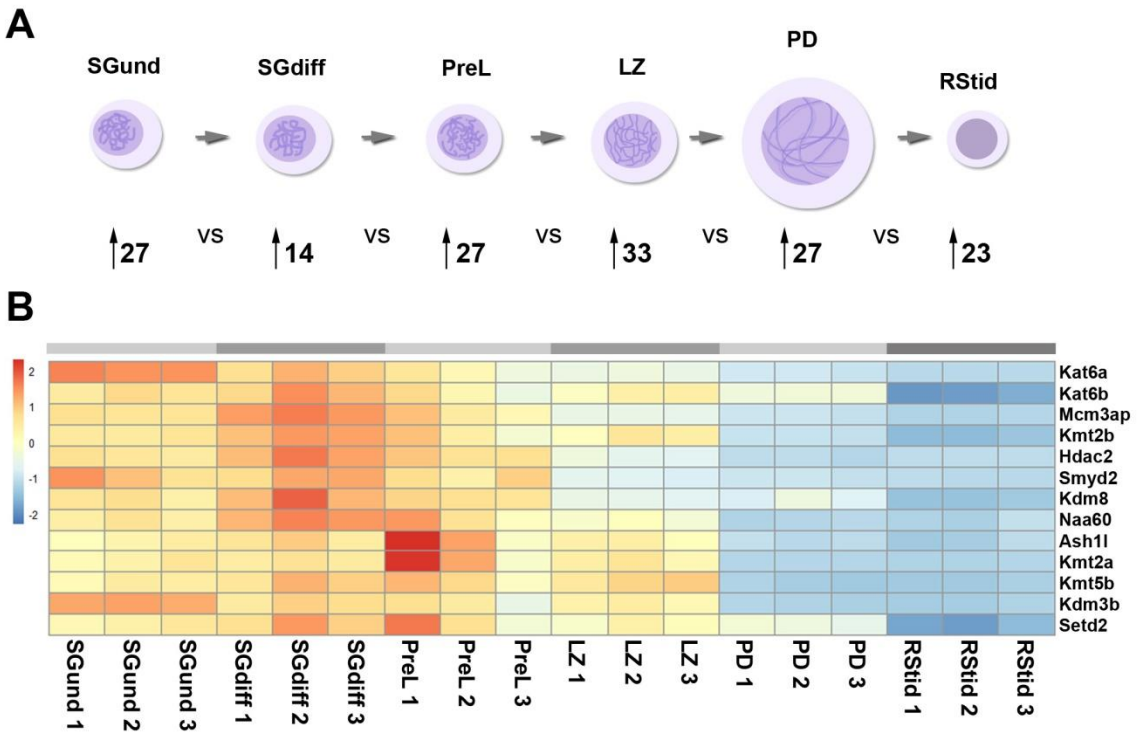


Figure 3. Histone K acetylation and methylation epigenetic enzymes expression during spermatogenesis A) Number of upregulated epigenetic enzymes for each germ cell stage.Differentiall gene expression analysis was conducted with DESeq2 on consecutive populations across differentiationn (SGdiff vs SGund, PreL vs SGdiff, LZ vs PreL, PD vs LZ and RStid vs PD), and upregulated genes selected with padj<0.05 and log2FC>0.5. B) Heatmap showing epigenetic enzymes not significantly upregulated at any germ cell stage across spermatogenesis. SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD:pachytenee/diplotene spermatocytes, RStid: round spermatid.

