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## **Biophysical ordering transitions underlie genome 3D re-organization during cricket spermiogenesis.**

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## ABSTRACT

Spermiogenesis is a radical process of differentiation whereby sperm cells acquire a compact and specialized morphology to cope with the constraints of sexual reproduction while preserving their main cargo, an intact copy of the paternal genome. In animals, this often involves the replacement of most histones by sperm-specific nuclear basic proteins (SNBPs). Yet, how the SNBP-structured genome achieves compaction and accommodates shaping remain largely unknown. Here, we exploited confocal, electron and super-resolution microscopy observations, coupled with polymer modeling simulations to identify the higher-order architecture of sperm chromatin in the needle-shaped nucleus of the emerging model cricket *Gryllus bimaculatus*. Accompanying spermatid differentiation and shaping, the SNBP-based genome was strikingly reorganized as ~25nm-thick fibers orderly coiled along the elongated nucleus axis. This chromatin spool was further found to achieve large-scale helical twisting in the final stages of spermiogenesis, favoring its ultracompaction. Through a combination of microscopy observations and polymer simulations, we revealed that these dramatic transitions may be recapitulated by a surprisingly simple biophysical principle based on a nucleated rigidification of chromatin linked to the histone-to-SNBP transition within a confined nuclear space. Our work highlights a unique, liquid crystal-like mode of higher-order genome organization in ultracompact cricket sperm completely distinct from nucleosomal chromatin, and establishes a multidisciplinary methodological framework to explore the diversity of non-canonical modes of DNA organization.

## **SIGNIFICANCE STATEMENT**

Animal sperm cells are highly compact and atypically shaped compared to other cell types. How DNA is packaged and organized in the 3D space of sperm cell nuclei to cope with these constraints is poorly understood. In this work, we identified an original and elegant solution to this problem in crickets, whereby DNA fibers orderly spool and twist to fit into ultracompact, needle-shaped sperm cells. To understand this reorganization, we modeled DNA fibers in the nucleus as polymers and found that a relatively simple mechanism through which fibers become more rigid bit by bit can largely recapitulate our observations. Our multidisciplinary work highlights a simple solution to compact DNA to extreme levels in specialized nuclei.

## MAIN TEXT

### INTRODUCTION

During spermiogenesis, post-meiotic spermatogenic cells differentiate to fulfill their main function of transporting an intact paternal genome into the egg. This imposes two critical constraints. First, this genome must fit into a cell that is morphologically optimized for the specific requirements of sexual reproduction in every species (1-3). Second, various genome activities are shut down and the genome is packed in a manner that minimizes damage and mutations<sup>1</sup>. Accommodating these constraints, there is a remarkable diversity in the morphology of sperm cells in animals. Notable examples include the falciform-shaped mouse sperm, the rod-shaped rooster sperm, the drill-bit-shaped sperm of certain sparrows and squids, or the needle-shaped sperm that is common among insects (2). To fit into these complex morphologies, the paternal nucleus typically undergoes a high level of compaction - ~10x to ~40x compared to somatic interphase cell nuclei, depending on the species (4,5). Abnormal compaction and/or morphology are systematically associated with reduced fertility (6-8), illustrating the physiological importance of these mechanisms.

Differentiating spermatogenic cells (spermatids) typically undergo re-shaping accompanied by removal of excess cytoplasm and formation of sperm-specific structures including the apical acrosome and the basally anchored sperm flagellum (9). Actin filaments and microtubules play a critical role in this remodeling process (9-10). The sperm head is at least in part shaped by a stack of F-actin hoops anchored at the acrosome-nucleus junction (a structure called *acroplaxome*) and a transient microtubule-actin basket-like structure known as the *manchette*, that encircles the nucleus. These structures are thought to act as scaffolding fibers contributing to constrained spermatogenic cell elongation by contractile forces and counter-selecting aberrantly shaped or condensed cells. Additionally, as the manchette forms, nuclear pores are redistributed caudally and are no longer found in fully mature spermatozoa (11). Concomitantly, the importance of multiple sperm-specific lamin, lamin-associated and LINC (linker of nucleoskeleton and cytoskeleton) proteins for sperm nuclear apico-basal polarization, shaping and compaction is well-documented (12-13).

In contrast, the link between sperm morphology establishment and genome reorganization has remained unclear. At the chromatin level, spermiogenesis in many animals involves widespread remodeling (14-16). In most eukaryotic cells, chromatin is organized as a string of nucleosomes, composed of ~146bp of DNA wrapped around an octamer of histone proteins. Histone proteins can further undergo post-translational modifications that alter their properties and enable their interaction with effector complexes (17). While nucleosomes are essential to organize chromatin and modulate its functions in somatic nuclei, the vast majority of histones are removed from chromatin during sperm differentiation and replaced by Sperm-specific Nuclear Basic Proteins (SNBPs) in many animal species, including mammals and insects (14-16). In mouse and *Drosophila*, histones first become broadly acetylated (18-20), a modification that entails changes in the biophysical properties of the chromatin, favoring DNA accessibility and increasing short-range rigidity (21). Acetylated histones are further recognized in both species by testis-specific bromodomain proteins (BRDTs) (22-25). Via BRDTs, acetylated histones are evicted and replaced on DNA by Transition Protein-like proteins (TPLs) and Protamines (in mammals) or Protamine-like proteins (PLs in insects). TPLs, Protamines and PLs are highly basic, fast-evolving small proteins (26-27). Contrasting with TPLs, Protamines and PLs usually feature multiple cysteine residues, which can form intra- and/or inter-molecular covalent disulfide bonds that participate in Protamines and PLs stability on DNA (28-30). Disulfide bond reduction at fertilization is thus required for paternal chromatin decompaction, PL removal, nucleosome assembly and paternal genome incorporation into the first zygotic nucleus (31). *In vitro*, Protamines acting as multivalent cations can organize DNA into ~150nm-wide tori (26,32-33). Extracted chromatin from vertebrate sperm confirmed the presence of torus- as well as rod-shaped structures (34). Yet, the actual *in situ* configuration of the genome in mature sperm is notoriously difficult to access, in large part due to the extreme density and insolubility of this substrate (35).

Important insights into this issue came from electron microscopy studies of insect spermatogenesis carried out over the last seventy years, which revealed an outstanding diversity in the large-scale re-configuration of the nuclear content. In insects, although sperm chromatin is generally diffuse in spermatocytes (meiotic germ cells) and highly compact in mature sperm, genome organization in intermediate spermatid stages can widely vary between species. A frequently observed feature is the progressive thickening of chromatin fibers, further organizing into lamellae, for example in sperm from the grasshopper *Chortophaga viridifasciata* (36), the firebrat *Thermobia domestica* (37), the cave cricket *Ceutophilus nigricans* (38), the bush katydid *Scudderia* or the treehopper *Ceresa diceros* (39). As a striking case in point, pioneering studies on the two-spotted cricket *Gryllus bimaculatus* revealed a highly regular configuration of DNA in maturing sperm, consisting of ~30nm-thick chromatin fibers that were bundled, stretched and coiled along the needle-shaped nucleus axis (40). This remarkable organization is arguably among the simplest solutions to higher-order genome folding in any cell type or species, thus providing a unique opportunity to dissect the mechanisms underlying large-scale remodeling of nuclear architecture.

Here, we investigate the underlying principles of such drastic spatial rearrangements of the genome in *G.bimaculatus* male germ cells. By exploiting confocal, electron and single-molecule localization microscopy, we identify the successive chromatin structural and architectural transitions that accompany spermiogenesis. To understand their mechanistic, physical bases, we establish a simple polymer modeling approach to simulate genome dynamics in the restricted sperm nuclear space. By iteratively confronting simulations and observations, we reveal that chromatin spooling can be explained by a nucleated, gradual increase in chromatin rigidity during the histone-to-protamine transition. We further uncover that the alignment of spooled fibers along the nuclear axis does not precede, but rather follows antero-posterior cell polarization and elongation, indicating that chromatin configuration may adapt to cell shape in a liquid crystal-like fashion. Finally, we provide evidence that spooled fibers twist around the nucleus axis during terminal differentiation to achieve an ultracompact configuration that is maintained by disulfide bonding. Our results demonstrate

how a relatively simple set of changes in the biophysical properties of the chromatin fiber may lead to radical tridimensional re-organization of the genome that allows its fitting into the sperm nucleus.

## RESULTS

### **Ordered coil configuration of chromatin fibers in sperm following the histone-to-protamine transition**

We first sought to better characterize spermiogenesis in *G.bimaculatus*, i.e. the differentiation of haploid post-meiotic germ cells. Based on nuclear morphology, by analogy to well-characterized spermiogenesis stages in *Drosophila* (14), we identified multiple steps in germ cell proliferation and differentiation including spermatocytes, round spermatids, canoe (elongating) spermatids and needle-shaped spermatids (Fig. 1A). As previously reported, the needle-shaped nuclei in mature cells were  $\sim 0,5\mu\text{m}$  in width and  $\sim 20\mu\text{m}$  in length (40). A previous study has shown that histone proteins are lost in mature sperm nuclei, where only a single species of acid-extractable SNBP remains (40). Accordingly, with immunofluorescence using an antibody that broadly targets histone core domains and H1, we confirmed that the strong histone signal in early elongating spermatids is undetectable at later stages (Fig. 1A,B). Consistently, histone H3 could be readily revealed by Western blot on testes extracts but was undetectable in mature sperm extracts (Fig. 1C). These results confirm a widespread histone-to-SNBP replacement in this species.

