Chapter 1
Sperm Chromatin: An Overview

Rod Balhorn

1.1 Sperm Chromatin: The First 50 Years

Sperm chromatin research began with the discovery of the two primary molecular components that fill the head of mature sperm cells—DNA and protamine. Only a year after Gregor Mendel reported his work on the laws of heredity in 1865 [1], Ernst Haeckel suggested that the nuclei of cells must contain the material responsible for the transmission of genetic traits [2]. Friedrich Miescher, working in Felix Hoppe Seyler's laboratory in Germany, had become intrigued by cells and began conducting experiments to determine their chemical composition. Working initially with lymphocytes obtained from blood and later enriched populations of leukocytes he obtained from hospital bandages, Miescher noticed a precipitate that formed when he added acid to the cell extracts he was using to isolate proteins [3]. While he and the rest of the scientific community were unaware that this material, which he called nuclein, was the genetic material Mendel and Haeckel had referred to, he became fascinated by this acid-insoluble component of cells and continued to study its properties [4]. Walther Flemming's work over the next decade introduced the scientific community to the cellular substructures called chromosomes and the concept of mitosis, and Flemming was the first to introduce the term chromatin [5]. It took another 30 years, however, before cellular biologists began to realize the importance of individual chromosomes as the carriers of genetic information.

Miescher, who began his research career isolating and characterizing proteins, spent the majority of his later efforts investigating nuclein (DNA). When he discovered he could not obtain enough of the nuclein from human cells to properly examine its properties, he turned to working with fish sperm. Salmon provided an abundance of sperm, and the sperm cells were considered ideal because they had almost no cytoplasm to contaminate his nuclear preparations with other proteins.

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In addition to being the first to isolate DNA, Miescher was also the first to isolate protamine, which he called protamin, and to discover its highly basic nature [6]. He discovered that nuclein and protamin made up the majority of the mass of the sperm head, and he also provided the first insight into the fundamental interaction that bound these two components together inside the sperm nucleus—that nuclein was bound in a salt-like state to protamin. As the interest in DNA and protamine grew, other researchers began to examine the molecules present in sperm. The majority of the initial work characterizing the composition of protamine molecules was carried out by Kossel and his group, not Miescher, over several decades spanning from about 1890 to the 1920s [7–10]. The proteins bound to DNA in sperm were distinguished from those found in other cells very early on, but the real significance of this difference was not appreciated until almost half a century later when more detailed studies of spermatogenesis and spermiogenesis revealed significant differences in DNA packaging and sperm chromatin compaction. Up until this time, sperm chromatin was considered by many to be similar to the chromatin found in somatic cells.

1.2 Spermatogenesis: Terminal Differentiation and Reprogramming of the Testicular Cell Genome

The testicular cells of men and other mammals undergo a radical morphological transformation as they progress through a process of differentiation called spermatogenesis. Undifferentiated spermatogonia begin the process when they differentiate into primary spermatocytes. Diploid spermatocytes containing two complements of the genome divide in meiosis to produce haploid spermatids that retain only a single copy of each chromosome. In addition to dramatic changes that subsequently occur in the structure of the spermatid at the cellular level (the shape of its nucleus and the development of the flagellum), the chromatin inside these cells also undergoes a series of structural and functional changes. In humans and other mammals, specific genes within the male genome are imprinted to identify their "parent of origin" [11–15], epigenetic modifications in the DNA and proteins packaging the genome prepare the chromatin for early embryonic development [16], and the chromatin is transformed from a highly functional, genetically active state characteristic of somatic cells (spermatogonia and spermatocytes) to a quiescent or completely inactive state found in the fully mature sperm cell.

One might think of this transformation as the testicular cell embarking on a path of terminal differentiation similar to the process that occurs when a stem cell begins to differentiate into a liver, kidney, or brain cell. The final cell not only differs structurally from the stem cell, but it also performs very different functions. Unlike the genome in most stem cells, however, the genome of these spermatids undergoes an additional step in the process, a transient stage in which the entire genome is deprogrammed and shut down. This genome-wide inactivation bears some similarity to processes of heterochromatinization that have been observed to occur with one X

chromosome in vertebrates [17, 18], the entire genome in avian erythrocytes [19], and one set of chromosomes in mealy bugs [20]. These changes, which are brought about by modifying or replacing the proteins that bind to and package DNA, enable the activity of the spermatid genome to be silenced, subsets of the genes to be marked for expression following fertilization, and the chromatin sequestered in a quiescent and protected state until the sperm enters the oocyte and its DNA is ready to be combined with the genome provided by the female to initiate embryogenesis. The process also provides a mechanism by which the genes contributed by the male can be reactivated in the proper temporal sequence and combinations to ensure the first cells function as embryonic stem cells, subpopulations of which later differentiate further into the other types of cells that are required for the development of a fully functional organism.

1.3 Chromatin Reorganization in Maturing Spermatids

Following the second meiotic division of mammalian spermatocytes, the chromatin of the haploid spermatids and their repertoire of functioning genes begin to change over a period of several days [21]. The entire genome of the early spermatid is initially packaged by histones in a manner that is identical to the structure of chromatin (Fig. 1.1) present in all other somatic cells [22]. Variants of histone H2B are