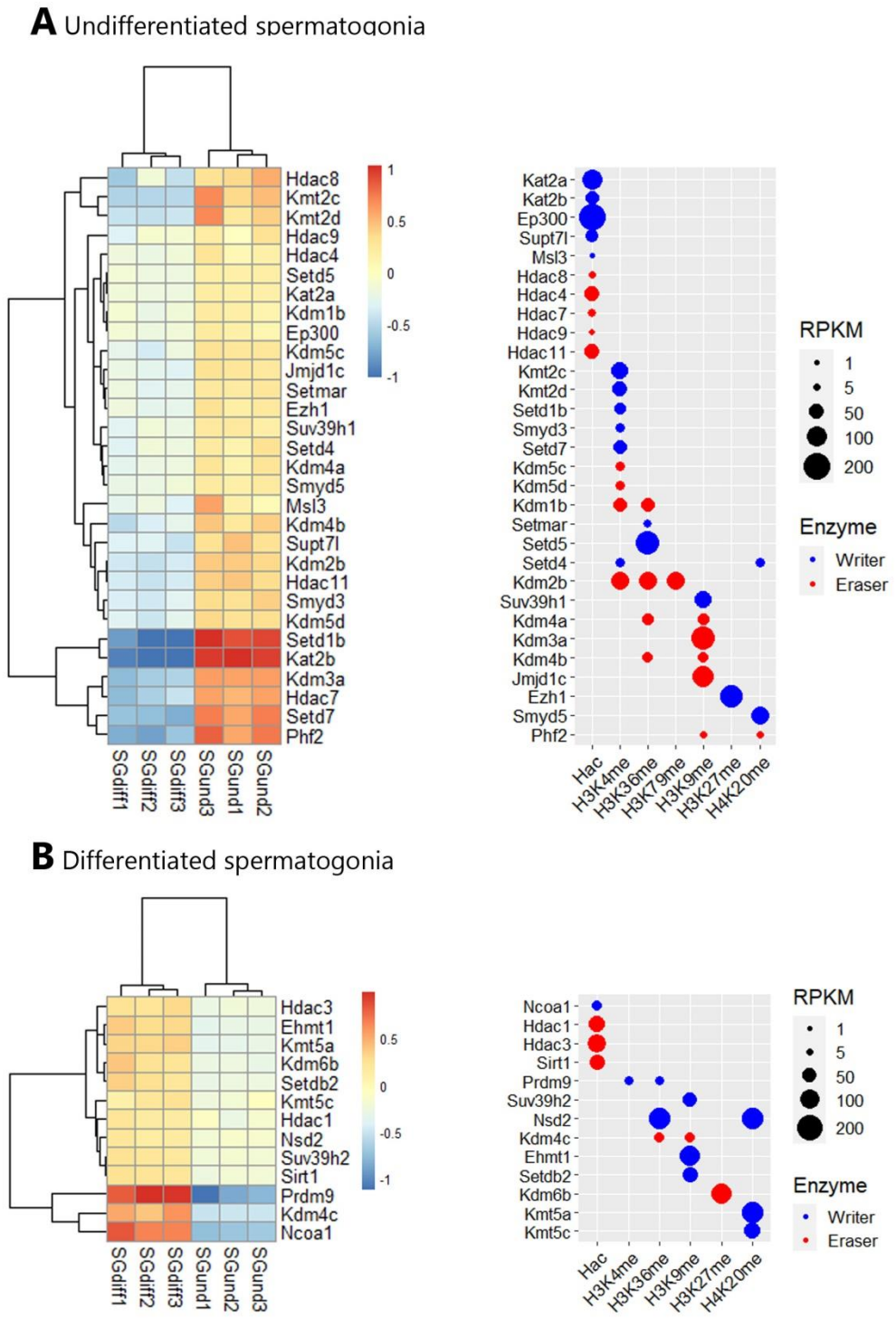


Figure 4. Epigenetic histone K writers and erasers upregulation at spermatogonial phase. The left panel indicates the heatmap showing z score values and the right panel indicates the Dot plot showing enzymes RPKM values in SGund (A) and SGdiff (B) population. Blue dots: writers, red dots: erasers. SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+.

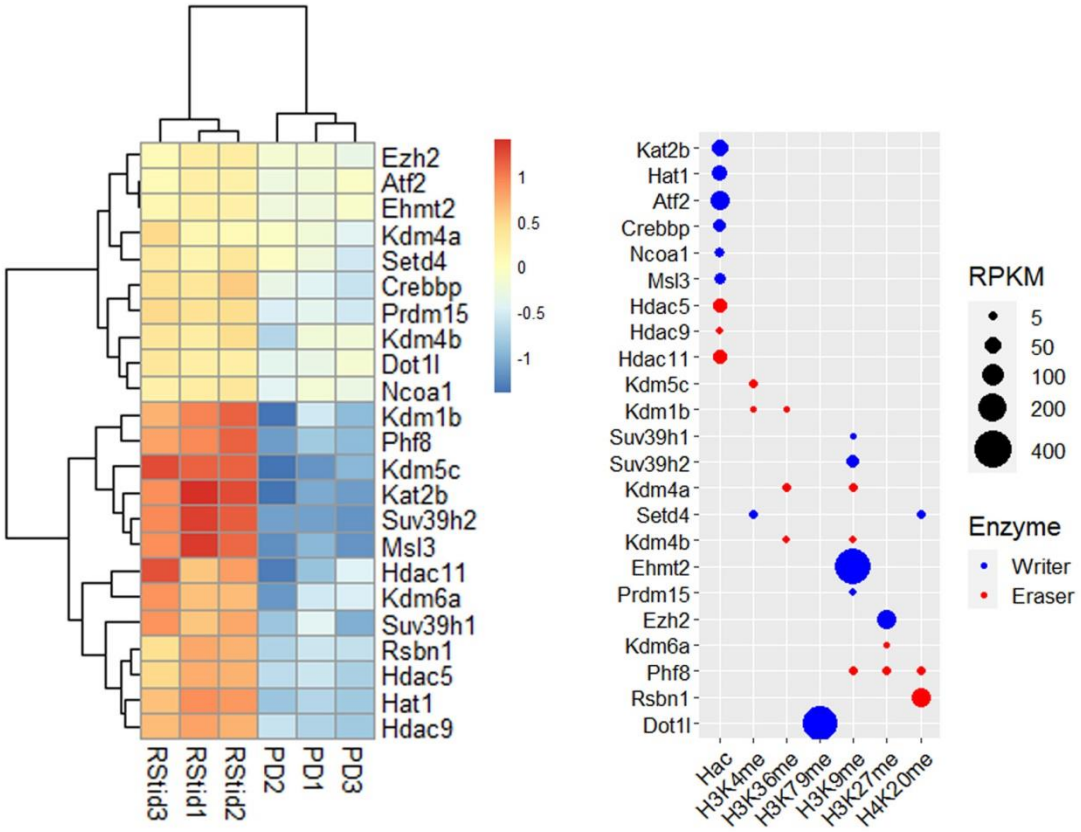


Figure 5. Epigenetic histone K writers and erasers upregulation at meiosis prophase I. The left panel indicates the heatmap showing z score values and the right panel indicates the Dot plot showing enzymes RPKM values, in PreL (A), LZ (B) and PD (C) spermatocytes. Blue dots: writers, red dots: erasers. PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD:pachytenee/diplotene spermatocytes.

