Revisiting chromatin packaging in mouse sperm

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ABSTRACT

Mammalian sperm exhibit an unusual and heavily-compacted genomic packaging state. In addition to its role in organizing the compact and hydrodynamic sperm head, it has been proposed that sperm chromatin architecture helps to program gene expression in the early embryo. Scores of genome-wide surveys in sperm have reported patterns of chromatin accessibility, histone localization, histone modification, and chromosome folding. Here, we revisit these studies in light of our recent finding that sperm obtained from the mouse epididymis are contaminated with low levels of cell-free chromatin. In the absence of proper sperm lysis we readily recapitulate multiple prominent genome-wide surveys of sperm chromatin, suggesting that these profiles primarily reflect contaminating cell-free chromatin. Removal of cell-free DNA, along with appropriate lysis conditions, are required to reveal a sperm chromatin state distinct from any yet reported. Using ATAC-Seq to explore relatively accessible genomic loci, we identify a landscape of open loci associated with genes expressed during late spermiogenesis. Histone modification and chromosome folding studies also strongly support the hypothesis that prior studies suffer from contamination, but technical challenges associated with reliably preserving the architecture of the compacted sperm head prevent us from confidently assaying true localization patterns for these epigenetic marks. Together, our studies strongly argue that our knowledge of mammalian chromosome packaging remains largely incomplete, and motivate future efforts to more accurately characterize genome organization in mature sperm.

INTRODUCTION

The packaging of an organism's genome into the limiting confines of a typical nucleus requires multiple hierarchical levels of organization, allowing relatively free access to gene regulatory elements in the face of extensive physical compaction. In eukaryotes, over the past half-century the key principles of chromatin organization have been elucidated: a repeating nucleoprotein complex known as the nucleosome serves to compact ~150 bp of DNA, resulting in the famous "beads on a string" polymer that is then further organized via loop extrusion and phase separation into larger scale structures such as genome "compartments" and topologically-associating domains (TADs) (Dekker and Misteli 2015; Friedman and Rando 2015; Klemm et al. 2019).

Although we have a relatively mature understanding of chromatin organization in typical somatic cell types, genomic organization in the highly compact sperm nucleus remains less well understood. At the most basic level, it has been known for decades that the vast majority of histones are removed from the genome during the process of spermatogenesis in mammals, to be replaced with small highly basic peptides known as protamines (Bellve et al. 1975; Calvin 1976; Balhorn 1982). Intriguingly, a small subset of nucleosomes are retained in mature sperm – between ~2% and ~15%, depending on the mammal (Gaucher et al. 2010; Moritz and Hammoud 2022). A number of studies have mapped the locations of retained nucleosomes, with the majority of reports in humans and mice finding nucleosomes retained at CpG island regulatory elements associated with key developmental genes (Arpanahi et al. 2009; Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013; Johnson et al. 2016; Jung et al. 2017; Jung et al. 2019), although a handful of studies instead find histones retained primarily in long stretches over "gene deserts" of low gene density (Carone et al. 2014; Yamaguchi et al. 2018). It appears at least one explanation for this discrepancy may be the extent of nuclease digestion used in mapping nucleosomes in sperm, although many other technical details – including fixation and "pre-swelling" steps prior to chromatin preparation – also differ between studies. Other features of sperm genome compaction that have been investigated in detail include covalent modifications of histones (Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013; Jung et al. 2017; Jung et al. 2019; Lismer et al. 2020; Yoshida et al. 2020; Lismer et al. 2021; Bedi et al. 2022), localization of various non-histone proteins (Jung et al. 2017; Jung et al. 2019), and 3-dimensional folding of the sperm genome (Battulin et al. 2015; Jung et al. 2017; Ke et al. 2017; Alavattam et al. 2019; Jung et al. 2019; Vara et al. 2019; Wang et al. 2019; Gou et al. 2020). In addition to the inherent interest in understanding how the genome folds under conditions of extreme compaction, it has been widely suggested that the organization of the sperm genome – the locations and modifications of retained histones, most notably – may also play a role in programming early gene expression in the preimplantation embryo and may thereby influence later phenotypes in offspring (Siklenka et al. 2015; Lesch et al. 2019).

In the mouse system, sperm are typically collected from the distal, or cauda, region of the epididymis, a long tube where sperm mature for ~10 days following testicular spermatogenesis. Interestingly, several groups have recently reported the abundant presence of cell-free chromatin in the proximal, or caput, epididymis (Galan et

al. 2021; Chen et al. 2022). Although both of these studies focused on this contamination in the caput epididymis, where it results in ~20-30% contamination of sperm cytosine methylation profiles, there is also a small amount of contaminating chromatin present in cauda sperm preparations – we typically observe ~2-3% contamination of these sperm preps; the extent of contamination differs somewhat from investigator to investigator. Importantly, ~2% contamination of cytosine methylation data is comparable to the precision of a typical cytosine methylation survey and is therefore essentially insignificant for most purposes. However, we reasoned that this contamination could be a much greater problem for measurements of sperm chromatin, as 2% genome equivalents of a fully nucleosomal genome would be comparable to the bona fide nucleosomal complement of sperm.

Here, we explored the hypothesis that many or most published studies of mouse sperm chromatin organization reflect contaminating cell free chromatin. We show that two experimental treatments – DNase I treatment prior to sperm lysis, and proper use of high levels of reducing agent to enable sperm lysis – are required to eliminate contamination and allow access to the sperm genome. We show that thousands of previously reported "open chromatin" regions result from contamination; our updated list of bona fide sperm accessible regions reveals a striking enrichment for loci associated with cytoskeletal genes expressed late in spermiogenesis. We find that most published histone modification maps are also likely to result from chromatin contamination; however, we have been unable to elicit antibody-specific signal from DNase-treated sperm and so resolution of the histone modification landscape of sperm awaits further protocol optimization. Similarly, previously reported chromosome compartments and TADs in sperm are lost following DNase treatment, although our data suggest that sperm chromatin is inefficiently crosslinked using formaldehyde, requiring future efforts using arginine-specific crosslinkers or alternative approaches to chromosome folding. Together, our data strongly imply that current views of the mouse sperm chromatin landscape are flawed, and motivate renewed focus on this important area of chromosome biology and epigenetics.

RESULTS

Probing sperm chromatin architecture by ATAC-Seq

In the course of studies focused on dietary effects on sperm chromatin, we carried out ATAC-Seq (Assay for Transposase-Accessible Chromatin by Sequencing – (Buenrostro et al. 2015)) to identify relatively accessible genomic loci across the sperm genome. Here and through the rest of this manuscript, we isolate sperm from the cauda epididymis via tissue dissection and sperm swim out, and we then wash sperm with a somatic cell lysis buffer. After washing, purified cauda sperm exhibit no detectable (<0.1%) contamination by intact somatic cells (**Fig. S1A**, left panels). Although our original goal was to exactly reproduce the conditions used in Jung *et al* 2017 (Jung et al. 2017), we noticed that the investigators in this study did not add sufficient reducing agent to properly lyse sperm. This was of concern as sperm are subject to extensive disulfide crosslinking during maturation, and failure to reverse these crosslinks via treatment with a reducing agent (DTT, β-ME, or TCEP) results in significant difficulties in

recovering genomic DNA from sperm. This is illustrated in **Fig. S1B** – even after overnight SDS and proteinase K treatment, scant genomic DNA is recovered in the absence of appropriate levels of reducing agent. Moreover, microscopic examination of untreated sperm revealed abundant intact sperm heads even after extensive incubation in detergent, whereas DTT-treated samples were almost entirely lysed following detergent treatment (**Fig. S1A**, right panels). Taken together, these findings suggest the concerning possibility that many assays of sperm chromatin architecture – including but not limited to those using inadequate levels of reducing agent to allow access to the sperm genome – could be contaminated by cell-free chromatin present in the epididymal lumen.

To test the hypothesis that published assays of sperm chromatin architecture might be contaminated by cell-free chromatin, we set out to characterize the impact of several treatments on sperm ATAC-Seq profiles. Four conditions were considered. First, purified and washed cauda sperm were subjected to the exact same ATAC-Seq protocol reported in Jung *et al* 2017 and used in several additional studies (Jung et al. 2017; Jung et al. 2019; Gou et al. 2020): we refer to this as the "untreated" case; given that no reducing agent was used to help permeabilize sperm, we predict that the resulting ATAC-Seq profiles should primarily – indeed, can *only* – reflect the chromatin state of cell-free chromatin. Second, we pre-treat sperm with DNase I to eliminate any cell-free chromatin prior to probing with Tn5; we predict that little to no genomic DNA should be available for transposition after removing cell-free chromatin but without properly opening sperm. Third, we add appropriate levels of DTT prior to Tn5 treatment to allow access to the sperm genome; under these conditions we anticipate a mixed profile resulting from both cell-free chromatin and the bona fide sperm genome. Finally, by pretreating sperm with DNase I, then adding DTT prior to Tn5 probing, we hope to reveal the landscape of accessible chromatin in mature spermatozoa without any confounding contamination.

Sperm ATAC-Seq profiles are sensitive to cell-free chromatin contamination

Our initial efforts to probe sperm chromatin architecture were carried out using the same "untreated" ATAC-Seq conditions reported by Jung *et al* (Jung et al. 2017). Under these conditions, we robustly reproduce the published ATAC-seq landscapes published in several prior reports (**Fig. 1A**), as expected. Analysis of library insert lengths revealed the typical pattern observed in somatic ATAC-Seq libraries, with a ~75 bp peak (after accounting for sequencing adaptor lengths) corresponding to paired Tn5 insertions into open regulatory elements, followed by a series of peaks every ~150 bp corresponding to insertions flanking mono/di/trinucleosomes (**Fig. 1B**).

Turning next to sperm samples pre-treated with DNase I to remove cell-free chromatin prior to Tn5 probing, we generally failed to recover enough transposed DNA to build a sequencing library (not shown). When we did obtain sequencing libraries we recovered very low yields, with <10% of the levels of amplified DNA that could be recovered in the untreated condition (**Fig. S1C**). Analysis of insert lengths revealed a complete loss of the nucleosome signature (**Fig. 1B**), and sequencing profiles from these libraries were almost entirely flat; almost all the ATAC-seq peaks recovered in

untreated sperm were lost after DNase treatment (**Fig. 2A-B**, **Fig. S2A**). These findings are consistent with the hypothesis that published ATAC-seq profiles generated using untreated sperm are most likely contaminated by cell-free chromatin.

Eliminating cell-free chromatin reveals bona fide open chromatin profiles from mature sperm

We next turn to ATAC-Seq libraries prepared in the presence of the high levels of reducing agent required to enable access to Tn5 and other protein probes of chromatin architecture. We focus first on sperm preparations treated with both DNase and DTT. This pre-treatment should recover bona fide features of sperm chromatin, since it depletes cell-free chromatin before permeabilizing the sperm head. Analysis of library insert sizes revealed a dominant peak corresponding to ~75 bp inserts (**Fig. 1B**), corresponding to short accessible loci, but no notable signature of nucleosome-sized footprints. This reflects a relatively flat landscape of Tn5 insertions across the primarily protamine-packaged genome, with any nucleosomal footprints expected to result from low level of nucleosome retention (~2%) in murine sperm being overwhelmed by this global background (albeit with potential implications for nucleosome positioning: see **Discussion**). Importantly, we find similar ATAC-Seq insert lengths in DNase-treated sperm that were lysed using other reducing agents such as β-ME or TCEP (**Fig. 1B**), further supporting our hypothesis that prior ATAC-Seq studies in murine sperm suffered from a failure to properly reverse disulfide crosslinks.

