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ADVANCED REVIEW

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Insights into the sperm chromatin and implications for male infertility from a protein perspective

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Abstract

Male germ cells undergo an extreme but fascinating process of chromatin remodeling that begins in the testis during the last phase of spermatogenesis and continues through epididymal sperm maturation. Most of the histones are replaced by small proteins named protamines, whose high basicity leads to a tight genomic compaction. This process is epigenetically regulated at many levels, not only by posttranslational modifications, but also by readers, writers, and erasers, in a context of a highly coordinated postmeiotic gene expression program. Protamines are key proteins for acquiring this highly specialized chromatin conformation, needed for sperm functionality. Interestingly, and contrary to what could be inferred from its very specific DNA-packaging function across protamine-containing species, human sperm chromatin contains a wide spectrum of protamine proteoforms, including truncated and posttranslationally modified proteoforms. The generation of protamine knock-out models revealed not only chromatin compaction defects, but also collateral sperm alterations contributing to infertile phenotypes, evidencing the importance of sperm chromatin protamination toward the generation of a new individual. The unique features of sperm chromatin have motivated its study, applying from conventional to the most ground-breaking techniques to disentangle its peculiarities and the cellular mechanisms governing its successful conferment, especially relevant from the protein point of view due to the important epigenetic role of sperm nuclear proteins. Gathering and contextualizing the most striking discoveries will provide a global understanding of the importance and complexity of achieving a proper chromatin compaction and exploring its implications on postfertilization events and beyond.

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male infertility, protamines, sperm, sperm chromatin, sperm epigenetics

1 | INTRODUCTION

Haploid paternal genome needs to be tightly wrapped into highly specialized structures for a proper sperm function. Sperm chromatin compaction is part of the complex process of male germ cell development named spermatogenesis. Spermatogenesis comprises three well-defined phases: (1) a mitotic phase, in which type A replicating spermatogonia are in charge of maintaining the germ cell niche, whereas type B spermatogonia commit to meiosis and divide mitotically one more time to generate primary spermatocytes, (2) a meiotic phase, in which primary spermatocytes undergo two consecutive meiotic divisions: the first one to generate two diploid secondary spermatocytes, and the second one without a previous DNA replication step to generate haploid round spermatids, and (3) a phase called spermiogenesis, in which postmeiotic round spermatids elongate through a series of marked nuclear, cytoplasmic, and morphological changes (Bolcun-Filas & Handel, 2018; de Kretser et al., 1998; Hecht, 1998; Oliva & Castillo, 2011; Oliva & Dixon, 1991).

Sperm nuclear compaction is achieved through the process known as nucleo-histone to nucleo-protamine (NH-NP) transition, which occurs during spermiogenesis (Christensen & Dixon, 1982; Grimes & Henderson, 1984; Hazzouri et al., 2000; Oliva et al., 1987; Oliva & Castillo, 2011; Oliva & Mezquita, 1982). Through this process, small, extremely basic protamines replace most of the histones, leading to highly packaged toroidal and rod-like structures that are able to compact 250 times more DNA than a nucleosome (Ausió et al., 2014; Balhorn, 2007; Bao & Bedford, 2016; Castillo, Amaral, & Oliva, 2014; Castillo et al., 2015; Gaucher et al., 2010; Torres-Flores & Hernández-Hernández, 2020; Vilfan et al., 2006; T. Wang, Gao, et al., 2019). A complex regulation is needed during NH–NP transition, involving posttranslational modifications (PTMs) and associated modulating proteins, in the context of a fine-tuned postmeiotic gene expression program of genes encoding the main proteins taking part in the process (Blanco & Cocquet, 2019; T. Wang, Gao, et al., 2019).

Despite being hydrodynamically shaped, testicular sperm are still immature and unable to fertilize on their own. Sperm maturation continues outside the testes during transit and storage through the male reproductive tract, where forward progressive motility is acquired, membrane composition and fluidity are modified, and sperm chromatin structure is stabilized. The contact of spermatozoa with fluids secreted by the epididymis and the accessory sex glands gives rise to fully mature and motile ejaculated sperm with fertilizing potential (Dacheux & Dacheux, 2014; Gervasi & Visconti, 2017; Jodar et al., 2017; Orgebin-Crist, 1969; Schoysman & Bedford, 1986; Yoshida et al., 2018).

Opposed to the traditional idea that the spermatozoon is a simple carrier of paternal genetic information, the application of high-throughput techniques has proven that the mature sperm cell contains a wide set of layers of information that are transmitted to the zygote and can impact fertilization and beyond (Castillo et al., 2018; Castillo, Amaral, Azpiazu, et al., 2014; Castillo, Amaral, & Oliva, 2014; Chen et al., 2016; Hammoud et al., 2009; Krawetz, 2005; Rando, 2012; Siklenka et al., 2015). In addition, alterations in the sperm epigenetic cargo, as well as impairments during the spermiogenic process, are associated with male infertility (Carrell et al., 2008; Castillo et al., 2015; Hammoud et al., 2010, 2011). When referring to the evaluation of the epigenetic potential of sperm chromatin, many studies have focused mainly on DNA methylation and chromatin organization (Hammoud et al., 2010; Laurentino et al., 2016; Leitão et al., 2020; Siklenka et al., 2015). However, advances in the description of the sperm proteome have revealed that the male gamete carries a substantial amount of proteins with epigenetic roles, being involved in gene expression regulation through the modulation of transcription, biogenesis of noncoding RNAs, and establishment of DNA and histone marks (Castillo et al., 2015, 2018; Castillo, Amaral, Azpiazu, et al., 2014; Castillo, Amaral, & Oliva, 2014; de Mateo, Castillo, et al., 2011). Although the presence of some of these proteins in mature sperm could represent simple leftovers of their spermatogenic activity, others may also have an impact during fertilization events or preimplantation embryo development, contributing to the early embryonic epigenome (Castillo et al., 2018). In the present review, we aim to highlight, from a protein point of view, the critical impact of the tightly regulated process of sperm chromatin compaction and its associated epigenetic marks toward the formation of a fully functional male gamete with a role beyond fertilization. To that end, we summarize the process of protamine-dependent histone removal to compile the latest findings on its orchestrated epigenetic regulation, giving rise to the complex final structure of sperm chromatin. In addition, we review the most recent knowledge on the most abundant protein components of mature sperm chromatin, the protamines, which have traditionally been less well characterized than histones in terms of their function, marks, and epigenetic potential. Finally, we propose our model of sperm chromatin structure and gene distribution according to the latest available evidence, based on a wide range of complementary techniques.

2 | THE SYNCHRONIZED MOLECULAR PROCESS OF NH–NP TRANSITION IN THE MALE REPRODUCTIVE TRACT

2.1 | Synergistic, rather than sequential, mechanisms of testicular histone replacement

The NH–NP transition is a unique process of chromatin remodeling taking place in male germ cells during spermiogenesis, which gives rise to tightly compacted nuclei necessary to produce functional sperm (Christensen & Dixon, 1982; Grimes & Henderson, 1984; Hazzouri et al., 2000; Mezquita, 1985; Oliva et al., 1987; Oliva & Castillo, 2011; Oliva & Mezquita, 1982). It comprises a gradual replacement of somatic cell-like core histones by testis-specific histone variants, followed by transition proteins 1 and 2 (TP1 and TP2, respectively) and, finally, by protamines (Balhorn, 2007; Barrachina et al., 2018; Castillo et al., 2011; Gatewood et al., 1987; Kimmins & Sassone-Corsi, 2005; Oliva, 2006; Oliva & Dixon, 1991; Rathke et al., 2014). Two protamines have been described in mammalian sperm, protamine 1 (P1), which is synthetized as mature form, and protamine 2 (P2), which arises from the proteolytic cleavage of a precursor (pre-P2) (Oliva, 2006). This tightly regulated process results in 85%–95% of the paternal human genome (92%–98% in mouse) being packed with protamines, giving rise to two differential chromatin regions: the nucleo-histone domain, containing the most accessible fraction of the genome; and the nucleo-protamine domain, inaccessible and silenced (Arpanahi et al., 2009; Castillo, Amaral, Azpiazu, et al., 2014; Hammoud et al., 2009).

The incorporation of histone variants contributes to nucleosome conformational changes and the destabilization needed to achieve an open chromatin state. Male germ cells express the largest number of histone variants, including unique testicular variants of H2A, H2B, and H3, as well as almost all histone variants present in somatic nuclei (Barrachina et al., 2018; Boussouar et al., 2008; Ding et al., 2021; Hoghoughi et al., 2018). Changes in amino acid sequence of testis-specific histone variants, such as TH2A, TH2B, and H2BFWT, and other histone variants, such as H3.3 and H3.4, are responsible for low-affinity binding to DNA (Bonisch & Hake, 2012; Boulard et al., 2006; Li et al., 2005; Padavattan et al., 2017; Tachiwana et al., 2010; Ueda et al., 2017; Urahama et al., 2016). Loss of TH2A and TH2B in mice has been related to impaired histone retention and sterility (Shinagawa et al., 2015), and depletion of the two H3.3 encoding genes (*H3f3a* and *H3f3b*) has been related to male subfertility and sterility in mouse, respectively (Tang et al., 2015; Yuen et al., 2014). Furthermore, novel histone variants have recently been discovered in testis, like H2B.K and H2B.N, without a specific function yet, likely redundant but vital for unusual chromatin packing (Raman et al., 2022).