We further corroborated by electron microscopy the remarkable organization of chromatin into bundled, coiled fibers (Fig. 1D(l,a)). We found that  $\sim 25\text{nm}$ -thick fibers stretch into  $\sim 200$  linear segments along the nucleus axis in canoe spermatids (Fig. 1D(t)). We observed that the flagellum cytoskeletal structures are lodged within an indentation at the basal-most end of the nucleus (Fig. 1D(b)). At the apical – acrosomal – end, we detected bending of the fibers, as previously described (Fig. 1D(a))(40). Sagittal sections of these  $\sim 25\text{nm}$ -thick



fibers (Fig.1D(s)) further revealed an internal structure in which DNA appears to be condensed within a protein cylinder featuring a central protein axis. Considering a genome of 1.66Gb in size, as recently described (41), we estimated a DNA linear density of  $\sim 415\text{bp/nm}$  along the fiber. With the volume of one base pair being of approximately  $1\text{nm}^3$ , the intra-fiber volume fraction of DNA is close to 100%, compared to  $\sim 10\%$  for nucleosomal chromatin fibers. Furthermore, assuming a 500nm thick and  $20\mu\text{m}$  long mature sperm nucleus, we evaluated that these fibers occupy  $\sim 70\%$  of the nuclear volume, while chromatin occupies  $\sim 10\%$  of the nuclear volume in typical somatic nuclei. Together, these observations confirm the remarkable organization of the *G.bimaculatus* sperm genome into an elongated, ordered coil of thick fibers that compact DNA to high levels and occupy most of the nuclear space.

### **Stiffening of a polymer in a confined space recapitulates chromatin nuclear re-localization.**

To understand the mechanical underpinnings of this striking transition, we developed a simple polymer model considering the specific features of sperm chromatin (see Methods). We modeled chromatin as a polymer consisting of identical 30nm monomers, whose kinetics are controlled by the interplay between thermal motion, steric repulsion and bending rigidity. This long chain is confined within a flexible and discrete nuclear membrane, modeled as a polymerized elastic shell. Positively charged SNBPs are expected to result in a higher stiffness of sperm chromatin compared to that of nucleosomal chromatin (21,29,42), which we translated into an increase in the local bending rigidity of the polymer. Thus, we represented the mature chromatin fiber as a worm-like chain of persistence length  $l_p \cong 1.5\ \mu\text{m}$ , qualitatively consistent with our microscopy imaging data in elongating spermatids. We further modeled the chromatin backbone connectivity via harmonic springs whose finite stiffness was adjusted to emulate a regime of moderate-to-high topoisomerase activity, in order to allow for large-scale rearrangements of the confined chain within computationally-accessible timeframes (see Methods).

First, we simulated the re-organization of a polymer upon a progressive, uniform increase in bending rigidity, starting from an isotropic, disordered conformation of an initially flexible chain. In these conditions, multiple segments of the polymer progressively coiled in different directions (Fig. 2A, left) and preferentially localized at the periphery of the nuclear space upon full rigidification (Fig. 2C, top). To evaluate the relevance of these predictions, we analyzed early stages in cricket spermatogenesis by electron microscopy. As expected, round  $\sim 10\mu\text{m}$  diameter cells (consistent with that of spermatocytes) displayed a uniform chromatin arrangement: the nuclear content was overall homogeneous, with the exception of a heterochromatin-like area located at the nuclear periphery (Fig. 2B). Upon progression towards the round spermatid stage, we found that round spermatids displayed a  $\sim 20\times$  smaller nuclear volume compared to spermatocytes (Figs. 1A-2B,C). Concomitantly, fluorescence and electron microscopy revealed an increasingly inhomogeneous distribution of nuclear material, characterized by a marked enrichment of chromatin at the nuclear periphery (Fig. 2C, bottom). As confirmed by our simulations, this loss of uniformity is characteristic of a strongly confined, stiff polymer, and becomes increasingly pronounced as the polymer persistence length increases relative to the decreasing radius of the confining cavity (Fig. 2A). Hence, the observed peripheral enrichment of chromatin may be attributed to the combination of the rigidification of the fiber with the reduction in nuclear dimensions measured at the round spermatid stage, consistent with the results of our model predictions.

To understand how this inhomogeneously oriented coiled polymer could fill the elongating nucleus during spermiogenesis, we imposed an arbitrary stretching of the membrane through an external radial force applied at two opposite poles (Fig. 2A, right). Upon stretching, the polymer coils persisted but did not align to the elongation axis. These predictions did not fit our observations in electron microscopy whereby early elongating nuclei instead already displayed a pronounced antero-posterior alignment of chromatin fibers (Figs. 2D,E). We conclude that a simple uniform polymer rigidification scenario only partially recapitulates the chromatin reorganization kinetics *in vivo* and, in particular, does

not account for the strikingly unidirectional orientation of chromatin fibers observed in maturing sperm.

### **Spooled fiber formation is consistent with a nucleated increase in chromatin rigidity**

To refine our model, we considered the possibility that changes in polymer properties did not occur simultaneously over the entire genome, but were instead nucleated at discrete regions and spread gradually in *cis* along the fiber (see Methods). In particular, we simulated the dynamical reorganization of chromatin when the local bending rigidity was first increased in a progressive fashion at a limited number of nucleation sites, initially located near the center of the nucleus (Figs. 3A,B). Upon full rigidification of the nucleation domains, this gradual stiffening kinetics was in turn propagated to their immediate adjacent regions along the chain, and the process was repeated iteratively until the entire polymer reached a homogeneous rigidity. Under these assumptions, locally stiffened chromosomal segments were found to be rapidly deported to the nuclear periphery, where they adopted a uniformly oriented coiled configuration (Figs. 3A,B). At intermediate stages, the more flexible, yet-unmodified regions were localized within a cylindrical core stretching across the nucleus with isotropic organization. This cylinder was surrounded by stiffened fibers that displayed a toroidal, spooled arrangement. Upon completion of the rigidification of the entire fiber, the polymer adopted a cylindrically-symmetric, spool-like overall structure, compatible with the peripheral localization of chromatin within the nucleus visualized in electron micrographs in Figure 2.

Since the nucleation and spreading of distinctive chromatin properties can notably occur via histone post-translational modifications, we next analyzed the spatio-temporal pattern of histone H4 acetylation (H4ac). H4ac accompanies and is required for the histone-to-protamine transition in other species, including *Drosophila* and mice. In these model species, H4 becomes broadly acetylated throughout the nucleus. In crickets, confocal imaging using an antibody that recognized multiple acetylated forms of H4 revealed a heterogeneous pattern (Fig.

3C). H4ac was only detected at small, discrete foci in early round spermatids, consistent with the nucleation of this mark at restricted segments in the genome. In early round spermatids, H4ac signal became clustered at the center of the nucleus, consistent with the early stages of nucleated stiffening in our simulations (Figs. 3A,C). In later-stage round spermatids, we observed a remarkable pattern, whereby the H4ac signal stretched directionally across two poles of the nucleus. These poles were enriched for Histone signal, while DNA staining was in contrast stronger in the complementary nuclear space surrounding H4ac/Histones (Fig. 3C). This particular configuration is strikingly similar to intermediate stages predicted by our polymer simulations (Figs. 3A,C). Finally, at early canoe stages, H4ac and Histone staining were found to globally and faintly mark the nucleus, and were no longer detected at later stages. Our observations thus support a model in which histone-to-SNBP exchange occurs via a nucleation-and-spreading mechanism that entails chromatin stiffening, and results in a uniformly coiled fiber organization.

Interestingly, the spool-like folded arrangement predicted by our simulations that arises from this rigidification process is characterized by a strong local alignment of the chromatin fibers (Fig. 3B), and does not involve any specific chromatin-chromatin or chromatin-membrane attractive forces. Hence, this self-organized state remains highly dynamic, and bears the hallmark features of a liquid crystal (43). Such liquid crystal phases are commonly observed in dense solutions of semiflexible polymers (44-45), and typically arise from the local tendency of stiff polymer segments to spontaneously align along a common axis to optimize packing. In our case, the presence of a spherical membrane interferes with this effect, preventing the establishment of a unique, uniform direction of fiber alignment throughout the entire nuclear interior. Thus, our cylindrically-symmetric spool may be generically explained in terms of the interplay between spherical confinement and orientational, liquid-crystalline order.