Comparing the DNase/DTT-treated ATAC-Seq landscape with the untreated landscape, we found that the majority of “untreated” ATAC peaks were lost and only a small fraction retained in DNase/DTT-treated sperm (**Figs. 2A-B**). Systematic peak calling identified 10,741 accessible loci in DNase/DTT-treated sperm samples, compared with 15,680 accessible loci in untreated samples. As in other cell types, ATAC peaks in sperm were overrepresented at promoters (**Fig. 2C**), although these promoters exhibited relatively modest CpG density compared to the extremely high CpG density found at the ATAC peaks from cell-free chromatin (**Fig. 2B**). Importantly, we found no evidence for well-positioned 150 bp ATAC-Seq fragments – diagnostic of nucleosome localization – surrounding accessible loci (**Figs. 1B, 2C**). This argues against a standard organization with accessible regulatory elements flanked by a pair of positioned nucleosomes (see **Discussion**). In terms of biological function, accessible peaks in the DNase + DTT dataset were enriched near genes involved in cytoskeleton organization (**Fig. S2B**). Dramatic reorganization of the cytoskeleton accompanies the process of spermatid elongation to form mature spermatozoa. Consistent with this, we found that accessible chromatin peaks in our DNase + DTT dataset were found near genes expressed late in spermatogenesis (**Fig. S2C**), suggesting that these accessible regions reflect remnants of the chromatin organization associated with the final stages of spermiogenesis.

Finally, we turn to ATAC-Seq libraries produced from DTT-only treated sperm. Consistent with these samples comprising a mixture of cell free chromatin and bona fide sperm chromatin, we found ATAC signal over both the peaks unique to untreated sperm as well as the DNase+DTT peaks (**Fig. 2B**). This is true globally, with DTT-treated

ATAC-Seq landscapes exhibiting a mix of both cell free accessibility peaks and bona fide sperm accessibility peaks (**Fig. 2B**, **Fig. S2A**). Moreover, metagene plots of open chromatin peaks and nucleosome signatures over transcription start sites revealed that DTT-only samples exhibited hybrid profiles between the untreated and DNase+DTT profiles (**Fig. 2C**), again consistent with these libraries representing a mixture of cell free chromatin and bona fide sperm.

DNase and DTT do not affect open chromatin in mESCs

Our findings in mature sperm are most consistent with the hypothesis that prior ATAC-Seq studies of the sperm genome primarily report on the status of cell-free chromatin, with this contamination being the only chromatin available when studies are performed in the absence of adequate reducing agent. However, we also considered the hypothesis that the DNase and DTT treatments might somehow drive artifactual changes to global chromatin architecture. To test this hypothesis, we performed ATAC-seq in a commonly-studied cell type – mESCs – treated with the same conditions used for sperm preparations. Our ATAC-Seq profiles from untreated mESCs broadly recapitulated known features of the ESC chromatin landscape, with widespread peaks of accessible chromatin, flanked by well-positioned nucleosomes, associated with promoters and enhancers (**Fig. 3**). This landscape of open chromatin was almost entirely unaffected by DNase, DTT, or DNase + DTT treatments (**Fig. 3**), confirming that these treatments do not grossly alter Tn5 transposition or chromatin organization in cell lines.

Contamination of histone modification profiles by cell-free chromatin

Our ATAC-Seq data provide strong evidence that epigenomic studies of untreated sperm report primarily on contaminating cell-free chromatin. This finding naturally raises questions about other published aspects of sperm chromatin architecture. Given that multiple studies have implicated sperm histone modifications in programming early gene expression in the preimplantation embryo (Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013; Jung et al. 2017; Jung et al. 2019; Lismer et al. 2020; Yoshida et al. 2020; Lismer et al. 2021), and perturbing histone modifications during spermatogenesis has phenotypic effects in the next generation(Siklenka et al. 2015; Lesch et al. 2019), we set out to explore whether the histone modification landscape of sperm might also be affected by cell-free chromatin in the epididymis.

We initially carried out CUT&RUN and CUT&Tag (Skene and Henikoff 2017; Kaya-Okur et al. 2019) to identify genomic loci associated with H3K4me3 or H3K27me3, histone modifications related to the Trithorax and Polycomb epigenetic memory systems (Schuettengruber et al. 2007) and which have been mapped in sperm in multiple prior studies (Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013; Jung et al. 2017; Jung et al. 2019; Lismer et al. 2020; Yoshida et al. 2020; Lismer et al. 2021). In both cases, we found that libraries prepared from untreated sperm recapitulated modification landscapes previously obtained using ChIP-Seq (Erkek et al. 2013; Jung et al. 2017) (**Fig. S3**), but that these profiles were completely lost following

DNase treatment. This again argues that prior histone modification maps were likely contaminated by cell-free chromatin. Curiously, we were unable to elicit antibody-specific CUT&RUN or CUT&TAG profiles from DTT-treated sperm (with or without DNase pretreatment – **Fig. S3**); we have thus far been unable to define conditions that yield antibody-specific profiles for these protocols.

Similar results were obtained from ChIP-Seq (Johnson et al. 2007) using formaldehyde-fixed sperm chromatin sheared via sonication (**Methods**). We focused on a sequence-specific DNA-binding protein, CTCF, and the well-studied histone modifications H3K4me3 and H3K27me3, as these epitopes are expected to exhibit distinctive localization profiles. For all three epitopes, ChIP-Seq profiles from DTT-treated sperm samples – where we expect to see a mixture of contaminating chromatin and bona fide sperm – yielded profiles consistent with prior mapping studies (Erkek et al. 2013; Jung et al. 2017) (**Fig. 4A**). Peaks from the CTCF dataset were strongly enriched for the CTCF binding motif; H3K4me3 and K27me3 profiles were associated with promoters and repressed developmental regulators, respectively (**Figs. 4B-C, Fig. S4A**).

However, all three ChIP-Seq profiles were completely altered in maps generated from DNase+DTT-treated sperm (**Fig. 4A**). In the case of CTCF, we obtained a flat landscape, and those peaks that were called from this dataset were not enriched for the CTCF motif (**Fig. 4B, Fig. S4B**). This flat landscape suggests either that CTCF is not in fact associated with the mature sperm genome, or that our ChIP conditions – whether incomplete fragmentation of the sperm genome (**Fig. S4C**), or fixation conditions that impact CTCF epitopes needed for immunoprecipitation – precluded accurate identification of CTCF localization. Similarly, DNase treatment also dramatically altered the localization landscape of the histone modifications here. Significantly, the localization of histone marks in DNase+DTT-treated sperm were distinct from the CTCF landscape; ChIP-Seq in bona fide sperm chromatin therefore exhibited antibody-specific profiles, unlike CUT&RUN or CUT&Tag. Nonetheless, all three ChIP datasets revealed relatively flat landscapes with a small number of low-amplitude peaks with little evident biological significance. Along with the nonspecific profiles seen for CUT&RUN and CUT&Tag, these data indicate that conditions for affinity-based chromatin analysis in bona fide sperm will require continued optimization to allow more efficient recovery of proteins of interest.

Contamination affects published chromosome folding maps from mature sperm

We finally turn to the question of how the sperm genome is folded in three-dimensional space, focusing on reports using the gold standard molecular approach to chromosome folding, Hi-C (Lafontaine et al. 2021). A number of studies have generated Hi-C maps for mature murine spermatozoa (Battulin et al. 2015; Jung et al. 2017; Ke et al. 2017; Alavattam et al. 2019; Jung et al. 2019; Vara et al. 2019; Wang et al. 2019), with the majority of these studies (but see (Vara et al. 2019): discussed in the **Fig. S5** legend) reporting maps with typical features of somatic cells – A and B compartments corresponding to euchromatin and heterochromatin, and TADs with flares and loop anchors characteristic of loop extrusion processes. These maps are quite surprising

given the compaction required to fit the genome into the small sperm nucleus, as well as the unusual physicochemical properties (Balhorn 1982; Balhorn 2007) of the major packaging proteins – the protamines – that organize the sperm genome. Notably, a review of methods sections of published sperm Hi-C papers revealed that the majority of these efforts neglected to add DTT or other reducing agents required to properly access the sperm genome, and are thus likely to assay only cell-free or other contaminating somatic cell chromatin architecture.

To test this hypothesis, we generated Hi-C maps for spermatozoa using DNase and/or DTT as in the various experiments detailed above. **Fig. 5** shows typical Hi-C contact maps for data from Jung *et al.* 2017 alongside our DTT only and DNase+DTT libraries, as indicated. Data from Jung *et al* reveal the typical "checkerboard" pattern of A/B compartment organization at megabase scales (top panels), along with boxes on the diagonal with dots at the corners diagnostic of loop extrusion-mediated organization of gene-scale domains into TADs (middle panels). In our DNase+DTT samples all of these features are lost, with the exception of an extremely faint signal of compartment organization (**Fig. 5**, bottom row). We note that although this absence of specific signal could plausibly arise from certain types of chromosome packaging behavior, a more likely possibility is that protamines are inefficiently crosslinked by formaldehyde and so standard Hi-C crosslinking conditions fail to capture the chromosomal contacts that do occur in the sperm nucleus (see **Discussion**).

Finally, as seen with our ATAC-Seq datasets, the DTT only samples exhibit behavior consistent with a mixture between the "empty diagonal" of the DNase+DTT sample with a small contribution from the contaminating signal seen in untreated sperm libraries. This again fits our predictions and further supports our contention that even using DTT to break sperm nonetheless allows for contamination by cell-free chromatin if it has not been eliminated prior to assay.

DISCUSSION

Here, we revisit the unusual genomic packaging state in mature mouse spermatozoa. Most importantly, we find that a wide variety of published analyses of sperm chromatin compaction are contaminated by cell-free chromatin. We show that DNase I treatment of sperm prior to lysis removes this contaminating material, and further show that inadequate use of reducing agents in many studies precludes analysis of bona fide sperm chromatin by numerous commonly-used methods, including ATAC-seq, CUT&RUN, CUT&Tag, ChIP-Seq, and Hi-C. Together, our findings force a reappraisal of the packaging of the haploid genome in the compact sperm nucleus and raise new questions about the potential role for sperm chromatin as a carrier of epigenetic information from fathers to offspring.

Sperm chromatin assays are contaminated by cell-free chromatin

We previously identified cell-free chromatin as a major (~20-30%) contaminant of immature sperm from the caput epididymis (Galan et al. 2021; Chen et al. 2022) based on cytosine methylation data. Definitive demonstration that unexpected cytosine

methylation patterns resulted from cell free DNA was obtained by showing that pretreating caput sperm with DNase was sufficient to restore cytosine methylation to the levels seen in other germ cell populations. Although we did not highlight potential contamination of cauda sperm, even in this population we found that DNase treatment resulted in a ~2-3% reduction in methylation at sites that are hypermethylated in cell-free chromatin compared to sperm (Galan et al. 2021). Further evidence for cell-free DNA contamination in cauda sperm can be seen in attempts to isolate sperm genomic DNA with and without DTT (**Fig. S1B**) – in the absence of DTT, only a light smear of DNA is obtained even after Proteinase K and SDS treatment overnight, with the yield of this DNA being ~2-5% of the DNA obtained when sperm are properly broken using reducing agents.

This subtle contamination is of little importance to studies of cytosine methylation (assuming sperm are obtained from a well-defined cauda epididymis dissection without substantial vas deferens), as extraordinarily deep sequencing is required to obtain precision greater than 2% in whole genome bisulfite sequencing. In contrast, studies of cauda sperm chromatin are particularly susceptible to this contamination thanks to two idiosyncrasies of sperm biology. The first is the aforementioned requirement for a reducing agent for effective sperm lysis, driven by the extensive disulfide crosslinking that occurs during sperm maturation (Calvin and Bedford 1971; Saowaros and Panyim 1979). Thus, any studies of sperm chromatin carried out in the absence of DTT or other reducing agents – including the majority of sperm Hi-C studies as well as a substantial number of 1-dimensional chromatin studies – can only possibly survey the landscape of contaminating chromatin. The second challenge, affecting even studies performed using DTT, is that the vast majority of nucleosomes are evicted during spermatogenesis, leaving a small population (~2% in mouse) of nucleosomes retained in mature spermatozoa. Thus, contamination by ~2-5% cell free chromatin, presumably completely nucleosome-associated or nearly so, is sufficient to overwhelm any true signal from sperm chromatin. This is of course even more problematic in scenarios where the bona fide sperm genome cannot be assayed for other technical reasons, as may occur for Hi-C (see below).

