Transcriptome profiling of histone acetylation and methylation writers and erasers families across male germ cell development and pre-cleavage zygote

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ABSTRACT

Accumulating evidence put on the focus that male spermatogenesis presents windows of vulnerability for epigenetic reprogramming by environmental stressors that not only can affect fertility but even transmit developmental, metabolic, and behavioral traits to offspring. This work analyzed the transcriptomic programs of epigenetic enzymes involved in histone K acetylation and methylation from undifferentiated spermatogonia to mature sperm and pre-cleavage zygote in the mouse. We dissected the peak expression at each stage for families and individual enzymes, their target histone PTMs functions and reported dynamics during spermatogenesis and early embryogenesis. This approach allowed us to identify epigenetic enzymes with known functions during spermatogenesis and, by the rule of "guilty by association", many others not even described yet in male germ cell development. Moreover, some of these enzymes' mRNA were detected higher in the pre-

cleavage zygote than in the transcriptionally silent MII oocyte, suggesting a paternal contribution. Our study contributes to the mechanistic aspects behind transgenerational epigenetics, described for the first time in such a comprehensive approach.

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 not only can affect fertility but 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 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 (Skvortsova et al., 2018).

Spermatogenesis is a finely regulated process where unipotent spermatogonia undergo meiosis and, subsequently, spermiogenesis, to become sperm cells. During 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, Carrel 2012). Importantly, a small fraction of histones in the sperm genome is retained on specific locations and carries several post-translational modifications (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, etc, 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, spermiogenesis and early embryo development 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 (Black et al., 2012).

The role of epigenetic enzymes comes to focus, as they are responsible for the histone's 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 (Fan et al., 2016; Nebbioso et al., 2018; Gonzalez et al., 2020). 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 of numerous diseases (Prachayasittikul et al., 2017) but the potential effect of these compounds on epigenetic inheritance is generally overlooked. Here, we curated transcriptomic data from spermatogonia to mature sperm populations and pre-cleavage zygote 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 the spermatogenesis in mice and the developmental windows when the transcription of these enzymes may be more susceptible to environmental disruption. Not only we expand the knowledge on enzymes with recognized roles in spermatogenesis, but provide evidence of

many more whose role in male germ cells development and zygote have not been described yet.

RESULTS

We curated RNA-seq data available in purified male germ cells from adult mice and selected the dataset of one of the most referenced studies regarding male germ cells epigenomic and transcriptomic profiles published by Hammoud et al. (2014). We analyzed the transcriptome from spermatogonia (SG), including the self-renewing and stem-like Thy1+/Kit- (SGThy1) and the committed Thy1-/Kit+ (SGKit) population, primary spermatocyte (SCyte), round spermatid (STid) and mature sperm stages.

1. Epigenetic writers and erasers involved in male germ cells histone K acetylation and methylation dynamics

Table 1 shows writers and erasers families for histone K acetylation and methylation, and the most representative targets described in mammalian cells. 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 (the H3K79-specific DOT1L) (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).

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		KDM2B - JHDM1B	H3K4me3
	NAA50 - NAT13	H4ac		HDAC5	Hac		KMT1F - SETDB2	H3K9me3			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	H3K4me1/2/3		KDM3B - JMJD1B	H3K9me1/2
	KAT7 - MYST2 - HBO1	H3/4ac		SIRT2	H4K16ac		KMT2F - SETD1A	H3K4me1/2/3		KDM3C - JMJD1C	H3K9me1/2
	KAT6A - MYST3 - MOZ	H3/4ac		SIRT3	H4K16ac		KMT2H - ASH1L	H3K36me2	KDM4	KDM4A - JMJD2A - JHDM3A	H3K9me2/3
	KAT6B - MYST4 - MORF	H3ac		SIRT5	Hac	KMT2 core	ASH2L	H3K4me1/2/3			H3K36me2/3
p300/CBP	KAT3A - CREBBP	H3ac		SIRT6	H3K9ac	KMT3	KMT3A - SETD2	H3K36me3		KDM4B - JMJD2B - JHDM3B	H3K9me3
	KAT3B - EP300	H3ac		SIRT7	H3K18ac		KMT3B - NSD1	H3K36me2			H3K36me3
SRC	NCOA1	H3/4ac	Class IV	HDAC11	Hac		KMT3C - SMYD2	H3K4me1/2/3		KDM4C - JMJD2C - JHDM3C	H3K9me3
	NCOA3	H3ac						H3K36me2			H3K36me3
Other	CLOCK	H3ac					KMT3D - SMYD1	H3K4me1/2/3		KDM4D - JMJD2D - JHDM3E	H3K9me2/3
	MSL3	H4K16ac					KMT3E - SMYD3	H3K4me2/3	KDM5	KDM5A - JARID1A	H3K4me2/3
	GTF3C4	H3ac					KMT3G - NSD2 - WHSC1	H3K36me2		KDM5B - JARID1B	H3K4me1/2/3
	BRCA2	H3ac					KMT3F - NSD3 - WHSC1L1	H3K36me2		KDM5C - JARID1C	H3K4me2/3
	MCM3AP	H3ac				KMT4	DOT1L	H3K79me1/2/3		KDM5D - JARID1D	H3K4me2/3
	SUPT7L	H3ac				KMT5	KMT5A - SETD8	H4K20me1	KDM6	KDM6A - UTX	H3K27me2/3
							KMT5B - SUV420H1	H4K20me1/2/3		KDM6B - JMJD3	H3K27me2/3
							KMT5C - SUV420H2	H4K20me1/2/3	KDM7	KDM7A - JHDM1D	H3K9me2
						KMT6	KMT6A - EZH2	H3K27me1/2/3			H3K27me2
							KMT6B - EZH1	H3K27me1/2/3			H3K9me1/2
						KMT7	KMT7 - SETD7	H3K4me1		KDM7B - JHDM1F - PHF8	H3K27me2
						KMT8	KMT8B - PRDM9	H3K4me1/2/3			H4K20me1
								H3K36me1/2/3		KDM7C- JHDM1E - PHF2	H3K9me2
							KMT8A - PRDM2	H3K9me1			H4K20me3
							KMT8D - PRDM8	H3K9me2	KDM8	KDM8 - JMJD5	H3K9me1
							KMT8C - PRDM6	H4K20me			H3K36me1/2
						SMYD	SMYD4	H3K4me1/2/3	KDM9	KDM9 - RSBN1	H4K20me2/3
							SMYD5	H4K20me3			
						SETD	SETD4	H3K4me1/2			
								H4K20me3			
							SETD5	H3K36me3			
							SETD6	H2AZK7me			
							SETMAR	H3K36me2			
						PRDM	PRDM15	H3K9me1			

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. The canonical histone methylation sites are found on H3K4/9/27/36/79 and H4K20, with well-established roles on chromatin functions (Husmann and Gozani 2019) (Figure 1A). For instance, H3K4me1/2/3, H3K36me3, H3K9me1, H3K27me1, H3K79me2, and H4K20me1 are found in enhancers, promoters, and gene bodies of active genes, whereas H3K9me2/3, H3K27me2/3, H4K20me3, and H3K79me3 are found in repressed chromatin such as heterochromatin, telomeric regions and inactivated X chromosome (Husmann and Gozani 2019) (Figure 1A). In addition, there are multiple non-canonical histone methylation sites on H2AZK7me, H3K23/63 and H4K5/12 with unknown functions. Figure 1B shows the most important histone K acetylation and methylation distribution during spermatogenic stages while Figure 1C the stacked RPKM accumulation

of enzymes families' expression associated with each histone mark. The accumulated expression patterns for writers and erasers families are consistent with global dynamics described for main histone K marks levels and functions during spermatogenesis, which will be further discussed below.