### **Chromatin orientation follows cell polarization**

The nucleation model results in spool that features a distinct axis imposed by the orientation of the spooled chromatin fiber. We thus wondered whether the definition of the antero-posterior axis of the cell preceded or rather was determined by chromatin orientation. Since the flagellum is a clear marker of cell posteriority, we performed immunofluorescent staining on  $\alpha$ -Tubulin and observed that flagellar microtubules start forming in early round spermatids (Fig. 4A), indicating that these cells already possess an antero-posterior axis at these stages. Further investigating electron micrographs of round spermatids, we identified cases where the nascent flagellum and acrosome were visible in multiple cells of the same cyst, which shared the same orientation (Fig. 4B). At this stage chromatin displayed a circular symmetry, with the periphery being denser than the center portion of the nucleus (Fig. 4B). Lack of chromatin orientation in these polarized cells importantly shows that sperm cells acquire an antero-posterior axis before chromatin does, suggesting that coiled fibers can reorganize to follow this axis in elongating nuclei.

To test whether chromatin orientation adapts to cell elongation, we similarly introduced an external stretching force acting on the spherical membrane encapsulating our spooled, stiff polymer (Figs. 2,4C). Our simulations show that the spooled fibers may readily align and stretch along the arbitrary elongation axis of the membrane, regardless of their own initial orientation. This alignment results from the expected tendency of rigid polymers to minimize bending by arranging along the directions of minimal curvatures. The nucleation-based model of chromatin spooling thus better recapitulates the unidirectional peripheral alignment of the fiber that we observed in early canoe spermatids (Figs. 2A,D,E). Transversal sections of this elongating configuration further reveal that the polymer retains a distinctive organization reflecting that of the previous spool-like configuration (Figs. 3A, 4D). Indeed, chromatin is found to stretch along the nuclear antero-posterior axis by forming two distinct peripheral domains separated by a core region - corresponding to the vestigial spool axis - featuring an orthogonal direction of fiber alignment. Supporting our simulations, we identified a strikingly similar configuration in electron microscopy images of elongating nuclei (Fig. 4D). These results further suggest that there is a temporal

overlap between progressive chromatin stiffening and nuclear elongation, which is supported by the fact that H4ac and histones in general can still be detected in early elongating nuclei (Fig. 3C). Together, we concluded that the orientation of coiled fibers follows the acquisition of the cell antero-posterior axis.

### **A twisted fiber configuration is observed in mature sperm**

Because of the extreme compaction in maturing sperm, electron microscopy only detects a homogeneous mass of dense material in their nuclei. To understand chromatin organization in the latest stages of spermiogenesis, we therefore turned to super-resolution fluorescence microscopy. We exploited a Hydroxymethyl-Silicon-Rhodamine (HMSiR) fluorophore coupled to the DNA dye Hoechst33382 (46) that readily labeled DNA *in situ* in whole mount testes. We performed single molecule localization microscopy (SMLM) detecting this dye and reconstructed high-resolution images of cricket nuclei (Figs. 5A,B). At advanced stages in spermatogenesis, SMLM revealed an additional level of chromatin organization. Indeed, in late canoe cells, we observed that DNA was twisted around the antero-posterior axis within the nucleus (Figs. 5A). We further confirmed that this helical organization was also present in fully mature sperm cells collected from dissected female spermatheca (Figs. 5B). Interestingly, previous work provided evidence for a helical configuration of DNA in mature sperm of the cricket *C.nigricans*, suggesting a conserved feature (38). Since early elongating nuclei did not display such twists, we inferred that the events underlying this higher-order structure occurred at the later stages of elongation.

Within our modeling hypotheses, we could not identify any simple and plausible changes in the polymer properties that could intrinsically generate this twist. Instead, we putatively implemented a geometric torsional field that, added to the elongation force, resulted in a twisted configuration (Fig. 5C). Importantly, a transversal section of this twisted polymer displayed a characteristic screw-like organization that was highly reminiscent of chromatin fiber organization in late spermatids visualized by electron microscopy (Fig. 5C). Interestingly, upon release of the imposed shaping forces (elongation and torsion), the twisted

polymer spontaneously tightened very rapidly, resulting in a highly compact structure consistent with that observed *in vivo* in mature sperm (Fig. 5C). This model suggests that the needle-like shape of the sperm nucleus could thus be further promoted by a lateral compaction effect induced by large-scale chromatin twisting. Searching for a putative mechanism for chromatin twisting, we confirmed previous observations that the basal part of the flagellum runs parallel to the nucleus (40). At the flagellum-nucleus junction, we noticed a structure reminiscent of a cytoskeletal mesh in physical contact with chromatin (Fig. 5D). Furthermore, we observed that the basal-most end of the flagellum is completely embedded in nuclear material (Fig. 5D, see also Fig. 1D). Further supporting flagellar activity in elongating cells, we found that near-mature sperm occasionally displayed a coiled nuclear shape (Fig. 5D), which may result from a torsional force applied to compacted, needle-shaped chromatin. Hence, we speculate that unidirectional rotations of the flagellum might transmit a torque to chromatin via physical contacts and/or viscous drag effects, and thus establish its twisted configuration and further bending the nucleus as a whole when chromatin becomes highly packed. Together, these observations reveal an additional level of sperm chromatin organization whereby spooled fibers twist around the nuclear axis in late spermiogenesis, potentially via a torque imposed by the flagellum, which may ultimately facilitate compaction.

### **Disulfide bonds stabilize the ultracompact chromatin organization**

In our simulations, this ultracompact configuration was unstable and transient. Indeed, upon the release of the applied extensional forces, the nucleus readily transitioned back into a relaxed state (Figure 6A). We took advantage of this situation to make a parallel with the chromatin decondensation dynamics that occur at fertilization, which can also be achieved experimentally by chemically treating sperm cells with reducing agents. In particular, exposing *Drosophila* mature sperm to Dithiothreitol (DTT) causes reduction of disulfide bonds between protamines, their eviction from chromatin, and a rapid decompaction of the needle-shaped nucleus (31). In our simulations, the unconstrained needle-shaped polymer as a whole shortened through lateral swelling and curling (Figure

6A). To test these predictions, along with the role of disulfide bonds in maintaining the sperm chromatin architecture, we treated mature sperm isolated from spermatheca with 25mM DTT for up to 30min (Figure 6B). Upon treatment, needle-shaped nuclei became progressively unwound and decompacted. Intermediate steps of chemically induced decompaction showed remarkable similarity with simulated decondensation steps and were fully compatible with the progressive loosening of the higher-order twisted organization. We concluded that the ultracompact needle configuration is locked *in vivo* by disulfide bonds, likely between SNBPs, that maintain and stiffen this structure in the absence of any external force, thus ensuring that sperm shape is preserved even when submitted to constraints such as flagellar movement, until the time of fertilization.

## DISCUSSION

### ***G. bimaculatus* offers a uniquely elegant solution to sperm genome packaging**

Packaging the genome in a compact configuration during spermiogenesis is a universal problem for which the coil organization we describe in *G. bimaculatus* is a simple and elegant solution. This arrangement maximizes space occupancy while minimizing chromosome entanglement, ensuring ultracompaction while facilitating genome unfolding at fertilization. Our simulations reveal that this ordered configuration can be achieved through relatively simple changes in the biophysical properties of chromatin, such as a nucleated rigidification of the fiber. In mice and *Drosophila*, a nucleus-wide wave of histone acetylation precedes their replacement with SNBPs (14-15,20). Both acetylation and SNBPs could cause chromatin stiffening. Illustrating its importance, failure in histone hyper-acetylation correlates with infertility in mice and men (47-48) and blocks histone replacement and sperm differentiation in *Drosophila* (49). In mice, histone hyperacetylation is thought to result from the activity of HATs including NUT-dependent p300 and CBP as well as Gcn5 (25,50), favored by a decrease in the levels of histone deacetylases (HDACs) in sperm (18,51). Accordingly, NUT or Gcn5 mutants display partial failure in histone-



protamine replacement, together with aberrant sperm compaction and shaping. Further insight into the mechanistic importance of this mark could emerge by identifying the enzymes responsible for acetylation in crickets, as well as the molecular identity of SNBPs. While our study indirectly addresses the existence and function of TPLs and PLs in *G. bimaculatus*, former studies suggest that there could be only a single SNBP in mature cricket sperm (40) - in contrast to two in most mammals and over five in flies - making this a simplified model for sperm chromatin compaction.

In crickets, we further show a histone acetylation pattern consistent with nucleation and spreading of this modification. The spreading of histone modifications has been well described for repressive heterochromatin methylation marks such as lysine 9 and lysine 27 on histone H3 (52). In mice and *Drosophila*, enzymatic complexes can both establish and recognize the methylation marks, therefore feeding a spreading mechanism on neighboring nucleosomes. These modifications alter the mechanical properties of chromatin and attract additional complexes, which favors the formation of nuclear sub-compartments (53-56) where these processes are further catalyzed (57). Histone acetylation can analogously target DNA regulatory elements (notably enhancers and promoters) via DNA sequence-specific transcription factors recruiting HATs and bromodomain proteins (58-59). It was further proposed that a feedforward loop involving NUT, p300 and BRDT helps spreading histone acetylation in mouse sperm (25). The targeting mechanisms for HATs acting on mouse or *Drosophila* sperm is unknown but the rather homogeneous nuclear distribution of acetylation favors a model in which histones are randomly and synchronously acetylated as a nucleus-wide wave, with the notable exception of genomic regions resisting the histone-protamine transition (20). Yet, these mechanisms must differ in cricket sperm, where early acetylation displays a sharp spatially defined sub-nuclear pattern. The nucleation of acetylation could be driven by a limited number of restricted cis-regulatory DNA segments capable of recruiting HATs, as for enhancers or promoters. A precise identification of genomic distribution of histone acetylation and HAT binding during spermiogenesis is needed to clarify this issue.