Our ATAC-Seq data confirm precisely our predictions based on the considerations above. In the absence of DNase or DTT, we robustly recapitulate prior ATAC-Seq studies performed under these same conditions (**Fig. 1**). Our contention is that these accessibility peaks reflect cell-free chromatin, a hypothesis that is further supported by the nearly complete loss of signal obtained when using sperm samples pre-treated with DNase I. Perhaps most compelling are the results from samples treated with DTT only, where we confirm the prediction that ATAC-Seq libraries should reveal a superposition of the cell-free chromatin peaks and the bona fide sperm peaks identified in DNase+DTT samples. Altogether our data are completely and parsimoniously explained by our hypothesis that sperm chromatin assays generated from untreated sperm samples can only capture aspects of cell free chromatin contamination.

An updated view of genomic accessibility in murine sperm

Considering our ATAC-Seq data that was generated from properly broken sperm, free of cell-free chromatin, we found thousands of peaks of genomic accessibility that were only observed in DTT-treated sperm but not in cell-free chromatin. These peaks, with lower CpG density than untreated peaks (**Fig. 2B**), were enriched at regulatory elements associated with genes involved in cytoskeletal reorganization. Biologically, this may reflect the gene expression program induced during late spermiogenesis that supports the major morphological remodeling that occurs during sperm elongation and individuation (**Fig. S2C**). In this view, sperm genome organization captures the last stages of active gene regulation that occur prior to complete transcriptional shutdown in spermatozoa, rather than providing evidence for anticipatory "programming" that facilitates early embryonic gene regulation. Nonetheless, we cannot rule out the possibility that some of the accessible loci identified in mature sperm may play functional roles in the early embryo, and this will be of great interest to explore in future studies.

Our data also provide some indirect evidence regarding nucleosome organization in mature sperm. Specifically, ATAC-Seq libraries not only capture relatively accessible genomic loci as relatively short ~75 bp DNA fragments, but also provide information on nucleosomes flanking accessible regions (Buenrostro et al. 2013). These ~140-150 bp fragments result from a pair of Tn5 transposition events, with one event occurring in a relatively open genomic locus along with a second insertion occurring at the far end of an adjacent nucleosome. Indeed, ~140-160 bp ATAC peaks have been used in several studies of mammalian sperm to infer the existence of nucleosomes flanking regulatory elements (CpG islands, etc.) (Jung et al. 2017; Jung et al. 2019). However, in our DNase+DTT ATAC-Seq libraries we find no discernable ~150 bp peak in the insert length distribution (**Fig. 1B**), and analysis of mapped 150 bp footprints reveals diffuse enrichment rather than any evidence for positioned peaks surrounding accessible genomic loci (**Fig. 2C**). These data are not evidence against the existence of nucleosomes in sperm – nucleosomes not adjacent to open chromatin require two Tn5 insertions into short linkers and are heavily underrepresented in ATAC-Seq libraries of somatic cells – but instead suggest that any sperm-retained nucleosomes are not highly enriched surrounding accessible regulatory elements.

Together, these considerations raise the question of the molecular nature of accessible genomic loci in mature sperm. Are these regions relatively depleted of protamines? Are they occupied by other proteins, or broadly protein-depleted? Given our concerns with affinity-based chromatin assays in sperm (below), answering these questions in molecular detail will be challenging and will require optimization of affinity-based chromatin assays suited to mature sperm.

The state of histone modification landscapes in mature sperm

Turning next to histone modification patterns, our data again suggest that the prevailing view of sperm histone modifications – that nucleosomes flanking CpG islands carry the "bivalent" pair of H3K4me3 and H3K27me3 histone modifications (Erkek et al. 2013; Zhang et al. 2016; Zheng et al. 2016; Jung et al. 2017) – may also reflect contamination by cell-free chromatin. Using three common affinity-based methods for histone

modification mapping – ChIP-Seq, CUT&RUN, and CUT&Tag – in untreated sperm we readily reproduce the findings of multiple prior histone modification surveys (**Figs. 4, S3**). As in the case of ATAC-Seq, these patterns are dramatically altered when sperm are pre-treated with DNase I to eliminate cell-free chromatin, again suggesting that extant histone modification profiles in sperm reflect cell-free chromatin contamination.

At present we have not identified conditions that allow for reliable affinity-based chromatin localization studies in sperm, as we find relatively flat landscapes for ChIP-Seq, CUT&RUN, and CUT&Tag using several antibodies. Further optimization will therefore be required to properly assess protein localization across the sperm genome using antibody-based approaches.

Absence of compartments and TADs in mature murine sperm

Finally, we turn to three-dimensional folding of the sperm genome, where multiple extant studies have reported the a priori unlikely finding that the sperm genome is organized nearly-identically to the genomes of fibroblasts and ES cells. As noted in the **Introduction**, all of the studies making this claim were performed in the absence of reducing agent, and thus can *only* represent cell-free chromatin. Consistent with this model, we note that multiple studies (Alavattam et al. 2019; Wang et al. 2019) have explored dynamic changes in chromosome folding during spermatogenesis, reporting that typical architectural features – compartments, TADs, loops – are present in spermatogonia and progressively lost during spermatogenesis, only to be inexplicably regained in mature spermatozoa and then immediately lost in the paternal pronucleus of the zygote. These data are consistent with dramatic alterations to chromosome folding occurring during spermatogenesis, combined with a failure to appropriately measure the chromosome folding state in mature spermatozoa thanks to contaminating extracellular chromatin.

Indeed, as with all the other assays of sperm chromatin, we find that the organization of the sperm genome into compartments, TADs, and loops, cannot be reproduced when sperm are properly broken (**Fig. 5**). We instead find an essentially featureless diagonal in the DNase+DTT condition. Importantly, here again – as with ATAC-Seq – we show that DTT only datasets exhibit a mixture of the bona fide sperm profile with the cell free chromatin profile, which provides compelling evidence for our overarching hypothesis. Parenthetically, one implication of this finding is that cell-free chromatin contamination in the epididymis must retain some of the folding features observed in somatic cells; we speculate that this results from loop extrusion complexes stalling in place in the absence of ATP, but interrogating this is outside the scope of this study.

The featureless diagonal seen in our DNase+DTT samples has two potential explanations. The first, which we do not favor, is that the sperm genome is compacted without any consistent architectural features, perhaps by protamine-mediated aggregation/liquid-liquid phase separation as observed in vitro (doi: https://doi.org/10.1101/2021.09.16.460631). Alternatively, we speculate that standard Hi-C methodology fails to effectively assay interactions between genomic loci in physical proximity in sperm. We suspect that this results from the requirement for

formaldehyde crosslinking to capture interactions between genomic loci in most 3C-derived protocols. Formaldehyde reacts efficiently with primary amines including the ε-amine moiety of lysine as well as primary amine groups on the nucleotide bases. Formaldehyde is expected to react much more inefficiently with the electron pair on arginine thanks to higher pKa (~12.5 vs 10) of the guanidino group making this residue much less nucleophilic. Thus, while formaldehyde very effectively crosslinks DNA bases to histone lysines, and histone lysines to one another, in the case of protamines – which are highly enriched for arginine and in many species are completely devoid of lysines – formaldehyde may not capture protamine-protamine interactions. Indeed, examination of interaction decay data for our Hi-C data is consistent with poor crosslinking (**Fig. S5**).

We therefore consider our data extremely strong evidence arguing that prior sperm Hi-C studies in mammals have been contaminated by cell-free chromatin. Beyond this, at present we feel our data do not provide any insight into how the mammalian sperm genome is in fact organized. For example, we find no evidence for the postulated 50-80kb solenoidal structure seen in in vitro studies of protamine-DNA complexes, which would be readily visible in Hi-C as a second diagonal 50-80 kb away from the primary diagonal. Whether such a structure occurs in vivo will be of considerable interest for future studies. Productive avenues for future chromosome folding studies in sperm can be envisioned using arginine-reactive or DNA-reactive crosslinkers (Jones et al. 2019; You et al. 2021), crosslinking-independent chromosome folding assays such as Hi-TrAC (Liu et al. 2022) or GAM (Beagrie et al. 2017), or super-resolution microscopy “walks” to probe the organization of megabase-scale domains (Wang et al. 2016).

Implications for sperm chromatin architecture studies in human

Finally, we note one intriguing finding that may provide important insights into differences between studies of human and mouse sperm chromatin. Briefly, Chen *et al* generated Hi-C maps from human sperm, finding a largely featureless diagonal like the one seen in our DNase+DTT dataset (Chen et al. 2019a). The authors performed a valuable and insightful mixing experiment in which they performed Hi-C in a mixture of ejaculated sperm and cauda epididymal mouse sperm, recovering the compartments and TADs reported in mouse sperm along with the flat diagonal they reported for human.

We consider two potential explanations. The first is that it has been claimed that human sperm are more readily permeabilized in the absence of DTT (Hisano et al. 2013), suggesting that although the mouse genome was inaccessible under the reported conditions and so mouse contacts could only be generated from cell-free chromatin, the human sample was properly permeabilized and assayed (with the caveat, above, regarding formaldehyde crosslinking as a tool for sperm chromosome folding). Alternatively, we believe it may be the case that ejaculated sperm are less contaminated by cell-free chromatin – in other studies (not shown) we have identified a DNase expressed at high levels in the seminal vesicle that may play a role in clearing the cell-free chromatin carried forward from the epididymis.

Given that many reported studies of sperm chromatin organization – such as the reported localization of H3K4 and K27 methylation to CpG islands – are broadly concordant between human and mouse samples, it will be important to consider the implications of our findings here for human sperm studies.

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MATERIALS AND METHODS

Ethics statement. Animal husbandry and experimentation was reviewed, approved, and monitored under the University of Massachusetts Medical School Institutional Animal Care and Use Committee (Protocol ID: A-1833-18).

Mouse husbandry and tissue collection. All samples were obtained from male mice of the C57BL/6J strain background, consuming control diet Ain-93g, euthanized at 19 weeks of age.

Cauda sperm isolation and purification. Cauda epididymis was briefly dissected in PBS to remove fat tissues. Cauda tissues were transferred to Donners medium and sperm were released by several cuts and gentle squeeze of the cauda tissues. The cauda was discarded, and medium was transferred to a 1.5 ml tube. Sperm swim-up was conducted at 37°C for 1hr. Next, the upper ~1.3 ml medium was recovered and filtered through a 40 μm strainer. Sperm were pelleted, washed with PBS, and resuspend/incubated in somatic lysis buffer (0.01% SDS, 0.005% Triton X-100) for 10 min on ice. Purified sperm were washed with PBS and subjected to downstream treatment.

DNase and DTT treatment of cauda sperm. Purified sperm pellet was resuspended in PBS. RDD buffer and DNase I (Qiagen) was added at 1:10 dilution and 2.5 μl/100μl, respectively. DNase treatment was carried out at room temperature for 10 min, followed by PBS wash. Next, sperm were treated with 50 mM DTT (50 mM TCEP or 1% β-ME) in PBS for 1 hr at RT and quenched by 100 mM NEM (N-Ethylmaleimide). Pretreated sperm were pelleted and wash with PBS and subjected to ATAC-seq.

ATAC-Seq. ATAC-seq was carried out using the Omni-ATAC-seq protocol (Corces et al. 2017) with several modifications. About 100,000 sperm were on-ice lysed with hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% NP40, 0.1% Tween-20, and 0.01% digitonin) for 10 min. Sperm were then resuspended in Tagmentation buffer (10 mM TAPS-NaOH pH 8.5, 5 mM MgCl2, 10% DMF, 0.05% Digitonin) and tagmentation was conducted by adding Tn5 transposase (Diagenode, C01070012) at 1:20 and incubated at 37C for 30min. Reaction was stopped by SDS and proteinase K and incubated at 55°C overnight. SDS was

quenched by tween-20 and library amplification was done with 1x NEBNext High-Fidelity PCR Master Mix containing 0.5 μM indexed primers using the following PCR conditions: 72°C for 5 min; 98°C for 3 min; and 14 cycles at 98°C for 15 s, 63°C for 30 s, and 72°C for 1 min. Libraries were pair-end sequenced on an Illumina NextSeq500 platform.