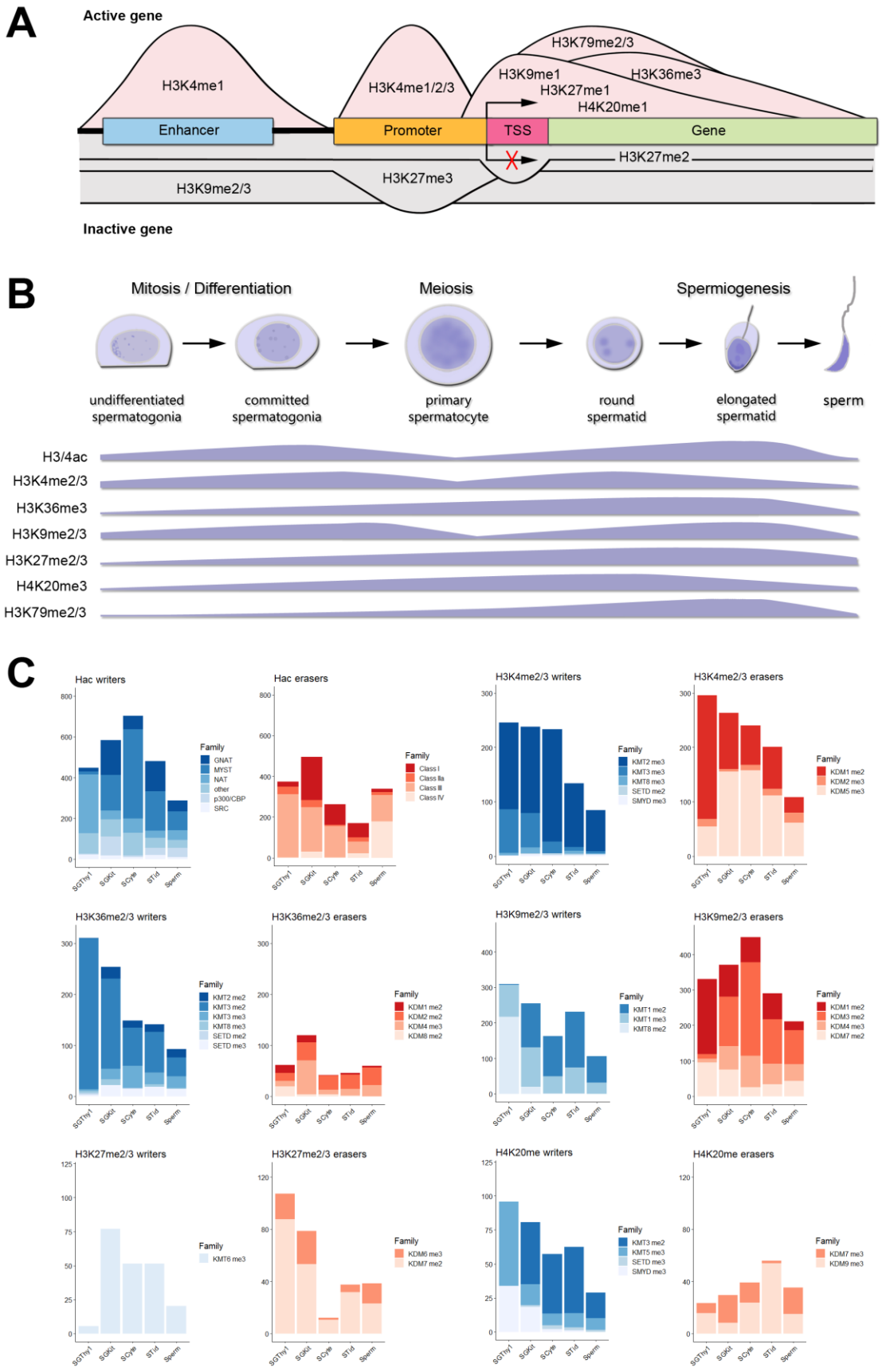

A
Active gene
H3K4me1
H3K4me1/2/3
H3K79me2/3
H3K9me1
H3K36me3
H3K27me1
H4K20me1
Enhancer
Promoter
TSS
Gene
H3K27me2
H3K9me2/3
H3K27me3
Inactive gene
B
Mitosis / Differentiation
Meiosis
Spermiogenesis
undifferentiated spermatogonia
committed spermatogonia
primary spermatocyte
round spermatid
elongated spermatid
sperm
H3/4ac
H3K4me2/3
H3K36me3
H3K9me2/3
H3K27me2/3
H4K20me3
H3K79me2/3
C
Hac writers
Hac erasers
H3K4me2/3 writers
H3K4me2/3 erasers
H3K36me2/3 writers
H3K36me2/3 erasers
H3K9me2/3 writers
H3K9me2/3 erasers
H3K27me2/3 writers
H3K27me2/3 erasers
H4K20me writers
H4K20me erasers
Family
SGThy1
SGKit
SCyte
STid
Sperm

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, Tatehana et al., 2020. C) Accumulated expression of writers/erasers for main canonical histone marks. Legend indicates the maximum number of methylations catalyzed by each enzyme family. SGThy1: spermatogonia Thy1+/Kit-, SGKit: spermatogonia Kit+, SCyte: primary spermatocyte, STid: round spermatid.

2. Clustering analysis of writers and erasers across male germ cell differentiation

Expression profiling analyses of male germ cells were conducted performing k-means partitioning clustering (k=5) (Figure 2A). Then, we searched in each cluster for epigenetic enzymes and marker genes for cell identity and developmental stage. Figure 2B shows that cluster 1 is enriched at SGThy1 cells, showing peak expression of undifferentiated SG marker genes *Thy1*, *Nanos1*, *Nanos2*, *Gfra1*, *Fgfr3*, *Mlh1* and *Cd9*. Cluster 2 is enriched in SGKit cells, showing differentiated SG marker genes *Kit*, *Stra8*, *Dazl*, *H2afv*, *Aldh2* and *Sycp3*. Cluster 3 is enriched in markers of meiosis characteristic of SCyte cells, such as *Sycp1/2*, *Top2a*, *Piwil1*, *Ddx4*, *Dkkl1*, *Spo11* and *H2Afx*. Clusters 4 and 5 correspond to the spermatic stage, characterized by peak expression of transition proteins (TNP) *Tnp1/2*, protamine (PRM) *Prm1/2*, *Izumo1*, acrosome proteins *Acr*, *Acrbp*, *Acrv1*, and *Pgk2*. Cluster 4 showed peak expression at round STid and cluster 5 at sperm cells.