While traditional approaches have shown that the incorporation of histone variants, TPs, and protamines during spermatogenesis, occurs in a purely sequential way (Balhorn, 2007; Bao & Bedford, 2016; Barrachina et al., 2018; Gaucher et al., 2012; Kimmins & Sassone-Corsi, 2005; Lewis et al., 2003; Meistrich et al., 2003; Oliva, 2006; Oliva & Castillo, 2011; Oliva & Dixon, 1991; Rathke et al., 2014), recent findings have pointed to a more synergistic than sequential NH–NP process (Arévalo et al., 2022; Barral et al., 2017; Rezaei-Gazik et al., 2022). It has been found that the synthesis of the spermatid-specific histone variant H2A.L.2, coincides with that of TPs, and that its depletion would lead to an aberrant loading of TPs in the chromatin and an inadequate processing of pre-P2. This suggests that H2A.L.2 creates an open conformation of the nucleosome that allows TP loading into chromatin (Barral et al., 2017). Furthermore, TPs are spatiotemporally co-expressed with pre-P2, which suggests their joint action during NH–NP transition (Rezaei-Gazik et al., 2022), and co-exist with protamines in late spermatids, when histones are still detectable (Meistrich et al., 2003; Zhao et al., 2004). Interestingly, recent evidence has shown that pre-P2 cleaved domain (cP2) mediates the correct interaction between TP1-H2A.L.2 complexes and pre-P2 (Arévalo et al., 2022). Therefore, under normal conditions, TPs are loaded into H2A.L.2-containing nucleosomes, not displacing histones but generating an open chromatin interface for protamine recruitment and pre-P2 processing, orientating protamines to finally displace histones (Arévalo et al., 2022; Barral et al., 2017). Indeed, the specific depletion of cP2 domain does not allow the correct incorporation of P2 into sperm chromatin, being P2 accumulated in the cytoplasm of spermatids and residual bodies (Arévalo et al., 2022). Besides, the specific loss of TP2 is responsible for the accumulation of pre-P2, which ends up in altered levels of P2 (Zhao et al., 2001). Knockout mouse models of the genes encoding TP1 and TP2 (*Tnp1* or *Tnp2*, respectively) are subfertile, producing sperm with abnormal chromatin condensation. Of note, these mice seem to compensate the lack of one of the TPs by overexpressing the other, so that only the simultaneous depletion of both genes would end in sterility (Shirley et al., 2004; Yu et al., 2000; Zhao et al., 2001). Despite that, testicular spermatids and few epididymal sperm, whose chromatin compaction is impaired, are generated in the double *Tnp1–Tnp2* knockout. This would support that protamines would be capable to replace histones without the participation of TPs (Shirley et al., 2004).

All this evidence highlights protamines as the main actors of histone displacement during spermiogenesis (Barral et al., 2017; Meistrich et al., 2003; Oliva et al., 1987; Oliva & Mezquita, 1986). In fact, it has been shown that the mere presence of protamines allows DNA to be compacted in vitro (Makita et al., 2011; Oliva et al., 1987; Oliva & Mezquita, 1986). For instance, human protamine 1 (P1) can displace nucleosomes from somatic chromatin, leading to a spermatid-like nuclear compaction in non-germ cell types (Iuso et al., 2015). Strikingly, this P1-compacted somatic nucleus can be reversibly loosened by maternal histones after in vitro fertilization of an oocyte, producing embryos that progress until blastocyst stage (Iuso et al., 2015). Similarly, P2 condensation ability has been proved in HEK cells, observing fully condensed nuclei after 48 h cell culture (Arévalo et al., 2022). Nonetheless, the process of paternal genome compaction is way more complex than simple affinity of protamines to DNA, with a tight epigenetic regulation underlying protamine incorporation.

2.2 | The regulation of the NH–NP transition through protein modification and implications in male fertility

2.2.1 | Posttranslational modification of proteins regulating the NH–NP transition

Global histone H4 hyperacetylation wave is crucial for a proper NH–NP transition, as it neutralizes the positivity of histone tails and destabilizes the histone–DNA and nucleosome–nucleosome interactions (Barrachina et al., 2022; Hazzouri et al., 2000; Oliva & Mezquita, 1982). In fact, acetylation on H4 Lys 5 and 8 (H4K5K8ac) is the first step toward nucleosome instability and histone eviction for large-scale remodeling of the chromatin. H4K5K8ac is recognized by the first bromodomain of the bromodomain testis-specific protein (BRDT), which triggers the NH–NP transition (Dhar et al., 2012; Goudarzi et al., 2014; Her et al., 2021; Manterola et al., 2018; Morinière et al., 2009; Pivot-Pajot et al., 2003; Shang et al., 2007). Together with H4 hyperacetylation, a specific wave of H3 Lys79 trimethylation (H3K79me3) also precedes the NH–NP transition, specifically, before TP1 incorporation, potentially contributing to its regulation (Dottermusch-Heidel et al., 2014; Moretti et al., 2017). Besides, residue-specific lysine acetylation of other histones, as described for H3, contributes to the final global histone hyperacetylation state, destabilizing nucleosomes and favoring their removal (Grimes & Henderson, 1984; Hazzouri et al., 2000; Nair et al., 2008; Steilmann et al., 2011; Tan et al., 2011).

In the last years, other less-known histone PTMs from the group of acylations have been identified in histones (Crespo et al., 2020; Sabari et al., 2017; Tan et al., 2011). Acylations such as propionylation, butyrylation, and crotonylation have similar physicochemical properties to those of histone acetylation, which would also lead to an open conformation of the nucleosome favoring histone eviction (Sabari et al., 2017). Interestingly, butyrylation on H4K5 (together with either acetylation or butyrylation on K8) is not recognized by the first bromodomain of BRDT, thus escaping histone detachment. This could lead either to a temporarily regulated histone replacement in specific nucleosomes during spermiogenesis, or to histone retention of specific gene sequences in mature sperm (Goudarzi et al., 2016). It has been shown that, in addition to the effect on relaxing nucleosomal conformation, PTMs also mark histones for a subsequent proteasomal degradation needed to achieve a proper histone replacement, as it is the case of histone (poly)ubiquitination of core histones H2A and H2B (Baarends et al., 1999; Gou et al., 2017; Guo et al., 2018; Meng et al., 2019; Shmueli et al., 2021; X. Wang, Kang, et al., 2019). The spermatoproteasome, a proteasome subunit alpha type-8 (PSMA8)-containing proteasome enriched in haploid cells, also plays a key role degrading histones in an acetylation-dependent manner (Gómez-H et al., 2019; Živković et al., 2022). This evidence on the tight regulation of histone replacement by PTMs highlights the need for further insight into the role during spermiogenesis of less well-characterized histone modifications, such as non-acetyl histone acylations. For instance, crotonylation of H3K27, although not confirmed as direct regulator of the NH-NP transition, has been detected in high abundance in postmeiotic cells, concomitant to H4 hyperacetylation and histone removal, as well as retained in sperm, which suggests a comparable function to that of H4K5 and K8 butyrylation (Crespo et al., 2020). Interestingly, crotonylated H3K27, together with acetylation in the same residue, induces the highest expression levels of spermiogenesis-specific genes, such as TP2 and P1 (Crespo et al., 2020).

Histone variants and TPs also bear PTMs, which affects their binding affinity to DNA (Gupta et al., 2015, 2017; Hazzouri et al., 2000; Kwak & Dohmae, 2016; Mishra et al., 2015; Pentakota et al., 2014; Pradeepa et al., 2009; Singh & Parte, 2021). Of note, TP2 acetylation reduces its ability to condense DNA (Pradeepa et al., 2009), suggesting that modified TPs may contribute to establish the final sperm epigenome.

Epigenetic regulation of spermiogenesis through the modification of specific protein residues has also been observed in protamines from different mammalian species, including human, mice, stallion, boar, bull, and ram (Arauz-Garofalo

et al., 2021; Brunner et al., 2014; Castillo et al., 2015; Chirat et al., 1993; Pirhonen, Linnala-Kankkunen, & Mäenpää, 1994; Pruslin et al., 1987; Soler-Ventura et al., 2020). The most relevant modification in protamines is phosphorylation, which regulates their binding to DNA. The negative charge of the phosphate group leads to a weaker affinity of phosphorylated protamines for negatively charged DNA, compared to unmodified protamines (Willmitzer et al., 1977a, 1977b). Thus, while protamine phosphorylation would be necessary to incorporate protamines into DNA, subsequent dephosphorylation is needed for tight sperm chromatin compaction (Itoh et al., 2019; Willmitzer et al., 1977a).

2.2.2 | The interplay between PTMs and their modulators during NH–NP transition

Protein modification and its implication on cellular processes are modulated by proteins that incorporate PTMs to specific amino acids (writers), recognize PTMs from specifically modified residues (readers), or remove PTMs from already modified amino acids (erasers; Jenuwein & Allis, 2001; Ruthenburg et al., 2007; Strahl & Allis, 2000). In the context of sperm chromatin compaction, histone acetyltransferases (HATs), which are writers of histone acetylation, develop an important role in the mediation of NH–NP transition. This process is mediated by the recruitment of Histone acetyltransferase p300 (p300 HAT) and CREB-binding protein (CBP) by Nuclear protein in testis (NUTM1, also known as NUT), which enhances H4K5/K8 acetylation (Shiota et al., 2018). Similarly, the components Enhancer of polycomb homolog 1 (EPC1) and Histone acetyltransferase Tip60 (TIP60) of the mammalian nucleosome acetyltransferase of H4 (NuA4) complex, are involved in histone hyperacetylation during spermiogenesis (Dong et al., 2017). It is also notable the role of BRDT as one of the main readers of histone PTMs during this last phase of spermatogenesis, which not only recognizes acetylated H4K5K8 to trigger and amplify the process of histone eviction, but also interacts with transcription factors in round spermatids to regulate gene expression of target genes essential for this developmental stage (Her et al., 2021). Modulators of histone methylation have been also shown to control the expression of TPs and protamine genes (Eelaminejad et al., 2017; Z. Liu et al., 2010; Okada et al., 2007, 2010; Zuo et al., 2018). Details of these proteins and additional examples, the deregulation of which has been related with male infertility and impaired chromatin compaction, can be found in Table 1.

Additionally, the epigenetic control of the NH–NP transition seems to be also affected by a crosstalk among different histone PTMs and modulators, suggesting a new layer of coordination. For instance, reduced expression of the Sypc3-like Y-linked (*Sly)* gene in mice, which is exclusively expressed in postmeiotic male germ cells, deregulates not only Histone–lysine N-methyltransferase, H3 lysine-79 specific (DOT1L) but also H4ac levels in elongated spermatids, leading to a global alteration of the state of chromatin compaction (Moretti et al., 2017). DOT1L also recognizes ubiquitinated H2B, enhancing a noncatalytic function of this methyltransferase on nucleosome destabilization, suggesting an interplay between H3 methylation and H2B ubiquitination toward nucleosome removal (Jang et al., 2019).

Although protamines and their PTMs have been historically less studied than histones, modulators of protamine phosphorylation have been also identified. SRSF protein kinase 1 (SRPK1) and Calcium/calmodulin-dependent protein kinase type IV (CAMK4) phosphorylate P1 and P2 in the testis, respectively (Papoutsopoulou et al., 1999; Wu et al., 2000). Additional candidates have been found in vitro, such as c-AMP-dependent protein kinase A (PKA), which phosphorylates P1, and protein kinase C (PKC), which phosphorylates P2 (Pirhonen, Linnala-Kankkunen, & Mënpää, 1994). In terms of erasers, while no P1-specific phosphatases have been described yet, the germ cell-specific isoform 2 of Serine/threonine-protein phosphatase PP1-gamma catalytic subunit (PP1γ2) has been found to be involved in the removal of phosphate groups from P2 in mice (Itoh et al., 2019). Depletion of the chaperone needed for recruiting *PP1γ2* to the sperm chromatin (Heat shock 70 kDa protein 4L, HS74L) leads to infertility in the knockout mice due to altered sperm head morphology, being normal phenotype rescued when the P2 phosphorylation site is substituted by a non-phosphorylatable residue. These interesting results suggest that PP1γ2-mediated dephosphorylation is essential to achieve mouse fertility avoiding abnormal P2 phosphorylation levels in epididymal sperm (Itoh et al., 2019).