ATAC-Seq data analysis. Paired-end ATAC-seq reads were adapter-trimmed and aligned to the mm10 genome using Bowtie2 (Version 2.3.2) with the parameters: -t -q -N 1 -L 25 -X 2000 no-mixed no-discordant. All unmapped reads, non-uniquely mapped reads and PCR duplicates were removed. MACS2 (Zhang et al. 2008) was used for peak calling with parameters: --nolambda --nomodel. Reads with insert size larger than 150 bp were separated and used for nucleosome positioning analysis. TSS enrichment analysis was done using Homer annotatePeaks (Heinz et al. 2010).

CUT&RUN. CUT&RUN libraries were prepared following the same protocol as previously described (Chen et al. 2019b) with minor modifications. Specifically, 0.1% NP40, 0.1% Tween-20, and 0.05% digitonin was used in the antibody incubation buffer and wash buffer, and SPRI beads were used for DNA extraction after proteinase K treatment. For H3K27me3 CUT&RUN, a rabbit anti-H3K27me3 antibody (1:100; Diagenode, C15410195) was used. For H3K4me3 CUT&RUN, a rabbit anti-H3K4me3 antibody (1:100; Diagenode, C15410003) was used. All CUT&RUN libraries were sequenced on an Illumina NextSeq500 platform.

CUT&Tag. CUT&Tag libraries were prepared following the same protocol as previously described (Kaya-Okur et al. 2019). Antibodies used were against H3K27me3 (1:50; ThermoFisher, MA5-11198), H3K4me3 (1:50; abcam, ab8580), Histone H3 (1:50; abcam, ab1791) and Serine 2 Phospho-Rpb1 CTD (1:50; Cell Signal, 13499S). All CUT&Tag libraries were sequenced on an Illumina NextSeq500 platform.

ChIP-Seq. ChIP-Seq experiments were conducted following the protocol described in (Chen et al. 2021) with some modifications. After lysing sperm in complete buffer containing detergent, equal volume of 2x RIPA buffer was supplied to make a 1x RIPA condition. Chromatin shearing was performed using Covaris S220 platform with parameters: peak power 140 W, duty factor 5% and 200 Cycles/burst for a total of 30 min. Sonicated sperm chromatin were directly subjected to immunoprecipitation with corresponding antibodies (H3K4me3: Diagenode, C15410003; H3K27me3: Diagenode, C15410195; CTCF: Millipore 07-792). Library preparation was carried out using NEBNext Ultra II DNA Library Prep kit (NEB E7645). All ChIP-Seq libraries were sequenced on an Illumina NextSeq500 platform.

Histone modification data analysis. For both CUT&RUN and ChIP-seq datasets, paired-end reads were trimmed using Trim Galore (version 0.4.5), followed by reads alignment to the mm10 genome using Bowtie2 (Version 2.3.2) with the parameters: -t -q -N 1 -L 25 -X 700 no-mixed no-discordant. PCR duplicates were removed using “MarkDuplicates” from Picard Tools (version 2.8.0) (https://broadinstitute.github.io/picard/). Only non-PCR duplicates and uniquely aligned reads (alignment records without “XS” tag) were used for downstream analyses. The RPKM values for each 100-bp bin were calculated following the formula “read counts/((bin_length/1000) × (total_reads/10^6)). Peak calling for H3K4me3 and H3K27me3

datasets were done by MACS2 with parameters: --nolambda --nomodel, and --broad-cutoff 0.1 --nolambda --nomodel, respectively.

Hi-C library construction. The sperm library collection was made as described before (Lafontaine et al. 2021) with some modifications. Briefly, cells were fixed in 1% formaldehyde and after washing in PBS, left for 1 hour in 50 mM DTT. After quenching with 100mM NEM (N-ethylmaleimide) and washing in PBS, cells were incubated with DpnII overnight at 37°C . After restriction digestion, cells were incubated for 4 hours at 23°C with the Klenow DNA polymerase I to fill in the 5' overhang and incorporate biotin-14-dATP followed by chromatin ligation for 4 hours at 16°C with T4 DNA ligase (Invitrogen). Then cells were left overnight in 400 ng/ml proteinase K and 0.5% SDS at 60°C overnight to reverse cross-linking. After DNA purification and precipitation, DNA was sonicated to 200-300 bp fragments and an additional size selection was done with Ampure beads. To repair DNA ends after sonication, T4 DNA polymerase (NEB) and Klenow DNA polymerase (NEB) were used together with T4 polynucleotide kinase (NEB). Biotinylated ligation products were pulled down using streptavidin beads (Invitrogen) followed by A-tailing and adaptor ligation for library construction. All Hi-C libraries were sequenced on an Illumina NextSeq500 platform.

Hi-C data analysis. Hi-C data was analyzed using the Open Chromosome Collective suite (https://github.com/open2c). Briefly, 50bp paired-end fastq files were mapped to mm10 mouse reference genome using the *distiller-nf* (https://github.com/mirnylab/distiller-nf), invoking bwa mem to map fastq pairs in a single-side regime (-SP). Aligned reads were allowed a 1bp flexibility for removal of optical and PCR duplicates using *pairtools* (https://github.com/mirnylab/pairtools). After removal of identical positions and strand orientations, remaining valid pairs were binned into contact matrices of various resolutions using *cooler* (Abdennur and Mirny 2020). An iterative balancing procedure (Imakaev et al. 2012) was applied to all matrices, ignoring the first 2 diagonals to avoid short-range ligation artifacts at a given resolution, and bins with low coverage were removed using MADmax filter with default parameters. Resultant ".cool" contact matrices were used in downstream analyses using *cooltools* (https://github.com/mirnylab/cooltools) and uploaded to a HiGlass server (Kerpedjiev et al. 2018) via the Reservoir Genome Browser (https://resgen.io/).

Contact probability (*P*(*s*)) plots & derivatives. We produced *P(s)* plots per chromosome arm as outlined in the "contacts vs distance" section of cooltools (https://cooltools.readthedocs.io/en/latest/notebooks/contacts_vs_distance.html). Briefly, mm10 chromosome sizes and arm locations were downloaded as ViewFrames from the UCSC database using Bioframe (https://bioframe.readthedocs.io/en/latest/index.html). For each arm, interaction counts (diagsum) were generated from a 1kb cooler file for each distance from the self-self diagonal. Counts for all chromosome arms were aggregated, logbin- smoothed (σ = 0.05) and *P*(*s*) curves were normalized for the total number of valid interactions in each data set. Corresponding derivative plots were generated using numpy gradient to estimate the slope from each *P*(*s*) plot.

Compartment analyses. When present, compartmentalization is the strongest signal and will be represented by the first eigenvector (EV1). We performed eigenvector decomposition on observed-over-expected contact maps at 100kb resolution separated for each chromosomal

arm using the cooltools package derived scripts
(https://cooltools.readthedocs.io/en/latest/notebooks/compartments_and_saddles.html).

FIGURE LEGENDS

Figure 1. ATAC-Seq profiles from sperm subject following DNase and DTT treatment

A) Genome browser tracks for ATAC-Seq data from untreated sperm (this study) as well as three published ATAC-Seq datasets for sperm (Jung et al. 2017; Jung et al. 2019; Gou et al. 2020).
B) Bioanalyzer traces of ATAC-Seq sequencing libraries prepared from sperm samples pre-treated with the indicated conditions prior to Tn5 transposition. Red arrows show ~75 bp inserts corresponding to open chromatin regions, while black arrows in "untreated" libraries show insert lengths corresponding to mono-, di-, and tri-nucleosome length inserts. Notable in all the DTT-treated libraries is a peak at ~1.3 kb. This peak reflects a landscape of random insertions across the sperm genome, with the size of the insert being an artifact of the PCR conditions used in library preparation. We confirmed this in two ways. First, we built a second set of ATAC-Seq libraries where the PCR extension time was extended to two minutes as compared to one minute in the libraries shown. Under these conditions, we find a ~2.5 kb peak, confirming that PCR extension time is responsible for this peak location. Second, we gel-isolated the 1.3 kb material from a DNase + DTT ATAC-Seq library, sheared the DNA and characterized the material by deep sequencing. Resulting sequencing reads revealed a genome-wide background (not shown).

Figure 2. Published ATAC-Seq profiles in sperm are dominated by cell-free chromatin contamination

A) ATAC-Seq browser tracks for sperm subject to the indicated conditions.
B) Heatmaps for the four indicated libraries, sorted into three categories: peaks specific to the untreated dataset (I; n= 13,605), shared peaks (II; n= 2,148), and peaks specific to DNase/DTT datasets (III; n= 8,627).
C) Metagenes for <100 bp inserts – diagnostic of open chromatin – or for 140-160 bp inserts – diagnostic of nucleosome footprints – aligned over all annotated TSSs.

Figure 3. DNase and DTT treatment do not affect ATAC-Seq profiles in mESCs

A) ATAC-Seq profiles for mESCs treated with the indicated conditions, as well as published ESC DHS data from (Vierstra et al. 2014).
B) Heatmaps show ATAC read counts aligned over peaks (n= 58,130) called from untreated cells.

C) Metagenes for <100 bp inserts, and ~150 bp inserts, as in **Fig. 2C**.

Figure 4. Histone modification profiling in DNase-treated sperm reveals a loss of specific signal

A) Browser tracks for H3K4me3, H3K27me3, and CTCF ChIP-Seq in sperm. In all three cases top panels show data from published sperm datasets (Jung et al. 2017), along with our data from DTT- and DNase+DTT-treated sperm underneath.
B) Metagenes aligned over the relevant peak locations (TSS, CTCF motif, and Polycomb-group (PcG) targets), showing H3K4me3, H3K27me3, and CTCF enrichment in our DTT only and DTT+DNase datasets.
C) Heatmaps show H3K4me3 and K27me3 enrichment over all peaks from Jung et al. 2017, with data shown for Jung *et al* alongside our DTT and DTT+DNase datasets.

Figure 5. Absence of compartment signals and TADs in bona fide sperm Hi-C maps

Hi-C contact maps for (from left to right) untreated sperm (data from (Jung et al. 2017)), DTT-treated sperm, and DNase and DTT-treated sperm. Top panels show a zoomout genome view covering chromosome 3, middle panels show a typical ~10 MB zoom in (Chr 3:30-42 MB). Bottom panels show A/B compartment calls (EV1: eigenvector 1) for the zoomed-in region.

SUPPLEMENTAL MATERIALS

Supplemental Figures S1-S5

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. DTT is required to lyse mature spermatozoa

A) DIC Images of typical cauda epididymal sperm samples before and after processing for ATAC-Seq. Top panels show sperm processed without the use of DTT, revealing unbroken sperm heads even after overnight incubation in SDS and proteinase K. Bottom panels show efficient sperm lysis for DTT-treated sperm under the same conditions.
B) DTT is required to recover genomic DNA from sperm. Cauda sperm were either left untreated or incubated with 50 mM DTT for one hour, then quenched with NEM. Both samples were then treated identically with lysis buffer and were incubated in SDS and proteinase K at 65°C for 16 hours, and lysed in Trizol for genomic DNA extraction.
C) ATAC-Seq library yields for sperm treated with the indicated conditions prior to Tn5 treatment. All yields (mean of DNA library yields from two replicates) from other conditions were normalized to untreated group.