The relative simplicity of the biophysical forces we uncover makes it all the more intriguing that, to our knowledge, this specific chromatin configuration has not been reported in any other cell type or species thus far. We propose that the precise spatio-temporal patterning of acetylation and histone-SNBP replacement in sperm cells is critical to determine chromatin organization. Notably, no particular higher-order genome organization is observed in electron micrographs from *Drosophila* sperm (60), which features a homogeneous histone hyperacetylation distribution. In mice, Hi-C maps of DNA-DNA contacts have yielded contrasting results, but usually revealed relatively modest differences in sperm cells compared to somatic cells (61-63). These surprising observations imply that the massive histone-SNBP replacement does not cause large-scale genome 3D rearrangements in this species. While these studies need further confirmation, developments of our modeling framework could help identify how histone acetylation and replacement patterns may result in this intriguing preservation of genome organization, which must clearly not occur in crickets. Alternatively, a more detailed analysis of the acetylation patterns in sperm cells of other animals could shed light into the underlying mechanisms of species-specific shaping and compaction.

At later stages of spermiogenesis, the twisting of the chromatin fiber spools may contribute to achieve maximum compaction. While we could not unequivocally identify its mechanistic bases, we suggest that unidirectional rotational movements of the flagellum may be involved. A limited number of turns may be enough to transmit sufficient rotational forces onto chromatin and the nucleus as a whole to explain the observed deformations. This finding would imply a nuclear structuring role for the flagellum, in addition to its motility and sensory roles. Whether flagellar components interact with chromatin and how they could exert such mechanical forces would deserve further experimental interrogation. Importantly, our results also suggest that additional processes may be required towards the final steps of spermiogenesis in order for sperm cells to acquire a robust needle-like morphology despite this persisting torque. Disulfide bonding between protamines could serve to stabilize and rigidify such a nuclear

configuration, so that flagellar movement may drive cellular motion without altering cell shape.

### **Sperm chromatin rearrangements are consistent with liquid-crystalline behavior**

The spool-like chromatin arrangement obtained at the outset of the rigidification of the full fiber is driven by the spontaneous alignment of neighboring chain segments within the near-spherical nuclear membrane, and arises from the sole competition between excluded volume and filament bending energy. It may therefore be identified as a self-organized, liquid-crystalline (nematic) state, characterized by the conjunction of local orientational order with a dynamic, liquid-like mode of molecular motion (43). A similar configuration, termed *condensed Hopf fibration*, has indeed been recently predicted by theory for long, semi-flexible polymers in tight spherical confinement (64), and is consistent with the textures displayed by polymer-based liquid crystals within finite spherical cavities (45,65).

The unique combination of order and fluidity provided by this original packing mechanism may in our case play a central functional role in the maturation of cricket sperm cells. Indeed, our results suggest that the strong orientational alignment of the chromatin fiber, associated with its ability to dynamically reorganize following changes in nuclear morphology and the application of torsional forces, may be key to achieve maximal levels of compaction and elongation in mature sperm nuclei. Hence, our findings would support a model in which the irreversible intermolecular interactions induced by disulfide protamine bonds should only be established at the very final stage of spermiogenesis, in order to “freeze” the resulting liquid-crystalline chromatin assembly into a glassy-like, crosslinked state resilient to external perturbations.

In this context, the potential influence of the detailed fiber structure on the observed chromatin arrangements would warrant further investigations. For instance, the chiral symmetry of many liquid-crystal-forming biopolymers such as

DNA is generally known to promote twisted, non-uniform patterns of orientational order, which may in turn give rise to a higher level of hierarchical organization (66). These so-called *cholesteric* liquid crystal phases are commonly observed in the mature sperm heads of many vertebrates (67), and have been more broadly proposed to fulfil a variety of biological purposes and functions (68). Such cholesteric interactions could in our case serve to further stabilize the twisted fiber configuration uncovered in mature spermatids, and thus contribute to the establishment and maintenance of optimal packing and elongation in the cricket sperm. Interestingly, similar cholesteric arrangements have also been reported in dinoflagellate chromosomes (69), which are characterized by unusually-large genome sizes, and feature a near-total absence of histones and histone-like proteins (70). Therefore, our results would more generally suggest the combination of histone replacement and liquid-crystalline organization as a convergent evolutionary process for the ultradense packaging of chromatin within highly-confined nuclear environments.

## **MATERIALS AND METHODS**

### **Cricket breeding and sample collection**

The *Gryllus bimaculatus* white-eyed mutant strain was a gift from C. Extavour. Crickets were reared at constant 29°C under a 12-hour light-dark photoperiod, fed with SAFE complete powdered medium, quenched with tap water, and allowed to lay eggs in soaked tissue paper. For imaging experiments and protein extraction, pairs of testes were collected from healthy adult males, while mature sperm was isolated from adult female spermatheca.

### **Confocal Microscopy**

For immunofluorescence staining, testes were dissected in PBS 0.1%, Triton X-100 (PBS-T) and fixed at room temperature in 4% formaldehyde in PBS for 30 minutes. Testes were washed three times (10 min each) with PBS-T and incubated with anti-H4ac (Merck Millipore #06-598, 1/500 dilution), anti-Histones (Millipore #MABE71; 1/2500 dilution) or anti- $\alpha$ Tubulin (Sigma #T9026) primary antibodies in the same buffer on a wheel overnight at 4°C. They

were then washed three times (10 min each) with PBS-T and secondary antibody incubations (1/1000 dilution) were performed identically. Testes and sperm samples were mounted in DAKO mounting medium containing DAPI (2 $\mu$ g/ml). Images were acquired on an LSM 800 confocal microscope (Carl Zeiss). Images were processed with Zen imaging software (Carl Zeiss) and FIJI software (71).

### **Single Molecule Localization Microscopy**

Fixed testes or sperm samples were mounted in PBS medium containing 100nM Hydroxymethyl Silicon-Rhodamine-Hoechst probe<sup>42</sup>. We acquired the streams using MetaMorph software (Molecular devices) on a home-built setup consisting in an IX81-ZDC2 microscope equipped with a temperature controlled incubation cage kept at 27°C. The intensity and wavelengths of the illumination were controlled through Labview to pilot an acousto-optical tunable filter (AOTF; Quanta Tech). Wide-field illumination was achieved by focusing the laser beams to the back focal plane of a 100x 1.49 numerical aperture (NA) oil-immersion apochromatic objective lens (Olympus). Data collection was obtained using an Evolve EMCCD camera (Photometrics) with continuous 642 nm light illumination, at high power. Images were reconstructed using the ThunderSTORM (72) Fiji plugin.

### **Transmission Electron Microscopy**

Testes were dissected water and fixed for 20h at 4°C in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate Ph7.35. After four washings of 12h at 4°C in 0.15 M sodium cacodylate, samples were postfixed 1 hour at RT in 1% OsO<sub>4</sub> / 0.15M sodium cacodylate. They were dehydrated in ethanol solutions (30° /50°/70°/80°/95°/100°) for 30 min each, and in propylene oxide 2 times 15 min. After 6 baths of substitution and 3 baths of impregnation, small blocks were embedded in epoxy resin in flat silicon molds and polymerized at 56°C for 48h. Ultrathin sections were cut with a UC7 Leica ultramicrotome. Ultrathin sections were contrasted in uranyl-acetate and lead citrate solutions. Sections were observed with a Philips CM120 transmission electron microscope at 120Kv (Centre des microstructures, Université Lyon 1, France).

## Western Blotting

Testes or sperm samples were homogenized by roto-douncing in 5% SDS, 50 mM TEAB, 50 mM DTT. The homogenates were incubated overnight at 37°C, pelleted and clear lysates were collected and quantified. 1 or 2µg of total protein were separated in 4-12% acrylamide gels, transferred to PVDF membranes and stained with Pierce Reversible Protein Stain Kit for total protein visualization. Stain was washed away, membranes were blocked in 5% non-fat milk and stained with an anti-H3 antibody (abcam #1791, 1/2500 dilution, overnight incubation at 4°C) in TBS-T with 1% non-fat milk. Membranes were washed in TBS-T, stained with HRP-coupled secondary antibody (Agilent Dako #P044801-2, 1/10000 dilution) for 2h at room temperature, and washed again with TBS-T. Proteins were revealed by chemiluminescence using Pierce SuperSignal West Pico substrate and imaged in a ChemiDoc imaging system.