All studies mentioned above reinforce the idea of a highly orchestrated process of epigenetic regulation through a complex interplay between histone PTMs and their writers, readers, and erasers.

2.2.3 | Posttesticular events involved in sperm chromatin stabilization

Interestingly, the formation of sperm chromatin does not finish when condensed spermatids (immature sperm) are released from the testis but continues through crucial events taking place in the epididymis (Yoshida et al., 2018). Cannabinoid

TABLE 1 Writers, readers, and erasers of posttranslational modifications of histone and protamines expected to be involved in the regulation of the nucleo-histone to nucleo-protamine transition

Name	Molecular role	Description	References
Effectors of histones and protamines PTMs			
BRD4	Bromodomain-containing protein, reader of histone acetylation	Acetylation reader, with preference for acetylated H4 over H3 in vivo. Associated with active genes in round spermatids. Differential gene expression through specific binding to residue-specific (H3K27) histone acetylation but not butyrylation, unlikely to bind crotonylation in the same residue. Potentially involved in nuclear proteins eviction linked to acrosome formation during spermiogenesis	(Bryant et al., 2015; Crespo et al., 2020; Ji et al., 2015; Vollmuth & Geyer, 2010)
BRDT	Bromodomain-containing protein, reader of histone acetylation	Reader of H4 acetylation. First bromodomain recognition of H4K5K8 is essential to trigger NH–NP transition, depletion leads to infertility (postmeiotic arrest). Gene expression regulation of postmeiotic genes	(Dhar et al., 2012; Gaucher et al., 2012; Her et al., 2021; Manterola et al., 2018; Pivot-Pajot et al., 2003; Shang et al., 2007)
CAMK4	Calcium/calmodulin-dependent kinase	Phosphorylates P2, depletion of Camk4 leads to infertility, impaired elongating spermatids with prolonged retention of Tnp2 and specific loss of P2	(Wu et al., 2000)
CDYL	Chromodomain-containing protein, reader of histone methylation	Histone crotonylation regulator facilitating TP1 and PRM2 incorporation. Depletion of CDYL caused changes in histone methylation and acetylation profiles and altered gene expression, giving rise to teratozoospermia and progressive infertility in adulthood	(S. Liu et al., 2017; Xia et al., 2019)
DOT1L	Histone methyltransferase	Methyltransferase in the testis of H3K79, which precedes the NH–NP transition, more precisely prior TP1 incorporation	(Dottermusch-Heidel et al., 2014; Moretti et al., 2017)
EPC1	Component of NuA4 histone acetyltransferase (HAT) complex	Its depletion, together with TIP60, leads to impaired histone H4 hyperacetylation and subsequent histone replacement	(Dong et al., 2017)
HDAC3	Histone deacetylase	Inactivates meiotic program and activates postmeiotic genes during meiotic exit. Depletion of HDAC3 leads to infertility due to meiotic/early-stages round spermatids arrest, and increased H3 acetylation in spermatocytes and round spermatids. Coordinates gene expression program with SOX30	(Yin et al., 2021)
MRG15	Component of the NuA4 HAT complex	Recognizes methylated H3K36 and regulates alternative splicing mediated by polypyrimidine tract binding protein PTB, part of the HAT and HDAC complexes balancing histone acetylation	(Hayakawa et al., 2007; Iwamori et al., 2016)
P300	Histone acetyl-transferase	Involved in histone H4 hyperacetylation and essential for histone-to-protamine exchange. Acetylation of TP2 by p300 leads to significant reduction in its DNA condensation	(Pradeepa et al., 2009; Reynoird et al., 2010; Shiota et al., 2018)

TABLE 1 (Continued)

Name	Molecular role	Description	References
PA200	Component of the proteasome, specifically recognizing acetylated histones, promoting degradation of core histones	Depletion leads to hypofertility with abnormal sperm, core histone retention in elongating spermatids due to defective acetylation-dependent histone proteasomal degradation. Its level of incorporation to the spermatoproteasome-containing PSMA8 subunit is 10-fold higher in spermatids than in early spermatogenesis, key for proteasomal activity during spermiogenesis	(Khor et al., 2006; Qian et al., 2013; Živković et al., 2022)
PHF7	Ubiquitin ligase	E3-ubiquitin ligase for H3K14 in postmeiotic cells, required for histone to protamine transition. Stabilizes BRDT by attenuating BRDT ubiquitination	(Kim et al., 2020; X. Wang, Kang, et al., 2019)
PP1γ2	Protein phosphatase	Dephosphorylates Ser56 of protamine 2 in mouse. HSPA4L-mediated chromatin localization of PP1γ2 is essential for fertility and maintenance of low oxidative stress levels during epididymal transit	(Itoh et al., 2019)
SIRT1	Histone deacetylase	Loss of Sirt1 causes defects on H4 Lys5, 8, and 12 acetylation, and subsequent histone removal	(Bell et al., 2014)
SRPK1	Serine/threonine kinase	Phosphorylation of protamine 1 in the testis, which favors protamine incorporation into the DNA	(Gou et al., 2020; Papoutsopoulou et al., 1999; Willmitzer et al., 1977a, 1977b)
TIP60	Catalytic subunit of NuA4 histone acetyltransferase (HAT) complex	Its depletion, together with Epc1, leads to impaired histone H4 hyperacetylation and subsequent histone replacement	(Dong et al., 2017)

receptor 1 (CB1) promotes the generation of disulfide bonds during epididymal transit, stabilizing the structure of sperm chromatin (Chioccarelli et al., 2020). Of note, CB1 depletion has been shown to reduce H4 acetylation and increase TP2 in the testis, as well as to induce increasing levels of sperm DNA fragmentation along the epididymis (Chioccarelli et al., 2010), suggesting that disulfide bonds formation is essential for posttesticular chromatin stability and DNA integrity. Other interesting approaches suggest additional mechanisms of chromatin stabilization during epididymal transit, such as protamine dephosphorylation which result in the release of zinc molecules acquired in the testes, mainly by P2, during protamine incorporation to DNA (Bianchi et al., 1992; Björndahl & Kvist, 2011; Dias et al., 2006; Gou et al., 2020; Itoh et al., 2019; Schneider et al., 2020). All this indicates that, even though the focus of the study of NH–NP transition has mostly been set on the testicular processes, attention should also be paid to posttesticular events that would contribute to the acquisition of a proper chromatin structure to protect the paternal genome from external damage (Box 1).

3 | THE UNIQUE FEATURES OF THE MATURE SPERM CHROMATIN CONTENT

3.1 | Different levels of DNA accessibility and protein affinity in the sperm chromatin with implications in male infertility

The interest in deciphering whether the peculiar chromatin structure remaining in the mature male gamete is predetermined and with a role beyond oocyte fertilization has been present since the 20th century. In fact, in 1987, it was published the first study reporting the presence of sequence-specific nucleo-histone and nucleo-protamine domains in human sperm (Gatewood et al., 1987). Although this was followed by many other studies applying conventional strategies and focusing on specific genes with complementary results (Gardiner-Garden et al., 1998; Nazarov et al., 2008;

BOX 1 Usefulness of deciphering the molecular mechanisms governing the NH–NP transition toward a complete in vitro development of human haploid male germ cells

The knowledge obtained from the molecular study of the NH–NP transition could be useful in the future to help developing complete human spermatogenesis in vitro. Functional sperm have been successfully generated from mouse testicular tissue culture (Sato, Katagiri, Gohbara, et al., 2011; Sato, Katagiri, Yokonishi, et al., 2011; Yokonishi et al., 2014). However, despite the great advances obtained using different approaches of in vitro human spermatogenesis, no elongated spermatids have been achieved yet (Baert et al., 2017; Medrano et al., 2018; Portela et al., 2019; Reda et al., 2016; Richer et al., 2021; Stukenborg et al., 2009). Even though meiosis is one of the most striking points to be overcome in these in vitro systems using testicular tissue (Lei et al., 2021), a low number of round spermatid-like haploid cells have been originated from human-induced pluripotent stem cells (Eguizabal et al., 2011). Still, they did not progress through differentiation, evidencing the importance and complexity of the postmeiotic phase regulation.

Wykes & Krawetz, 2003; Zalenskaya et al., 2000), it was not until the application of high-throughput techniques for DNA sequencing that the main advances in this field were obtained. In 2009, it was proposed for the first time, in two independent studies, the specific association of nucleosomal DNA to regulatory regions of developmental genes characterized by high CpG density in both human and mice (Arpanahi et al., 2009; Hammoud et al., 2009). These and subsequent studies applying similar strategies confirmed those previous results suggesting a nonrandom DNA distribution through sperm chromatin domains and pointing to its role as an epigenetic mark with impact on early embryo development (Brykczynska et al., 2010; Castillo et al., 2015; Castillo, Amaral, Azpiazu, et al., 2014; Erkek et al., 2013; Saida et al., 2011; Vavouri & Lehner, 2011).