Supplemental Figure S2. Functional enrichment in sperm open chromatin

A) Heatmaps showing the datasets from **Fig. 2B**, with panels I-V showing data over ATAC-Seq peak locations called from Jung et al (I; n=51,353), DNase-only (II; n= 600), DTT only (III; n= 21,756), DNase+DTT (IV; n= 10,741), or DNase hypersensitivity maps (Lu et al. 2016) (V; n= 1,439).

B) Bar graph shows p values (expressed as -log10 of the p value) for various Gene Ontology categories enriched in genes found near bona fide sperm ATAC-Seq peaks.

C) Heatmap shows expression (in TPM) of genes located near DNase+DTT ATAC-Seq peaks at the indicated stages of spermatogenesis (Hammoud et al. 2014).

Supplemental Figure S3. Lack of antibody specificity in sperm CUT&RUN and CUT&TAG profiles

A) CUT&RUN profiles for the indicated histone modifications, for sperm either untreated or treated with DNase I and DTT. Published ChIP-Seq profiles from the indicated studies (Erkek et al. 2013; Jung et al. 2017) are shown above the CUT&RUN libraries for comparison.

B) Heatmaps showing H3K4me3 signal for the indicated libraries, aligned over peaks called from Erkek *et al* (Erkek et al. 2013)

C) Metagene showing averaged H3K4me3 enrichment for a 4 kb window surrounding all transcription start sites.

D) As in (A), for CUT&Tag libraries. Note that four distinct epitopes, including one which should be absent from mature sperm (Pol2S2P), all exhibit nearly identical localization landscapes. This localization resembles our ATAC-Seq data (not shown) and thus presumably reflects untargeted Tn5 activity in the CUT&Tag protocol.

Supplemental Figure S4. Features of sperm ChIP-Seq datasets

A) Heatmaps of H3K4me3 and H3K27me3 data for the DTT only and DNase+DTT libraries, aligned over all promoter regions (n = 57,102;TSS +/- 2.5 kb), organized from high to low enrichment of H3K4me3 signals.

B) Sequence logos for enriched motifs at CTCF ChIP-Seq peaks called from DTT-only and DNase+DTT libraries, as indicated.

C) Limited solubilization of the sperm genome following sonication. Formaldehyde-fixed sperm were sonicated to fragment the genome for ChIP-Seq. Following sonication, resulting material was centrifuged (10,000 xg, 10 min) and DNA was extracted from pellet and supernatant fractions and characterized by agarose gel electrophoresis.

Supplemental Figure S5. Hi-C scaling plots

Top panel shows interaction frequency between two loci at increasing genomic distances (x axis) for the indicated datasets. Bottom panel shows the derivative of these

curves. The relatively flat profile seen in the DNase+DTT dataset is typical for poorly-crosslinked Hi-C libraries, potentially owing to the paucity of lysines in sperm DNA-associated proteins like the protamines.

We note that although the majority of published Hi-C studies of mouse sperm exhibit typical somatic features including A/B compartments and TADs (Battulin et al. 2015; Jung et al. 2017; Ke et al. 2017; Alavattam et al. 2019; Jung et al. 2019; Wang et al. 2019), TADs were notably absent in (Vara et al. 2019). Although this would be explained parsimoniously by the use of DTT in that study – generating maps dominated by bona fide sperm rather than contaminating chromatin, as seen in our **Fig. 5** data – this does not appear to have been the case based on the Methods section.

Instead, two technical choices may provide a potential explanation. First, Vara *et al* state that testis, epididymis, and epididymal sperm were co-incubated during testicular dissociation, conditions which include DNase I treatment (DNase treatment is a typical part of testicular dissociation protocols). Of course this would eliminate the cell-free chromatin contamination present in the cauda epididymis, as demonstrated throughout this manuscript, but raises the question of what material was captured in the resulting Hi-C libraries. This highlights the second technical choice, where Vara *et al* snap freeze FACS-sorted sperm prior to all fixation and permeabilization steps. In our experience, MNase-Seq data are dramatically altered when the assay is performed on frozen and thawed sperm vs. freshly isolated sperm used immediately (not shown). We speculate that this reflects ice damage to sperm frozen without cryoprotectant, potentially allowing later enzyme access to fractured sperm heads. In this scenario Vara *et al* would be able to generate Hi-C maps, free of cell free chromatin, from sperm broken by freezing.

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Figure 1

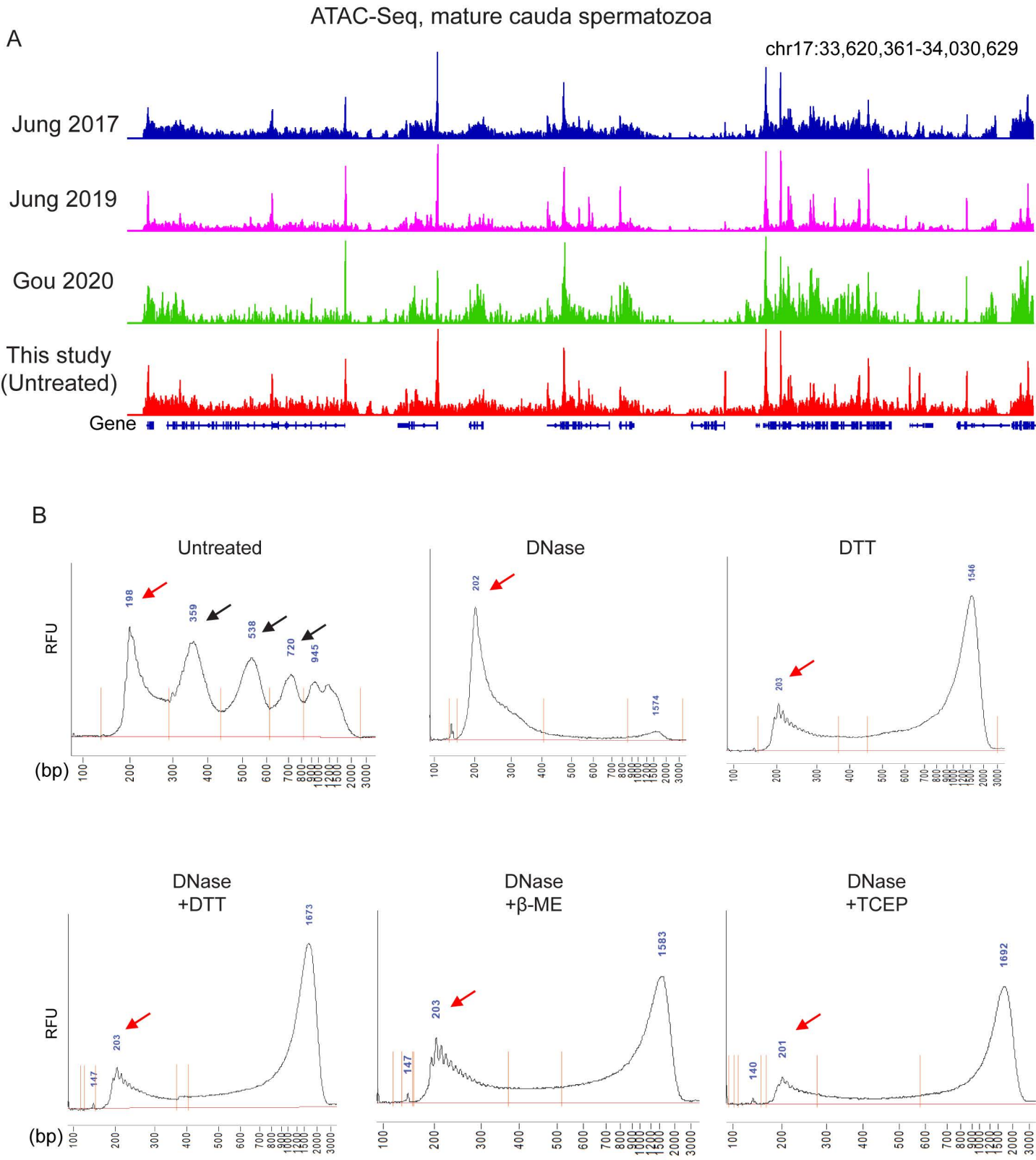

ATAC-Seq, mature cauda spermatozoa
A
chr17:33,620,361-34,030,629
Jung 2017
Jung 2019
Gou 2020
This study
(Untreated)
Gene
B
Untreated
198
359
538
720
945
DNase
202
1574
DTT
203
1546
DNase
+DTT
203
147
1673
DNase
+β-ME
203
147
1583
DNase
+TCEP
201
140
1692
RFU
(bp)
100
200
300
400
500
600
700
800
1000
1200
1500
2000
3000

Figure 2

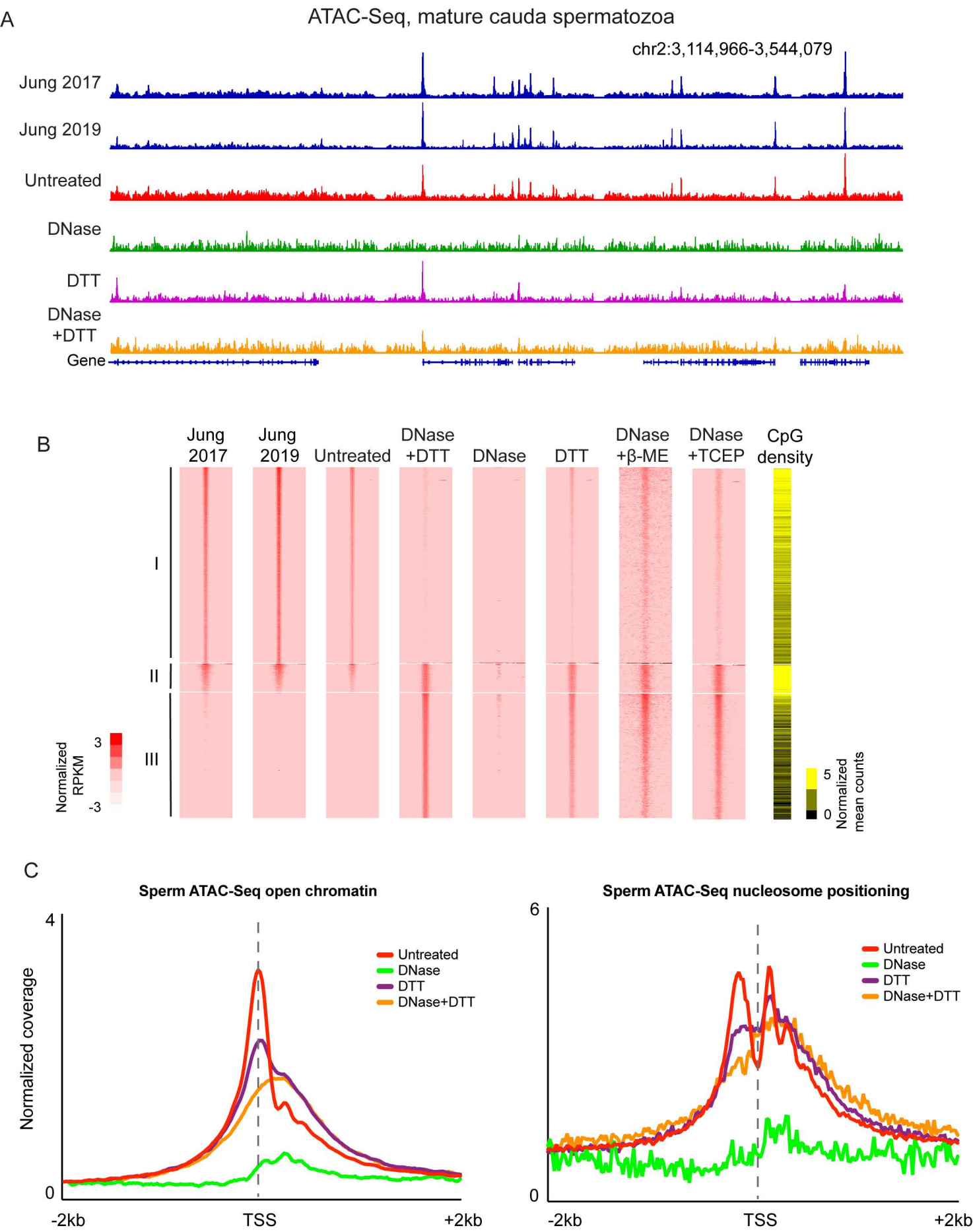

A
ATAC-Seq, mature cauda spermatozoa
chr2:3,114,966-3,544,079
Jung 2017
Jung 2019
Untreated
DNase
DTT
DNase +DTT
Gene
B
Jung 2017
Jung 2019
Untreated
DNase +DTT
DNase
DTT
DNase +β-ME
DNase +TCEP
CpG density
I
II
III
Normalized RPKM
3
-3
5
0
Normalized mean counts
C
Sperm ATAC-Seq open chromatin
Sperm ATAC-Seq nucleosome positioning
Normalized coverage
4
0
6
0
Untreated
DNase
DTT
DNase+DTT
-2kb
TSS
+2kb