## Chemically induced decondensation assays

Sperm samples were isolated from female spermatheca, spread in PBS onto microscopy slides. Excess liquid was removed and sperm was mounted in PBS-Triton 0,3% containing 5µg/ml of Hoechst with 25mM DTT for immediate visualization.

## Numerical model

The nuclear membrane was described as a cross-linked biopolymeric shell consisting of a fixed number  $N_v$  of vertices, which were initially distributed uniformly on the surface of a sphere of radius  $R$  (45). Pairs of neighboring vertices were linked via springs of stiffness  $k_{memb}$  and rest length  $r_0$ , as represented by the harmonic potential

$$U_{memb}^{bond}(r_{vv}) = \frac{k_{memb}}{2} (r_{vv} - r_0)^2,$$

with  $r_{vv}$  the inter-vertex distance. The set of connected vertices, which defines the topology of the membrane, was constructed by Delaunay triangulation of the initial vertex positions through the Quickhull algorithm (73), and was kept fixed throughout the simulations to mimic the static cross-links of the membrane lamina network (74). Self-avoidance was achieved through the use of a truncated and shifted Lennard-Jones potential with exclusion radius  $r_0$  and depth  $\epsilon$ ,

$$U_{r_0}^{exc}(r) = \begin{cases} 4\varepsilon \left[ \left(\frac{r_0}{r}\right)^{12} - \left(\frac{r_0}{r}\right)^6 + \frac{1}{4} \right], & r < 2^{1/6}r_0, \\ 0, & r \geq 2^{1/6}r_0 \end{cases}, \quad (1)$$

which was applied to all pairs of unconnected vertices.

The chromatin fiber was modelled as a single linear polymer comprised of  $N_m$  monomeric subunits of effective diameter  $\sigma$ , joined together by harmonic springs of stiffness  $k_{chrom}$ ,

$$U_{chrom}^{bond}(r_{mm}) = \frac{k_{chrom}}{2}(r_{mm} - \sigma)^2,$$

with  $r_{mm}$  the distance separating a pair of consecutive monomers along the chain. Excluded volume effects were emulated through the use of a soft repulsive potential of depth  $\varepsilon_{chrom}$ ,

$$U_{chrom}^{exc}(r) = \begin{cases} \varepsilon_{chrom} \left[ 1 + \left(\frac{r}{\sigma}\right)^{12} \left(\frac{6r^2}{\sigma^2} - 7\right) \right], & r < \sigma, \\ 0, & r \geq \sigma \end{cases},$$

which similarly acts on all pairs of non-contiguous monomers. The bending rigidity of the chromatin fiber was represented by a standard angular potential of stiffness  $k_{bend}$ ,

$$U_{chrom}^{angle}(\theta) = k_{bend}(1 - \cos \theta), \quad (2)$$

where  $\theta$  denotes the angle formed by the two bonds linking a triplet of adjacent monomers. The mutual repulsion between chromatin and the encapsulating membrane was finally described by an interaction term  $U_{\Sigma}^{exc}$  of the same form as Eq. (1) for each monomer-vertex pair. In this case, the exclusion radius was set to  $\Sigma = r_0 + \sigma$ , which effectively ensured that the membrane surface could not be crossed by the chromatin chain in any configuration.

The effective diameter of the chromatin fiber was set to  $\sigma = 30 \text{ nm}$ , which serves as the model unit of length. Each monomer thus contains about  $\sim 15 \text{ kbp}$ , based on our estimation of the fiber linear DNA density (see main text). For computational feasibility, a number  $N_m = 10,000$  of monomeric units were considered, which corresponds to a chromatin fiber length  $L = N_m \sigma = 300 \mu\text{m}$ . This value amounts to a fraction  $f \sim 10\%$  of the total chromatin content, as estimated by electron microscopy imaging of canoe-stage spermatids (see main text). Accordingly, the initial membrane radius was set to  $R = R_{round} \times f^{1/3} = 0.7 \mu\text{m}$  to ensure the

conservation of chromatin density, where  $R_{round} \cong 1.5 \mu m$  is the experimentally-measured nuclear radius at the early round spermatid stage. An arbitrarily-high number  $N_v = 5,560$  of vertices were used in the calculations to provide a fine-mesh discretization of the membrane surface, and the spring rest length was set to the associated mean inter-vertex distance in the uniform initial state, i.e.,  $r_0 = (8\pi R^2 / \sqrt{3} N_v)^{0.5} \cong 35 \text{ nm}$ .

Molecular dynamics (MD) simulations were run on multiple graphics processing units via the HOOMD-blue software package (75,76). A standard Langevin thermostat was used to keep the system temperature fixed at  $T = 293 \text{ K}$ , and the model unit of energy was set to  $\varepsilon = k_B T$ , with  $k_B$  the Boltzmann constant. Correspondingly, a height  $\varepsilon_{chrom} = 5\varepsilon$  was employed for the chromatin-chromatin repulsive barrier, and the stiffness of the chromatin backbone springs was taken as  $k_{chrom} = 15 \varepsilon / \sigma^2$ . This choice of parameters was specified to allow for occasional self-crossings of the chromatin chain, and thus mimics moderate topoisomerase activity. The stiffness of the membrane springs was set to  $k_{memb} = 2500 \varepsilon / \sigma^2 \cong 10 \text{ mN/m}$ , which matches the typical Young's modulus of the bare nuclear lamina network estimated from micropipette aspiration experiments (77). A vanishing bending rigidity  $k_{bend} = 0$  was assumed in the initial state to emulate the high compliance of the nucleosomal chromatin fiber at the onset of the round spermatid stage.

### **Relaxation and rigidification runs**

The chromatin chain was initialized as a dense, self-avoiding random walk encapsulated within the bounds of the initial spherical membrane conformation. Relaxation runs of  $\mathcal{O}(10^8)$  MD steps were performed to achieve a homogeneous chromatin density throughout the nuclear interior. For simulations of uniform fiber rigidification, the chromatin bending modulus was gradually raised from  $k_{bend} = 0$  to  $k_{bend} = 50\varepsilon$  in  $\mathcal{O}(10^2)$  linear increments. This latter value corresponds to a chromatin bare persistence length  $l_p \cong 1.5 \mu m$ , qualitatively consistent with electron microscopy observations of the fiber at the canoe stage (Figure 2).  $\mathcal{O}(10^8)$  MD steps were performed following each parameter update in order to reach a stationary state.



In the case of nucleated stiffening simulations, all loci located within a distance  $d = 150 \text{ nm}$  of the center of the nucleus – corresponding to about 2% of the total chromatin content – were tagged in the relaxed initial configuration to mimic the transient action of a putative localized acetylation factory. A state-dependent bending rigidity  $k_{bend}$  was then used, in which the stiffness of all bonds involving tagged loci was similarly raised from 0 to  $50\varepsilon$  in an incremental fashion over  $\mathcal{O}(10^8)$  MD steps, while the bending modulus associated with triplets of adjacent untagged loci remained set to  $k_{bend} = 0$  (c.f. Eq. (2)). This process emulates a progressive rigidification of the chromatin fiber through the gradual eviction of acetylated histones, as discussed in the main text. Upon full stiffening of the tagged genomic regions, a number  $N_{spread} = \mathcal{O}(10)$  of consecutive monomers were subsequently tagged on either side of the boundaries of each acetylated domain, to reproduce a slow *cis*-spreading of acetylation marks along the chain. The above protocol was then repeated over  $\mathcal{O}(10^2)$  iterations, until the complete rigidification of the whole fiber was achieved.

### **Nuclear elongation simulations**

To simulate the directional elongation of the nuclear membrane, two antipodal vertices were identified along an arbitrary stretching axis  $\vec{u}_{stretch}$  in the final configuration of the chromatin rigidification runs. An outward-directed force gradient  $\vec{F}_{stretch} = \pm \|\vec{F}_{stretch}\| \times \vec{u}_{stretch}$  was then applied in the vicinity of the two vertices, whose magnitude was given by  $\|\vec{F}_{stretch}\| = F$  at each extremal vertex, and  $\|\vec{F}_{stretch}\| = F/2$  at each of their respective connected neighbors. The magnitude of the force was raised from  $F = 0$  to  $F = 2 \times 10^5 \varepsilon/\sigma \cong 27 \text{ nN}$ , consistent with typical values reported in nuclear stretching experiments<sup>72</sup>, over  $\mathcal{O}(10^2)$  iterations. The system was similarly evolved over  $\mathcal{O}(10^8)$  MD steps following each force increment.

### **Twisting and decondensation simulations**

To model the effects of geometric twist on spermatid morphology, a uniform torsional field of axis  $\vec{u}_{stretch}$  and total twist angle  $10\pi$  was applied to the

simulated nuclei at various levels of elongation, which yielded a local thread angle compatible with single-molecule localization microscopy observations of late-stage spermatids (Figure 5). The system was then allowed to relax over  $\mathcal{O}(10^5)$  MD steps, which resulted in a significant lateral compaction of the encapsulated chromatin (Figure 5C). For decondensation simulations, the magnitude  $F$  of the external force acting on the membrane vertices was subsequently set to 0, and the kinetics of nuclear recovery towards the spherical reference elastic state of the membrane were monitored over  $\mathcal{O}(10^7)$  MD steps.