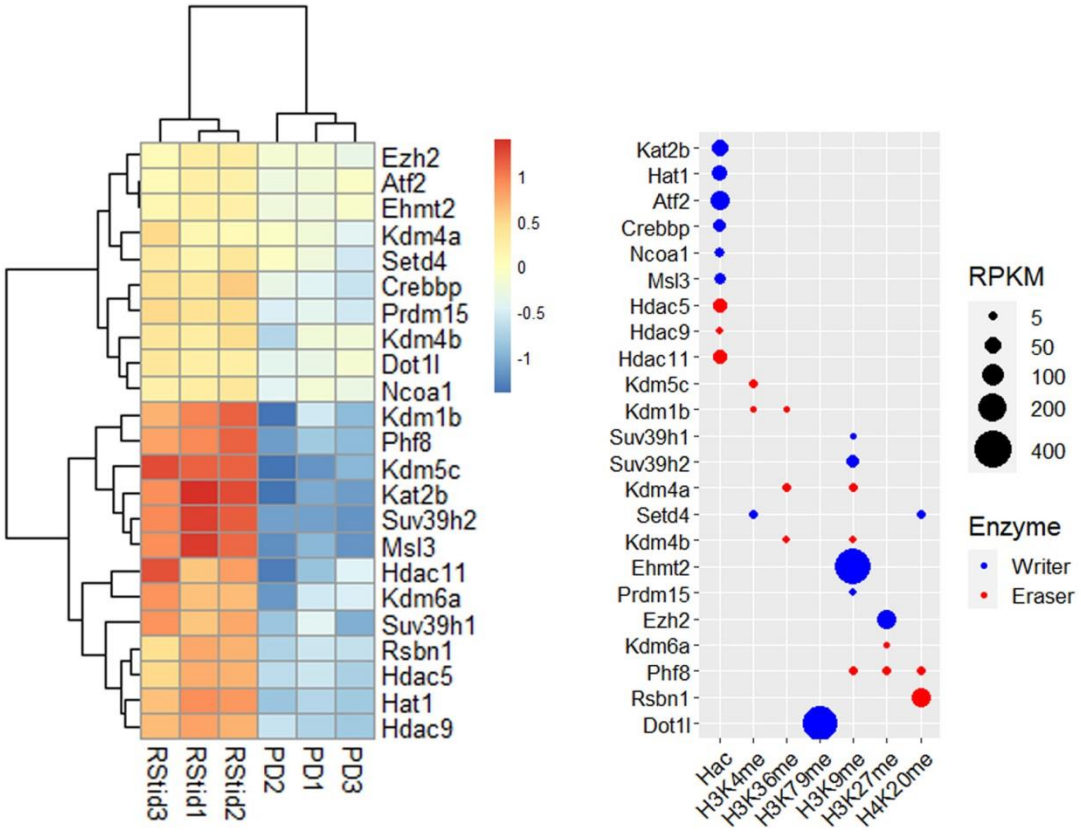


Figure 6. Epigenetic histone K writers and erasers upregulation at spermiogenesis. The left panel indicates the heatmap showing z score values and the right panel indicates the Dot plot showing enzymes RPKM values in RStid. Blue dots: writers, red dots: erasers. RStid: round spermatid, PD: pachytene/diplotene spermatocytes.

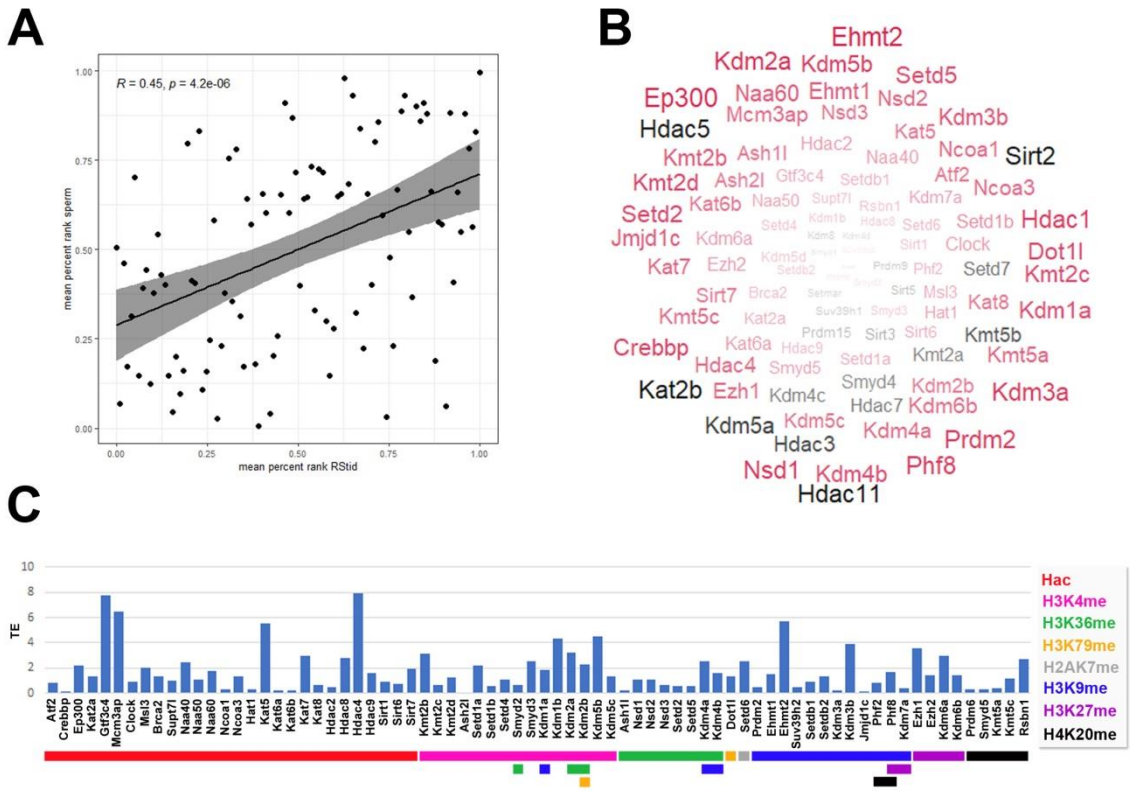


Figure 7. Epigenetic histone K writers and erasers detected at mature sperm RNA cargo and 1-cell embryo translatome. A) Pearson correlation between epigenetic enzymes mean percent rank detected at RStid and sperm. B) Mature sperm word cloud for epigenetic enzymes indicating the enzyme abundance (word size = mean percent rank) and translation efficiency (TE) detected at 1 cell embryo (red: TE > 0, black: TE=0). C) Epigenetic enzymes TE in mouse pre-implantation embryos (TE = RNA RPKM at ribosome / total RNA RPKM, RPKM>0.5)

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Table 1. Writers and erasers families for histone K acetylation and methylation and the targets described.