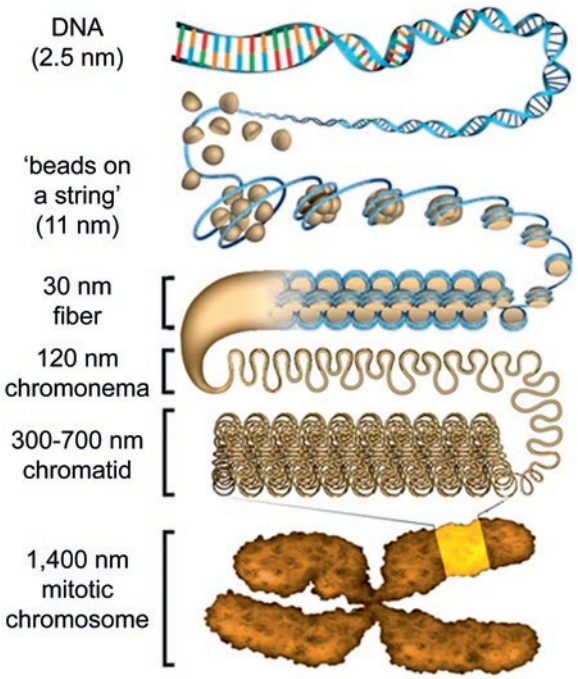

Fig. 1.1 The structure of chromatin (From Ou et al. [215]. Reprinted with permission from AAAS)

incorporated into the chromatin replacing a subset of their somatic counterparts prior to meiosis [23]. Other histones are posttranslationally modified by acetylation and methylation to modulate the interaction of the histone with DNA [24–29]. Acetylation of H3 has been observed to occur throughout spermatogenesis and is considered to relax the interaction of H3 with DNA. H4 acetylation appears to increase specifically during spermatid elongation [30, 31]. While the function of H4 acetylation has not been confirmed, it is thought to play a role in making it easier to displace the histone from DNA during spermiogenesis and also loosen the structure of the histone bound to regulatory regions of genes important for early embryonic development. H4 is increasingly methylated throughout spermatogenesis as spermatogonia differentiate into spermatids [24, 27, 32] and then this methylation is reduced during spermatid elongation [31]. H3 methylation, which occurs predominantly in round spermatids, has been reported to mark regulatory sites [26, 27, 33] on developmentally repressed genes [34] that play a role both in gamete differentiation and early embryonic development.

Only a small fraction of the chromatin in mature sperm retains its histone packaging [33–41]. This histone-containing subset of the genome appears to be present in the sperm of all mammals and is small, comprising not more than 1–2% of the sperm genome in mice and bulls. In human sperm, however, the fraction of DNA bound by histones is significantly larger, possibly as high as 10–15% [41–45]. The H2A, H2B, H3, and H4 histones and their variants that remain associated with DNA in the chromatin of fully mature sperm [43, 46], which will be described in more detail in Chap. 2, have been reported to be associated with centromeric and telomeric DNA [47, 48], matrix-associated regions [49], genes for epsilon and gamma globin [38], paternally imprinted genes [34, 50], retroposons [40], microRNA clusters [34], regulatory sequences [33, 51], genes that produce rRNA [52] and transcription factors such as those in the Hox family [34], genes known to be transcribed in the final stages of spermatogenesis [52], and the transcription initiation sites of a number of genes expressing signaling proteins important for early embryonic development [34, 35, 37, 38, 53]. Some studies suggest the retained histones may be associated with the transcription sites of all genes in sperm [54, 55], preserving the epigenetics of the paternal genome and providing markers to guide their expression in the early embryo [33, 34, 54, 56].

Although the incorporation of the histone variants and the acetylation and methylation of H3 and H4 do not visibly alter the structure of chromatin, a structural change is observed when two small basic transition proteins, TP1 and TP2, are expressed and incorporated into the spermatid chromatin (in human, step 1 for TP2 and step 3 for TP1) [57]. Coincident with the binding of TP1 and TP2 to DNA, the majority of the somatic histones are replaced, and the chromatin becomes more compact. The TP1 protein, which is half the size of a histone, appears to loosen the structure of the nucleosome and facilitate the displacement/replacement of the histones [58]. The larger protein TP2 has two bound zinc atoms and has been reported to stabilize and compact the DNA showing a preference for CG-rich sequences [59, 60].

During their short period of residence in spermatid chromatin, both TP1 and TP2 are posttranslationally modified at multiple sites by phosphorylation, acetylation, and methylation [61, 62]. In contrast to the methylation sites in histone, which occur on lysine residues, both lysine and arginine residues are methylated in the TPs. The presence of these modifications in the protein is likely to have a significant impact on the protein's ability to bind to DNA. Phosphorylation of the serine residues in the C-terminal domain of TP2, which has been shown to be the region of the protein that condenses DNA when it binds, reduces the ability of TP2 to condense DNA [63]. Since TP2 was found to be a poor substrate for phosphorylation when bound to DNA, it has been hypothesized that the phosphorylation of TP2 is more likely to occur prior to DNA binding. Lysine residues in this same region of TP2 have also been shown to be acetylated. In addition to reducing TP2 ability to condense DNA, the acetylation of TP2 has been shown to block the protein's ability to interact with NPM3, a histone chaperone similar to nucleoplasmin [64].

While the binding of TP1 and TP2 affect DNA differently and the two proteins appear to perform different functions, gene knockout studies have suggested the TP1 and TP2 proteins may work together with each being capable of compensating for the other in effecting the displacement of the majority of the somatic histones from the spermatid's DNA [65, 66]. As TP1 and TP2 displace the histones, both proteins also appear to facilitate the repair of DNA damage incurred as the genome is repackaged [67, 68]. Exactly how the TPs remove the histones and whether they work directly or indirectly to induce or initiate DNA repair are not yet known.

HMGB4 and the rat variant HMGB4L1 (previously identified as TP4 [69]), two members of the high mobility group box protein family, have also been observed to be synthesized and deposited in chromatin near the basal pole in elongating spermatids [70] around the same time as TP1 and TP2. Rat spermatids produce both proteins, while neither the HMGB4L1 gene nor the HMGB4L1 protein have been observed in the spermatids of mice or men. HMGB4 has also been detected in spermatocytes and in brain and neuronal cells [71]. RNA profiling and histological analyses in human and mouse testes suggest HMGB4 may play a role in the organization of chromatin in X and Y chromosomes [72]. Other studies have suggested it may also participate in regulating the transcription of genes through the posttranslational modification of histones [71].