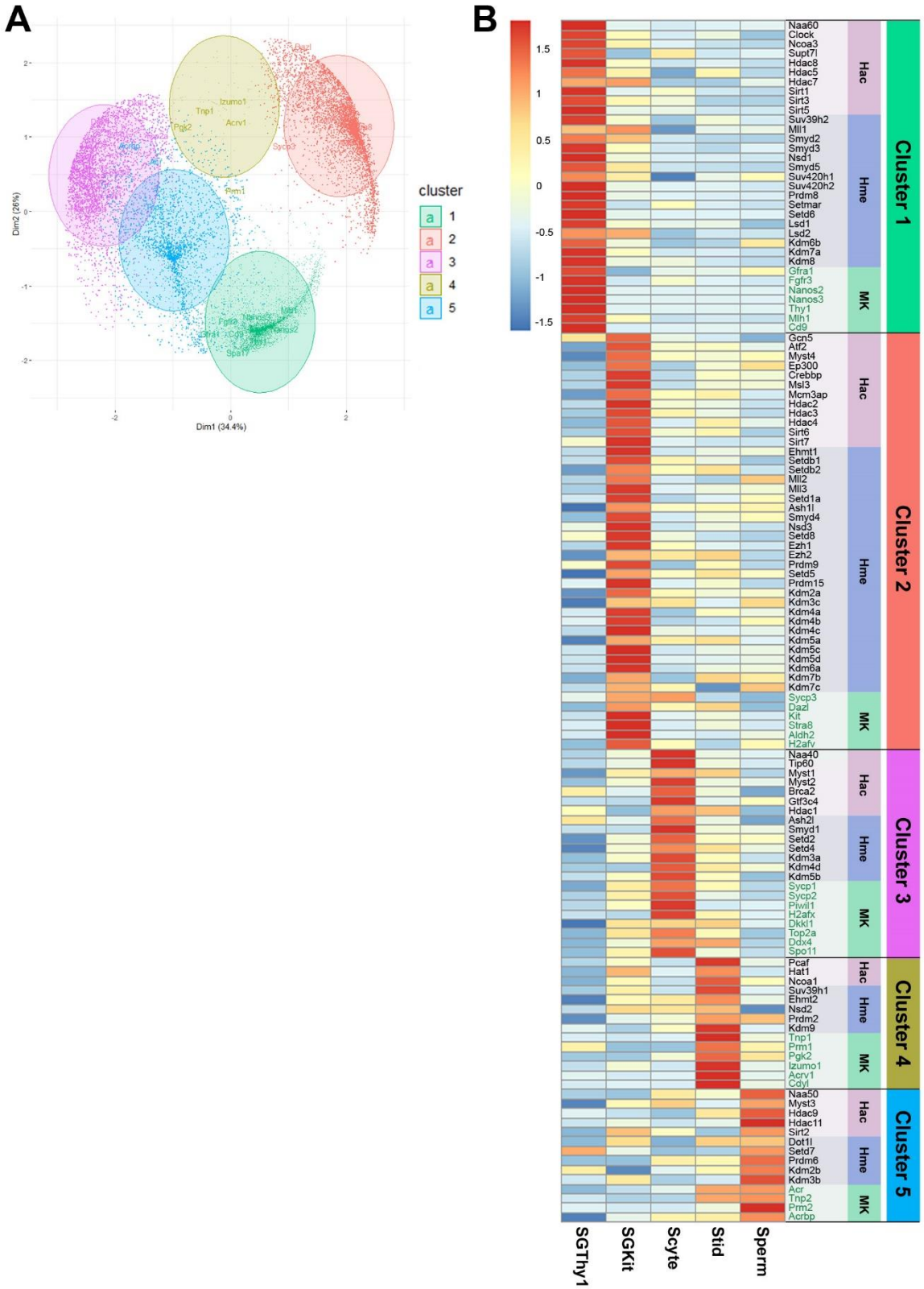


Figure 2. Clustering analysis (k-means) of male germ cells transcriptome. A) Principal component analysis (PCA) plot showing partitioning clustering results. B) Heatmap showing z-

score from RPKM values of histone K acetylation (Hac) and methylation (Hme) writer/eraser enzymes (black), and marker genes for male germ cell development (green). SGThy1: spermatogonia Thy1+/Kit-, SGKit: spermatogonia Kit+, SCyte: primary spermatocyte, STid: round spermatid.

2.1 Enzymes with peak expression at SGThy1 population

Spermatogonial stem cells (SSC) are characterized by a mitotic activity to maintain testicular homeostasis through self-renewal during spermatogenesis. Some of these germ cells are subject to extensive chromatin reorganization and the epigenetic landscape of SG shifts dramatically to differentiate to committed SGKit. Few variations were found in DNA methy1ation at promoter regions between SGThy1 and SGKit, suggesting that histone PTMs may be responsible for the transition between self-renewal and differentiation (Hammoud et al., 2014). Accordingly, we observed that SGThy1 population showed different epigenetic enzymes families expression patterns compared to SGKit, having most of them a peak expression that seems to decrease after SGKit stage.

SGThy1 cluster (Figure 3A) showed peak expression of HATs *Naa60*, *Clock*, *Ncoa3* and *Supt7l*, none of which have been described yet in the SG population. CLOCK is a central component of the circadian pacemaker complex that contributes to chromatin-remodeling events implicated in circadian control of gene expression (Hirayama et al., 2007). SUPT7L and NCOA3 were found to interact with transcription factors to allow the RNA polymerase II preinitiation complexes on protein-encoding genes (Demény et al., 2007; Percharde et al., 2012). Moreover, NCOA3 was related to stem cell self-renewal, reprogramming, and pluripotency maintenance (Percharde et al., 2012; Percharde and Azuara, 2013). *Naa60*, which showed the highest RPKM value, was linked to acetylation of free histone H4K79/91ac and the promotion of nucleosome assembly (Yang et al., 2011). Among the HDACs family, we found peak expression and high RPKM of *Sirt1/3/5*, and also *Hdac8* and *Hdac5/7*. SIRT1 is a key regulator of SG differentiation, as 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). SIRT1 also prevents abnormal DNA methylation of developmental genes by antagonizing DNA methyltransferase DNMT3L (Geo et al., 2017). SIRT3/5 are mainly mitochondrial but also found in the nucleus linked

to H4K16ac, a mark involved in the formation of higher-order chromatin (Scher et al., 2007; Giblin et al., 2021). Finally, class IIa HDACs were found to induce pluripotency by repressing the function of MEF2 (Myocyte Enhancer Factor 2) (Luo et al., 2019).

It is established that undifferentiated SG typically lacks heterochromatin, which forms as they differentiate to SGKit population committed to meiosis (Chiarini-Garcia et al., 2002). Self-renewal and maintenance of the undifferentiated SG pool require the transcriptional repressor PLZF (Promyelocytic Leukaemia Zinc Finger), which mediates the deposition of repressive marks H3K9me2/3, H3K27me2/3 and H4K20me3 at specific perinuclear foci (Costoya et al., 2004; Payne and Braun, 2006). These repressive marks distribution, reflect the epigenomic landscape associated with chromatin silencing necessary to maintain the stem state and suppress somatic cell differentiation (Payne and Braun, 2006; Eun et al., 2010). Here, we show that SGThy1 exhibited high expression of H3K9me2/3 writers *Suv39h2* and *Prdm8*, and H4K20me3 writers *Suv420h*1, *Suv420h2* and *Smyd5*, pointing to these enzymes as important drivers of repressive chromatin. We also found peak expression of *Kdm8*, which was associated with H3K36me erasing at centromeric regions and mitosis regulation (He et al., 2016), pointing to a novel role of this enzyme at SGThy1 self-renewal.