Acetylation						Methylation					
WRITERS			ERASERS			WRITERS			ERASERS		
FAMILY	NAME	TARGET	FAMILY	NAME	TARGET	FAMILY	NAME	TARGET	FAMILY	NAME	TARGET
GNAT	KAT2A - GCN5	H3ac	Class I	HDAC1	Hac	KMT1	KMT1A - SUV39H1	H3K9me3	KDM1	KDM1A - LSD1	H3K4me1/2
	KAT2B - PCAF	H3ac		HDAC2	Hac		KMT1B - SUV39H2	H3K9me3			H3K9me1/2
	HAT1	H4ac		HDAC3	Hac		KMT1C - EHMT2 - G9a	H3K9me1/2		KDM1B - LSD2	H3K4me1/2
	ATF2	H4/2Bac		HDAC8	Hac		KMT1D - EHMT1 - GLP	H3K9me1/2	KDM2	KDM2A - JHDM1A	H3K36me2
NAT	NAA40 - NAT11	H4ac	Class IIa	HDAC4	Hac		KMT1E - SETDB1	H3K9me3			H3K4me3
	NAA50 - NAT13	H4ac		HDAC5	Hac		KMT1F - SETDB2	H3K9me3		KDM2B - JHDM1B	H3K36me2
	NAA60 - NAT15	H4K79/91ac		HDAC7	Hac	KMT2	KMT2A - MLL1	H3K4me1/2/3			H3K79me2/3
MYST	KAT5 - TIP60	H4/ H2AZac		HDAC9	Hac		KMT2B - MLL2	H3K4me1/2/3	KDM3	KDM3A - JMJD1A - JHDM2A	H3K9me1/2
	KAT8 - MYST1 - MOF	H4K16ac	Class III	SIRT1	H4K16ac		KMT2C - MLL3	H3K4me1		KDM3B - JMJD1B	H3K9me1/2
	KAT7 - MYST2 - HBO1	H3/4ac		SIRT2	H4K16ac		KMT2D - MLL4	H3K4me1		KDM3C - JMJD1C	H3K9me1/2
	KAT6A - MYST3 - MOZ	H3/4ac		SIRT3	H4K16ac		KMT2F - SETD1A	H3K4me1/2/3	KDM4	KDM4A - JMJD2A - JHDM3A	H3K9me2/3
	KAT6B - MYST4 - MORF	H3ac		SIRT5	Hac		KMT2G - SETD1B	H3K4me1/2/3			H3K36me2/3
p300/CBP	KAT3A - CREBBP	H3ac		SIRT6	H3K9ac		KMT2H - ASH1L	H3K36me2		KDM4B - JMJD2B - JHDM3B	H3K9me3
	KAT3B - EP300	H3ac		SIRT7	H3K18ac	KMT2 core	ASH2L	H3K4me1/2/3			H3K36me3
SRC	NCOA1	H3/4ac	Class IV	HDAC11	Hac	KMT3	KMT3A - SETD2	H3K36me3		KDM4C - JMJD2C - JHDM3C	H3K9me3
	NCOA3	H3ac					KMT3B - NSD1	H3K36me2			H3K36me3
Other	CLOCK	H3ac					KMT3C - SMYD2	H3K4me1/2/3		KDM4D - JMJD2D - JHDM3E	H3K9me2/3
	MSL3	H4K16ac						H3K36me2	KDM5	KDM5A - JARID1A	H3K4me2/3
	GTF3C4	H3ac					KMT3D - SMYD1	H3K4me1/2/3		KDM5B - JARID1B	H3K4me1/2/3
	BRCA2	H3ac					KMT3E - SMYD3	H3K4me2/3		KDM5C - JARID1C	H3K4me2/3
	MCM3AP	H3ac					KMT3G - NSD2 - WHSC1	H3K36me2		KDM5D - JARID1D	H3K4me2/3
	SUPT7L	H3ac					KMT3F - NSD3 - WHSC1L1	H3K36me2	KDM6	KDM6A - UTX	H3K27me2/3
						KMT4	DOT1L	H3K79me1/2/3		KDM6B - JMJD3	H3K27me2/3
						KMT5	KMT5A - SETD8	H4K20me1	KDM7	KDM7A - JHDM1D	H3K9me2
							KMT5B - SUV420H1	H4K20me1/2/3			H3K27me2
							KMT5C - SUV420H2	H4K20me1/2/3			H3K9me1/2
						KMT6	KMT6A - EZH2	H3K27me1/2/3		KDM7B - JHDM1F - PHF8	H3K27me2
							KMT6B - EZH1	H3K27me1/2/3			H4K20me1
						KMT7	KMT7 - SETD7	H3K4me1		KDM7C- JHDM1E - PHF2	H3K9me2
						KMT8	KMT8B - PRDM9	H3K4me1/2/3			H4K20me3
								H3K36me1/2/3	KDM8	KDM8 - JMJD5	H3K9me1
							KMT8A - PRDM2	H3K9me1			H3K36me1/2
							KMT8D - PRDM8	H3K9me2	KDM9	KDM9 - RSBN1	H4K20me2/3
							KMT8C - PRDM6	H4K20me			
						KMT9	KMT9 - N6AMT1- HEMK2	H4K12me1			
						SMYD	SMYD4	H3K4me1/2/3			
							SMYD5	H4K20me3			
						SETD	SETD4	H3K4me1/2			
								H4K20me3			
							SETD5	H3K36me3			
							SETD6	H2AZK7me			
							SETMAR	H3K36me2			
						PRDM	PRDM15	H3K9me1			

The HATs can be divided into six major families: 1) the GNATs (GCN5-related N-acetyltransferases), 2) the NATs (N-terminal acetylases, which also possess a GNAT domain), 3) the MYST, 4) the p300/CBP, 5) the SRC steroid receptors coactivators, and 6) other HATs (Wapenaar and Dekker, 2016). HATs actions are counteracted by the HDACs, which include zinc-dependent class I, class IIa, and class IV HDACs, and NAD-dependent class III Sirtuins (Wapenaar and Dekker, 2016). On the other hand, the KMTs present two domains with annotated lysine methyltransferase activity: 1) SET domains, that catalyze all the canonical methylation sites except for H3K79, and 2) 7βS domains (DOT1L and N6AMT1) (Mosammaparast and Shi, 2010). The KDMs can be divided according to their catalytic activity in: 1) amine oxidases (KDM1), and 2) Jumonji C demethylases (KDM2/3/4/5/6/7/8) (Mosammaparast and Shi, 2010).