Nevertheless, this remarkable enrichment in genic regions with crucial developmental functions contrasts with the fact that the male gamete contains only a small proportion of the whole genetic material associated with histones (approximately 2% and 8% in mouse and human, respectively), which is the most vulnerable and exposed DNA to damaging factors. In this regard, several studies have suggested an opposing composition of the sperm nucleo-histone domain. While the specific distribution seems to be maintained, some groups suggested that the association of nucleosomal DNA to regulatory gene regions was minimal, while the major part localizes on repetitive sequences and intergenic regions (Carone et al., 2014; Samans et al., 2014). This agreed with previous studies showing the association of histones with telomeric DNA by using methods based on DNA probes and Southern hybridation (Zalenskaya et al., 2000), as well as to centromeric and pericentromeric regions using immunostaining (Govin et al., 2007; van der Heijden et al., 2006). As a potential explanation for these contrary reports regarding the content of the sperm nucleosomal DNA, it has been suggested the presence of technical artifacts at the levels of chromatin dissection and sequencing data analysis (Carone et al., 2014; Royo et al., 2016). DNA digestion by micrococcal nuclease (MNase) has been the preferred method to isolate sperm histone-associated DNA (Arpanahi et al., 2009; Brykczynska et al., 2010; Carone et al., 2014; Castillo, Amaral, Azpiazu, et al., 2014; Erkek et al., 2013; Hammoud et al., 2009; Ozturk et al., 2021; Saida et al., 2011; Samans et al., 2014; Table 2). Nevertheless, the amount of enzyme used for chromatin dissection has been suggested as a critical source of variability. By comparing light (<10 U per 100 million sperm) and longer (>10 U per 100 million sperm) MNase digestions in crosslinked sperm chromatin, Carone and collaborators reported the presence of two types of nucleosomes: (1) a minor group with higher stability located around promoters and (2) a predominant group more sensible to the method and associated to gene-poor regions (Carone et al., 2014). According to this, sperm DNA enriched in regulatory regions of developmental genes would represent an infrequent population of highly stable nucleosomes with specific histone modifications and variants. However, it is important to remark that two studies have reported human and mouse sperm nucleosomal enrichment in DNA surrounding transcriptional start sites using light MNase digestions (Arpanahi et al., 2009; Castillo, Amaral, Azpiazu, et al., 2014). This fact leaves the only difference between these studies and Carone's protocol limited to a previous formaldehyde-crosslinking of the sperm in the latter, a technical factor that might be also considered to induce variability on the results (Table 2). Of note, one of these studies applying light MNase digestions with no previous crosslinking showed the presence of two nucleosomal particles, differing on size and located in nonoverlapping sites (Castillo, Amaral, Azpiazu, et al., 2014). In spite of this, both types of nucleosomes showed a preference association to CpG enriched regions and transcriptional start sites (Castillo,

TABLE 2 Type of major genomic sequences associated with histones in sperm, according to the experimental procedure followed for nucleosomal DNA extraction in experiments applying DNA sequencing analyses. MNase, Micrococcal nuclease; NPM, nucleoplasmin

MNase-based digestion				Alternative strategies		
Light digestion (<10 U/100 million)		Longer digestion (>10 U/100 million)		NPM-protamine depletion	0.65 M NaCl-histone depletion	ATAC-seq
No crosslinking	Crosslinking	No crosslinking	Crosslinking			
Regulatory gene regions[1,7]	Gene deserts/ repetitive DNA[6]	Regulatory gene regions[2,3,4,5] Gene deserts/ repetitive DNA[8] Dual[12]	Regulatory gene regions[6]	Gene deserts/ Repetitive DNA[10]	Regulatory gene regions[1]	Regulatory gene regions[9] Gene deserts/ repetitive DNA[11]

Note: 1. (Arpanahi et al., 2009; human/mouse); 2. (Hammoud et al., 2009; human); 3. (Brykczynska et al., 2010; human/mouse); 4. (Saida et al., 2011; mouse); 5. (Erkek et al., 2013; mouse); 6. (Carone et al., 2014; mouse); 7. (Castillo, Amaral, Azpiazu, et al., 2014; human); 8. (Samans et al., 2014; human/bovine); 9. (Jung et al., 2017; mouse); 10. (Yamaguchi et al., 2018; mouse); 11. (Luense et al., 2019; mouse); 12. (Ozturk et al., 2021; human).

Amaral, Azpiazu, et al., 2014). As another technical artifact influencing the results, Royo and colleagues pointed to the approach for DNA sequencing data analyses as a potential source of bias. According to this work, the alignment of the reads to multiple locations in the genome would lead to an overrepresentation of repetitive sequences, while allowing only a single alignment per mapped read would derive on better identification of genic regions (Royo et al., 2016).

All these data obtained by independent groups reveal the complexity of the sperm DNA accessibility and maintain the debate about whether nucleosome-associated DNA is enriched in developmental genes, repetitive sequences, or poor genic regions as the main question to be solved about sperm chromatin function and relevance on male fertility.

To deep into this matter, it is important to review the results obtained using no MNase-based techniques (Table 2). Since DNA compaction might impede the accessibility of the enzymes, some groups have designed smart approaches based on the depletion of either histones (Arpanahi et al., 2009) or protamines (Yamaguchi et al., 2018) before chromatin immunoprecipitation of specific histone variants or modified histones. Removal of histone–DNA associations by 0.65 M NaCl followed by endonuclease restriction enzyme digestion is a classical method for sperm chromatin dissection that supports the hypothesis of sperm nucleosomal association to promoters, as well as an enrichment in CTCF binding sequences (Arpanahi et al., 2009). Yamaguchi and colleagues, in contrast, decided to address this issue differently by ensuring nucleosome solubility depleting protamines with nucleoplasmin, an oocyte protein responsible for paternal DNA remodeling after fertilization (Philpott & Leno, 1992). Following this protocol, a histone modification-dependent retention was detected, being H4, H3K9me3, and the N-terminal part of the H3 (corresponding to 14 amino acids) preferentially mapped to distal intergenic regions and gene deserts, whereas H3K4me3 and cleaved H3 (with no presence of the N-terminal part) were associated to regulatory regions of developmental genes (Figure 1). Despite this dual behavior, gene coding regions represented a minority of the nucleosomal DNA (Yamaguchi et al., 2018). More recently, some studies applied ATAC-seq technology based on the use of the Tn5 transposase to reveal accessible and inaccessible regions of the sperm chromatin (Buenrostro et al., 2013). Interestingly, this novel approach showed a distinct association of nucleosome-retained DNA characterized by the presence of DNA bound to CTCF and cohesin similarly to round spermatids and embryonic stem cells, and a retention in intergenic regions that seems to not increase along spermatogenesis (Jung et al., 2017; Luense et al., 2019; Table 2). Nonetheless, as seen with other technical approaches, ATAC-seq studies did not show consensus data regarding the main type of histone-associated genomic sequences in the mature sperm (Table 2).

It is important to remark on the detection in mature sperm cells of predetermined enhancers and super-enhancers associated with elements involved in the 3D organization of the chromatin that are functional in embryonic and adult tissues (Jung et al., 2017). Interestingly, sperm genomic 3D organization revealed many similarities to that of somatic cells, such as fibroblasts and embryonic stem cells, in which DNA is distributed in two compartments called A and B and associated with active and repressive chromatin regions, respectively. In addition, topologically associated domains (TADs) are partially conserved (Battulin et al., 2015; Jung et al., 2017; Ke et al., 2017; Y. Wang, Wang, et al., 2019). Nonetheless, sperm possess a major number of long-range interactions (>2 Mb), most of them between TADs, that could contribute to either establishing or maintaining the highly compacted state of its chromatin. Indeed, 3D

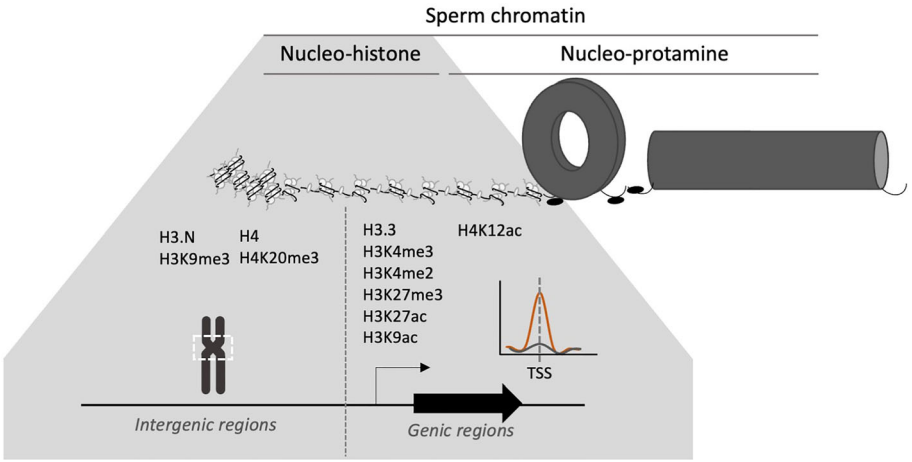

FIGURE 1 Modification-dependent retention of sperm histones to either intergenic (gene deserts or repetitive DNA) or genic regions (regulatory elements and transcriptional start sites, TSS). ChIP-based studies targeting histone variants and modified histones revealed a modification-dependent preferential association along the nucleosomal DNA (Nucleo-histone domain, highlighted in gray). Histone H4 and the N-terminal part of histone H3 (H3.N), as well as the modified H3K9me3 and H4K20me3, have been detected associated with intergenic regions and repetitive DNA, as those found on the chromosomal centromeres (highlighted with a white-line box). Histone 3.3 and modified histones H3K4me3, H3K4me2, H3K27me3, H3K27ac, H3K9ac, and H4K12ac are preferentially associated with promoters and genic regions characterized by the enrichment of transcriptional start sites (TSS; enrichment represented as an orange line). Based on results derived from Arpanahi et al. (2009), Carone et al. (2014), Erkek et al. (2013), Hammoud et al. (2009), Jung et al. (2017), and Ozturk et al. (2021).

conformation of sperm DNA packaging from non-protamine-containing species lacks TADs, resembling mitotic chromosomes (Gibcus et al., 2018; Wike et al., 2021). This A-B and TADs compartmentalization correlates with retained histones in sperm. Some controversy arises from the use of current 3D-chromatin technologies in such compacted and inaccessible genome, which would potentially bias the results from the real in vivo genomic architecture (Moritz & Hammoud, 2022).

Therefore, when globally evaluated, the results obtained so far lead to the hypothesis of a nonrandom dual behavior of the sperm nucleo-histone DNA, with a main enrichment of intergenic regions and repetitive DNA principally associated with centromeric loci, and the presence of promoters of developmental genes that would represent a minor proportion of the nucleosomal DNA (Castillo et al., 2015; Luense et al., 2019; Ozturk et al., 2021; Vavouri & Lehner, 2011; Yamaguchi et al., 2018; Figure 1). Of note, when focussing on the protein fraction of the human sperm chromatin, two different sets of chromatin-associated proteins are also detected, according to their affinity to the paternal genome. Protamines and structural proteins are strongly associated with DNA, supporting the most condensed fraction of sperm chromatin. Histones, in contrast, are more easily detached simultaneously with a remarkably rich population of transcription factors and chromatin modifying proteins, which would add another layer of regulatory elements to the complex structure of human sperm chromatin (Castillo, Amaral, Azpiazu, et al., 2014).

The specific distribution of the sperm chromatin, establishing a mark of epigenetic information exclusive for the male gamete, is then robustly supported, as well as its impact on sperm function. In fact, infertile men seem to lose the specificity of the most accessible sperm DNA, showing a random nucleosomal retention and a diminished association of H3K4me or H3K27me at developmental transcription factors (Hammoud et al., 2011). Alterations in sperm histone retention, as well as in the abundance of other proteins involved in the correct packaging of sperm DNA, have also been associated with the inability to achieve a successful pregnancy after application of assisted reproduction technologies (Azpiazu et al., 2014). It has been shown in mouse that an impaired poly (ADP-ribose) metabolism during spermiogenesis give rise to sperm with an altered number of retained histones, which, remarkably, seem to promote differences also on the expression of specific embryonic genes in the offspring (Ihara et al., 2014). In addition, sperm influence on the correct embryo gene expression has been robustly demonstrated in Xenopus, showing how the specific loss of sperm histone marks results in paternally derived embryo transcriptional de-regulation with an effect on its development (Teperek et al., 2016).