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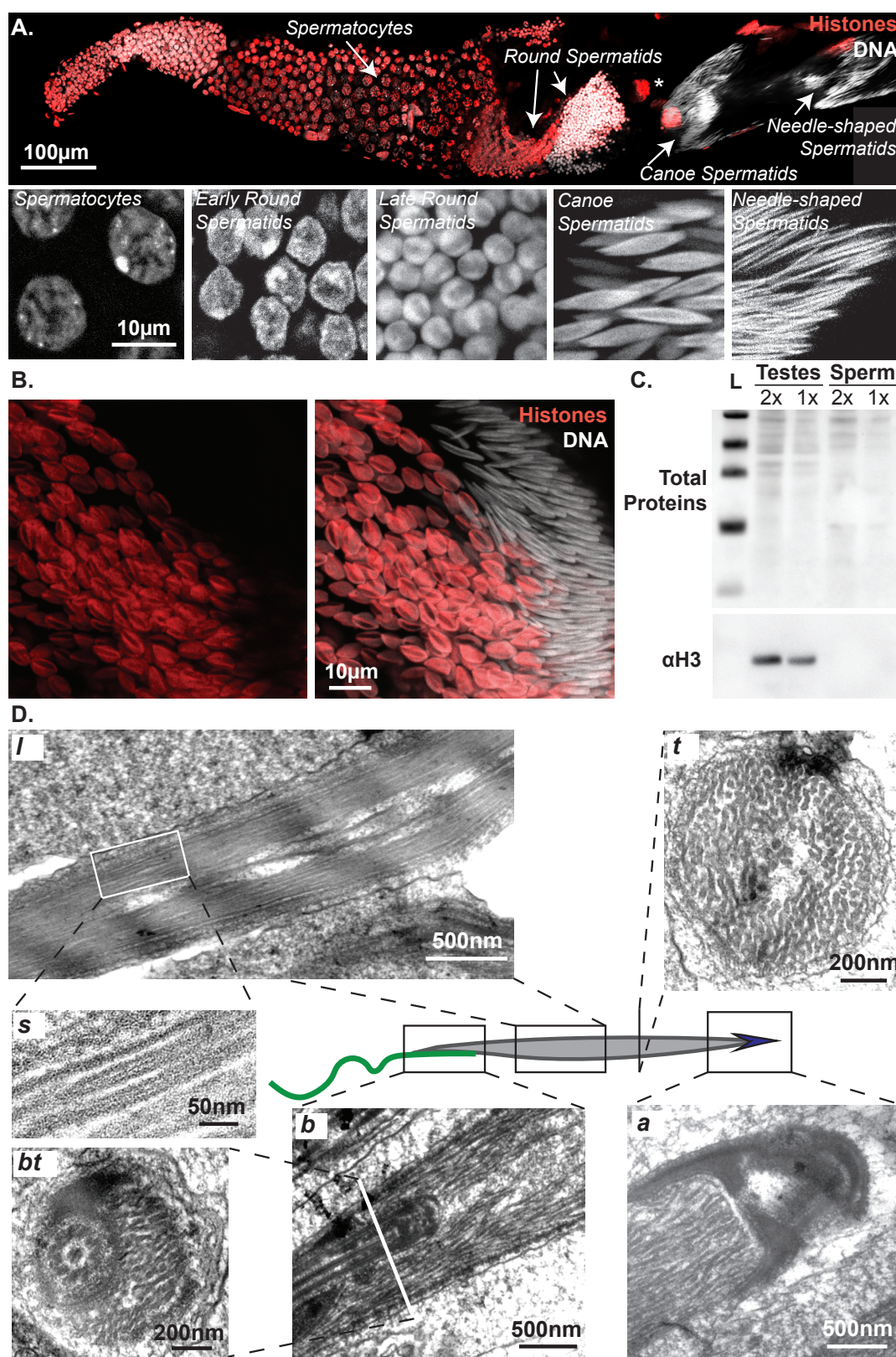
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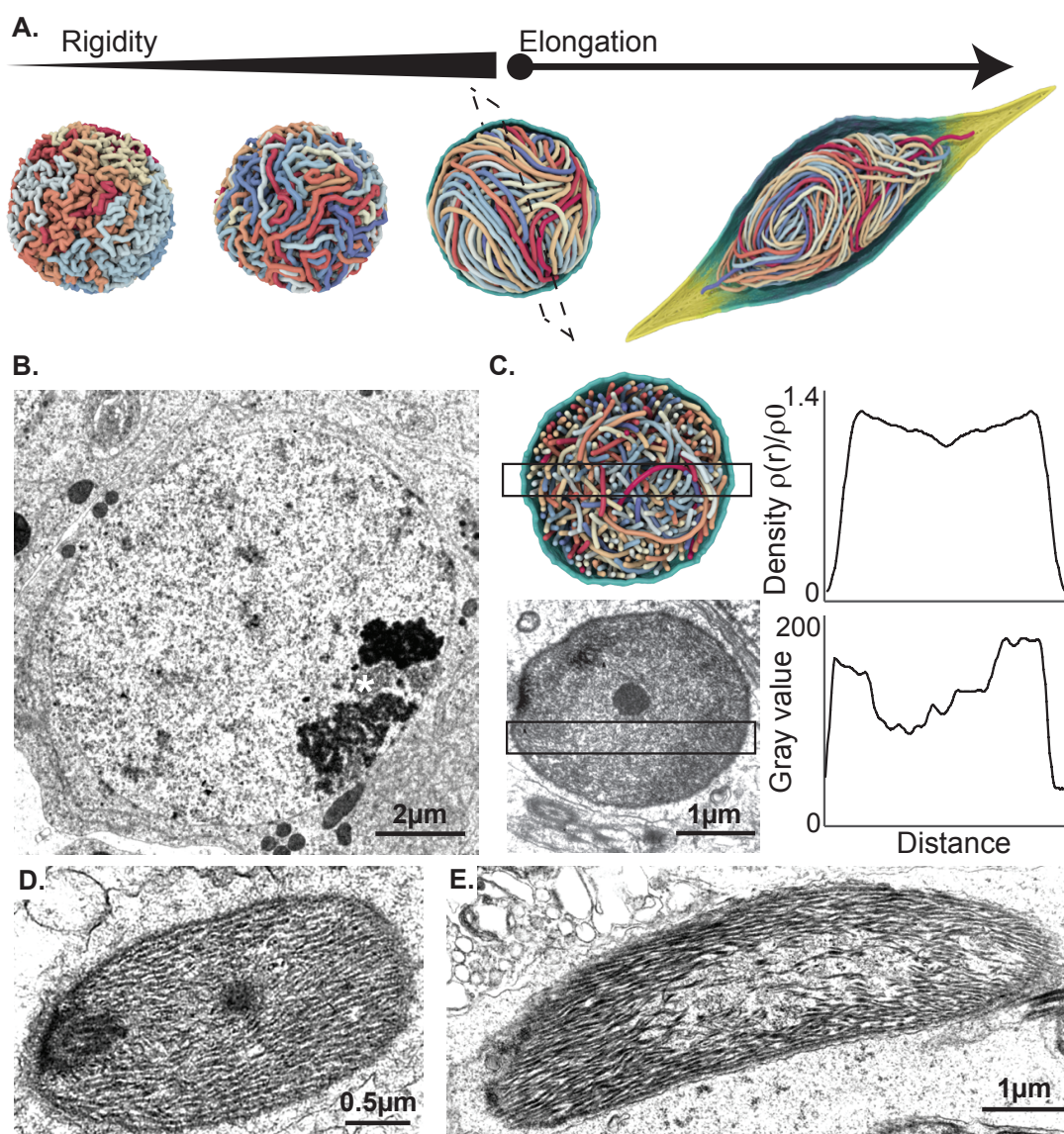
## FIGURES