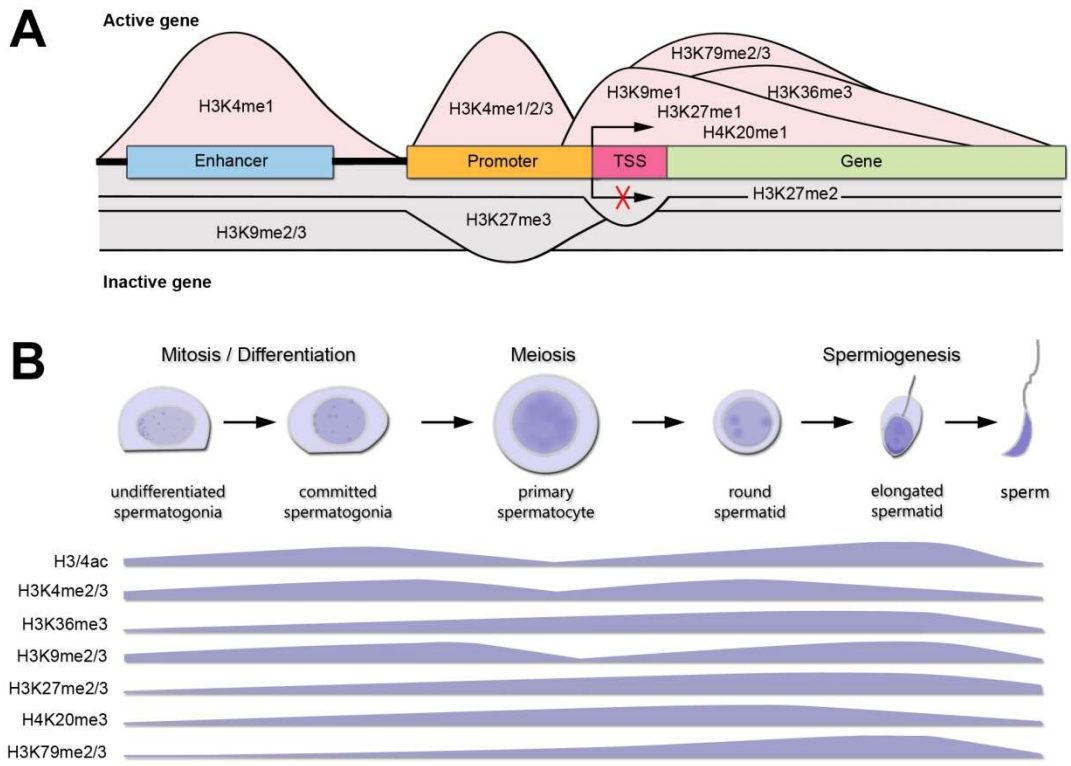
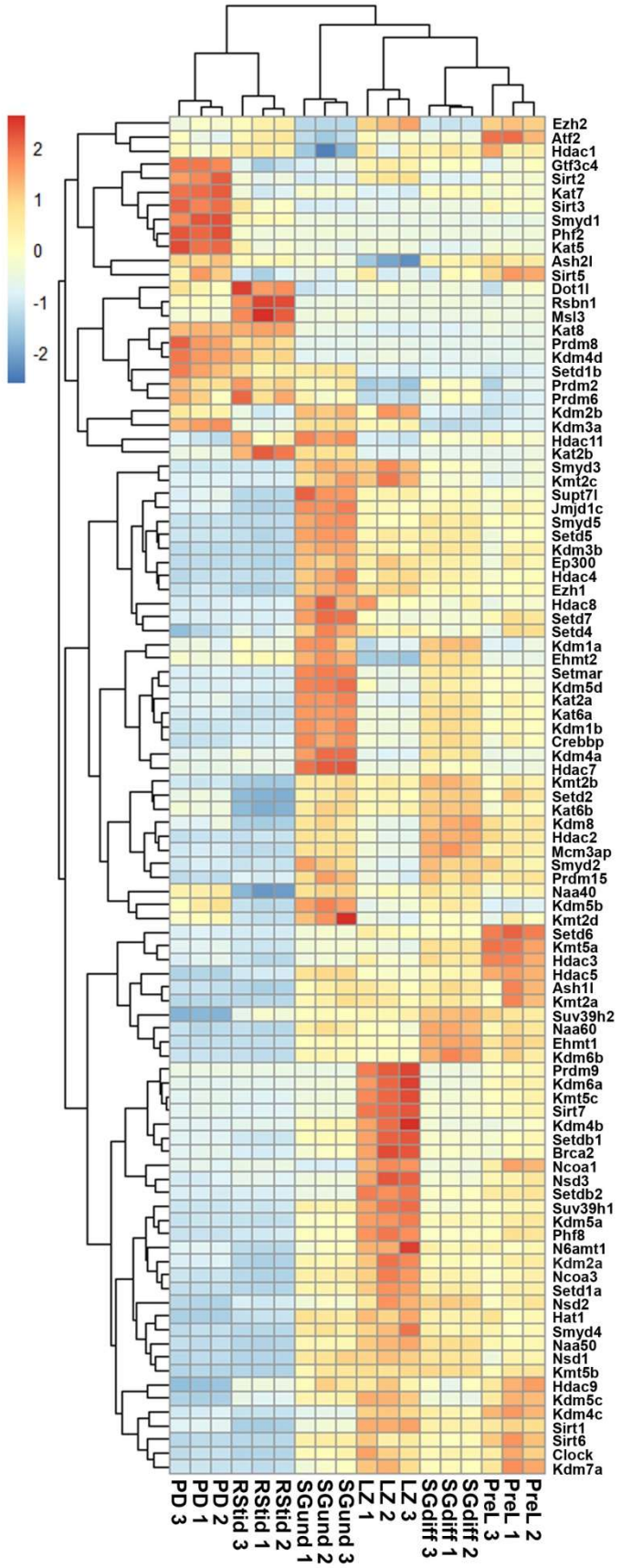


Figure 1. Canonical histone K marks and writers/erasers families distribution during spermatogenesis. A) Histone K methylation PTMs distribution at DNA elements and functional roles in gene expression for an active gene (pink shading) or inactive gene (gray shading). TSS: transcription start site. Only K modifications with sufficient and consensual information about distribution across genes are shown. B) Main histone K PTMs levels involved in chromatin remodeling during spermatogenesis. The data presented is summarized from the following references: Song et al., 2011; Godmann et al., 2007; Zuo et al., 2018; Luense et al., 2016; Wang et al., 2021). SGund: spermatogonia Thy1+/Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD: pachytene/diplotene spermatocytes, RStid: round spermatid.


2
1
0
-1
-2
Ezh2
Atf2
Hdac1
Gtf3c4
Sirt2
Kat7
Sirt3
Smyd1
Phf2
Kat5
Ash2l
Sirt5
Dot1l
Rsbn1
Msl3
Kat8
Prdm8
Kdm4d
Setd1b
Prdm2
Prdm6
Kdm2b
Kdm3a
Hdac11
Kat2b
Smyd3
Kmt2c
Supt7l
Jmjd1c
Smyd5
Setd5
Kdm3b
Ep300
Hdac4
Ezh1
Hdac8
Setd7
Setd4
Kdm1a
Ehmt2
Setmar
Kdm5d
Kat2a
Kat6a
Kdm1b
Crebbp
Kdm4a
Hdac7
Kmt2b
Setd2
Kat6b
Kdm8
Hdac2
Mcm3ap
Smyd2
Prdm15
Naa40
Kdm5b
Kmt2d
Setd6
Kmt5a
Hdac3
Hdac5
Ash1l
Kmt2a
Suv39h2
Naa60
Ehmt1
Kdm6b
Prdm9
Kdm6a
Kmt5c
Sirt7
Kdm4b
Setdb1
Brca2
Ncoa1
Nsd3
Setdb2
Suv39h1
Kdm5a
Phf8
N6amt1
Kdm2a
Ncoa3
Setd1a
Nsd2
Hat1
Smyd4
Naa50
Nsd1
Kmt5b
Hdac9
Kdm5c
Kdm4c
Sirt1
Sirt6
Clock
Kdm7a
PD 3
PD 1
PD 2
RStid 3
RStid 1
RStid 2
SGund 1
SGund 2
SGund 3
LZ 1
LZ 2
LZ 3
SGdiff 3
SGdiff 1
SGdiff 2
PreL 3
PreL 1
PreL 2