Since the relevance of this exceptional epigenetic mark on sperm function and embryo development is clear, further studies should focus on still unsolved questions regarding its effect on male infertility, such as whether differences in

DNA accessibility can be detected among individual cells within the ejaculate, or on the possibilities to overcome aberrant histone deposition in infertile patients.

3.2 | The complexity of the sperm protamines

3.2.1 | The remarkable number of protamine proteoforms in the normal sperm

Protamines represent the vast majority of the protein content of male gamete nucleus, despite more than 6000 different proteins having been identified (Castillo et al., 2018). This is due to the highly relevant function of protamines, whose main role is to compact DNA in a semi-crystalline structure that allows sperm to achieve a hydrodynamic shape needed to reach the oocyte, as well as to protect paternal genome from external damage during this journey (Barrachina et al., 2018; Oliva, 2006; Oliva & Castillo, 2011).

The packaging capacity of protamines is higher than that of histones. In particular, while a nucleosome is able to compact around 200 bp of DNA, it has been estimated that the structural unit formed by the union of protamines and DNA may contain at least 50,000 bp (Castillo, Amaral, & Oliva, 2014; check further details on the structure of nucleo-protamine in Section 3.3 of this review). This is possible thanks to the extreme physicochemical properties of protamines, which are very small and extremely basic proteins, and are able to form inter- and intramolecular disulfide bonds to stabilize the nucleo-protamine structure (Balhorn, 2007; Oliva, 2006). Protamine function and properties are maintained among mammals, which might point also to a high stability in the amino acid sequence and low isoform variety within the same species. However, the fact is that a notable number of different protamine proteoforms have been identified in human and mouse spermatozoa (Arauz-Garofalo et al., 2021; Brunner et al., 2014; Castillo et al., 2015; Soler-Ventura et al., 2020). Protamines from human sperm, as well as from other mammals, are divided in two main groups: protamine 1 (P1) and protamine 2 families (P2; Balhorn, 2007; Barrachina et al., 2019; Jodar & Oliva, 2014; Oliva, 2006; Oliva & Castillo, 2011). Human P1 is translated from a unique gene, named *PRM1*, as a mature protein of 6823 Da, with 51 amino acids and a 47.1% of arginine, which confers an extremely positive charge (pI = 12.08; UniProtKB database https://www.uniprot.org/). Human P2, in contrast, is translated from the *PRM2* gene as a precursor (pre-P2) of 102 amino acids that is processed by proteolysis in the N-terminal part giving rise to three mature components, HP2 (the most abundant one), HP3, and HP4 (Jodar & Oliva, 2014; Oliva, 2006). Interestingly, the human mature sperm not only contains P2 mature forms in its chromatin, but also retains pre-P2 and other immature proteoforms, named HPI1, HPI2, HPS1, and HPS2, the function of which has not been revealed yet (de Mateo, Ramos, et al., 2011). In addition to these P2 immature proteoforms generated by proteolysis and slightly differing between them in the number of amino acids in the N-terminal part, there is evidence at protein level of another isoform generated by alternative splicing (Soler-Ventura et al., 2020). This protein, named P2 isoform 2, contains 140 amino acids and differs from pre-P2 by the incorporation of 50 amino acids between positions 91 and 102 of the canonical sequence (Soler-Ventura et al., 2020). Of note, in the same work it was also identified an RNA corresponding to an additional splice variant, the P2 isoform 3, although it was not detected at protein level (Soler-Ventura et al., 2020). Both P2 isoforms generated by alternative splicing seem to be only conserved among higher primates but not in other mammals such as mice and rats, and no specific function has been suggested for any of them yet (Soler-Ventura et al., 2020). In global, these results reveal a high diversity of protamine amino acid sequences in human sperm, which raises the interest on deciphering whether this is the result of a simple inefficient processing of pre-P2, it responds to the need of redundancy to ensure protamine function, or there is any proteoform-specific functional implication still unknown.

The complexity of protamine proteoform composition in the sperm cell is even higher when considering posttranslational modifications. As indicated in Section 2.2.1. of this review, protamine phosphorylation is required for a proper deposition of protamines in the sperm chromatin and displacement of histones (Gou et al., 2020; Papoutsopoulou et al., 1999; Willmitzer et al., 1977a, 1977b; Wu et al., 2000). Despite this function during spermatogenesis, it has been shown in different mammalian species that the sperm cell retains phosphorylated protamine proteoforms (Arauz-Garofalo et al., 2021; Brunner et al., 2014; Castillo et al., 2015; Chirat et al., 1993; Pirhonen, Linnala-Kankkunen, & Mäenpää, 1994; Pruslin et al., 1987; Soler-Ventura et al., 2020). Our group has recently determined the most complete profile of phosphorylated protamine proteoforms to date in the normal human spermatozoon, by adapting mass spectrometry approaches and proteoform identification algorithm to the special properties of protamines (Arauz-Garofalo et al., 2021; Soler-Ventura et al., 2020). In particular, mono-, di-, and tri-phosphorylated proteoforms were detected for P1, while P2 was identified with one or two phosphorylations in its sequence. Of note, pre-P2 showed the highest

incorporation of phosphate groups, since mono-, di-, tri-, and tetra-phosphorylated proteoforms were identified (Soler-Ventura et al., 2020). In an attempt to localize the phosphor groups in the protamine sequences, several target residues have been suggested, being some of them maintained among different studies, such as S9 and S11 in P1, counting the first methionine, and S37 and S59 for P2 (Chirat et al., 1993; Hornbeck et al., 2015; Soler-Ventura et al., 2020). From those, P1 S9 and P2 S59 were also identified in the corresponding homolog protamine sequences in mice, increasing the interest on the functional role of these specific residues and their modification in the male gamete (Brunner et al., 2014). By using mouse sperm as a model, acetylation and methylation groups were also identified in protamine sequences, either as a single modification or in combination, demonstrating that protamines can bear multiple PTMs in a single molecule (Brunner et al., 2014). The relevance of some of these modifications on sperm functionality and its impact on sperm chromatin formation has been achieved using mouse models, as discussed in the following section of this review. This, together with the identification of potential alterations in specific groups of infertile patients, will not only allow increasing the knowledge on sperm chromatin compaction, but also to find novel causes of male infertility.

3.2.2 | Protamine-deficient mouse models and implications for fertility status

A substantial proportion of our knowledge about the importance of the role of sperm protamines in male fertility has been obtained from functional studies in transgenic mouse models (Table 3). Early studies on protamine genes disruption in chimeric mice suggested that haploinsufficiency of Prm1 and/or Prm2 genes resulted in male infertility (Cho et al., 2001, 2003; Takeda et al., 2016). Even so, the recent introduction of sophisticated CRISPR/Cas9 technology has clearly demonstrated that while loss of a single Prm2 allele was well tolerated, disruption of both Prm2 alleles resulted in infertile mice (Schneider et al., 2016). The precision and efficiency of CRISPR/Cas9 technology in gene editing without disturbing gene structure or regulatory DNA sequences could explain these differences. Sperm evaluation of targeted Prm2−/− mice revealed normal epididymal sperm counts but with very drastic morphological alterations in both flagellum and acrosome structures, resulting in immotile sperm (Schneider et al., 2016). However, apparently, no morphological changes were observed in testicular sperm, suggesting that sperm defects in Prm2−/− mice are acquired during epididymal transit (Schneider et al., 2020). In fact, Prm2-deficient testicular sperm showed a success rate to develop blastocyst after ICSI as that of wild-type testicular sperm. Upregulation of P1 expression is observed in deficient Prm2−/− mice to compensate for the loss of P2, but even though it seems enough to properly package testicular sperm DNA, it is insufficient to generate functional epididymal spermatozoa. It has been suggested that P2 incorporates zinc into its structure to stabilize sperm chromatin in the testis (Bianchi et al., 1992; Björndahl & Kvist, 2011), but zinc molecules are released during sperm epididymal maturation (Dias et al., 2006). Recent findings have proposed that zinc released by P2 is incorporated into the protein structure of the antioxidant protein SOD1 in the epididymis, conferring stability and ensuring an optimal antioxidant activity inside spermatozoa during sperm maturation (Schneider et al., 2020). Therefore, loss of P2 could result in a low antioxidant capability of epididymal sperm that might promote alterations in sperm DNA, membranes, and morphology.

CRISPR/Cas9 technology has also been used to target *Prm1* gene. Contrary to the observed for *Prm2*+/−, disruption of just one allele of *Prm1* shows a negative impact on male fertility (Mashiko et al., 2013; Merges et al., 2022). In contrast, the phenotype of Prm1−/− mice is very similar to that of Prm2−/− mice, both being infertile with dramatic alterations in epididymal spermatozoa motility and morphology. Interestingly, accumulation of pre-processed P2 is observed when one or both alleles of Prm1 are disrupted, suggesting that P1 is required for proper P2 processing. This fact could explain why Prm1+/−, but not Prm2+/−, are subfertile. Indeed, disruption of the cleaved part of pre-P2 (cP2 domain) in one or both alleles (Prm2+/Δc and Prm2−/Δc, respectively) results in immotile and inviable sperm, similar to Prm2−/− and Prm1−/− spermatozoa (Arévalo et al., 2022). Additionally, testicular sperm from Prm2+/Δc and Prm2−/Δc transgenic mice already show DNA fragmentation that increase throughout epididymal transit, contrasting with the phenotype observed in Prm2−/− mice. These different results could be explained by a disruption of the synergistic process of NH-NP mediated by H2A.L.2–TP complexes potentially induced by the loss of cP2, which may end with a random incorporation of P2 in the sperm chromatin (Arévalo et al., 2022). Nonspecific binding of protamines to DNA lead to strong DNA hypercondensation, which could result in strand breaks in testicular spermatozoa. Moreover, it seems that TPs are retained in sperm chromatin in absence of cP2, therefore avoiding P1 and P2 incorporation. Strong oxidative DNA damage in cauda epididymis and complete epididymal sperm DNA fragmentation were reported in both Prm2+/Δc and Prm2−/Δc mice. Of note, the Prm2−/Δc model displayed a more severe phenotype, showing not only alterations on sperm motility and morphology, but also reduced sperm counts.