Figure 3

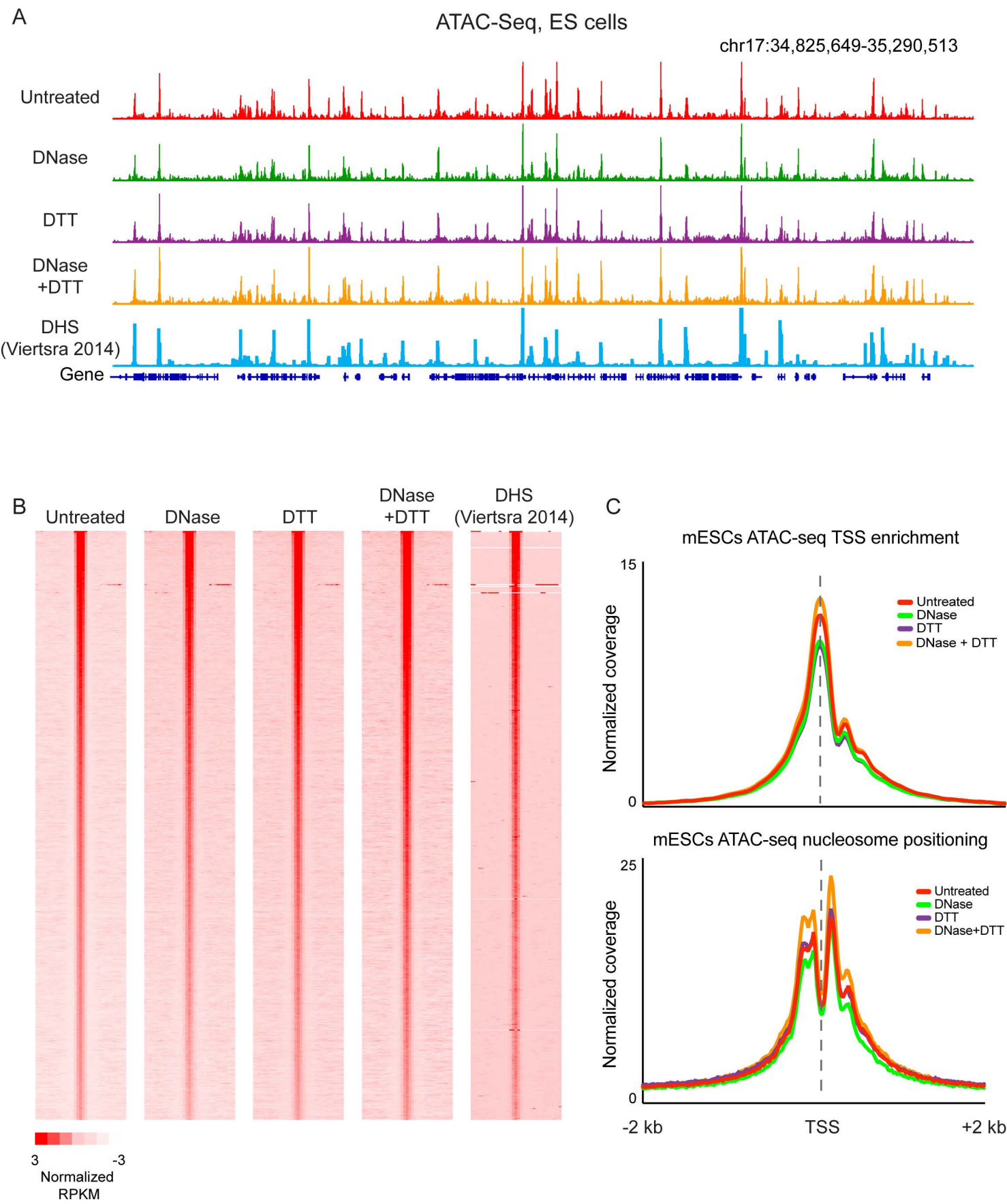

A
ATAC-Seq, ES cells
chr17:34,825,649-35,290,513
Untreated
DNase
DTT
DNase +DTT
DHS (Viertsra 2014)
Gene
B
Untreated
DNase
DTT
DNase +DTT
DHS (Viertsra 2014)
3
-3
Normalized RPKM
C
mESCs ATAC-seq TSS enrichment
Normalized coverage
15
0
Untreated
DNase
DTT
DNase + DTT
mESCs ATAC-seq nucleosome positioning
Normalized coverage
25
0
Untreated
DNase
DTT
DNase+DTT
-2 kb
TSS
+2 kb

Figure 4

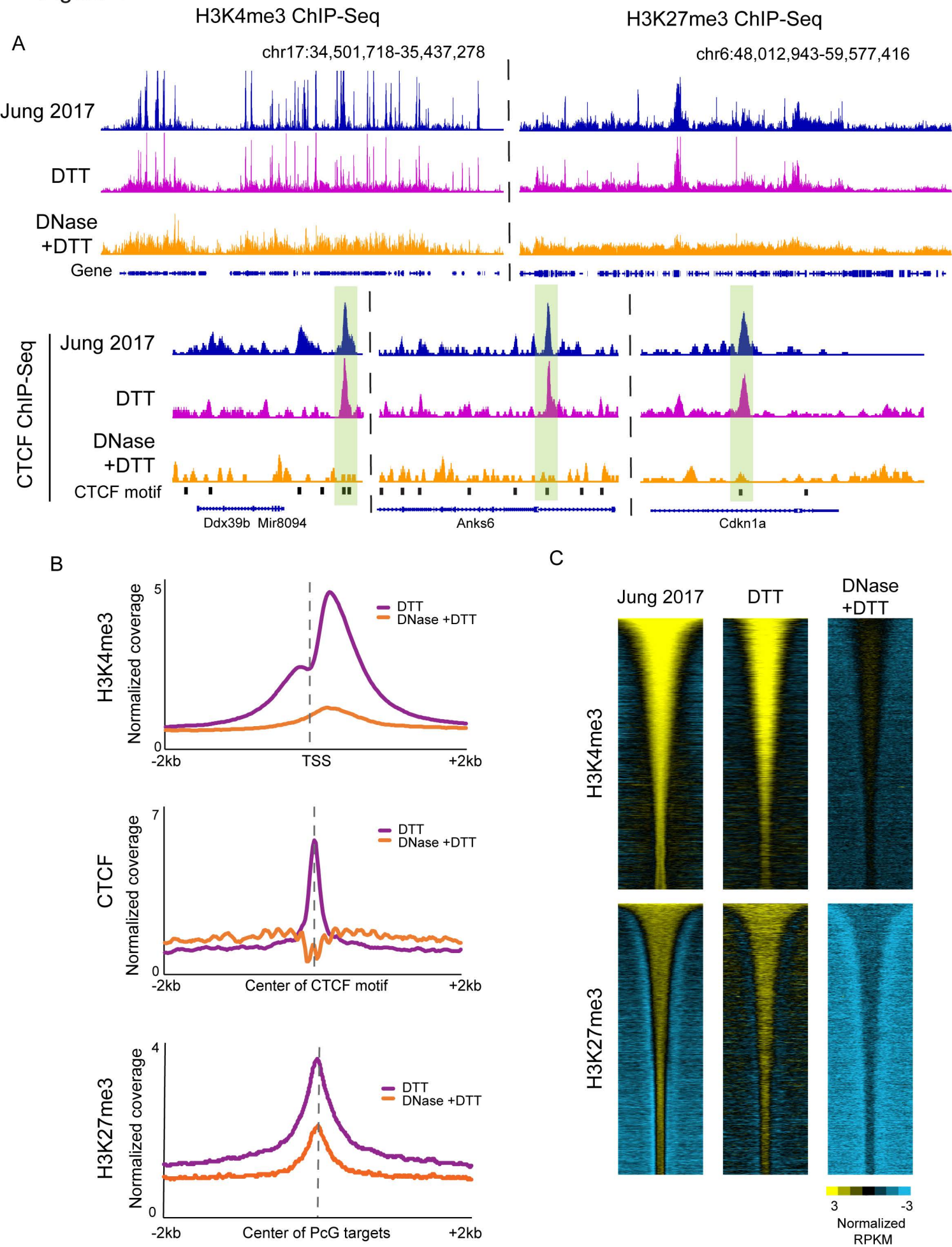

A
H3K4me3 ChIP-Seq
H3K27me3 ChIP-Seq
chr17:34,501,718-35,437,278
chr6:48,012,943-59,577,416
Jung 2017
DTT
DNase +DTT
Gene
CTCF ChIP-Seq
Jung 2017
DTT
DNase +DTT
CTCF motif
Ddx39b Mir8094
Anks6
Cdkn1a
B
H3K4me3
Normalized coverage
5
0
DTT
DNase +DTT
-2kb
TSS
+2kb
CTCF
Normalized coverage
7
0
DTT
DNase +DTT
-2kb
Center of CTCF motif
+2kb
H3K27me3
Normalized coverage
4
0
DTT
DNase +DTT
-2kb
Center of PcG targets
+2kb
C
Jung 2017
DTT
DNase +DTT
H3K4me3
H3K27me3
3
-3
Normalized RPKM