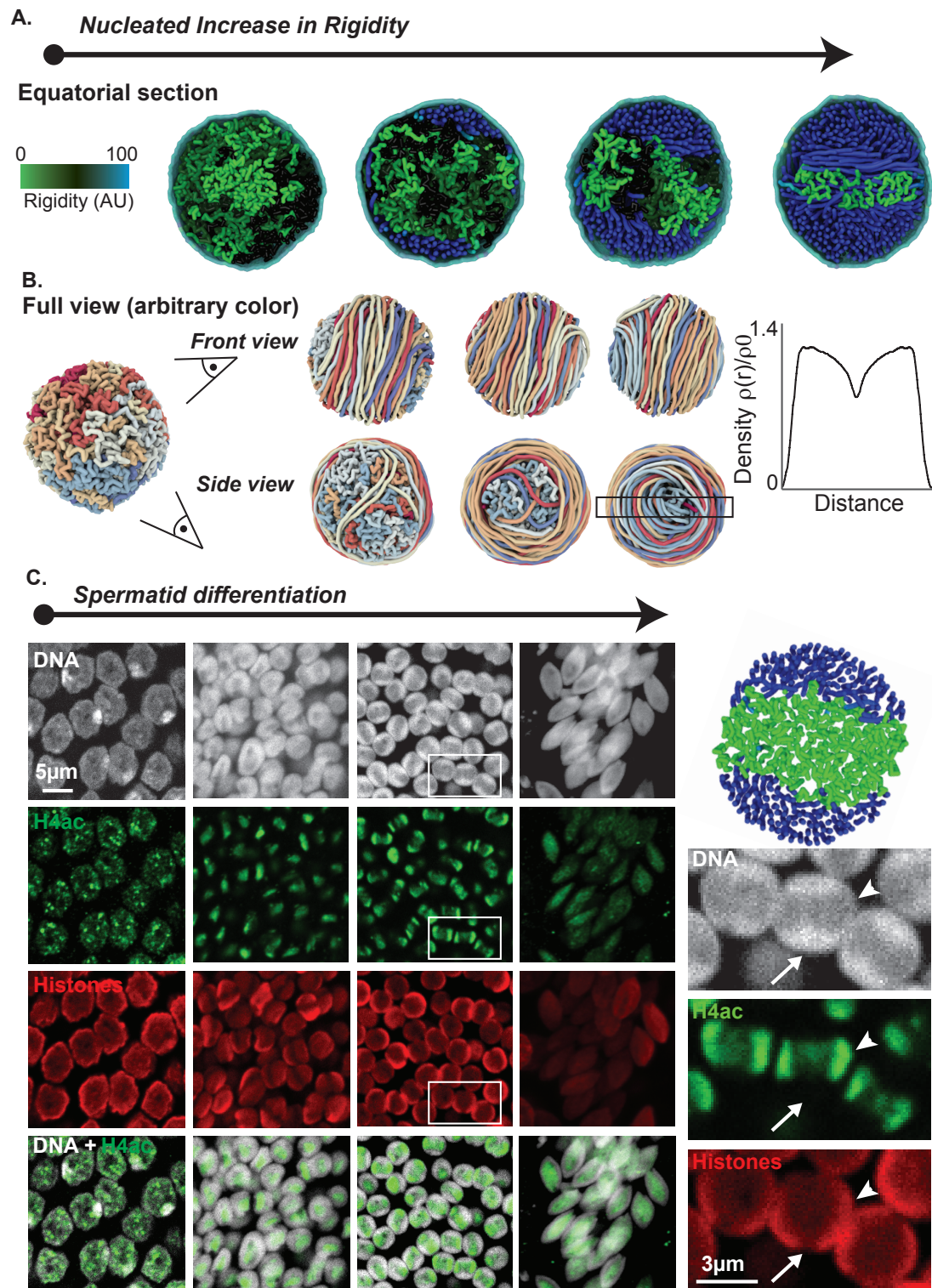
**Figure 1: Coiled organization of chromatin fibers in *Gryllus bimaculatus* sperm. (A):** Overview of spermiogenesis in *Gryllus bimaculatus*. Confocal microscopy image of a single follicle from an adult testis stained for DNA (white) and histones (red). Multiple cysts are visible in which male germ cells differentiate synchronously. From our estimations, the



premeiotic cysts contain 512 primary spermatocytes and post-meiotic cysts must then contain about 2048 spermatids. By analogy to *Drosophila*, we distinguish successive stages in spermiogenesis including spermatocytes, round spermatids, canoe (elongating) spermatids and needle-shaped spermatids. \*: a polyploid somatic nucleus. **(B-C)**: Sperm chromatin undergoes a Histone-SNP transition. Confocal image showing early elongating (left side) and late elongating (right side) spermatids stained for DNA (white) and histones (red) confirm massive histone eviction during spermatid elongation (B). Western blot of protein extracts from testes and sperm revealing whole proteins and histone H3 confirm undetectable histone levels in mature gametes (C). L: ladder, 2x 1x: two-fold dilution of protein extract. **(D)**: Chromatin organizes as coiled fibers in spermatids. Electron microscopy images show different sections of spermatids around the canoe stage. A longitudinal section (l) of the spermatid nucleus shows chromatin fibers in alignment with the its antero-posterior axis. A sagittal section (s) of individual chromatin fibers reveals a tubular internal structure. At the basal end (b, bt), an overlap between the nucleus and flagellum is observed. Note that images in (b) and (bt) are from different cells. Bending of chromatin fibers is observed at the apical end (a). Approximately ~200 individualized fibers are distinguished in a transversal section (t).

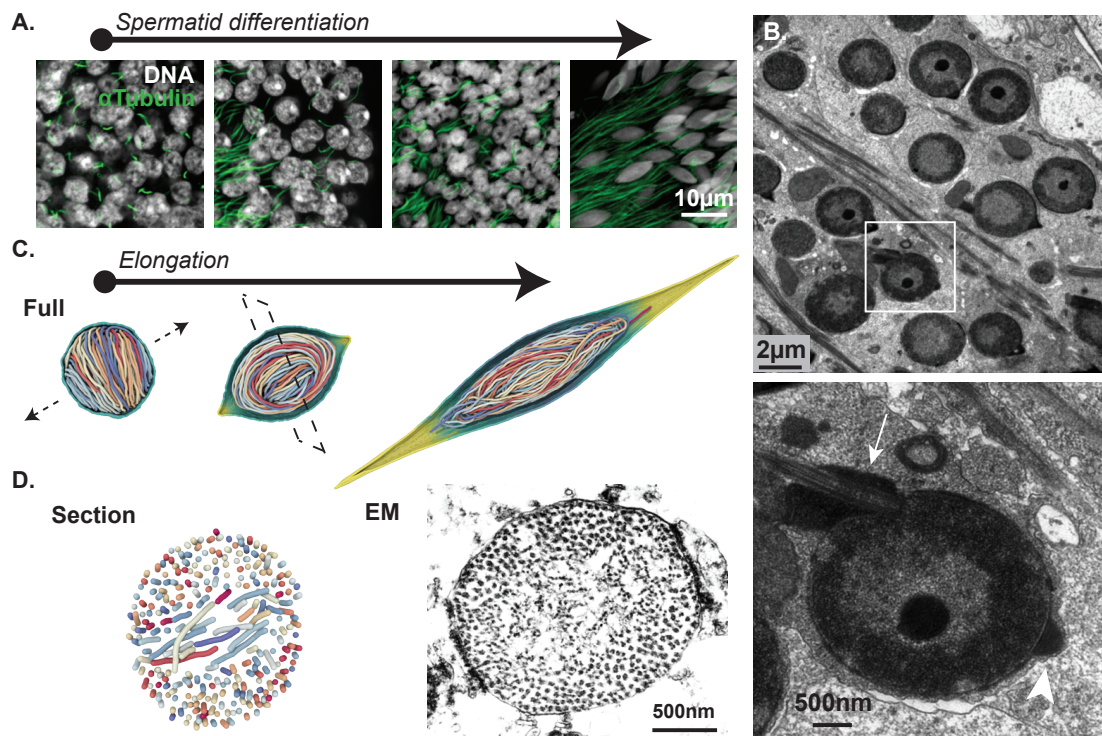


**Figure 2: Polymer modeling of rigid fiber dynamics recapitulates features of chromatin localization in sperm. (A):** Chromatin re-organization via a uniform, homogeneous increase in local fiber rigidity in simulated models. Polymer simulations of the stiffening process results in a yarn-ball-like state, which is relatively disordered and prevents the establishment of a coherent, homogeneous direction of fiber alignment upon the onset of nuclear elongation. **(B-E):** Chromatin re-organization during spermiogenesis *in vivo*. Electron microscopy images of spermatocytes (B), round (C), early elongating (D) and elongating (E) spermatids show progressive reorganization of chromatin by fiber individualization and alignment. In (C), density profiles of simulated intermediate stages and round spermatids observed *in vivo* show a similar accumulation at the nuclear periphery. In (B), ‘\*’ indicates a heterochromatin mass.