TABLE 3 Sperm evaluation and fertility status of the available protamine-deficient mouse models

	Generation of protamine mice mutants	Sperm concentration	Sperm motility	Sperm morphology	Sperm DNA damage	Fertility	Others	References
Chimeras *Prm1*+/− Chimeras *Prm2*+/−	Disruption of either *Prm1* or *Prm2* coding sequence of one allele in ES cells by homologous recombination and ES injection into blastocysts	Normal	Reduced	Flagellum tightly wrapped around the head or with elongated heads having a reduced ventral flexure	Altered sperm chromatin integrity, higher sperm DNA fragmentation	No sire produced	Only P1 protein was reduced. Abnormally processed P2 P1 and mature P2 were reduced. Abnormally processed P2	(Cho et al., 2001, 2003)
Prm1+/−	*Prm1*-deficient female chimeric mice carrying *Prm1*-deficient oocytes	Normal	Reduced	Abnormal morphology mainly in the tail	Altered sperm chromatin integrity, higher sperm DNA fragmentation	No sire produced by natural mating or IVF but by IVF with zona-free oocytes	Enhanced acrosome reaction and reduced mitochondrial membrane potential. Reduced P1 and mature P2. Abnormally processed P2	(Takeda et al., 2016)
Prm1+/−	CRISPR/Cas9 mediated gene-editing in zygotes	Normal	Drastically decreased	Normal (no drastic head defects)	Low but detectable DNA fragmentation	Subfertile (low pregnancy rate, reduced litter size)	Accumulation of P2 precursor. Impaired flagellar structure.	(Merges et al., 2022)
Prm1−/−		Normal	Immotile	Head defects (lose of hooked-shape, smaller, elliptic, thinner), membrane damage, disrupted acrosome	Altered sperm chromatin integrity. Almost all DNA is fragmented, some part remains intact	No sire produced	Accumulation of P2 precursor, increased histone retention, defects on chromatin hypercondensation. Impaired flagellar structure and membrane	
Prm2+/− *Prm2*−/−	CRISPR/Cas9 mediated gene-editing in oocytes	Normal Normal	Normal Immotile	Normal No acrosome, midpiece wrapped around sperm head	Normal Altered sperm chromatin integrity, sperm DNA fully fragmented	Fertile No sire produced	Reduced P2 No P2 and upregulation of P1. Declined viability	(Schneider et al., 2016, 2020)

(Continues)

TABLE 3 (Continued)

	Generation of protamine mice mutants	Sperm concentration	Sperm motility	Sperm morphology	Sperm DNA damage	Fertility	Others	References
Prm2+/Δc	CRISPR/Cas9 mediated gene-editing in oocytes	Normal	Drastically decreased	(a) Slimmer less-hooked nucleus, and (b) smaller hookless nucleus	Completely fragmented DNA	Infertile	Drastically decreased viability. Switched P1/P2 ratio, P2 precursor accumulation. Incorrect eviction of P2 from spermatid nucleus.	(Arévalo et al., 2022)
Prm2−/Δc		Reduced	Drastically decreased	Similar but more severe nuclear defects than $Prm2^{+/\Delta c}$	Completely fragmented DNA, even more severe	Infertile	Drastically decreased viability. More pronounced switch of P1/P2 ratio. Additional P2 signal in spermatid cytoplasm and residual bodies.	
P1S9A/+	CRISPR/Cas9 mediated knock-in mutations in specific phosphorylation sites of P1	Normal	NA	Normal	Normal DNA compaction	Fertile	–	(Gou et al., 2020)
P1S43A/+		Normal	NA	Normal	Normal DNA compaction	Fertile		
P1S9S43A/+		Slightly reduced	NA	Normal	Normal DNA compaction	Drastic Reduced		
P2S56A/A	CRISPR/Cas9 mediated knock-in mutations in specific phosphorylation sites of P2	NA	NA	NA	NA	Fertile	–	(Itoh et al., 2019)
P2S56D/D		NA	NA	NA	NA	Fertile		

3.2.3 | Alterations on human protamines and association to male infertility

It is difficult to establish the phenotypic effects of protamine disruption in humans, since there is too little genetic variation among human protamine genes (Jodar et al., 2011). Among population variation of the harmonized sequencing data of 125,748 exome sequences and 15,708 whole genome sequences provided by gnomAD v2 1.1, we found 24 and 11 alleles with a frameshift or stop gain variant in *PRM1* and *PRM2* genes, respectively. To the best of our knowledge, just one infertile patient has been described to be carrier of the nonsense variant c.248C>T (p.Q50*) in the *PRM2* gene in heterozygosis (Tanaka et al., 2003). Although this infertile patient did not show any sperm in the ejaculate, the pathogenesis of the variant could not be confirmed since no functional or segregation studies were performed in this case. However, according to the results in disrupted *Prm1* and *Prm2* mice described in the section above, this nonsense variant in heterozygosis does not seem to be the cause of the azoospermia observed in this patient.

Additionally, pathogenic missense variants in protamine genes are extremely rare in infertile patients and are always identified in heterozygous state (Jodar et al., 2011). In fact, from the 2992 and 795 alleles with a missense variant in *PRM1* and *PRM2* genes, respectively, identified in the general population, only 31 and 4 individuals carried a missense variant in homozygosis (Figure 2). Of those, the most frequent genetic variant is c.102G>T (p.Arg34Ser) in *PRM1* gene with an allelic frequency of 0.9% and a total of 30 homozygotes detected in the general population. Although some studies suggested this variant as a potential risk factor for male infertility, due to the replacement of an arginine residue by a serine, which could represent a new potential phosphorylation site on P1, this hypothesis was not supported by the meta-analysis performed by our group (Jodar et al., 2011). This likely silent effect agrees with the fertile phenotypes observed in transgenic mice with knock-in mutations at specific phosphorylated residues of P1 and P2 (Gou et al., 2020; Itoh et al., 2019). Specifically, these knock-in mice were generated to evaluate whether loss of specific sites of phosphorylation on protamines would affect fertility, and no adverse effects were observed for single mutations. These results suggest a redundancy within protamine phosphorylation sites, with no individual direct impact on fertility. Nevertheless, a minimum level of phosphorylation on P1 must be reached for proper sperm functionality, since drastically reduced fertility was observed in mice when two phosphorylation sites were simultaneously depleted on P1 (Gou et al., 2020). In contrast to the redundancy associated with the protamine phosphorylation sites, protamine dephosphorylation, at least for P2, has been revealed as crucial for fertility. In particular, male infertility together with abnormal sperm morphology has been observed in mice when dephosphorylation of residue 56 of P2 is disrupted, being the phenotype rescued when the same residue is modified to avoid phosphorylation (Itoh et al., 2019). A correct dephosphorylation of the new phosphorylation site created by the variant c.102G>T in P1 mentioned above could explain why it has no impact in fertility. Therefore, the search for alterations in the PTMs of protamines, rather than in the unmodified residues, seems to provide more answers about the impact of protamines on male fertility.

Apart from the search of single-nucleotide variants in protamine gene sequences, the relative protein levels of P1 and P2 have traditionally been evaluated as an indicator of sperm chromatin status and male infertility. It has been proposed that mature sperm from fertile men have a P1/P2 ratio of around 1, suggesting a similar abundance of P1 and P2 components at the protein level (Balhorn et al., 1988; de Yebra et al., 1993; Mengual et al., 2003; Nanassy et al., 2011). Alterations in the P1/P2 ratio, as well as in the individual abundance of P1 and P2, have been associated with altered seminal parameters (sperm count, motility, and morphology), increased DNA damage, and lower success rates of assisted reproduction technologies (ART), as summarized in Jodar and Oliva (2014) and Soler-Ventura et al. (2018).

Not only protein alterations in mature P1 and P2, but also in pre-P2 seem to compromise male fertility, either due to incomplete processing of pre-P2 or the total absence of mature P2 (Carrell & Liu, 2001; de Mateo et al., 2009; de Mateo, Ramos, et al., 2011; Rezaei-Gazik et al., 2022), with impact on increased DNA damage, lower sperm count, reduced motility, abnormal morphology, and worse ART outcomes (Rezaei-Gazik et al., 2022; Soler-Ventura et al., 2018). It has recently been reported that increased retention of pre-P2 in sperm is associated with fertilization failure, as well as to impaired nucleosome disassembly during spermatogenesis (Rezaei-Gazik et al., 2022), which could explain the origin of the observed sperm defects.

Overall, these findings support a role of protamines in the production of fully functional sperm, ART success, and postfertilization events, evidencing that alterations in the relative amount between P1 and P2, as well as in pre-P2 processing, have a direct impact on male infertility. It is important to remark that the threshold for the P1/P2 ratio has been defined by conventional techniques based on acid-urea polyacrylamide gel electrophoresis (acid-urea PAGE), which limits quantification to the main bands corresponding to P1 and P2. Therefore, this approach would not encompass the whole amount of protamine proteoforms that have been described by mass spectrometry (Arauz-Garofalo