The final proteins to be synthesized and deposited in late-stage spermatid chromatin are the protamines. In the mouse, synthesis of protamine P1 begins in step 12 spermatids approximately 24–30 h earlier than protamine P2 [73]. Unlike most mRNAs, those for protamines P1 and P2 are transcribed several days earlier [74–77], their translation is delayed, and protamine synthesis and its deposition into spermatid chromatin only begin after TP1 and TP2 have successfully replaced the majority of the histones. This delay has been shown to be essential for the proper completion of the histone-TP-protamine transition [78]. Without it, the synthesis of the protamines causes early condensation of the spermatid DNA, incomplete processing of the protamine P2 precursor, and induces the formation of abnormally shaped sperm heads. The binding of these protamines to DNA during the final steps of spermatid maturation completes the process of chromatin reorganization, packaging the male's haploid genome into a highly compact, genetically inactive state programmed for reactivation once the sperm head enters an oocyte.

1.4 Protamines P1 and P2

Two different types of protamines have been isolated from mammalian sperm. Protamine P1, the smaller of the two proteins, is found in the late-step spermatids and mature sperm of all mammals [79]. The P1 protamine of placental mammals is a small protein containing only 46–51 amino acids [80]. In marsupials and monotremes, the protamine P1s are slightly larger (typically 57–70 residues). Protamine P2, which is almost twice the size of P1 (typically 100–107 residues), is expressed at significant levels only in the spermatids and sperm of a subset of placental mammals. These include primates, most rodents, lagomorphs, and perissodactyls [79].

Protamine P1 and P2 are similar to the protamines isolated from the sperm of salmon, tuna, and many other fish in that they all contain a series of (Arg)n sequences that wrap around the phosphodiester backbone and bind the protein to duplex DNA. In protamine P1, these anchoring sequences, which are typically separated by one or two uncharged amino acids, make up a central DNA-binding domain that is very similar to the entire sequence of the fish protamines. In contrast to fish protamines, protamine P1 sequences from placental mammals also contain two N- and C-terminal peptide domains that do not bind to DNA [80, 81]. Both of these domains contain serine, threonine, or tyrosine residues that are phosphorylated shortly after the protein is synthesized [82–84]. These domains also contain multiple cysteine residues that form a series of inter- and intraprotamine disulfide bonds and link each protamine molecule to its neighbor as the spermatid matures and passes through the epididymis [81]. The formation of these disulfide cross-links occurs sequentially with the intraprotamine disulfides forming first, beginning in late-step spermatids and nearing completion by the time the sperm enter the caput epididymis [81, 85]. Once formed, the intraprotamine disulfides do not prevent the protein from being dissociated from DNA. As the sperm traverse the epididymis, a series of interprotamine disulfides are formed, cross-linking all the protamines to each other in such a manner that the protamines cannot be removed from the DNA. The resulting network of disulfide cross-links [81, 86] stabilize the structure of the completed sperm chromatin complex making it more difficult for other proteins (e.g., transcription factors and other enzymes) to gain access to the DNA until the protamines are removed following fertilization. With one exception, monotreme (platypus and echidna) [87] and marsupial [88–91] protamines differ from the P1 protamines of placental mammals in that they do not contain any cysteine residues. A shrewlike marsupial in the genus *Planigales* has been found to produce protamines that contain five to six cysteines [90, 92], a number similar to the number of cysteines that are typically found in the P1 protamines of placental mammals.

Unlike protamine P1, P2 is synthesized as a larger precursor protein (101 residues in human, 106 residues in mouse) that is deposited onto DNA and subsequently shortened over a several-day period [93]. Processing of the P2 precursor, which does not begin until several hours after its synthesis and deposition onto DNA, occurs by progressive and sequential cleavage (Fig. 1.2) and removal of a series of short peptide fragments from the amino-terminus of the precursor [93–97]. Each intermediate processed form of the protein persists for several hours before being

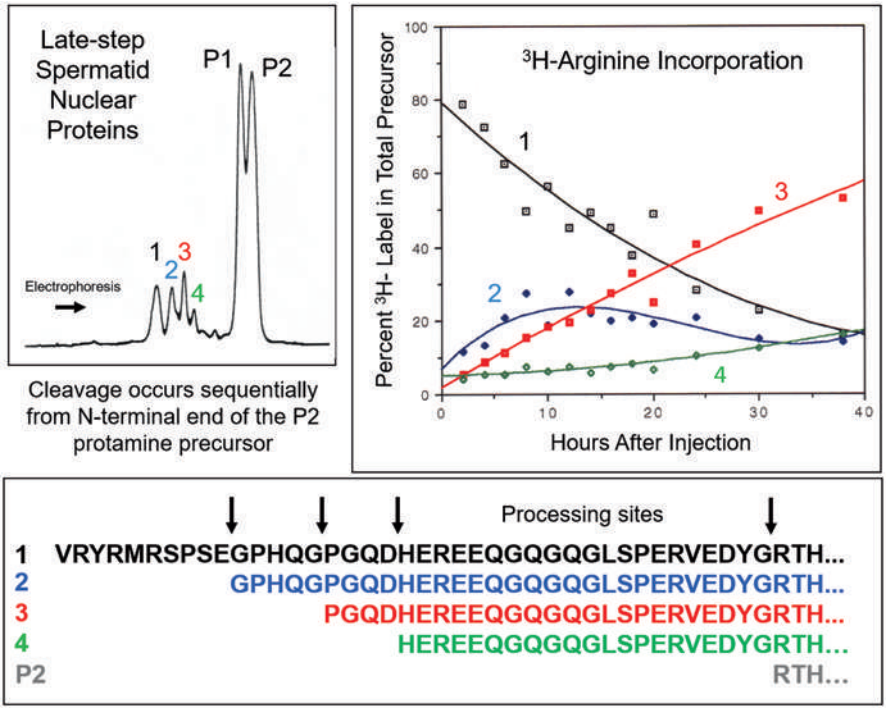

Fig. 1.2 Processing of the P2 precursor, which does not begin until several hours after its synthesis and deposition onto DNA, occurs by the sequential cleavage and removal of a series of short peptide fragments (black arrows) from the amino-terminal domain of the protamine P2 precursor. The intact P2 precursor (1, black) and partially processed forms of P2 (2, blue; 3, red; 4, green) migrate more slowly than P1 and P2 in acid-urea gels. Changes in tritium labeling of the intact P2 precursor and its partially processed forms isolated from the spermatids of mice injected with 3H-arginine show the radiolabel appears first in the intact precursor (1, black; highest ^{3}H-arginine content at 2 h). As the labeling decreases in the intact precursor over time, the label moves next into processed form 2 (blue), followed by processed form 3 (red), and then processed form 4 (green)