Accumulating evidence points to H3K4me2 in the maintenance of transcriptional states during cell development and, its removal by erasers such as KDM1A/LSD1 is a key step for epigenetic reprogramming and cell fate (Martinez-Gamero et al., 2021). This is consistent with the low expression of H3K4me writers found at the SGThy1 stage, as opposed to the high levels of *Kdm1a* and *Kdm1b* (Figure 1C and 2). In germ cells, KDM1A is part of a repressive complex that mediates chromatin compaction and transcriptional repression (Zhang et al., 2013). It was found that KDM1A mediates SG commitment and differentiation, as testicular deletion of *Kdm1a* leads to progressive germ cell loss and abnormal accumulation of meiotic SCytes (Lambrot et al., 2015; Myrick et al., 2017). SGThy1 cluster also showed peak expression of H3K36me2 writers *Nsd1*, *Smyd2*, and *Setmar*. In embryonic stem cells (ESC), NSD1-mediated H3K36me2 was found to prevent H3K27me3 deposition by polycomb repressive complex 2 (PRC2), modulating polycomb-mediated H3K27me domains demarcation (Streubel et al., 2018). Moreover, NSD1 was found to safeguard a subset of genes against H3K27me3-mediated transcriptional silencing

and to have a critical role in de novo DNA methylation in prospermatogonia (Shirane et al., 2020). In line with this, adult SSC exhibits low H3K27me2/3 levels that increase with cell differentiation and meiotic progression (Payne and Braun, 2006). In agreement, we found the highest total H3K36me and lowest H3K27me writers families expression at SGThy1 population (Figure 1C), suggesting that H3K36me2 landscape could be set prior to increased polycomb activity from SGKit onward. H3K27me erasers *Kdm6b* and *Kdm7a* showed peak expression in the SGThy1 population and may participate in maintaining low H3K27me levels. In this context, KDM6B was found to regulate the fragmentation of spermatogonial cysts, and its down-regulation promotes an increase in undifferentiated SG without affecting their differentiation (Iwamori et al., 2013). Interestingly, we also observed high expression of *Sedt6*, a writer that specifically deposits H2AZK7me1, which in ESC was found with H3K27me3 close to differentiation marker genes and removed upon retinoic acid (RA) signal, suggesting that SETD6 may have a role in self-renewal and differentiation of SG (Binda et al., 2013).

2.2 Enzymes with peak expression at SGKit population

SGKit population is highly committed to gametogenesis and express RA-induced STRA8 (stimulated by retinoic acid 8), consistent with known roles for RA in committing Kit+ germ cells to meiosis. SGKit population is characterized by the activation of transcriptional programs that control meiosis entry and post-meiotic differentiation, consisting of genes that increase and maintain their expression from committed SG, and genes that are transiently silenced during meiosis and regain expression at round STids (Chen et al., 2018).

Germ cell differentiation and meiosis entry is driven by increased levels of H3/4ac, H3K4me2/3 and H3K36me3 (Song et al., 2011; Godmann et al., 2007; Zuo et al., 2018) (Figure 2B). Here, SGKit population (Figure 3B) showed peak levels of specific HAT families including GNATs *Gcn5* and *Atf2*, p300/CBP *Ep300* and *Crebbp*, and *Myst4*, *Msl3* and *Mcm3ap*. Among them, GCN5, CBP and p300 are co-activators for several transcription factors and nuclear receptors including AR and ERα (Jin et al., 2011). Moreover, *Gcn5* removal by CRE under *Stra8* promoter disrupted histone eviction, promoted nucleosome retention in sperm, and affected fertility (Luense et al., 2019).

P300/CBP was linked to metabolism programming of pre- and post-meiotic male germ cells (Boussouar et al., 2014) and to chromatin reorganization at STids (Pradeepa et al., 2009). Importantly, it was shown that MSL3, a member of the NSL complex, binds to H3K36me3 marked sites and deposits H4K16ac, promoting changes in higher order chromatin structure that prepares meiosis entry and enables STRA8 functions (McCarthy et al., 2022). In line with this, we also found peak expression of H3K36me2 writers *Ash1l* and *Nsd3*, and a specific increase of H3K36me3 writers, including *Setd2/5* and *Prdm9*. PRDM9 and ASH1L were found strongly linked to meiosis, as both enzymes are responsible for H3K4me3 and H3K36me3 that mediate the double-strand breaks (DSBs) formation at meiotic hot spots (discussed below) (Sun et al., 2020; Getun et al., 2017).

SGKit population also expressed peak levels of *Hdac2/3*, *Hdac4* and *Sirt6/7*. It was shown that class IIa HDACs enzymatic activity depends on their recruitment into a complex containing HDAC3 and RA nuclear receptor (Parra 2015; Fischle et al., 2002), suggesting that these HDACs could mediate chromatin silencing induced by RA signal. HDAC3 was also found increased in the last stages of meiosis, inducing repression of meiotic/spermatogonial genes and activation of postmeiotic haploid gene programs during meiotic exit, with associated histone acetylation alterations (Yin et al., 2021). On the other hand, SIRT6 and SIRT7 were found associated with heterochromatic regions and nucleoli, respectively (Michishita et al., 2005). 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 pathway (Barber et al., 2012; Wu et al., 2018).

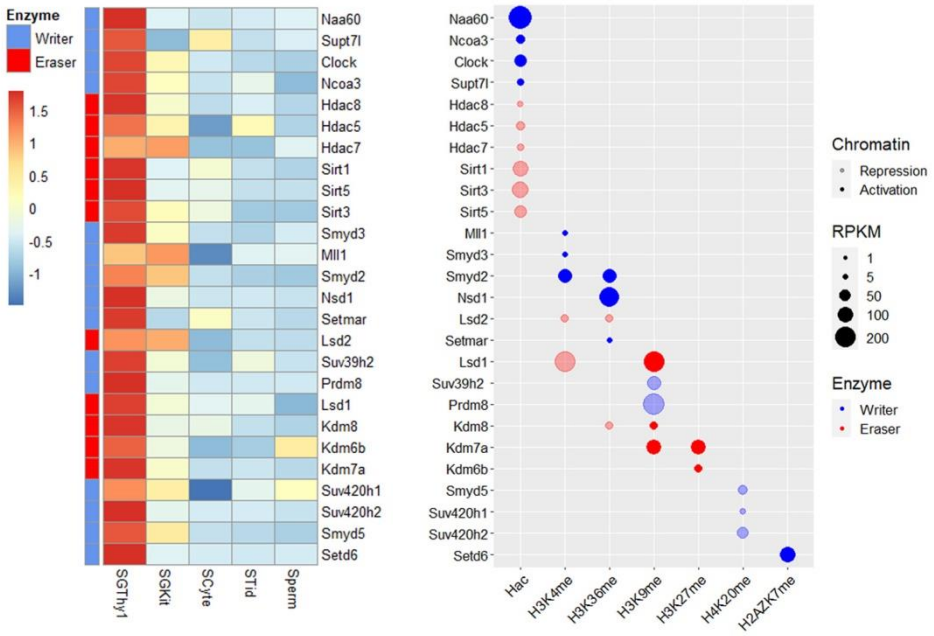
SGKit population showed peak expression of the KMT2 members *Setd1a*, *Mll2* and *Mll3*, which are part of the COMPASS complex family that display both redundant and non-redundant H3K4me deposition. In mammalian cells, SETD1A/B reportedly catalyzes most of the H3K4me3 (Couture and Skiniotis, 2013) while MLL3 deposits H3K4me1 at enhancers (Herz et al., 2012) and MLL1/2 H3K4me2/3 at developmental genes (Hu et al., 2017; Rickels et al., 2016). Importantly, targeted loss of H3K4me methylation in SSC by reduction of MLL2 activity results in a dramatic reduction in the number of SCytes (Glaser et al., 2009), and manipulating H3K4me3 patterns can induce transgenerational inheritance (Lismer et al., 2020). During the SGKit stage, certain genes such as *Stra8*, *Aldh2* and *Spo11*

gain expression by losing H3K27me3 bivalency and transitioning from a bivalent to active chromatin state (Hammoud et al., 2014). In line with this, we detected peak expression of H3K27me2/3 erasers *Kdm6a* and *Kdm7b* in the SGKit population. Interestingly, KDM6A is also a component of the MLL2/MLL3 COMPASS complex that mediates enhancer activation via simultaneous H3K4me3 deposition and H3K27me2/3 removal (Fagan and Dingwall, 2019). Moreover, the COMPASS complex promotes H3K27ac by interacting with p300/CBP (Fagan and Dingwall, 2019). Taken together, these data could reveal the importance of MSL and COMPASS complexes during meiosis entry, deserving further future attention.