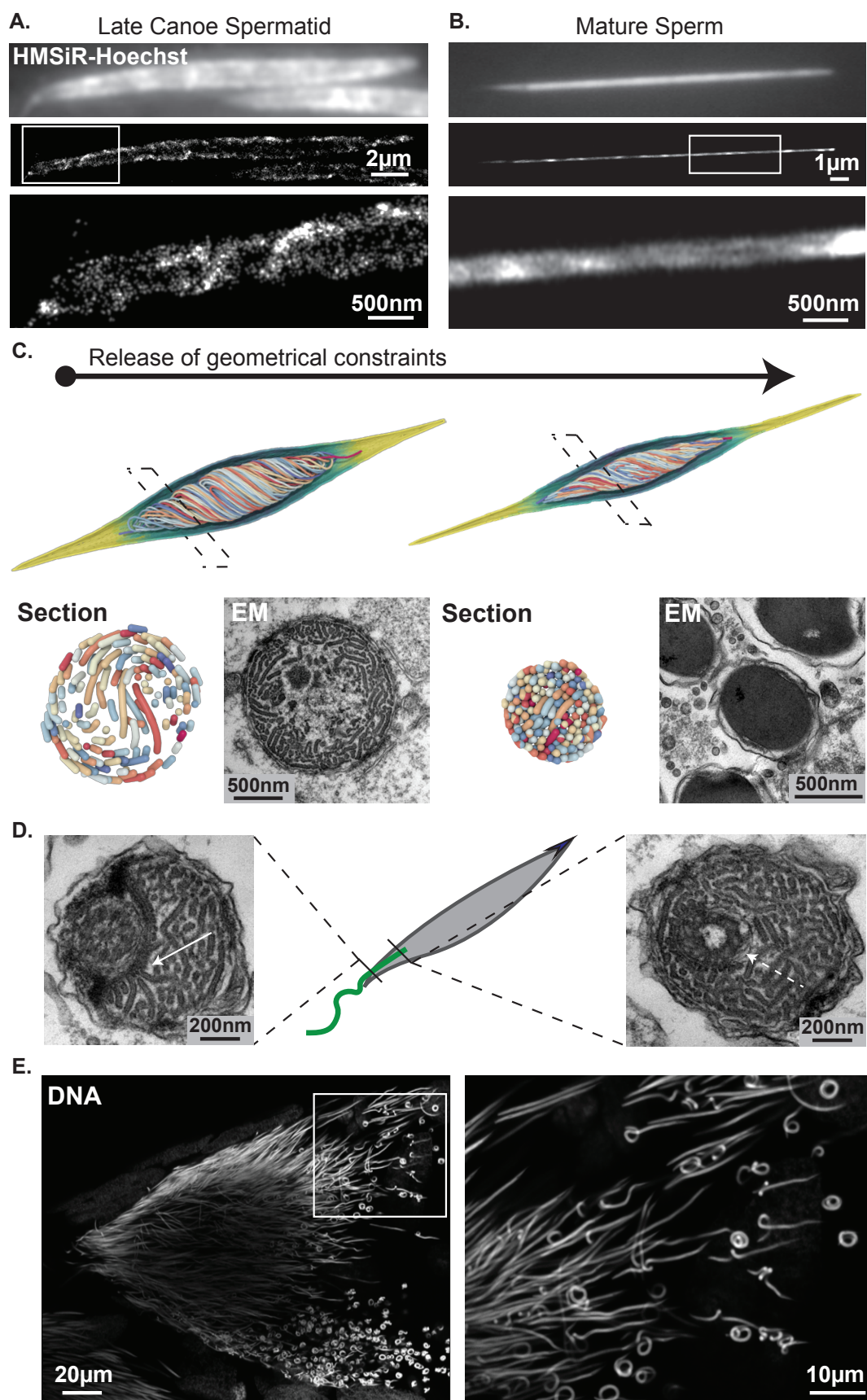


**Figure 3: Nucleated chromatin rigidification results in a spool-like organization.** (A): Chromatin re-organization via a nucleated, *in cis* propagation of fiber rigidification. Fiber segments undergoing gradual stiffening are marked in green, denoting a putative peak in acetylation activity. Fully-rigidified segments are depicted in blue, representing SNBP-based chromatin. Unmodified chromatin regions are rendered in black. (B): Same as Fig. 3a in full-fiber view. The segregation of rigidified chromatin segments into a toroidal structure at the nuclear periphery is clearly visible, and leads to the formation of a spool-like ordered structure towards the late round spermatid stage. (C): Histone

acetylation patterns in vivo are consistent with the rigidification dynamics predicted by our model. Confocal images of spermatids at four early, consecutive stages in spermiogenesis stained for DNA (white), pan-acetylated histone H4 (H4ac, green), and total histones (red). H4ac appears as punctate nuclear foci that cluster at the nucleus center, subsequently spreading as a two waves directed towards opposite nuclear poles (see arrowheads in magnified panels on the right). At this stage, DNA is enriched in the complementary nuclear space surrounding the central region (arrows). This organization is consistent with our simulations. Early elongating spermatids show dispersed and weaker H4ac and histone signals.

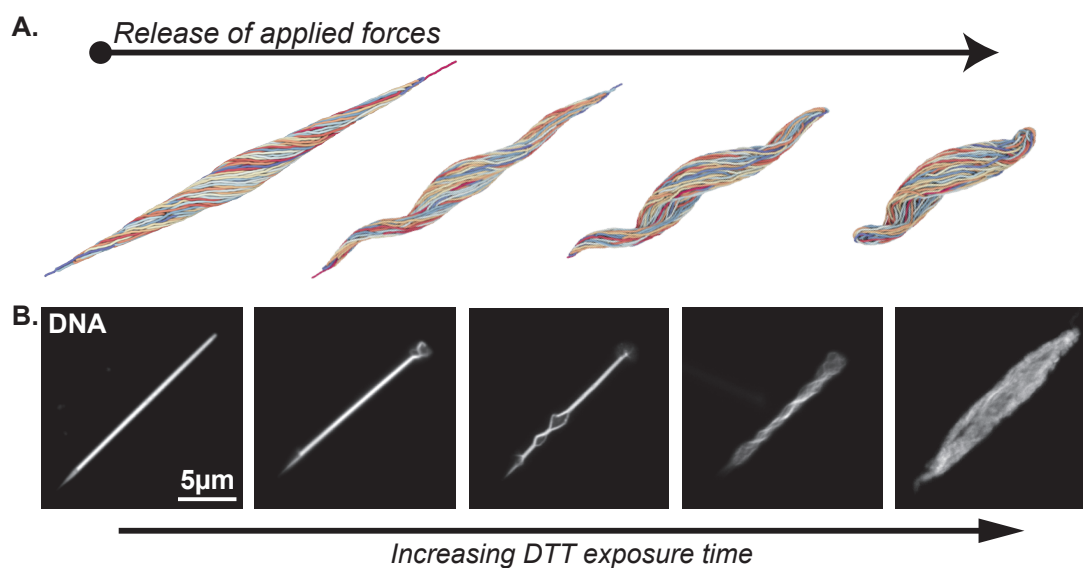


**Figure 4: Cellular polarization precedes chromatin orientation. (A-B):** Round spermatids feature nascent flagella and acrosomes. **(A):** Confocal microscopy images of testes stained for DNA (white) and  $\alpha$ Tubulin (green) show nascent flagellar microtubules in early spermatids. **(B):** Electron microscopy image of a round spermatid cyst showing multiple nuclei with chromatin homogeneously enriched at the nuclear periphery flanked by nascent flagellar (arrow) and acrosomal (arrowhead) structures, indicating that cellular antero-posterior poles were defined before chromatin became oriented. **(C):** In polymer simulations, nuclear elongation drives the spontaneous reorientation of the chromatin spool axis perpendicular to the antero-posterior line, and leads to the distinct unidirectional alignment of the chromatin fiber coils. **(D):** Elongating spool organization observed in simulations and in vivo. Transversal sections of nuclei during early elongation in polymer simulations (left) and in electron microscopy images (right) show a similar configuration whereby the vestigial spool axis perpendicular to the elongation axis is still apparent.



**Figure 5: Chromatin twists around the nucleus axis in late spermiogenesis. (A-B):** DNA twisting in late spermatids. Single Molecule Localization Microscopy (SMLM) images

of late canoe (A) and fully mature (B) spermatids labeled with an HMSiR-Hoechst probe to reveal DNA with nanoscopic resolution. Top panels show the epifluorescence image; middle and bottom panels show SMLM images and close in. DNA twists around the nucleus axis over several turns. Boxed regions are zoomed in bottom panels. **(C)**: In simulated data, a geometric twist imposes an additional extensional strain on the peripheral chromatin fibers, which leads to an increased degree of lateral compaction upon mechanical relaxation. Chromatin configuration and density in simulated polymers before and after relaxation recapitulate that observed in vivo by electron microscopy in late canoe (bottom left) and mature (bottom right) spermatids respectively. **(D)**: Evidence for contacts between the flagellum and chromatin. Electron microscopy transversal sections of the basal end of canoe spermatids reveal a proteinaceous structure connecting the flagellar axoneme and chromatin fibers (full arrow). Close to its organizing center, the flagellum fully penetrates the nucleus and is fully embedded by chromatin (dashed arrow). **(E)**: Whole-nucleus looping in late spermatids. Confocal microscopy images of late needle-shaped spermatid cells in testes labeled for DNA with DAPI, showing occasional curling. The boxed region is zoomed in the right panel.



**Figure 6: Disulfide bridging maintains nuclear compaction and shaping. (A):** Nuclear decondensation is modeled by the release of applied extensional forces, and leads to a rapid relaxation towards a spherical membrane shape via a transient, corkscrew-like intermediary morphology. **(B):** Chromatin is readily decondensed upon treatment with reducing agent DTT. Epifluorescence images of mature spermatids isolated from female spermatheca, labeled for DNA and exposed to 25mM DTT. Chromatin decondensation pattern is consistent with simulated data and suggests that protamine disulfide bridges maintain the needle-like nuclear morphology.