processed further [81]. The final processing step occurs approximately 24–30 h after the intact precursor is synthesized and deposited onto DNA. While the function of this processing remains unknown, the sequential nature of segment removal and the observed delay in each step suggest the amino-terminal sequence being removed may have some time-dependent function that facilitates P2's integration into chromatin or modulates the protein's interaction with DNA or other protamines. P2 is also posttranslationally modified by phosphorylation [81, 98] on serine and threonine residues. Both the unprocessed precursor and the fully processed P2 are phosphorylated [98]. The P1 and P2 protamines appear to be phosphorylated prior to their binding to DNA and then repeatedly dephosphorylated and re-phosphorylated until they are properly bound to DNA. The level of phosphorylation progressively declines as the spermatids traverse the epididymis [98]. Once the sperm reach the vas deferens, the majority of the phosphorylation has been removed [83].

Precisely how the final processed form of P2 interacts with DNA has not yet been determined, but analyses of the protamines packaging sperm chromatin in several species suggest the majority of the length of the P2 molecule binds to DNA [42]. The "footprint" of P1 when bound to DNA has been estimated to be 10–11 base pairs, or one full turn of DNA, while the "footprint" of P2 appears to be larger (15 base pairs) [42]. Similar to protamine P1, the final processed form of P2 also contains a series of (Arg)n anchoring sequences that are used to bind the protein to DNA. These segments are shorter than those found in the DNA-binding domain of P1, and they are distributed throughout much of the length of the P2 sequence. P2 also contains multiple cysteine residues that participate in the formation of the disulfide bonds that interconnect all the protamines (both P1 and P2) late in spermiogenesis.

In addition to phosphorylation, mouse protamines P1 and P2 are posttranslationally modified by acetylation on lysine and serine residues and by methylation on lysine residues [99]. All three types of modifications change the charge state of the affected amino acid side chain in such a way that their presence could impact the conformation of the region of the protein containing the modification or disrupt its binding to DNA. In contrast to the acetylated and methylated sites, which can be found in the same protein molecule, phosphorylated and acetylated residues were not found in the same protamine molecule, suggesting they may have separate and possibly exclusive functions.

Protamine P2 also differs from protamine P1 in that it binds zinc [100–102]. Particle-induced X-ray emission (PIXE) analyses of individual mouse and hamster caudal sperm and ejaculated human and stallion sperm have shown the amount of zinc present in the sperm chromatin of each of these species is consistent with a single zinc atom being bound to each P2 molecule [101]. This zinc is already bound to hamster protamine P2 in late-step spermatids isolated from testes, and the stoichiometry (1 Zn/1 P2 protamine) remains constant as hamster sperm traverse the epididymis (Table 1.1). Nuclear zinc has been reported to increase once the sperm enters the zinc-rich seminal fluid [103, 104], but what role this zinc plays in sperm chromatin is currently unclear.

While analyses of zinc bound to protamine P2 in solution have suggested the coordination of zinc by histidine and cysteine residues may bear some similarity to zinc finger proteins [102, 105], other studies conducted with DNA-bound protamine P2 peptides and with intact hamster spermatid and sperm heads indicate zinc is

Table 1.1 Zinc is bound to hamster protamine P2 in late-step spermatids, and the stoichiometry (1 Zn/1 P2 protamine) remains constant as hamster sperm traverse the epididymis

Sperm source	DNA (pg)	P2 (10^{-15} mol)	Zn (10^{-15} mol)	Zn:P2
Cauda epididymis	3.3 ± 0.1	0.131 ± 0.007	0.16 ± 0.04	1.2 ± 0.3
Caput epididymis	3.4 ± 0.1	0.132 ± 0.008	0.15 ± 0.04	1.1 ± 0.3
Testis	3.4 ± 0.1	0.142 ± 0.008	0.15 ± 0.04	1.0 ± 0.3

DNA, protamine P2, and zinc contents were determined by PIXE analysis of individual spermatids and sperm using the nuclear microprobe at Lawrence Livermore National Laboratory as described previously [102]

Table 1.2 X-ray absorption fine structure (XAFS) analysis of zinc coordination by protamine P2 in sonication resistant Syrian hamster testicular sperm and caudal sperm

Chromatin source	Ligand	Coordination distance
Sperm (cauda)	1 S	At 2.33 Å
	3 N/O	At 2.04 Å
Testicular sperm	3 S	At 2.33 Å
	1 N/O	At 2.05 Å
MSH-treated sperm (cauda)	1 S	At 2.33 Å
	3 N/O	At 2.08 Å

coordinated differently when the protein is bound to DNA. Two potential zinc-binding sites were identified in the human protamine P2 peptide [100], one located near the amino-terminus of P2 and a second site near the carboxy-terminus. Only one zinc-binding site near the carboxy-terminus of hamster protamine P2 fits the hamster spermatid and sperm results [106]. What the analyses of P2-bound zinc in hamster sperm and spermatids also showed is that the amino acids in protamine P2 coordinating the zinc change during epididymal transit [106]. Extended X-ray absorption fine structure analyses of sonication-resistant (late-step) hamster spermatids have shown that, prior to the formation of the intraprotamine disulfides, the zinc is coordinated by three cysteines and one histidine or carboxyl group in protamine P2 (Table 1.2). Only one site located near the carboxy-terminal end of P2 (Fig. 1.3, structure A) has three cysteine residues and a histidine in close enough proximity to each other to coordinate zinc [106]. Once all the protamines have been deposited in sperm chromatin and the sperm pass through the epididymis, the coordination of zinc changes with two of the three cysteine residues coordinating zinc in late-step spermatids being replaced by two histidines or carboxyl groups (Fig. 1.3, structure B) in mature sperm. Following treatment of mature sperm chromatin with a reducing agent in vitro, the amino acids coordinating zinc change back to the arrangement observed in late-step spermatids. This change in amino acids coordinating zinc, which occurs as the inter-protamine disulfide bonds are being formed, may reflect the initial protection and sequestration of specific cysteine residues until they are needed late in spermiogenesis for inter-protamine disulfide bond formation.