Figure 5

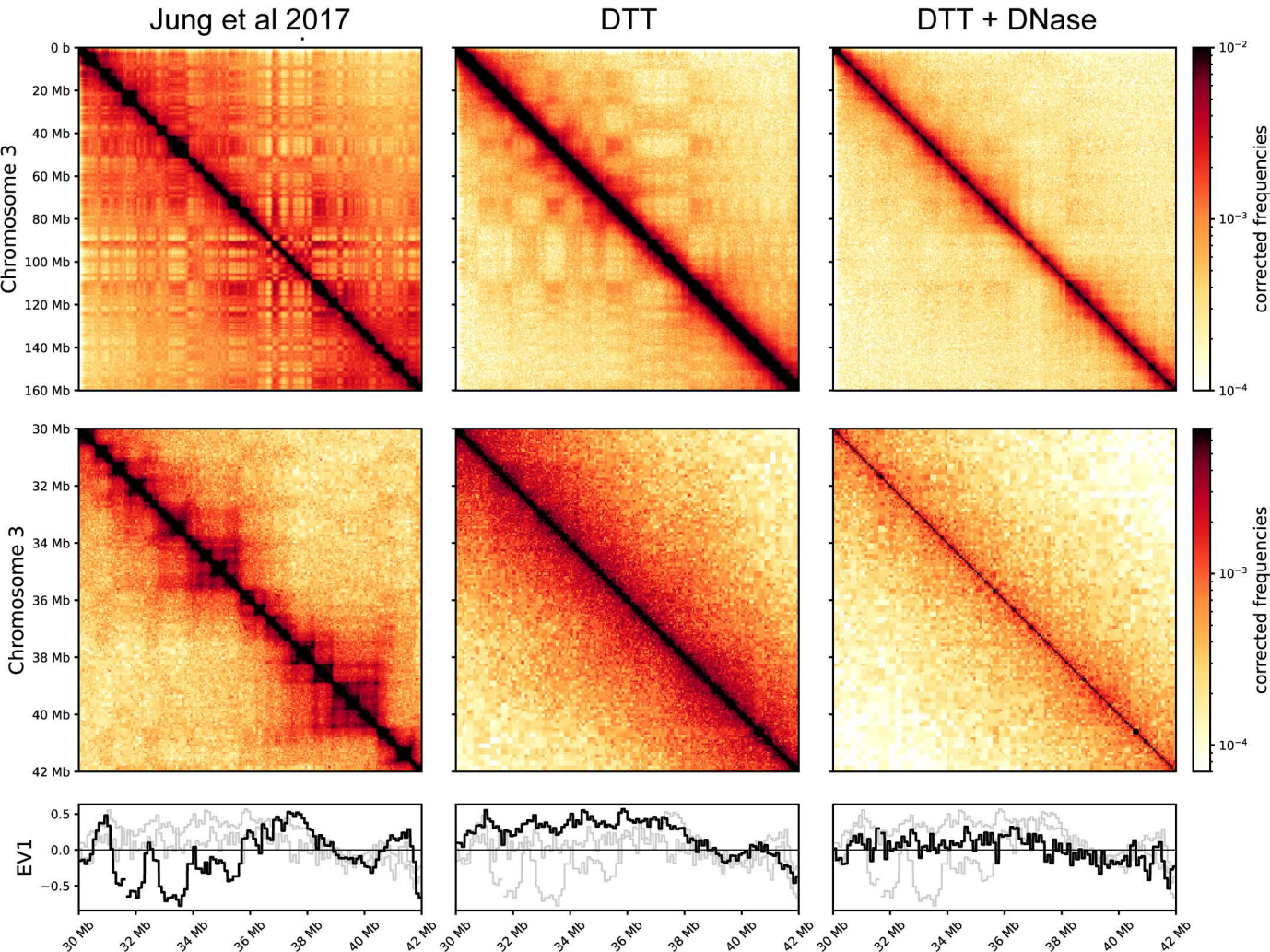
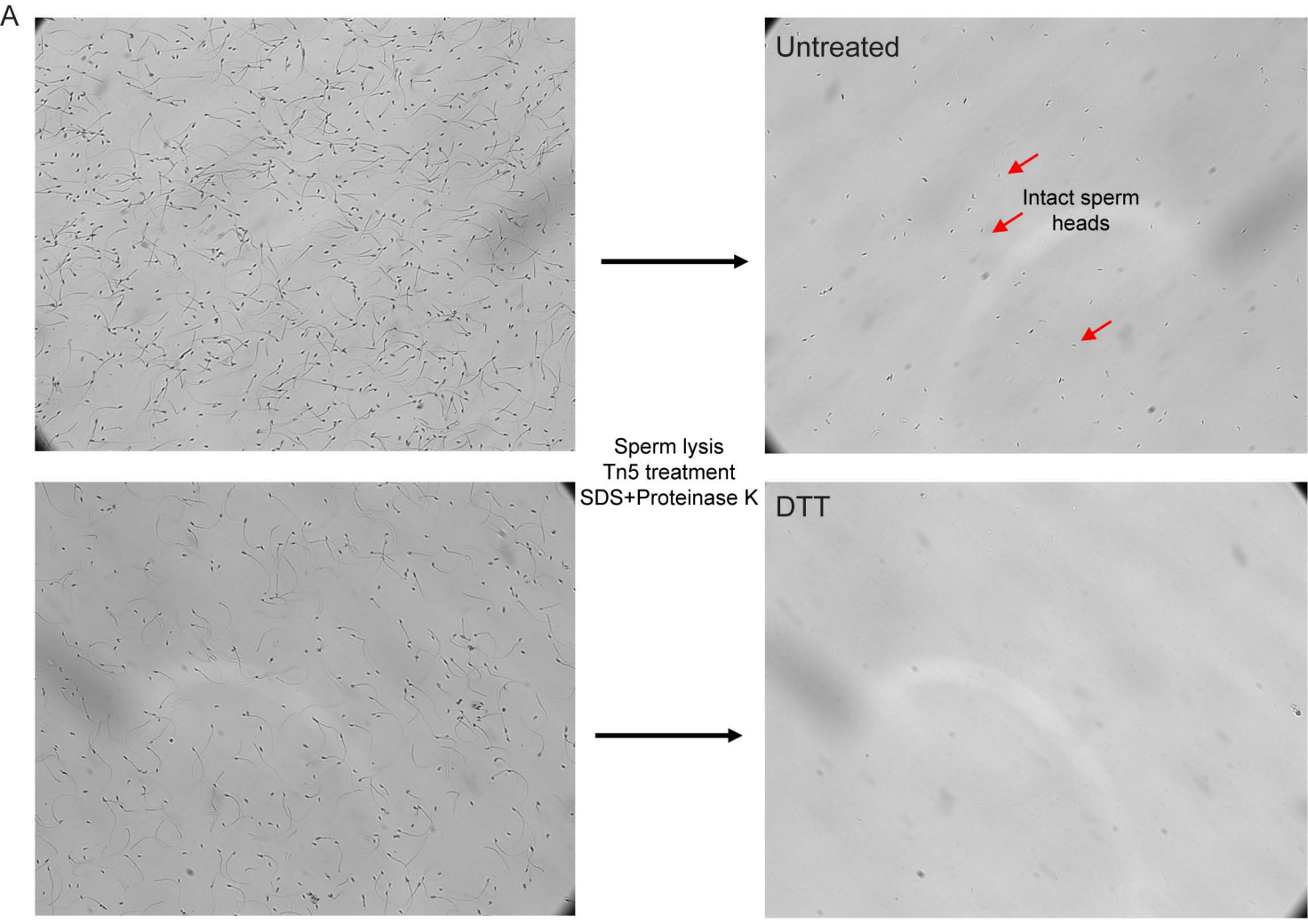
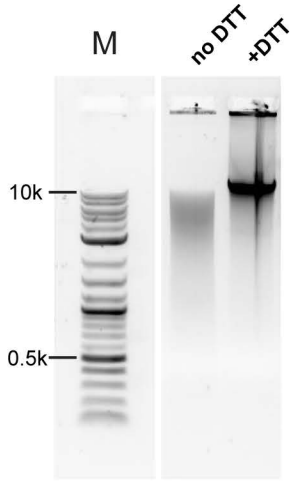

Jung et al 2017
DTT
DTT + DNase
Chromosome 3
0 b
20 Mb
40 Mb
60 Mb
80 Mb
100 Mb
120 Mb
140 Mb
160 Mb
corrected frequencies
10^{-2}
10^{-3}
10^{-4}
Chromosome 3
30 Mb
32 Mb
34 Mb
36 Mb
38 Mb
40 Mb
42 Mb
corrected frequencies
10^{-3}
10^{-4}
EV1
0.5
0.0
−0.5
30 Mb
32 Mb
34 Mb
36 Mb
38 Mb
40 Mb
42 Mb

Figure S1

A



B



C

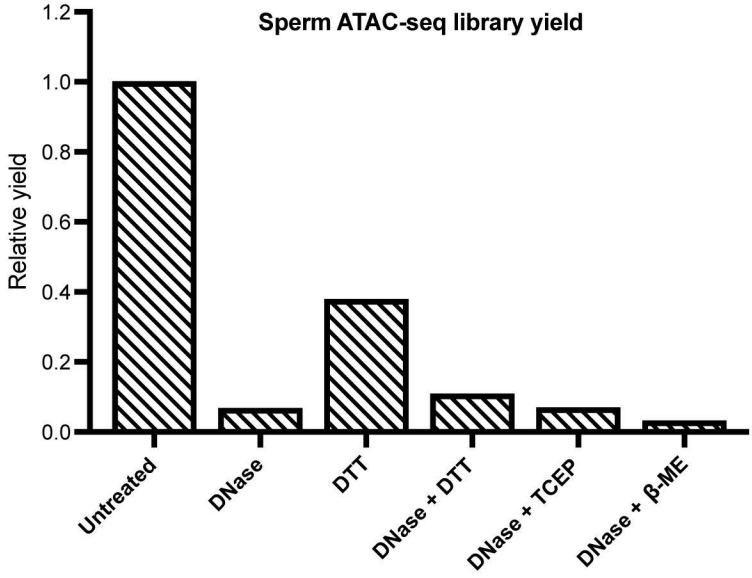


Figure S2

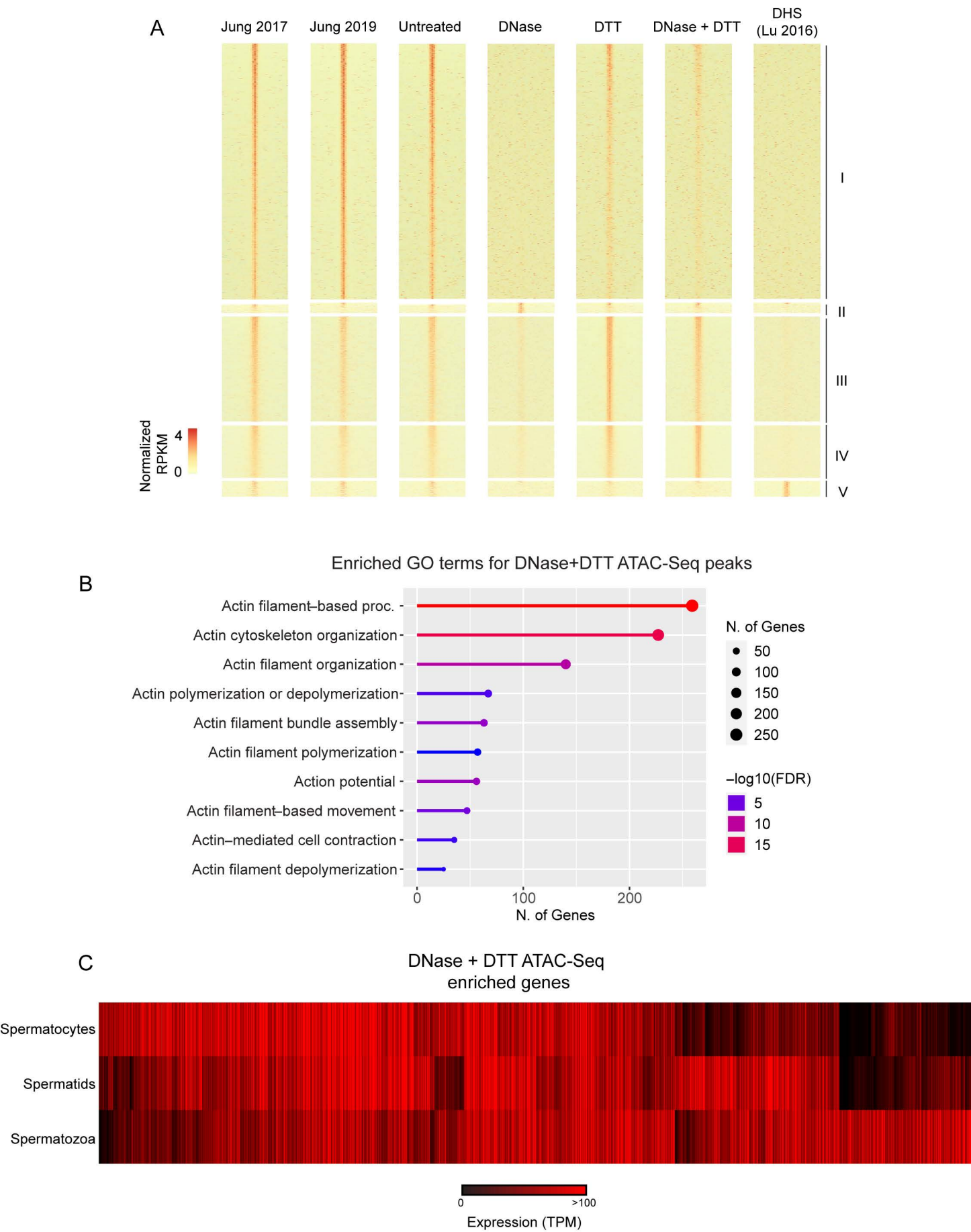

A
Jung 2017
Jung 2019
Untreated
DNase
DTT
DNase + DTT
DHS (Lu 2016)
I
II
III
IV
V
Normalized RPKM
4
0
B
Enriched GO terms for DNase+DTT ATAC-Seq peaks
Actin filament–based proc.
Actin cytoskeleton organization
Actin filament organization
Actin polymerization or depolymerization
Actin filament bundle assembly
Actin filament polymerization
Action potential
Actin filament–based movement
Actin–mediated cell contraction
Actin filament depolymerization
0
100
200
N. of Genes
N. of Genes
50
100
150
200
250
–log10(FDR)
5
10
15
C
DNase + DTT ATAC-Seq enriched genes
Spermatocytes
Spermatids
Spermatozoa
0
>100
Expression (TPM)