SGKit stage also showed peak expression of repressive marks writers indicative of facultative heterochromatin and cell differentiation, including KMT1 H3K9me2 *Ehmt1* and H3K9me3 *Setdb1/2*, and KMT6 H3K27me2/3 polycomb *Ezh1/2* families. At this stage there is de novo formation of bivalent domains by H3K27me deposition at developmental genes (Maezawa et al., 2018). Her, we detected peak expression of the H4K20me1 writer *Setd8*, which was found to physically interact with STRA8 and to mutually control each other transcription (Niu et al., 2020). Moreover, SETD8, H4K20me1 and PCNA co-localize with STRA8, indicating a role for SETD8 in the cell cycle transition from mitosis to meiosis (Niu et al., 2020). We also found peak expression of H3K36me *Kdm2a* and H3K9/K36me *Kdm4a/b/c* erasers. KDM2A was strongly linked to heterochromatin maintenance, transcriptional repression of repetitive DNA elements and inactive genes, and the proper segregation of chromosomes during mitosis (Frescas et al., 2008), but the exact roles during spermatogenesis remain to be further explored. On the other hand, the KDM4 family participates in DNA replication control, where KDM4B is linked to core transcription factors NANOG and SOX2 and KDM4C to PRC2 and Myc regulatory modules (Das et al., 2014). Interestingly, in meiotic and post-meiotic SCytes, KDM4A/B were found to remove H3K36me2/3 after binding to H3K23me3, a poorly understood histone modification recently shown to be enriched in heterochromatin at meiosis onset (Su et al., 2016). Here, we also detected high expression of the KDM5 family which removes H3K4me2/3. KDM5A is an integral, conserved component of the Notch/RBP-J repressor complex and fundamental for the switch-off of the Notch target genes that control cell fate transitions (Liefke et al., 2010.). Among the KDM5 family, KDM5A/D were found

strongly related to spermatogenesis progression and fertility (Nishio et al., 2014; Gegenschatz-Schmid et al, 2019). Importantly, KDM5D forms a complex with the MSH5 DNA repair factor that controls synapsis and crossing-over at meiotic prophase and locates to condensed DNA during the leptotene/zygotene stage (Akimoto et al., 2008).

A *Cluster 1*: SGThy1 population



B *Cluster 2*: SGKit population

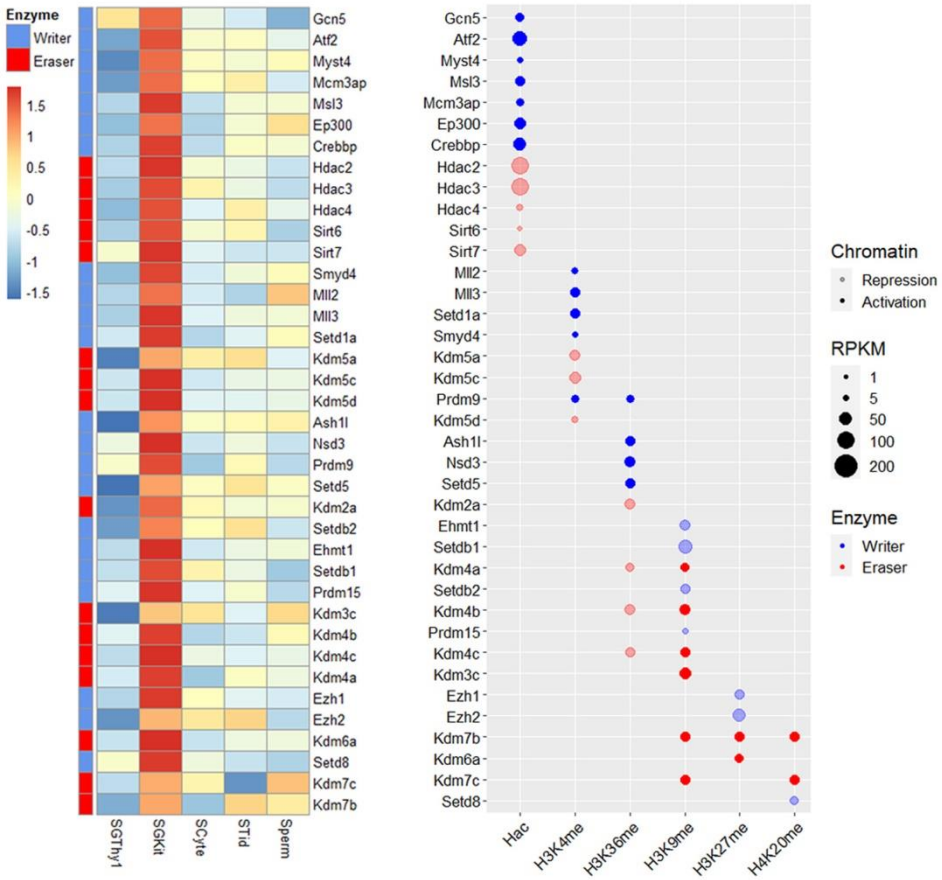


Figure 3. Cluster 1 and 2 epigenetic enzymes showing peak expression at SGThy1 (A) and SGKit population (B). Left: heatmap showing z score values for each male germ cell population. Right: Dot plot showing enzymes RPKM values in SGThy1 (A) and SGKit (B) population, repectively. SGThy1: spermatogonia Thy1+/Kit-, SGKit: spermatogonia Kit+, SCyte: primary spermatocyte, STid: round spermatid.

2.3 Enzymes with peak expression at primary SCyte population

Meiotic division is associated with specific chromatin changes that enable genetic exchange at meiotic recombination: i) DNA DSBs deposition and repair at synapsed chromatin, and ii) epigenetic silencing of unpaired chromatin (MSUC), including meiotic sex chromosome inactivation (MSCI). Several studies showed that histone PTMs contribute to synapsis formation, recombination at hot spots and to MSCI and sex body formation (Buard et al., 2009; Baudat et al., 2010; Parvanov et al., 2010).

Hot spot cores of SG and pre-leptotene-stage cells harbor H3K9/18/23ac and H4K5/8/12/16/91ac that are associated with open chromatin (Getun et al., 2017; Chioccarelli et al., 2020). We observed that SCyte cluster (Figure 4A) showed peak expression of MYST family *Myst1*, *Myst2* and *Tip60*, and also *Naa40*, *Brca2* and *Gtf3c4*, showing most of them high RPKM values. Accordingly, MYST enzymes were previously found strongly related to meiosis control, increasing their expression from early pachytene through diplotene stages (Getun et al., 2017). Moreover, MYST1/MOF, a specific H4K16ac writer, was found involved in DSBs repair and crossover numbers (Jiang et al., 2018). In line with this, H4K91ac was also linked to chromatin assembly of repair machinery (Ye et al., 2005). In addition, we detected peak expression of the H3K36me3 writer *Setd2*, which has been also associated with DSBs establishment and repair in transcribed genes (Jha et al., 2014). Importantly, *Setd2* knockout in germ cells results in complete loss of H3K36me3, decreased expression of genes encoding PRMs and altered spermiogenesis with acrosomal malformation at the round STid stage (Zuo et al., 2018).