515

516 **Figure 2. Unsupervised hierarchical clustering analysis of male germ cells transcriptome**.
517 Heatmap showing z-score values of histone K acetylation and methylation writer/eraser enzymes.
518 SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene spermatocytes, LZ:
519 leptotene/zygotene spermatocytes, PD: pachytene/diplotene spermatocytes, RStid: round spermatid.
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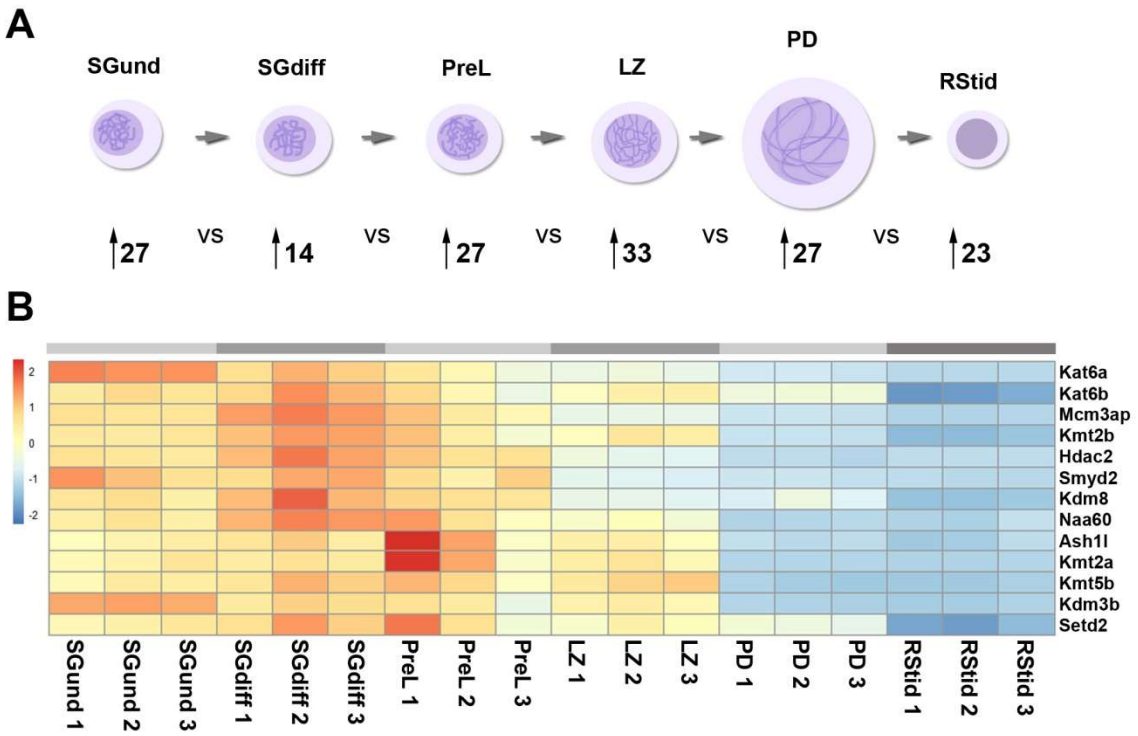


521
522 **Figure 3. Histone K acetylation and methylation epigenetic enzymes expression during**
523 **spermatogenesis** A) Number of upregulated epigenetic enzymes for each germ cell
524 stage.Differentiall gene expression analysis was conducted with DESeq2 on consecutive
525 populations across differentiationn (SGdiff vs SGund, PreL vs SGdiff, LZ vs PreL, PD vs LZ and
526 RStid vs PD), and upregulated genes selected with padj<0.05 and log2FC>0.5. B) Heatmap
527 showing epigenetic enzymes not significantly upregulated at any germ cell stage across
528 spermatogenesis. SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+, PreL: pre-leptotene
529 spermatocytes, LZ: leptotene/zygotene spermatocytes, PD:pachytenee/diplotene spermatocytes,
530 RStid: round spermatid.
531
532

