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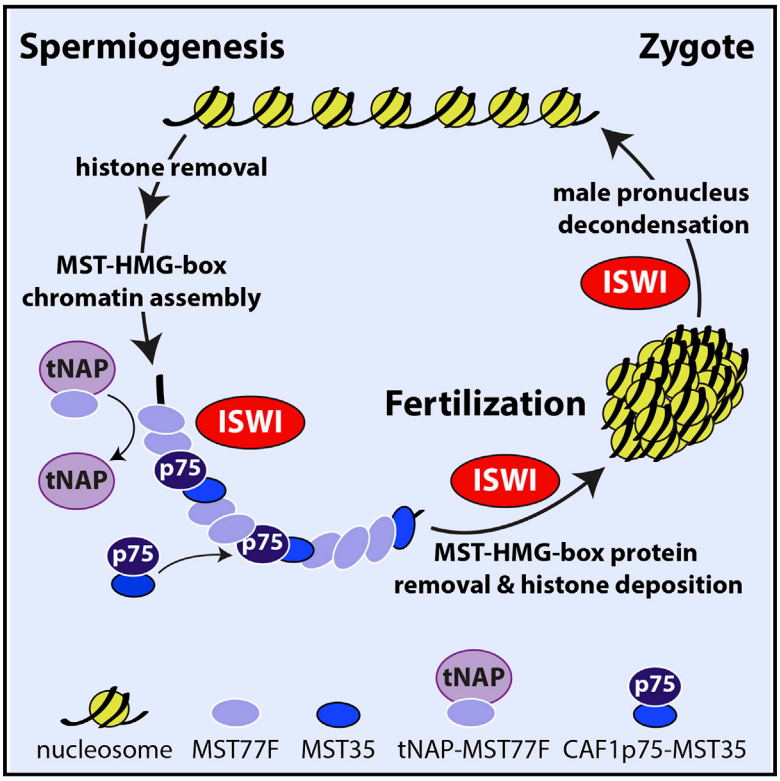
Report

A Testis-Specific Chaperone and the Chromatin Remodeler ISWI Mediate Repackaging of the Paternal Genome

Graphical Abstract



Highlights

- *Drosophila* sperm chromatin is based on MST-HMG-box proteins
- tNAP is a testis-specific chaperone of the MST-HMG-box protein MST77F
- ISWI mediates the assembly of MST-HMG-box chromatin
- ISWI is required for de-condensation of the paternal chromatin at fertilization

Authors

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In Brief

The packaging of the male genome in sperm cells is fundamentally different from the nucleosome-based organization in somatic cells. Doyen et al. found that *Drosophila* sperm chromatin formation involves a testis-specific NAP chaperone and the remodeler ISWI. Moreover, ISWI is required for de-condensation of the paternal chromatin at fertilization.

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Report

A Testis-Specific Chaperone and the Chromatin Remodeler ISWI Mediate Repackaging of the Paternal Genome

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SUMMARY

During spermatogenesis, the paternal genome is repackaged into a non-nucleosomal, highly compacted chromatin structure. Bioinformatic analysis revealed that *Drosophila* sperm chromatin proteins are characterized by a motif related to the high-mobility group (HMG) box, which we termed male-specific transcript (MST)-HMG box. MST77F is a MST-HMG-box protein that forms an essential component of sperm chromatin. The deposition of MST77F onto the paternal genome requires the chaperone function of tNAP, a testis-specific NAP protein. MST77F, in turn, enables the stable incorporation of MST35Ba and MST35Bb into sperm chromatin. Following MST-HMG-box protein deposition, the ATP-dependent chromatin remodeler ISWI mediates the appropriate organization of sperm chromatin. Conversely, at fertilization, maternal ISWI targets the paternal genome and drives its repackaging into de-condensed nucleosomal chromatin. Failure of this transition in ISWI mutant embryos is followed by mitotic defects, aneuploidy, and haploid embryonic divisions. Thus, ISWI enables bi-directional transitions between two fundamentally different forms of chromatin.