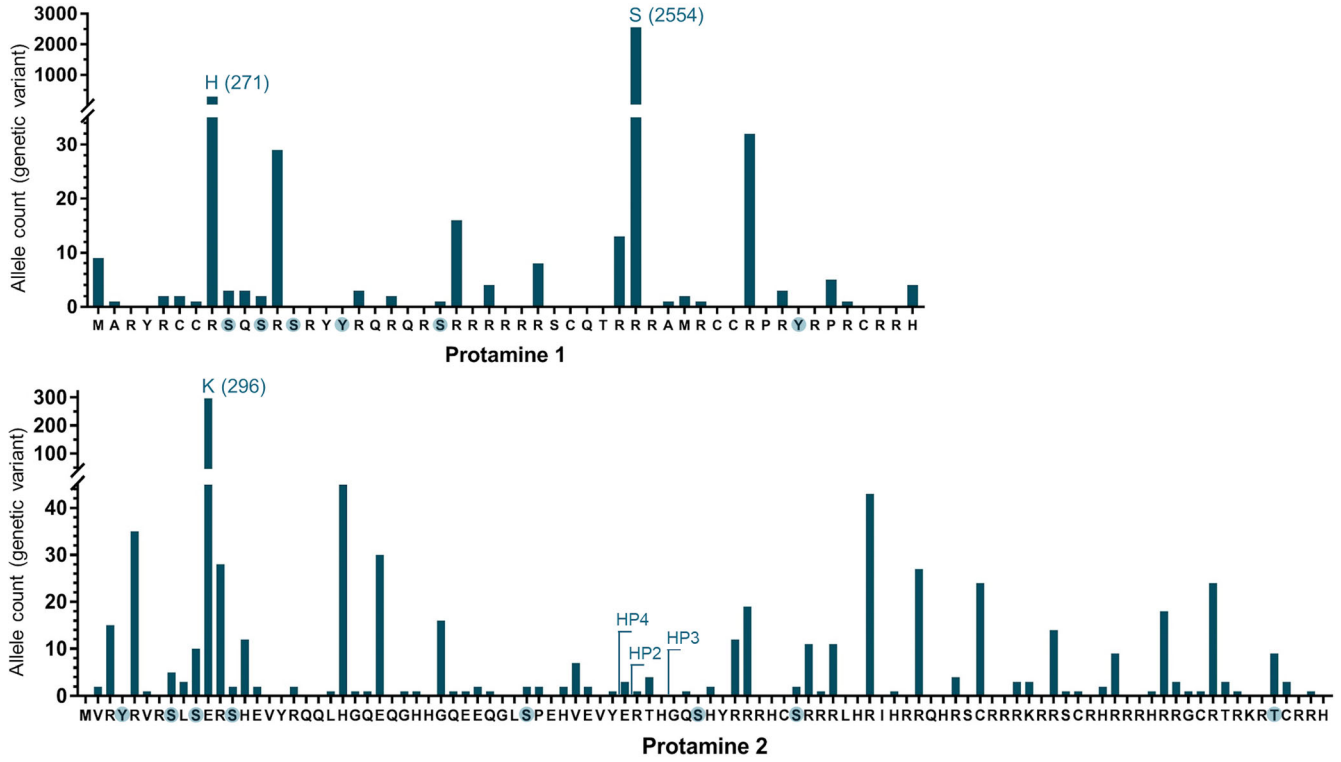

FIGURE 2 Number of alleles presenting missense variants identified in human protamine genes in the general population. A total of 2992 alleles have been found with missense variants in protamine 1 (upper plot), being p.Arg8His and p.Arg34Ser the two most abundant changes. Of the 795 alleles with a missense variant in protamine 2 (lower plot), p.Glu11Lys is the most abundant one, found in the catalytic part of the protamine 2 precursor. Most of the variants reported herein are found in heterozygosis. The most frequent amino acid resulting from the missense variant is shown at the top of the bars corresponding to the most abundant alleles (specific count shown in brackets). Amino acid sequence is shown on the *X*-axis for both protamines, and annotated phosphorylation sites are highlighted with a blue circle. Cleavage sites for human protamine 2, 3, and 4 (HP2, HP3, and HP4, respectively) are shown on the plot.

et al., 2021; Soler-Ventura et al., 2020; see previous Section 3.2.1, for more information), which could explain the variability in P1/P2 levels detected among studies (Ni et al., 2016).

3.3 | The nucleo-protamine structure model: Knowledge derived from different complementary methodological approaches

The current model of the human sperm chromatin structure is summarized in Figure 3 and is the result of the application of a wide range of methodologies. The first clues to understand the structure of nucleo-protamine were derived from knowledge of the chemical composition of its constituents (protamines and DNA) together with the extremely condensed structure of the sperm nucleus, as observed by microscopy (Figure 3b). Protamines were one of the first proteins to be isolated more than a century ago by Friedreich Miescher, who extracted a basic component that he called "protamin" coupled to an acidic component that he called "nuclein" (later known as DNA) from salmon sperm (Dahm, 2005; Miescher, 1874). The extreme basic nature of "protamin" and the acidic nature of "nuclein" readily suggested a condensed structure based on strong electrostatic interactions that explained the highly condensed structure of the sperm nucleus observed by microscopy methods (Figure 3b). Despite that, while protamines were one of the first proteins to be isolated, study of the details of the nucleo-protamine structure, composition, and function started much later (Bloch, 1969; Dixon, 1972).

When comparing among species, protamines are found to be highly variable proteins, since many vertebrates either have no protamines in their mature spermatozoa or, when present, the amino acid sequences show low homology (Kasinsky et al., 1987, 2011; Mezquita, 1985; Oliva & Dixon, 1991; Soler-Ventura et al., 2018; Subirana, 1983). This diversity contrasts with the extremely well-conserved eukaryotic somatic chromatin packaged by nucleosomal particles

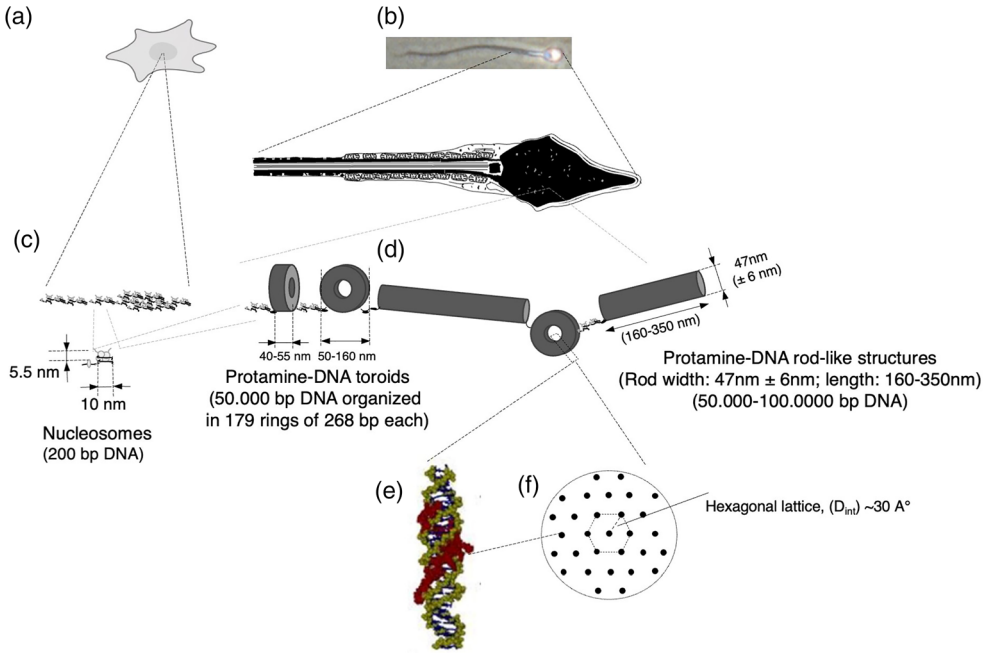

FIGURE 3 Current model of the mammalian nucleo-protamine structure. At the top of the figure, scale representations of a human somatic cell (a) compared to an optical microscopy image of a human spermatozoa (b) and an enlarged section of the head and midpiece, where it is possible to appreciate a reduction in the size of the sperm head, are shown. (c) Schematic representation of the nucleo-histone structure of somatic cells where each nucleosome organizes approximately 200 bp of DNA. (d) Current model of the nucleo-protamine structure formed by toroidal and rod-like structures. (e) Favored model where protamines would bind longitudinally located in the major groove of the DNA. (f) Evidence indicates that linear complexes of DNA and protamine are associated side-by-side in a close-packed hexagonal arrangement with an interaxial spacing (dint) representing the actual distance between DNA helices of 30.1 Å (see text for references and more details).

(Figure 3C). Possibly due to its high conservation, nucleo-histone structure was elucidated much earlier than that of the nucleo-protamine by a combination of electron microscopy techniques. This approach evidenced "beads on a string" images, combined with information derived from partial micrococcal nuclease digestion of chromatin and size determination of the generated DNA ladders, which allowed determining the nucleosomal elementary size (Subirana et al., 1985). Furthermore, nucleosome crystallization allowed determining its high-resolution structure by *X*-ray diffraction (Azorin et al., 1980; Subirana, 1997). In contrast, no nucleo-protamine crystals have been obtained so far. Thus, the information and models available on the nucleo-protamine structure, either extracted in vivo or reconstituted in vitro, are based on the combination of a wide range of microscopic techniques and X-ray diffraction in solution.

Different reports applying electron microscopy and/or atomic force microscopy to the study of human, mouse, and rooster sperm chromatin have evidenced a combination of toroidal structures with an external diameter of 50–160 nm and a width of 40–55 nm, and rod-like structures with a thickness of 40–55 nm and a length of 160–350 nm (Figure 3d; Allen et al., 1993, 1997; Ausió et al., 2014; Balhorn, 2007; Hud et al., 1993, 1995; Vilfan et al., 2006). Of note, similar toroidal structures were obtained in vitro upon DNA condensation with purified protamines and other cations (Conwell et al., 2003; Conwell & Hud, 2004; DeRouchey & Rau, 2011).

At its finer structure, one of the initial approximations was based on a tRNA–protamine crystal and x-ray diffraction which led to the proposal of a model where protamine molecules would bind to the narrow groove of double helical portion of tRNA (Warrant & Kim, 1978). Nevertheless, results based on tRNA–protamine complexes are not necessarily extrapolated to infer the DNA–protamine complex structure. In fact, the results derived from x-ray diffraction studies using in vitro reconstituted nucleo-protamine in solution or using native sperm chromatin, suggest a model favored by most studies where protamine molecules would align along of DNA and bind to the major groove (Figure 3e; Balhorn, 1982; Fita et al., 1983; Vilfan et al., 2004).

Furthermore, molecular dynamics simulations and free energy calculations with full atomistic details have indicated that protamine molecules located in the major groove rather utilize the DNA backbone as a binding site, which represents the best compromise between enthalpy and entropy gains (Mukherjee et al., 2021).

These linear complexes of DNA and protamine have been also proposed to be associated side-by-side in a close-packed hexagonal arrangement (Figure 3f, right; Allen et al., 1993; Balhorn, 1982; Fita et al., 1983; Hud & Downing, 2001; Hud & Vilfan, 2005; Sartori Blanc et al., 2001). Accurate measurements using small angle x-ray scattering (SAXS) concluded that in this hexagonal lattice, the equilibrium interaxial spacing (Dint) representing the actual distance between DNA helices is 30.1 Å for bull sperm nuclei and 29.2 Å for salmon sperm nuclei, both in solution under near physiological salt conditions (150 mM NaCl), without added reducing agent and at a temperature of 20°C (Hutchison et al., 2017; Figure 3f). A different approach based on an infrared spectroscopy study of salmon and squid protamines complexed with DNA also concluded that DNA-bound protamine in sperm nuclei contains large amounts of defined secondary structure that is stabilized by intramolecular hydrogen bonding and that efficiently promotes tight hexagonal packing of DNA molecules (Roque et al., 2011).

In addition to the strong electrostatic interactions derived from the positively charged arginine residues in protamines, and the negatively charged phosphates of the DNA backbone, an additional layer of stabilization occurring in cysteine-containing protamines is provided by the formation of inter- and intra-molecular disulfide bonds and the formation of zinc bridges (Balhorn, 2007; Björndahl & Kvist, 2011; Hutchison et al., 2017; Vilfan et al., 2004). As discussed in other sections of this review, this tightly packed nucleo-protamine structure condenses most of the sperm DNA, is nonrandomly distributed in the genome, and intercalates with regions retaining histones and nucleosomes (Figure 3d), providing an epigenetic richness that is transferred to the oocyte upon fertilization (Castillo et al., 2015; Castillo, Amaral, Azpiazu, et al., 2014; Gatewood et al., 1987; Hammoud et al., 2009; Oliva, 2006).