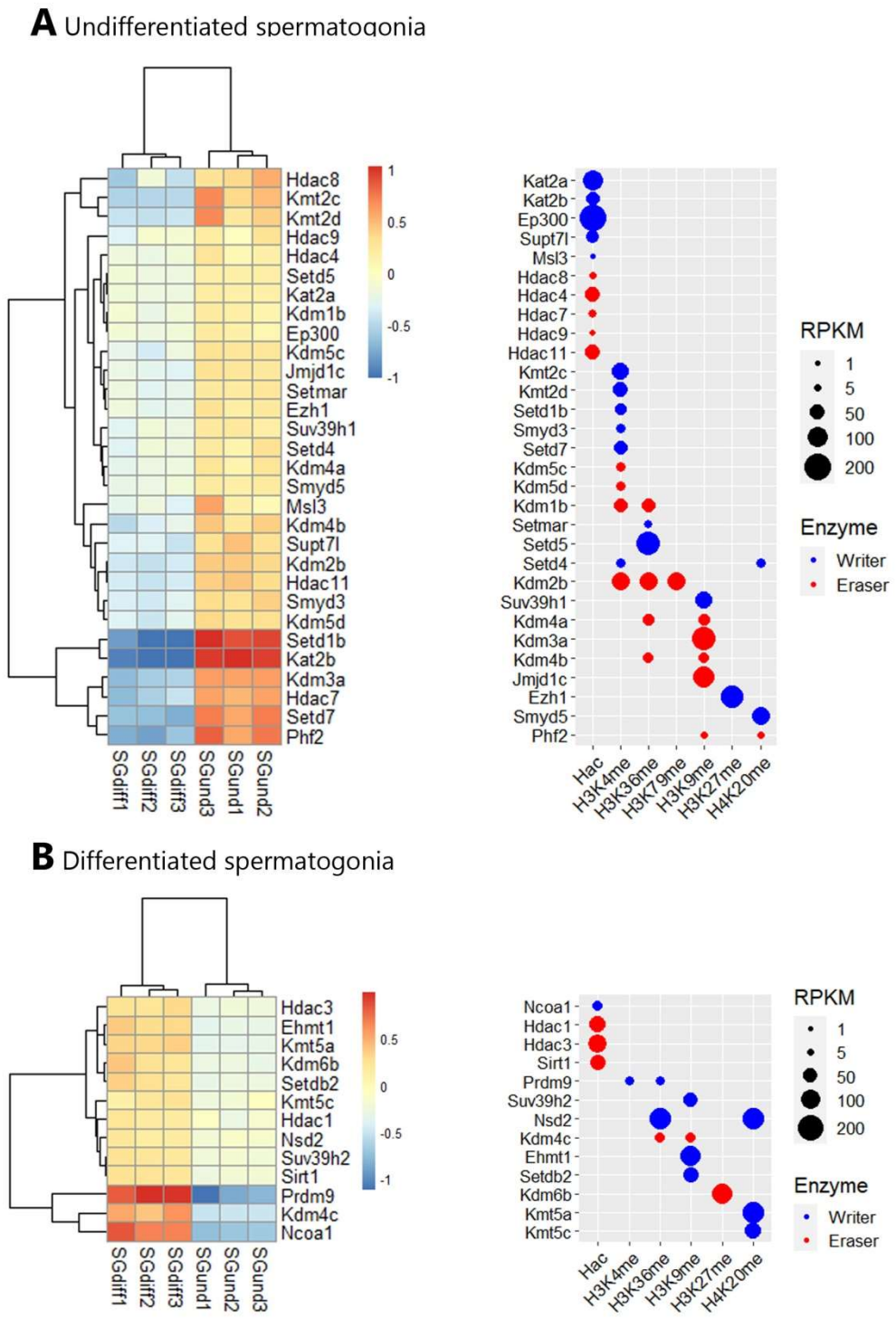


Figure 4. Epigenetic histone K writers and erasers upregulation at spermatogonial phase. The left panel indicates the heatmap showing z score values and the right panel indicates the Dot plot showing enzymes RPKM values in SGund (A) and SGdiff (B) population. Blue dots: writers, red dots: erasers. SGund: spermatogonia Kit-, SGdiff: spermatogonia Kit+.

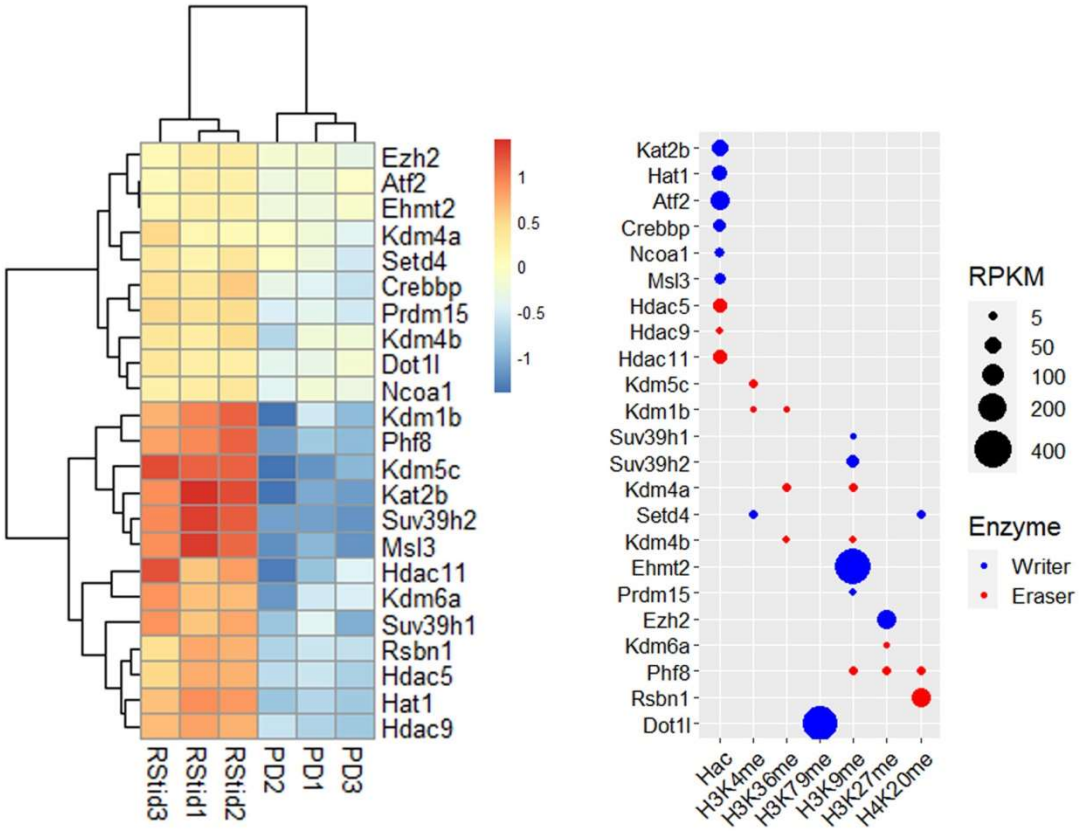
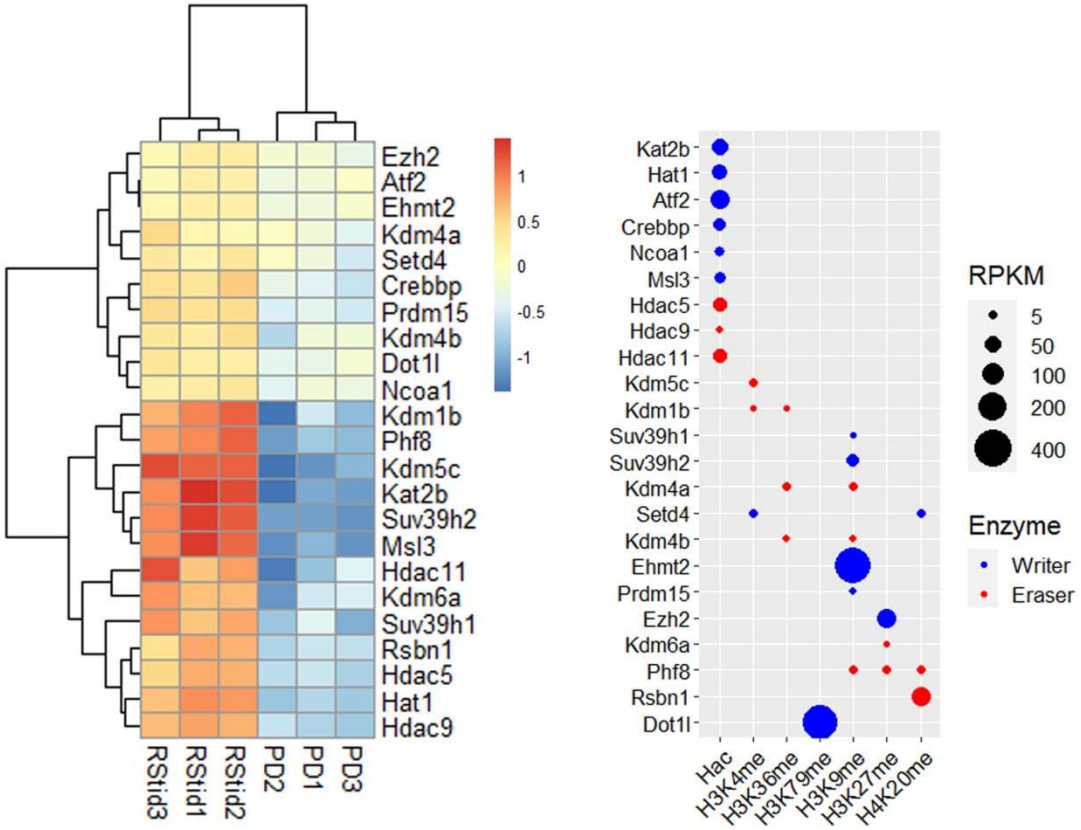