One interesting alternative theory proposed by Bjorndahl et al. [107] suggests that inter-protamine disulfides may not cross-link protamines together during the final stage of sperm chromatin maturation, but that the final step in the stabilization of the DNA-protamine complex is instead brought about by zinc forming inter-protamine zinc-dithiolate cross-links between neighboring protamine molecules [103, 107]. While this is unlikely to happen in the sperm of species whose DNA is packaged only by protamine P1 (all current studies indicate P1 does not bind zinc), zinc-mediated cross-linking of neighboring protamine P2 molecules (on same strand of DNA or neighboring strands in coiled toroid) could occur (Fig. 1.3, structures C and D) and might explain why the sperm chromatin containing protamine P2 is more easily decondensed in species that use protamine P2 to package their DNA [108]. Other cases have been reported in which tetrahedral zinc coordination by two different protein subunits or partners is used to stabilize an interaction [109, 110].

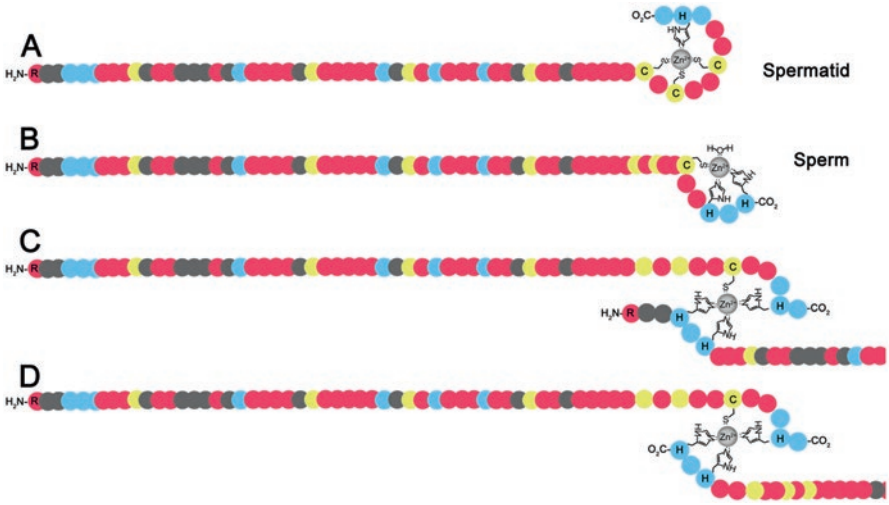

Fig. 1.3 Proposed mode of zinc coordination by protamine P2 in the Syrian hamster and changes that occur during the final stages of sperm chromatin maturation as the sperm traverse the epididymis. The site located near the carboxy-terminal end of Syrian hamster P2 has three cysteine and three histidine residues in close enough proximity to each other to coordinate zinc. The proposed zinc coordination shown in **A** is consistent with XAFS data obtained from analyses of Syrian hamster late-step spermatids (Table 1.2) which show the zinc bound to protamine P2 is coordinated by three cysteines and a histidine. By the time the sperm pass through the epididymis and reach the cauda, the amino acids coordinating the zinc in mature sperm have changed to a structure consistent with that shown in **B** where the zinc is coordinated by a single cysteine, two histidine residues, and a water molecule. Possible inter-protamine P2 zinc-mediated cross-links that could form between **C** the carboxy-terminal domain of one P2 and the amino-terminal domain of a different P2 or **D** the carboxy-terminal domain of one P2 and the carboxy-terminal domain of a different P2. Such zinc-coordinated cross-links could form between adjacent P2 molecules bound to the same DNA or between P2 molecules on different strands of DNA (such as those packed together during the coiling of the DNA-protamine complex into a toroid)

Artificial complexes with similar structures have also been generated to test the feasibility of using zinc coordination by a pair of partners to create novel peptide assemblies [111].

1.5 Protamine-DNA Interactions and Structure of the Complex

Differences in P1 and P2 protamine sequence, the synthesis of P2 as a precursor and its subsequent processing, the binding of zinc to P2 but not P1, the observation that sperm chromatins containing P2 are less stable than those containing only P1 [108], and the fact that no species has been identified that produces sperm with its DNA packaged only by protamine P2 suggest there are likely to be important differences

in the way the P1 and P2 protamines bind to and "package" DNA. The observation that many species of mammals produce sperm with their DNA packaged only by protamine P1, while none are known to use only protamine P2, also suggests that P1 may be sufficient for the final packaging and that P2 contributes to the process but cannot be substituted entirely for P1. In those species whose sperm contain both protamines P1 and P2, the relative amounts of the P1 and P2 protamines found in the sperm chromatin of mammals varies widely between mammalian genera, but the relative proportion of the two proteins packaging the DNA appears to be conserved among the species within a genus [79].

Beyond the knowledge that both protamines P1 and P2 bind to DNA in some manner that allows the two proteins to be cross-linked together by disulfide bridges during the final stage of sperm maturation, we know very little about how protamines P1 and P2 are distributed along a segment of DNA. Experiments analyzing chemically cross-linked protamines and the disulfide bonds that are formed within and between protamine molecules in rodents, taken together with the variability in proportion of P2 present in the sperm of different mammalian species, have suggested protamine P2 molecules are likely to be clustered together along the DNA as dimers [112, 113]. This would be consistent with the observed conservation of cysteine residues (numbers and locations) in protamine sequences, and it could also explain how a similar pattern of disulfide bond formation could be used to interlink all the protamines together irrespective of the relative proportion of P1 and P2 [112, 113].