4 | PATERNAL CHROMATIN REMODELING BEYOND FERTILIZATION: THE ZYGOTIC EVENT OF PROTAMINE-TO-HISTONE TRANSITION AS A MODEL TO STUDY THE PROCESS OF CHROMATIN (DE-)COMPACTION

The interplay between histones and protamines is not limited to male germ cell development. As it can be seen in the scheme of male germ cells' chromatin remodeling through DNA (de-)protamination in Figure 4, once the nucleoprotamine structure has been established in mature sperm, the male pronucleus must be de-compacted after fertilization to start activation of the embryonic genome. Nucleoplasmins are a protein family that plays an essential role in male pronuclear decompaction (Philpott & Leno, 1992). Interestingly, some of the key players involved in the NH–NP transition, or in its regulation, have been found to be crucial for the inverse process of protamine-to-histone transition in the zygote. A very elegant study demonstrated the crucial role of SRPK1-mediated protamine phosphorylation in accelerating the process by recruiting nucleoplasmin to H3.3-retained nucleosomes, which would be the starting point of the protamine removal (Gou et al., 2020). Additionally, phosphorylated protamines would allow recruitment of Protein HIRA to deposit H3.3 into newly free DNA regions, which is essential for de novo nucleosome assembly and required for proper nuclear envelope formation in the zygote (Gou et al., 2020; Inoue & Zhang, 2014; Lin et al., 2014; van der Heijden et al., 2005). Histone chaperone ASF1 assists in the loading of H3.3–H4 dimers into HIRA, before their incorporation into the paternal chromatin (Horard et al., 2018). Other histone variants such as TH2A, TH2B, and H2A.X (Montellier et al., 2013; Nashun et al., 2010; Shinagawa et al., 2014) and histone PTMs such as H3K4 methylation mediated by the histone–lysine N-methyltransferase (MLL) complex MLL3/4 (Aoshima et al., 2015) also play a crucial role in paternal genome reprogramming guiding genome-wide chromatin transitions.

These findings also suggest a coordinated epigenetic regulation of the process, that somehow resembles that of the NH–NP transition. Therefore, it could be thought that impairments in the nucleo-protamine structure, derived from a non-efficient process of nuclear compaction, from poor structural stabilization during sperm maturation in the male reproductive tract, or from an altered composition of protamine proteoforms in the sperm chromatin, could also impact de-protaminization rhythms in the zygote and potential effects on paternal nuclear reprogramming and beyond.

The study of this reverse process could be of high interest as a model to find new regulators of the spermiogenic process. For example, H4R3 methylation has been found to play a role in de-protaminization of the male pronucleus (Hatanaka et al., 2017), the potential role of which is still unknown during spermiogenesis. Recent findings have identified arginine methylation of histone H3 prior to phosphorylation of H3T3 by Serine/threonine-protein kinase haspin (Haspin) during spermiogenesis, but with unknown function (Soupsana et al., 2021). Also, H3 arginine dimethylation in the core histone weakens histone affinity to DNA (Casadio et al., 2013). It is known that some arginine methylations on the histone tail are related with transcriptional activation (i.e., open chromatin states), and H4R3me2, in particular, triggers a subsequent acetylation of H4 histone tails by p300 (H. Wang et al., 2001). H4 arginine methylation is indeed

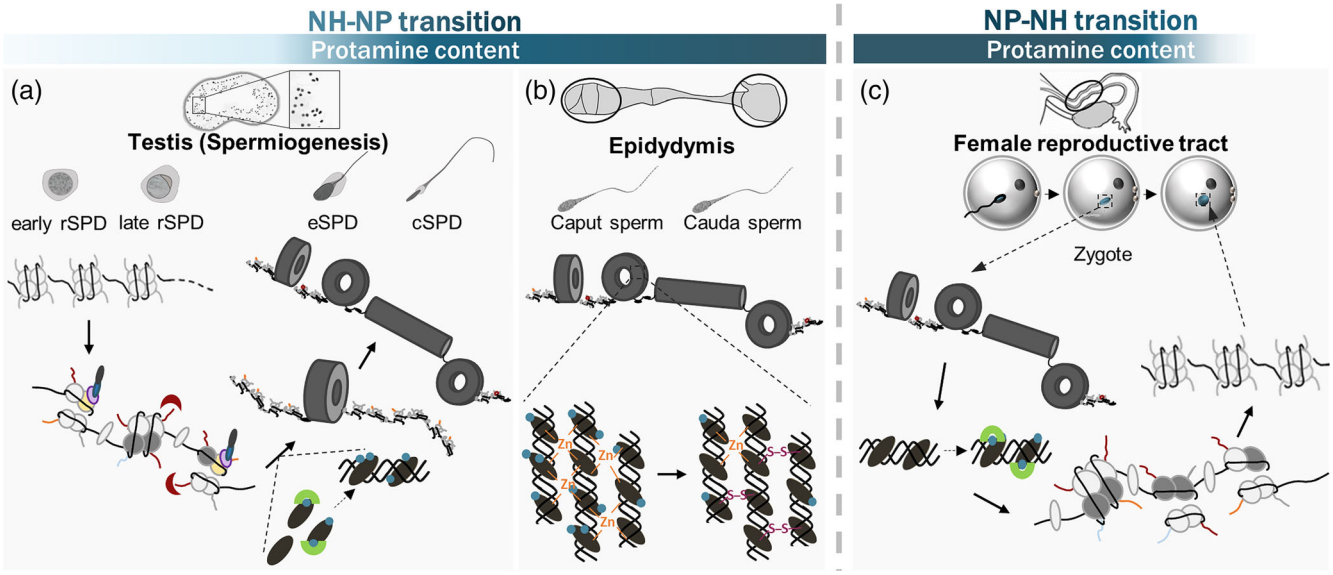

FIGURE 4 Schematic representation of male germ cell chromatin compaction through the nucleo-histone to nucleo-protamine transition, and the reverse postfertilization process of male pronuclear decompaction through the nucleo-protamine to nucleo-histone transition in the zygote, based on the current literature. (a) During spermiogenesis, core histones (light gray) are replaced by H2A.L.2 (in yellow), other histone variants (dark gray), and transition proteins (purple), building the template for the processing of immature protamine 2 (elongated forms in blue and gray). Other histone PTMs (represented as multi-colored histone tails), and their writers, readers, and erasers (moon-shaped, in red) assist nucleosome removal. Protamine (elongated shape, dark gray) incorporation is facilitated by phosphorylation (blue dots) added by kinases (in green), allowing protamine accommodation to originate higher-order structures (in gray). Some nucleosomes bearing specific histone PTMs are retained. (b) In the epididymis, part of zinc bridges (Zn, in orange) are lost, and the chromatine is stabilized through disulfide bonds (S—S, in magenta), coupled to a potential protamine dephosphorylation wave. (c) The reverse process occurs in the zygote, where some essential proteins to achieve a proper NH–NP transition play a role in reprogramming the male genome.

regulated by the crosstalk of its modulators with other H4 coil PTMs, such as acetylated or butyrylated K5, or phosphorylated S1 (Fulton et al., 2022), which is involved in histone accessibility and mitotic chromatin compaction and is essential for histone deacetylation after DNA-breaks repair (Krishnamoorthy et al., 2006; Utley et al., 2005). This may lead us to envisage a role for histone arginine methylation on nucleosome destabilization during spermiogenesis coordinated with acetylation, phosphorylation, and their writers, readers, and erasers.

5 | CONCLUSION

In this article, we review all advances made, from the use of conventional techniques to the most novel approaches, to deeply explore the formation of sperm chromatin during sperm development and the final nucleo-protamine structure from the protein point of view, with a special focus on the lead actors, the protamines, and the implications in male infertility. We provide evidence on the complexity of the chromatin compaction process, in which orchestrated events involving different levels of protein regulation through histone PTMs and their modulators allow the displacement of canonical nucleosomes from the paternal genome and the incorporation of protamines. Besides the well-known regulators of the NH–NP transition during spermiogenesis, we gather new findings on other histone PTMs, such as the lesser-described non-acetyl histone acylations and the protamine PTMs, and highlight the importance of their readers, writers, and erasers as a new layer of regulation in postmeiotic sperm development. Even after leaving the testis, the process of nuclear compaction continues to fine-tune the final genome packaging in sperm, stabilizing the structure through inter- and intra-molecular disulphide bonds during epididymal maturation. This exceptionally complex scenario evidences the handicaps that need to be overcome to develop human in vitro spermatogenesis models and obtain fully functional male gametes. There is still need to increase the knowledge on the processes of DNA protaminization and de-protaminization, during postmeiotic cell development and zygotal male pronuclear reprogramming, respectively, to be able to properly mimic the process of protamine incorporation in humans in vitro.

Apart from that, the own aminoacidic nature of the small and arginine-rich protamines is what gives them their intrinsic unique way of compacting sperm DNA. However, top-down proteomic studies on purified protamines have revealed a much more complex population of protamine proteoforms than previously thought, including mature, immature, and truncated forms of P1 and P2, also bearing PTMs. Such diversity raises the question of whether this is the result of a need of functional redundancy to cover protamine demand during the short window of spermatid elongation, or it is revealing the presence of protamine proteoforms with specific roles. In this regard, it is interesting to highlight that although some levels of protamine phosphorylation are needed for proper sperm development, what has been revealed as essential to achieve fertility potential is the dephosphorylation of specific residues at least for P2. Therefore, although many details about the implication of protamine proteoforms remain to be discovered, this complex population points to potential specific roles for some of the candidates, either in protamine deposition on DNA, or in achieving higher-order chromatin structures. Indeed, the nucleo-protamine structure model evidences that the protamine-packaged genome does not result in a unique way of chromatin compaction, but in a combination of toroidal and *rod-like* structures. Investigating whether these two different high-order chromatin organizations arise from a differential composition of protamine proteoforms would suppose a milestone in understanding paternal genome packaging and protection, and subsequent implications for male fertility.

Taking together the role of sperm chromatin-associated and regulatory proteins needed to achieve a successful postmeiotic germ cell development and to contribute to the mature sperm epigenome, as well as the implications of their alterations on sperm functionality, we provide evidence on the importance of maintaining a proper chromatin structure and composition to support male fertility. Therefore, we highlight the importance of monitoring sperm chromatin quality, on its many different levels, toward the successful generation of a new individual.

AUTHOR CONTRIBUTIONS

Alberto de la Iglesia: Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal). **Meritxell Jodar:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal). **Rafael Oliva:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal). **Judit Castillo:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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