During MSCI, both sex chromosomes undergo extensive H3/4 deacetylation and H3K9me2/3 marking to create the sex body (Khalil et al., 2004). At the SCyte stage, we detected peak expression of *Hdac1* that was shown to form a complex with DNA methyltransferase DNMT3L to regulate X chromosome compaction (Zamudio et al., 2011;

Deplus et al., 2002). Upon meiosis completion, there is an increase in H3K4me3 and a drop in H3/H4ac and H3K9me3 (Figure 1B). Here, we found high RPKM values for H3K9me2/3 erasers *Kdm3a* and *Kdm4d* that remain elevated in the STid stage. In line with this, H3K9me demethylation by KDM3A was found essential for post-meiotic chromatin organization, enabling the transcriptional activation of TNP1 and PRM1 (Okada et al., 2007). SCyte stage also showed peak expression and high RPKM values of the H3K4me writer *Ash2l*, a key core member of the WRAD complex that interacts with KMT2 COMPASS writers to confer methyltransferase activity (Ernst and Vakoc, 2012). Interestingly, this enzyme also showed high expression at SGThy1, consistent with previous work showing that *Ash2l* controls ESC maintenance and differentiation (Wan et al., 2013). Among H3K4me erasers, we found high RPKM values of *Kdm5b*, strongly linked to spermatogonial differentiation. Moreover, *Kdm5b* is highly expressed in pre-meiotic SG and pachytene-diplotene SCytes, where it is thought to repress the expression of genes that would otherwise cause the SG to remain in the mitotic phase (Madsen et al., 2003).

2.4 Enzymes with peak expression at round STid population

Spermiogenesis is the final phase of spermatogenesis, where haploid round STids undergo dramatic morphological changes to transform into elongated STids and sperm. During this phase, extreme chromatin remodeling takes place to facilitate the condensation and packaging of the paternal genome. Spermiogenesis involves transient DSBs formation and repair, specific histone variants incorporation and H3/4 hyperacetylation to destabilize the nucleosome, and subsequent replacement of 95-99% histones by TNPs and PRMs (Rajender et al., 2011, Carrel 2012).

During spermiogenesis, H4K16ac is essential for histone eviction which does not occur in the absence of this specific mark (Lu et al., 2010). Here, SCyte cluster (Figure 4A) showed peak transcriptional levels of MYST enzymes *Tip60* and *Myst1*, which were linked to H4ac prior to histone eviction (Dong et al., 2017, Lu et al., 2010). In line with this, it was previously reported that pachytene SCytes show higher expression of genes related to spermiogenesis than round STids, showing a gap between the time of peak transcription and the execution of certain programs (Da Cruz et al., 2016). The existence of translational

delays in spermatogenesis is well known and has been associated with a strategy to regulate the timing of protein synthesis in STids, as transcription in these cells is silenced after histone eviction (Kleene et al., 2001; Idler and Yan, 2012; Braun 1998; Lee et al., 1995). A characteristic of STid enzymes is that they also show high expression at SGKit with or without transient silencing at SCyte stage, in line with the notion that many STid genes begin to express at SG population (Jan et al., 2017). In a very interesting study, Bell et al., (2014) found that SIRT1 deletion by CRE under protamine promoter has no effect, but CRE under *Stra8* promoter not only dampens SG differentiation but STids show reduced H4K5/8/12ac and TNP2 do not localize in the nucleus leading to chromatin condensation defects in sperm. Similar results were reported for GCN5 (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.

Histone eviction happens in an initial wave when nucleosomes become accessible at the round STid stage followed by a "major" wave of nucleosome accessibility at elongated STids (Luense et al., 2019). The specific epigenetic programs controlling the histone eviction waves are not fully understood, but different HATs activities are likely to be involved. It is important to note that SGKit and SCyte cluster HATs show moderate/high expression at STid population as well, suggesting they could be involved in the acetylation waves prior to histone eviction, except for p300/CBP that has been already discarded (Boussouar et al., 2014). Here, STid cluster (Figure 4B) showed peak expression of *Pcaf*, *Ncoa1* and *Hat1*. PCAF functions as a co-activator for ERα and RA receptor (RAR) (Blanco et al, 1998), and can interact with NCOA1 to promote transcription (Spencer et al., 1997). Interestingly, p300/CBP and PCAF were found to regulate histone eviction by acetylation of TNP2, affecting DNA condensation properties and interaction with histone chaperones (Pradeepa et al., 2009). Concerning *Hat1* expression, a cytoplasmic HAT responsible for acetylating newly synthesized H4, it 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).

STid population showed peak expression of H3K9me2/3 writers *Ehmt2* and *Suv39h1*, which have been previously found related to heterochromatin formation during meiosis (Tachibana et al., 2007). Importantly, deficiency of these enzymes totally disrupts SCytes

maturation and induces cell arrest (Tachibana et al., 2007, Peters et al., 2001). Therefore, given their crucial role in the meiotic stage, the involvement of these KMT1 enzymes in STids may have been overlooked. At this stage, 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). We also observed high expression of KMT *Prdm2* that was found in an activation complex with p300 that drives Hac upon ER binding (Di Zazzo et al., 2013). *Nsd2* and *Ezh2* presented highly similar RPKM values in SGKit, SCyte and STid population, and we actually consider that the real peak expression of these enzymes comprises the three stages. At STid stage there is specific H3K27me deposition to stablish bivalency at developmental genes (Maezawa et al., 2018), and proteomic studies found substantial H3K27me3/K36me2 double marking in STid and sperm (Brunner et al., 2014; Luense et al., 2016). EZH2 and NSD2 were found to form a regulatory axis that promotes the oncogenic phenotype, where EZH2 acts upstream NSD2 regulating its expression and H3K36me2-mediated chromatin activation by a microRNA network (Asangani et al., 2013; Popovic et al., 2014). Hence, through this axis, EZH2 repressive marking leads to deposition of activating H3K36me2 marking, showing yet another level of complexity in the epigenetic landscape dynamics of cells. Additionally, the γH2AX-MDC1 signaling pathway is involved in the recruitment of NSD2 to DSBs, where it catalyzes H4K20me2 methylation involved in DNA damage repair pathway (Pei et al., 2011).

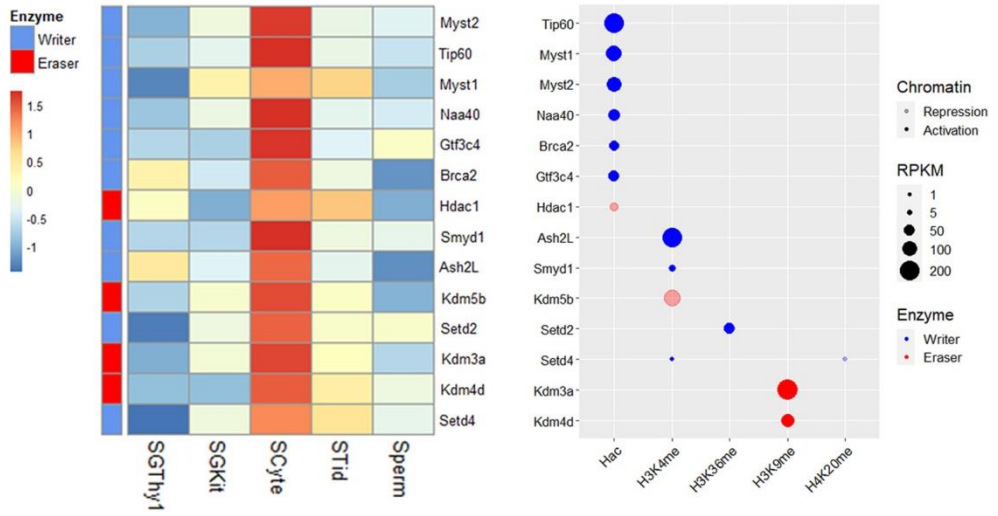
2.5 Enzymes with peak expression at mature sperm population