Protamines are unusual in that they are unstructured in solution [114] and only adopt a specific conformation when they bind to DNA. At the molecular level, protamines bind to duplex DNA in a manner that has been shown to be independent of base sequence [115, 116]. The primary interactions are electrostatic and involve the binding of the positively charged guanidinium groups in the arginine residues present in the DNA anchoring domains of protamine to the negatively charged phosphates that comprise phosphodiester backbone of DNA. The high affinity of binding is derived from two aspects of these interactions, the formation of a salt bridge and hydrogen bond between the guanidinium group and the phosphate and the multivalency achieved through the binding of multiple arginine residues in the DNA-binding domain of protamine to an equivalent number of phosphate groups in DNA. Computer modeling, X-ray scattering, and other experimental studies [114, 117–119] have provided evidence to suggest that the DNA-binding domain of protamine P1 wraps in an extended conformation around the DNA helix (Fig. 1.4), positioned above and stretching across the major groove. Adjacent arginine residues in the (Arg)n anchoring domains bind to phosphates on opposite strands of the duplex DNA molecule, interlocking the relative positions of the bases together and preventing strand separation or changes in DNA conformation throughout the period that the protamines remain bound to DNA. In order for alternating arginine residues in the (Arg)n anchoring sequences to bind to phosphates in opposing strands of the DNA helix, the amide backbone of the DNA-binding domain is forced into a unique conformation similar to a gamma-turn [114]. Following the binding of the protamines to DNA, a neutral, highly insoluble chromatin complex is produced that enables DNA strands to be packed tightly together without charge repulsion.

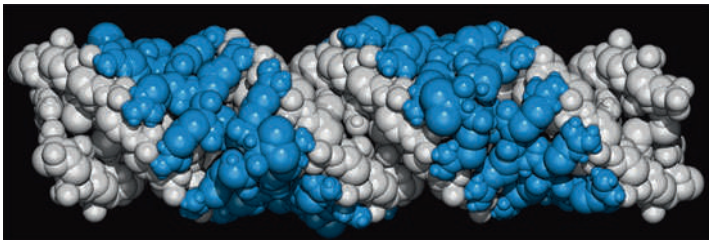

Fig. 1.4 A computer-generated molecular model of a DNA-protamine P1 complex shows how the DNA-binding domain of protamine P1 wraps in an extended conformation around the DNA helix, positioned above and stretching across the major groove. The structure contains four turns of DNA (white) and two bull protamine DNA-binding domains (blue)

Low-angle X-ray scattering experiments performed on intact sperm heads have confirmed the close packing of the DNA within sperm chromatin, showing the center-to-center distance between adjacent DNA molecules is approximately 2.7–3 nm [117, 120, 121]. To achieve this tight packing, the protamine-bound molecules must be organized in a hexagonal arrangement [86] with only 0.7 nm distance of separation between the surfaces of adjacent molecules. This is achieved by coiling the DNA into toroidal structures approximately 100 nm in diameter that contain ~50,000 bp of DNA. High-resolution EM studies of individual toroidal subunits [122] have confirmed the individual DNA molecules are tightly packed in a hexagonal arrangement, consistent with what has been observed by low-angle X-ray scattering.

1.6 Higher-Ordered Organization of Chromatin in Mature Sperm

Electron microscopy (EM) images of the chromatin in differentiating spermatids have shown that the DNA is initially organized similar to somatic chromatin (~11 nm nodules/nucleosomes and 30 nm fibers [22, 123]), which subsequently transforms into nodular structures or fibers in late-step spermatids with diameters (50–100 nm) much larger than individual nucleosomes. As the protamines are deposited in the chromatin, dephosphorylated and their disulfide bonds begin forming, these nodules coalesce into increasingly larger masses or fibers that eventually become so tightly packed and electron dense that the individual structures can no longer be resolved. Both the extreme degree of compaction of the DNA into the toroidal subunits and the insolubility of the DNA-protamine complex have made it difficult to obtain more detailed information about the higher-ordered arrangement of toroids and nucleosomes packed inside the nucleus by direct analyses of mature sperm.

Additional information has been obtained, however, by partially disrupting sperm chromatin using polyanions, reducing agents, high ionic strength, or following partial digestion by nucleases [112, 124–130]. EM and scanning probe microscopy

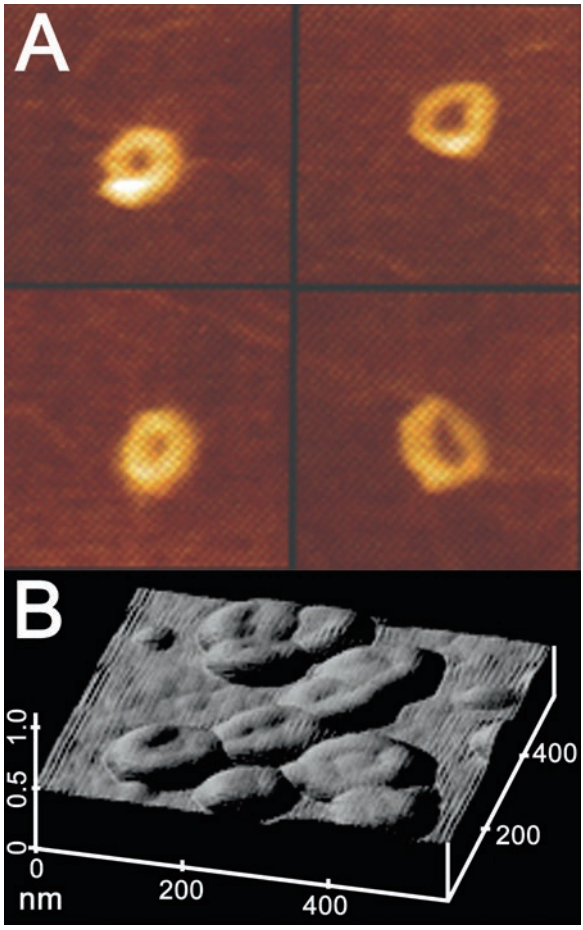

Fig. 1.5 Scanning probe microscopy images of **A** toroidal structures (100 nm) with lifesaver-like features generated in vitro when bull protamine P1 is added to dilute solutions of DNA (7.5 kb plasmid) loosely bound to a Mg+2 treated mica surface (dimensions of each toroid image is 325 × 325 nm) and **B** globular lifesaver-shaped toroid structures in dispersed human sperm chromatin. These native DNA-protamine toroids are approximately 100–150 nm in diameter and 20 nm thick with a hole or depression in the center