Figure S3

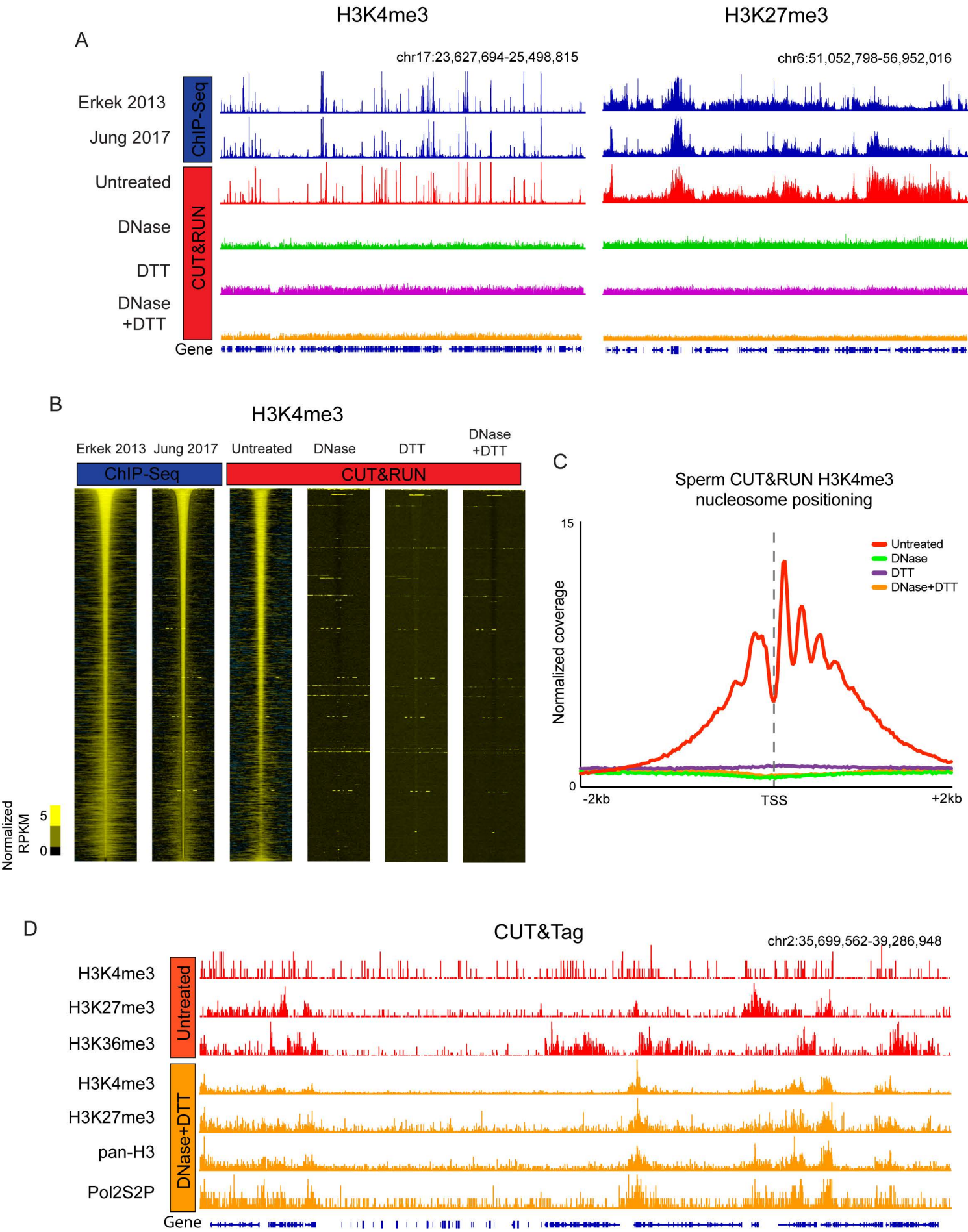
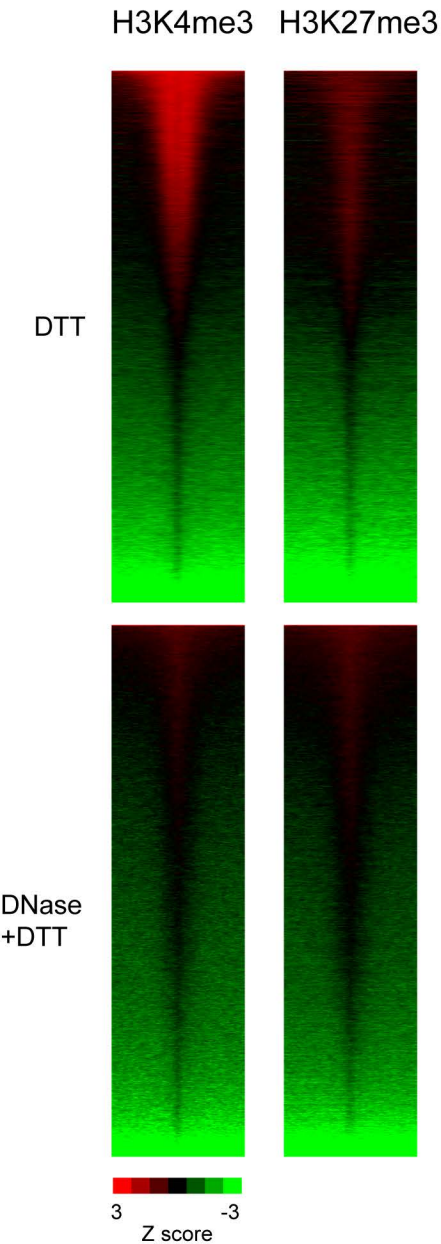
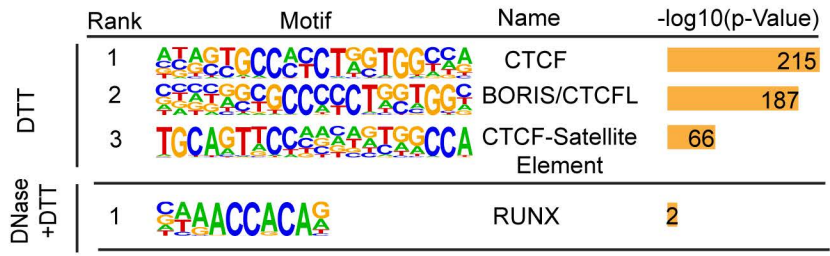

A
H3K4me3
H3K27me3
chr17:23,627,694-25,498,815
chr6:51,052,798-56,952,016
Erkek 2013
Jung 2017
ChIP-Seq
Untreated
DNase
DTT
DNase +DTT
CUT&RUN
Gene
B
H3K4me3
Erkek 2013
Jung 2017
Untreated
DNase
DTT
DNase +DTT
ChIP-Seq
CUT&RUN
Normalized RPKM
5
0
C
Sperm CUT&RUN H3K4me3 nucleosome positioning
Normalized coverage
15
0
Untreated
DNase
DTT
DNase+DTT
-2kb
TSS
+2kb
D
CUT&Tag
chr2:35,699,562-39,286,948
H3K4me3
H3K27me3
H3K36me3
Untreated
H3K4me3
H3K27me3
pan-H3
Pol2S2P
DNase+DTT
Gene

Figure S4

A



B



C

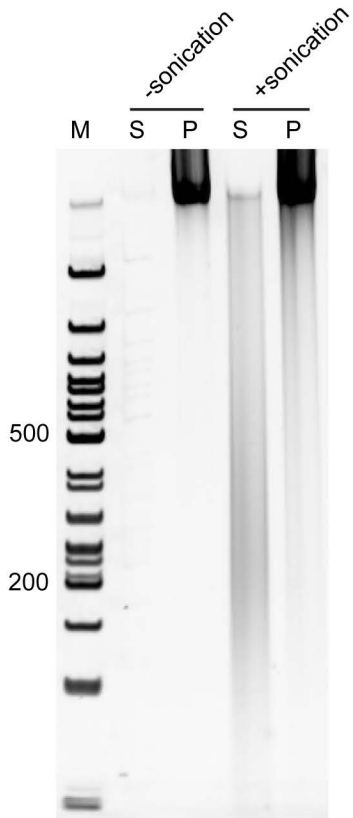
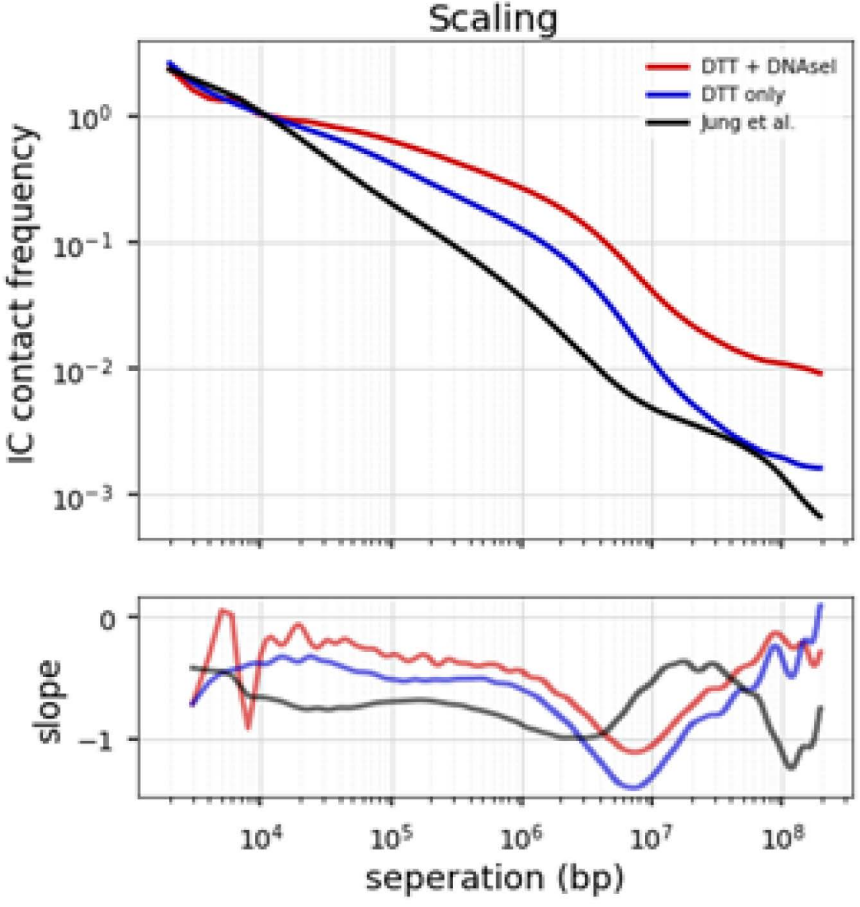


Figure S5


Scaling
DTT + DNAseI
DTT only
Jung et al.
IC contact frequency
10^0
10^{-1}
10^{-2}
10^{-3}
slope
0
−1
10^4
10^5
10^6
10^7
10^8
seperation (bp)