The mature sperm transports a cargo of RNAs that have been the focus of intense attention, composed of non-coding RNAs like miRNAs, lncRNAs, tRNAs and circRNAs, and protein-coding mRNAs (Guo et al., 2017; Jodar et al., 2013). Some of these RNAs are remnants of the last stages of elongated STids, and others are acquired along the passage through the epididymis, as sperm absorb epididymosomes released by somatic cells (Trigg et al., 2019). Sperm RNA sequencing shows a set of mRNAs, from marker genes of the spermatogenic process (Figure 2B), known as the “memory of spermatogenesis”, and many

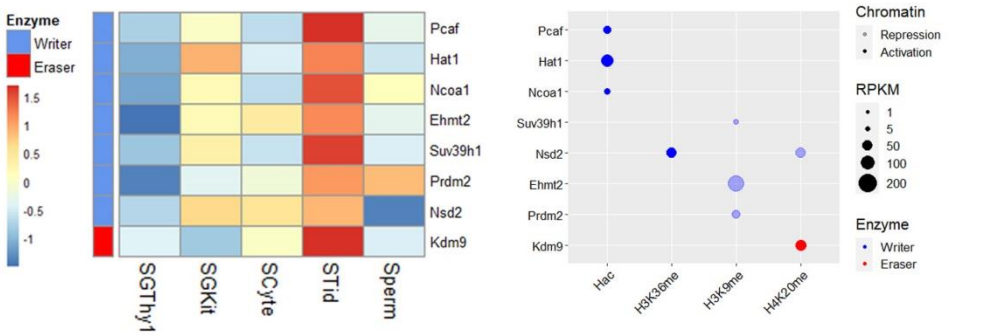
other RNAs that were found to carry instructions for early embryo development and metabolic and behavioral traits in the offspring (Guo et al., 2017; Jodar et al., 2013).

We found a very specific pattern in sperm cluster (Figure 4C) of HDACs including *Hdac9*, *Sirt2*, and *Hdac1*, and peak RNA values for HATs *Naa50* and *Myst3*. Importantly, HDACs are also highly detected in human late STids from integrated single-cell RNAseq data (Figure S1). There is no information concerning possible roles for the HATs and HDACs of the sperm cluster, but HDAC11 was linked to early embryo development, as will be discussed below. On the other hand, we observed peak expression of the H3K79me writer *Dotl1*, which was found expressed throughout embryogenesis and with apparently higher levels in the ovary and testis (Barry et al., 2010). Moreover, in ESCs, DOT1L may play a role in telomere maintenance since H3K79me2 is enriched at constitutive telomeric and centromeric heterochromatin (Jones et al., 2008). The DOT1L binding to the H4 tail is regulated by H4K16ac, and also deletion or overexpression of DOT1L leads to mislocalization of the SIRT complex, causing silencing defects (Farooq et al., 2016). Here, we also detected the H3K9me1/2 eraser *Kdm3b*, which would reflect other contributions than spermatogenesis, as this enzyme is highly expressed by the epididymal epithelium (Liu et al., 2015) and therefore, likely enriched in epididymosomes (Trigg et al., 2019). Finally, we also detected *Kdm2b* expression, which erases H3K4/K36me and was found to protect the polycomb-silenced promoters against ectopic de novo methylation (Boulard et al., 2015). Furthermore, KDM2B is identified as one subunit of non-canonical PRC1.1 complex (Wang et al., 2018), consistent with the last stages of spermiogenesis, when Polycomb KMT6 family and H3K27me2/3 expression are the highest (Figure 1B, C). Importantly, KDM2B was recently found to erase H3K79me2/3 and to induce transcriptional repression via SIRT1 chromatin silencing (Kang et al., 2018). Here, we also found *Setd7*, previously associated with mesoderm lineage differentiation, muscle and cardiac development (Bultman et al., 2019).

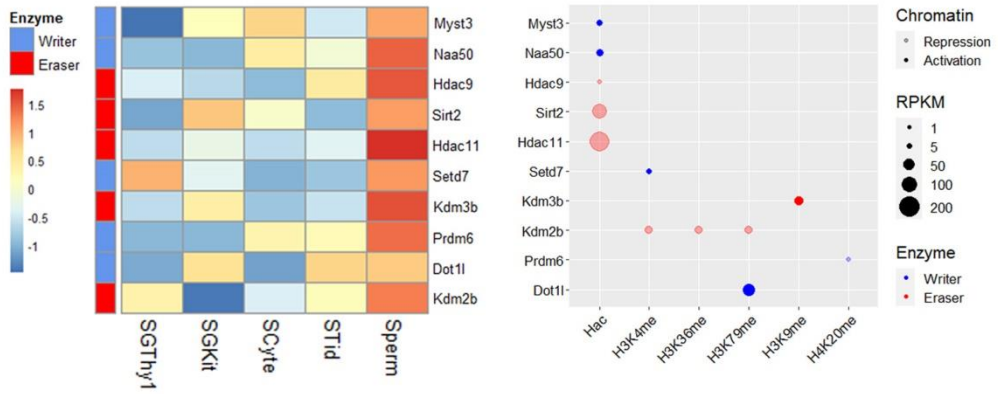
A *Cluster 3*: primary SCyte population



B *Cluster 4*: round STid population



C *Cluster 5*: mature Sperm population



482

Figure 4. Cluster 3, 4 and 5 epigenetic enzymes showing peak expression at primary SCyte (A), round STid (B) and mature sperm (B) populations. Left: heatmap showing z score values for each male germ cell population. Right: Dot plot showing enzymes RPKM values in primary SCyte (A), round STid (B) and sperm (C) population, respectively. SGThy1: spermatogonia Thy1+/Kit-, SGKit: spermatogonia Kit+, SCyte: primary spermatocyte, STid: round spermatid.

2.6 Sperm enzymes: potential contribution to early embryo development

Recent findings indicate that the sperm delivers RNAs to the oocyte at fertilization which stay stable until the activation of the embryonic genome, having putative roles at zygote formation (Boerke et al., 2007; Smith and Spadafora, 2005). Early molecular processes in the zygote include differential epigenetic reprogramming of the parental genomes, where PRMs are quickly released from the paternal genome to be repackaged by maternal nucleosomal histones (McLay and Clarke, 2003). The zygote genome is translationally 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). In this sense, Guo et al. (2017) characterized that sperm-carried RNAs are enriched in genes associated with the ontologies of embryonic cleavage and blastocyst formation. For example, it was shown that coding mRNA for the egg-activating factor PLC-zeta, is inherited by the father and participates in early embryo development (Yao et al., 2010; Sone et al., 2005).