images of decondensed human sperm (Fig. 1.5) have revealed the presence of two types of structures, small subunits similar in diameter (~10 nm) and thickness (~5 nm) to somatic nucleosomes and larger globular lifesaver-shaped structures approximately 100 nm in diameter and 20 nm thick with a hole or depression in the center [112]. Toroidal structures with lifesaver-like features and similar dimensions (100 nm) are also spontaneously generated in vitro when protamine (Fig. 1.5) [112, 116], viral proteins involved in DNA encapsulation [131, 132], and other polycations are added to dilute solutions of DNA or to individual DNA molecules [133–135]. The size of the toroids, which have been generated using a wide variety of lengths of DNA and condensing agents, appears to represent a minimal energy state for DNA condensed by protamines and other polycations [122, 134, 136]. The toroids formed by protamine binding to DNA contain approximately 50,000 bp of DNA [112, 116]. Closely packed structures with diameters similar to these toroids were found by Koehler to comprise the lamellar sheets of chromatin packed inside rat, rabbit, bull, and human sperm [127, 128, 137]. Such a packing arrangement for

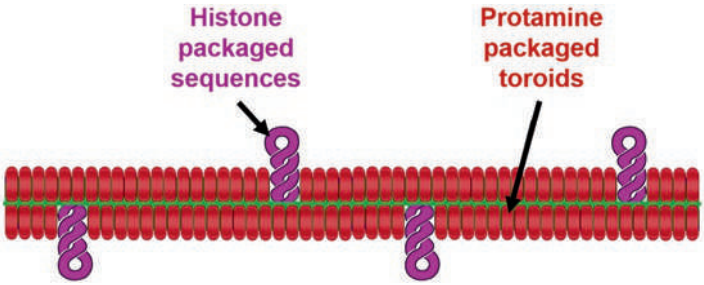

Fig. 1.6 Model showing arrangement of protamine-packaged DNA toroids (red) stacked side by side in sperm chromatin with interspersed regions of DNA packaged by histones (purple) as proposed by Ward et al. [49] (See Chap. 3, Fig. 3.2, for the complete model)

DNA would be consistent with the microscopy data obtained from stallion sperm heads [138] if the toroidal structures are stacked tightly together as lifesavers (Fig. 1.6 and see Chap. 3, Fig. 3.2) similar to the models presented by Ward [49] and Vilfan et al. [86]. While areas of less densely packed DNA similar to nucleosomal containing somatic chromatin were not observed by Koehler, images obtained from scanning probe microscopy studies of human and mouse sperm chromatin do show clusters of nucleosome-sized structures interspersed between much larger toroidal chromatin domains packaged by protamines [112].

1.7 Preservation of DNA Domains and Nuclear Matrix Associations

Following the multitude of nuclear protein transitions and the final compaction of the spermatid genome by protamines into a densely packed chromatin "particle," several aspects of somatic chromatin architecture still appear to be retained inside the sperm head. Confocal microscopy of somatic cells has shown in a number of cases that the DNA molecules that comprise individual chromosomes are not randomly distributed throughout the nucleus, but each appears to be confined to a specific domain or territory inside the interphase nucleus [139–143]. Similar observations have been made regarding the distribution of chromosomal DNA inside the heads of human, bull, mouse, echidna, and platypus sperm [47, 144–147]. While studies conducted with sperm from placental mammals have not provided strong evidence that the chromosomes are arranged in any particular order relative to each other, there is some evidence for a specific arrangement in echidna and platypus sperm.

Two other organizational features retained in sperm cell nuclei are the chromatin loop domains and the attachment of the chromatin to a nuclear protein scaffold or nuclear matrix [124, 148–151]. While protein content of the nuclear matrix changes as the spermatid differentiates [148], the DNA remains bound to the matrix at a very large number of sites (~50,000). This matrix appears in EM images as a network of dense protein filaments filling the interior of the head of

the spermatid and sperm bounded by a peripheral structure, the lamina. The DNA in between the sites of attachment to the matrix retain the loop organization present in somatic cells [152, 153]. These loops, which contain ~40,000–50,000 bp of DNA in both the somatic and sperm nucleus, are anchored to the matrix through specific chromatin domains, called nuclear scaffold attachment regions/matrix attachment regions (SARs/MARs). The retention of the matrix and its associations with DNA in sperm are important to maintain because their presence would facilitate and speed up the process of genome reactivation following fertilization and the initiation of the first cycle of DNA replication in the male pronucleus [154, 155]. The loop domains play important roles in transcriptional regulation, DNA replication, and chromosome organization both prior to spermiogenesis and after fertilization. In sperm these loops may also aid in the packing of the DNA by protamines into toroids which also contain ~50,000 bp of DNA ([49, 112, 116] and (Fig. 1.6 and Chap. 3, Fig. 3.2).

1.8 Reactivation of Paternal Chromatin Following Sperm-Oocyte Fusion

Fertilization and the entrance of the haploid male genome into the oocyte trigger a cascade of events [156] that rapidly convert the genome back to nucleosome-organized chromatin and activate sets of genes within the male genome required for the first steps in embryonic development. Removal of the protamines and paternal histones and deposition of the histones provided by the oocyte onto DNA appears to be accomplished by a histone chaperone [157–165] similar to the nucleoplasmin first identified in frogs [162, 166, 167]. Sequence analyses of the frog and related mammalian nucleoplasmins have shown these proteins contain a series of polyglutamic acid sequences that may facilitate the removal of the protamines from the DNA prior to loading it with histones [168] by forming a series of salt bridges with the (Arg)n DNA-binding domains of the protamines [160, 164, 165]. The (Arg)n segments in the protamines, which have a higher affinity for polyglutamic acid than the phosphodiester backbone of DNA, would then release from the DNA and allow the chaperone to deposit the histones and regenerate the nucleosomal organization required to reactivate the new embryo's genome.