Figure 5. Epigenetic histone K writers and erasers upregulation at meiosis prophase I. The left panel indicates the heatmap showing z score values and the right panel indicates the Dot plot showing enzymes RPKM values, in PreL (A), LZ (B) and PD (C) spermatocytes. Blue dots: writers, red dots: erasers. PreL: pre-leptotene spermatocytes, LZ: leptotene/zygotene spermatocytes, PD:pachytenee/diplotene spermatocytes.



546

547 **Figure 6. Epigenetic histone K writers and erasers upregulation at spermiogenesis.** The left
548 panel indicates the heatmap showing z score values and the right panel indicates the Dot plot
549 showing enzymes RPKM values in RStid. Blue dots: writers, red dots: erasers. RStid: round
550 spermatid, PD: pachytene/diplotene spermatocytes.

551

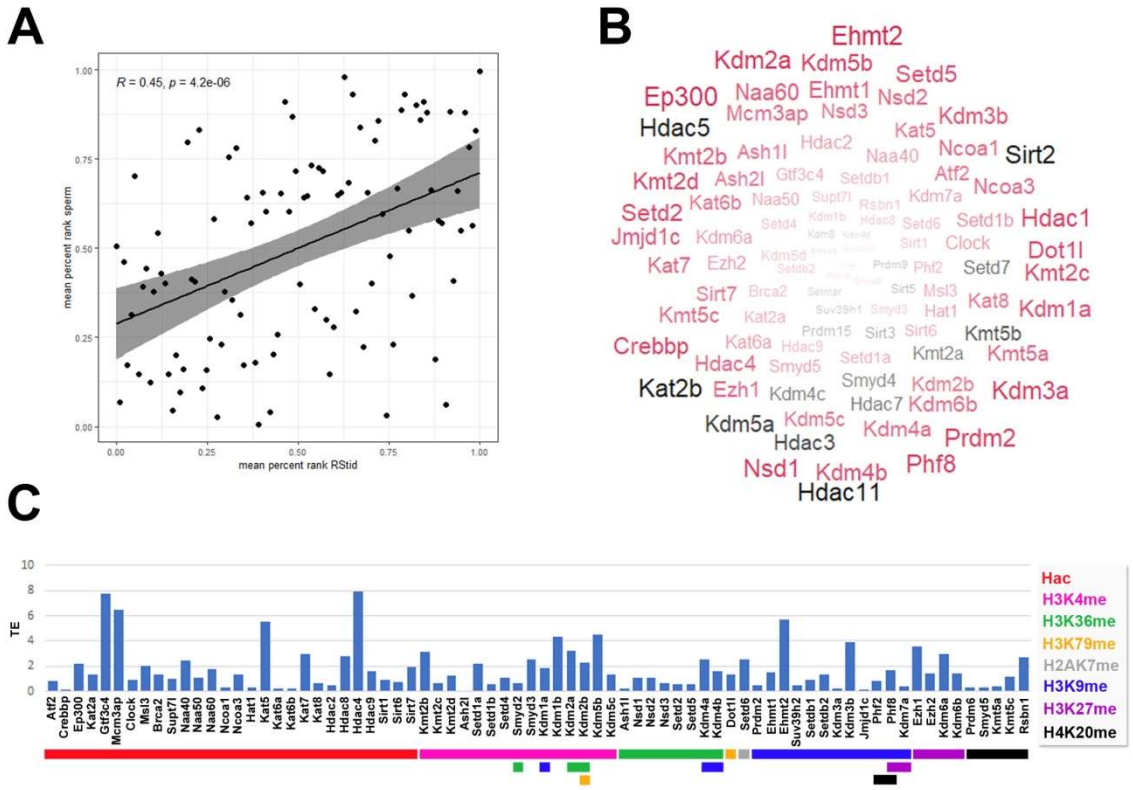


Figure 7. Epigenetic histone K writers and erasers detected at mature sperm RNA cargo and 1-cell embryo translatome. A) Pearson correlation between epigenetic enzymes mean percent rank detected at RStid and sperm. B) Mature sperm word cloud for epigenetic enzymes indicating the enzyme abundance (word size = mean percent rank) and translation efficiency (TE) detected at 1 cell embryo (red: TE > 0, black: TE=0). C) Epigenetic enzymes TE in mouse pre-implantation embryos (TE = RNA RPKM at ribosome / total RNA RPKM, RPKM>0.5)