Here, we evaluated the possible paternal contribution of epigenetic enzymes to the zygote, by analyzing RNAseq data from meiosis II (MII) oocytes and pre-cleavage zygotes (Wu et al., 2016). We searched for enzymes showing increased RPKM values in zygotes compared to MII-oocytes (Fold > 1, Table 2), indicative of potential paternal contribution to the zygotic mRNA pool. This approach allowed us to detect many marker mRNAs previously described as high in sperm and with roles early in embryo development (Guo et al., 2017), as well as many enzymes with peak expression in the different clusters that were also present in sperm population (Table 2). Remarkably, we detected several enzymes from the sperm cluster, including *Naa50*, *Hdac11*, *Sirt2*, and *Setd7*. In this context, previous work *in silico* identified *Hdac11* as a paternally-inherited mRNA that could potentially interact co-operatively and synergistically with maternal factors during zygote formation (Ntostis et al., 2017). Among enzymes of clusters 1-4, we found *Hdac1* expression, which

was previously linked to cell cycle regulation and ZGA during pre-implantation (Ma et al., 2008). We also detected key enzymes involved in chromatin structure organization: the H4K20me2/3 writer *Suv420h2*, the H3K9me3 writers *Suv39h1/2* and *Setdb2*, and H3K9me2 writer *Ehmt2*. In line with this, the active mark H3K4me3 is detected in the male pronucleus, but H3K9me2/3, H3K27me3 and H4K20me3, are mostly absent (Lepikhov et al., 2010). The parental pronuclei have asymmetric reprogramming capacities and the reprogramming factors reside predominantly in the male pronucleus. 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 SUV420H2 deposits H4K20me3 to allow the timely and coordinated progression of replication after fertilization (Eid et al., 2016). Finally, we also detected several other writers and erasers with unknown functions at pre-cleavage embryo.

Symbol	Sperm	MII_oocyte	Zygote	Fold
Clock	3.14	11.86	12.37	**1.04**
Myst1	28.90	9.98	25.49	**2.55**
Gtf3c4	18.18	0.24	0.27	**1.13**
Pcaf	6.12	0.03	0.07	**2.45**
Naa50	14.01	35.67	99.97	**2.80**
Hdac1	1.91	0	1.73	**-**
Hdac11	178.31	0	0.04	**-**
Sirt1	3.63	2.23	3.84	**1.73**
Sirt2	96.57	0.36	1.96	**5.45**
Sirt5	1.96	1.82	2.79	**1.53**
Sirt7	2.64	6.89	10.22	**1.48**
Ash2l	48.31	9.88	13.33	**1.35**
Setd7	4.14	0.01	0.01	**1.44**
Setd4	1.29	7.11	13.96	**1.96**
Nsd1	10.31	14.68	20.44	**1.39**
Nsd3	7.16	1.90	2.35	**1.24**
Prdm9	1.14	0.06	0.25	**4.27**
Setd5	14.71	0.71	1.05	**1.47**
Ehmt2	67.83	0	0.03	**-**
Suv39h1	1.36	1.54	3.61	**2.34**
Suv39h2	13.08	4.73	9.82	**2.08**
Setdb2	7.09	6.53	11.54	**1.77**
Suv420h1	7.64	0.47	1.30	**2.78**
Kdm5a	11.82	0.19	0.29	**1.50**
Kdm5b	42.30	0.74	1.30	**1.76**
Kdm5d	1.06	0	0.02	**-**
Kdm4c	3.32	0.02	0.10	**6.43**
Kdm7a	5.53	20.61	21.80	**1.06**
Aatf	61.530	2.866	4.098	1.430
Pik3ca	4.663	2.301	2.395	1.041
Rpl7l1	7.437	0.631	3.203	5.073
Rad51	1.937	66.832	78.557	1.175
Nek2	64.205	10.929	34.466	3.154
Itch	18.287	5.405	5.926	1.096
Serpine1	0.740	0	0.016	-
Sp1	10.705	0.728	2.144	2.945
Cacna1g	0.579	0.008	0.017	2.157
Pnrc1	8.158	6.300	38.548	6.119
Ltbp1	1.727	0	0.007	-
Map1lc3a	1858.090	0.566	1.415	2.501
Zfp821	495.639	5.905	6.054	1.025

528

Table 2: RPKM values of writers and erasers in mature sperm, meiosis II oocytes and pre-cleavage zigotes. Fold represent the ratio between zygote and MII oocytes RPKMs. Blue: writers, red: erasers, green: marker genes for paternal contribution to zygote RNA pool.

DISCUSSION

A new trend in research points toward sperm epigenome contribution to embryonic development and offspring health. However, few links were established between alterations in sperm histone PTMs, the specific enzymes responsible, and the developmental window where these marks may be reprogrammed. We show for the first time a comprehensive evaluation of all known families of histone acetylation and methylation writers and erasers from undifferentiated SG to mature sperm. Not only we accurately detected germ cell markers genes, but also by the rule of "guilty by association", enzymes with known functions and many others not even described yet in male germ cells. 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.

Many studies have implicated different histone PTMs in sperm chromatin as substrates for transgenerational inheritance (Skvortsova et al., 2018, Brunner et al., 2014, Hammoud et al., 2009; Brykczynska et al., 2010). Altered H3K4me3 in sperm was associated with diet-induced phenotypes in the offspring and developmental defects (Lismer et al., 2021). In *C elegans*, inheritance of H3K27me3-lacking sperm chromatin resulted in lost repression of neuronal genes from sperm-inherited alleles, which primed germ cells for losing their identity and adopting a neuronal fate (Kaneshiro et al., 2019). In *Drosophila*, paternal restraint stress affected offspring metabolism via ATF2-dependent mechanisms (Seong et al., 2020). H4K8/12ac was found prior to full decondensation of the sperm nucleus, suggesting that these marks are transmitted to the zygote (van der Heijden et al., 2006). Moreover, it was recently reported that alterations in the expression of paternal KDM1A/LSD1 and KDM6A enzymes were able to induced defects that persisted transgenerationally (Siklenka et al., 2015, Lesch et al., 2019).

Finally, we report that mature sperm carries several histone-modifying enzymes mRNAs with moderate to high RPKM values. Moreover, some of these enzymes' mRNAs were detected higher in the zygote than in the transcriptionally silent MII oocyte, pointing to a paternal contribution. Many pieces of evidence showed that sperm RNAs, in particular miRNAs and tsRNAs, can mediate intergenerational transmission of paternally acquired phenotypes such as diet-induced metabolic and neurological phenotypes (Chen et al., 2016). Moreover, it was proposed that their mechanisms involve changes in chromatin structure and DNA methylation. Dynamic chromatin remodeling by histone PTMs occurs after fertilization to facilitate the transition from gametic-to-embryonic chromatin, and inherited mRNAs from the gametes are selectively translated before the ZGA. The data presented here, 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, cleaning and processing

Data was manipulated using Rstudio (v1.2.1033) and Tidyverse packages Dplyr and ggplot2 (Wickham et al., 2019). Male germ cell processed RNA-seq data tables (RPKM) were downloaded from NCBI GEO database, deposited by Hammoud et al., (2014), under accession number GSE49622. Data contained one replica of SGThy, spermatogonia SGKit and mature sperm, and 5 replicas of primary SCyte and round STid. We removed 54 extra genes in SCyte, STid and sperm that were not included in the SG tables. The mean value for each gene was calculated in SCyte and STid samples, and generated a data matrix by merging the mean SCyte and mean STid with SGThy1, SGKit and sperm samples (RPKM matrix). The accumulated RPKM for each enzyme family was calculated and generated stacked bar plots. To perform clustering analysis, RPKM matrix was converted to z score by gene and performed k-means clustering from the R Base Stats package, selectig k=5 and nstart = 25. We generated an enzyme metadata table, and retrieved the genes of interest RPKM values and cluster assigned. Heatmap plots were plotted using pheatmap package from R base. The complete samples tables, enzymes metadata table and R scripts used in

this study are available for download and further editing at https://github.com/Gonzalez-Lab/Gonzalez-2022-germ-cells. Finally, meiosis II oocyte and zygote accessed RNA-seq data tables (RPKM) were downloaded from NCBI GEO database, deposited by Wu et al., (2016), under accession number GSE66582.

Author contributions

B.G and C.R.G conceived the project, analyzed the data and wrote the manuscript. 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.

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