Another early event associated with the unpacking of the sperm chromatin that occurs almost immediately after removing the protamines is the initiation of a period of DNA synthesis associated with DNA damage repair [169–172]. This repair synthesis is required to repair DNA strand breaks and remove DNA adducts or other damage that is acquired during spermiogenesis and epididymal transit and storage when repair activities could not be performed due to the packaging of the genome by protamines. Studies have shown that the majority of the damage brought into the oocyte by the sperm is repaired during this period of DNA synthesis, and this process is considered to be critical for maintaining the integrity of the male genome and for ensuring normal embryonic development.

1.9 Consequences of Disrupting Sperm Chromatin Remodeling

A number of the chromatin protein-related changes associated with the repackaging of spermatid genome have been shown to be important for male fertility. These include the removal of the majority of the somatic histones by transition proteins TP1 and TP2, the deposition and posttranslational marking of a subset of paternal histones, and the replacement of the TP proteins by protamine. Numerous studies have suggested there is a positive correlation between male subfertility or infertility and elevated levels of histone in mature human sperm [95, 120, 173–178]. Alterations in the expression and/or translation of the protamine genes leading to a change in the proportion of the P1 or P2 proteins present in sperm chromatin have been shown to not only be linked to infertility [179–189] abnormal sperm head morphology and high levels of DNA fragmentation [186, 190] but to also adversely impact IVF (in vitro fertilization) outcome and early embryonic development [191–195]. The observed differences in protamine content of sperm obtained from infertile males ranged from sperm chromatin containing very little protamine to having too little protamine P1 or too little protamine P2. Defects in protamine P2 precursor processing, which have also been observed in infertile males [196, 197] and a male experiencing a high fever during an episode of influenza [198], may indirectly contribute to the reduction in the amount of P2 (the fully processed form of the P2 precursor).

Other studies have shown that the timely formation of the protamine disulfide cross-links that occur during the final stages of sperm maturation are also important for fertility. In mammals, both protamines P1 and P2 contain multiple cysteine residues. The thiol groups of these cysteines are in the reduced form (free thiols) when the protamines are synthesized and deposited onto DNA, and they remain reduced until the final stage of spermiogenesis when they participate in the formation of both intra-and intermolecular protamine disulfides as the spermatids elongate and the sperm pass through the epididymis [81, 199–202]. A number of cases of human, stallion, and bull infertility have been associated with what appear to be errors in disulfide cross-linking among the protamines. In the sperm of fertile males, the formation of the disulfide bonds is believed to stabilize the chromatin and protect it from physical damage. An equally feasible possibility is that these disulfide bonds not only stabilize the chromatin, but they also prevent the thiol groups from being oxidized or alkylated during the long period of time required for spermatid maturation and sperm storage prior to fertilization. This might be important if the cysteine residues in mammalian protamine also participate in the process of protamine removal from DNA after fertilization. Cysteine-free thiols are excellent free radical scavengers and are susceptible to oxidation to cysteic acid. If functional free thiols are required for efficient protamine removal, the oxidation or alkylation of even a few cysteines could potentially complicate or prevent the efficient removal of the modified protamine from the male genome, and its retention would block the gene it was bound to from being transcribed or replicated later in development.

Mice exposed to alkylating agents prior to protamine disulfide bond formation have been shown to produce sperm with alkylated protamine thiols [203–205]. Matings conducted with the treated males resulted in the production of embryos that died early in development from dominant lethal mutations [204]. The sperm containing the protamines with alkylated protamines succeeded in fertilizing oocytes and inducing embryonic development, but at some point the embryo died when a key gene could not be turned on.

Male infertility has also been linked to deficiencies in sperm chromatin-associated zinc. Zinc is known to be essential for several aspects of sperm development, ranging from contributions to structural elements in the tail to roles in chromatin organization and protamine structure and function [107]. A deficiency in zinc can affect the developing sperm directly, or it can impact the function of other testicular cells that contribute to or play a role in spermatid maturation, such as Sertoli cells. Because zinc plays multiple roles in spermatogenesis and testicular function, it has been difficult to decipher how sperm chromatin-bound zinc impacts the functionality of the sperm cell. Since sperm chromatin-associated zinc is almost exclusively bound to protamine P2 in mammals [101], it has been suggested that the coordination of the zinc by protamine P2 may influence the binding of the protamine to DNA [102, 105] or to other protamines [107].

An alternative possibility is that zinc coordination by cysteine residues in protamine might also protect the thiol groups and prevent their oxidation [206, 207] until it is time for the cysteines to form inter- and intramolecular disulfide bonds. Several studies have also suggested that exposures to other metals, such as copper and lead, may result in these metals binding to the cysteines in protamine in place of zinc (or prior to disulfide bond formation) and their being transported into the oocyte upon fertilization [201, 208–210]. In addition to potentially disrupting the function of sperm by altering chromatin decondensation or protamine P2 function, the delivery of these and other toxic metals into the oocyte would also be expected to have an adverse impact on early embryonic development.

DNA damage incurred during spermatid chromatin reorganization, deficiencies in transition protein synthesis and posttranslational modification, and defects in a number of epigenetic processes that contribute to imprinting [211, 212] and the reprogramming of the haploid genome during spermatogenesis have also been shown to adversely impact male fertility and the postfertilization function of the male genome [213]. Because sperm histones provide epigenetic information that regulates the transcription of genes in the two-cell embryo, environmental perturbations have the potential to change the pattern of gene expression in embryos via changes/differences in sperm chromatin composition during the reactivation of the male genome. Several of these processes will be described in the chapters that follow. A number of excellent reviews have been published describing others [14, 15, 30, 214].

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