INTRODUCTION

Gametes are uniquely specialized cells dedicated to the transfer of genetic information from one generation to the next. Gametogenesis involves dramatic changes in virtually all cellular structures, including the packaging of the genome (Fuller, 1998; Lesch and Page, 2012; Rathke et al., 2014). The DNA in most eukaryotic cells is assembled onto nucleosomes, comprising a histone octamer core with ~147 bp DNA wrapped ~1.7 turns around it (Luger et al., 1997). During spermatogenesis, the nucleosomal packaging of the paternal genome is typically replaced by a non-nucleosomal structure based on sperm-specific chromosomal proteins (Balhorn, 2007; Rathke et al., 2014; Sassone-Corsi, 2002). This genome-scale repackaging yields a highly compacted, closed chromatin structure that is not permissive to transcription or DNA replication. In *Drosophila*, this process allows a striking 200-fold compression of the *Drosophila* sperm nuclear volume compared to that of a typical somatic cell (Fuller, 1998; Tokuyasu, 1974). Genomic compaction is believed to provide hydrodynamic advantages to the sperm and to protect the structural integrity of the paternal genome. In *Drosophila*, a set of male-specific transcripts (*Mst*) encodes sperm chromatin proteins that organize the paternal genome into a densely compacted architecture (Andrews et al., 2000; Russell and Kaiser, 1993; Rathke et al., 2014). MST77 is an integral component of mature sperm chromatin that is essential for nuclear shaping and male fertility (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2010). *Mst35Ba* and *Mst35Bb* encode two closely related sperm chromatin proteins (MST35Ba/Bb) that help to protect the paternal genome from DNA damage but are not essential for male fertility (Rathke et al., 2010). Thus, although less studied, MST77F plays a more crucial role during spermatogenesis than MST35Ba/Bb. However, the molecular machinery that controls its incorporation into sperm chromatin remains unknown.

At fertilization, the *Drosophila* male and female gametes form a single cell containing two separate nuclei, referred to as pronuclei. In order to become replication-competent and participate in the formation of zygotic nuclei, sperm chromatin proteins must be removed from the paternal DNA and replaced by maternally provided histones (Callaini and Riparbelli, 1996; Poccia and Collas, 1996). Following histone deposition, the male pronucleus continues to de-condense. Concurrently, the male and female pronuclei migrate toward each other and appose but do not fuse to form a single zygotic nucleus. Instead, both pronuclei replicate their DNA and then enter the first embryonic mitosis as two separate sets of chromosomes (Callaini and Riparbelli, 1996). The paternal and maternal genomes will only merge at telophase and then form two zygotic daughter nuclei, thereby completing the cycle of germline chromatin reprogramming.

Histones are deposited onto the DNA by a variety of chaperones that prevent their nonproductive aggregation (Eitoku

et al., 2008). However, the function of histone chaperones is not restricted to just histone deposition. Chaperones, such as the histone H2A/H2B chaperone NAP1, participate in a wide range of chromatin transactions, which include the control of histone modifications and sister chromatid resolution (Eitoku et al., 2008; Moshkin et al., 2009, 2013). The molecular mechanisms that drive the dramatic genomic chromatin reprogramming in the male germline and early embryo remain poorly defined. Recently, we established that the p75 subunit of the replication-coupled nucleosome assembly factor CAF1 is an essential loading factor for MST35Ba/Bb and is itself a component of mature sperm chromatin (Doyen et al., 2013). In fertilized oocytes, the histone chaperone HIRA and the chromatin remodeler CHD1 play crucial roles in the deposition of the histone variant H3.3 onto the paternal genome (Konev et al., 2007; Loppin et al., 2005). The proper assembly of H3.3 is essential for the subsequent participation of the paternal genome in zygotic mitosis (Konev et al., 2007). However, neither CHD1 nor HIRA is required for the removal of sperm chromatin proteins.

Below, we describe the male-specific transcript high-mobility group box (MST-HMG box), a protein motif that characterizes *Drosophila* sperm chromatin proteins. We identified a testis-specific paralog of NAP1 as a chaperone required for the formation of MST-HMG-box-based sperm chromatin. Our results suggest that the ATP-dependent chromatin remodeler ISWI catalyzes key steps in the genome-wide repackaging of the paternal genome during spermatogenesis and at fertilization.

RESULTS

Identification of the MST-HMG-Box in *Drosophila* Sperm Chromatin Proteins

Given its fundamental role in sperm chromatin formation, we decided to study the molecular mechanism of MST77F deposition onto the paternal genome. An interactive search for MST77F homologs in 12 sequenced *Drosophila* species with the *jackhammer* program (Eddy, 2011) revealed an amino acid (aa) motif resembling a truncated form of the canonical HMG box (pfam: PF00505), which we named MST-HMG box. Using the aligned *Drosophila* sequences, we build an initial hidden Markov model (HMM) for the HMG-Mst box. Next, we used this HMM to search metazoan genomes for the presence of an MST-HMG box with *hmmsearch* (Eddy, 2011). We identified 132 MST-HMG-box motifs in 25 species, all belonging to the *Neoptera* taxon. Based on this set of MST-HMG-box motifs, we build the final HMM (Figure 1A). In *Drosophila melanogaster*, we identified eight different MST-HMG-box-encoding genes (along with 11 pseudogenes), which include the known sperm chromatin components MST77F, MST35Ba, MST35Bb, and the transition protein TPL94D (Rathke et al., 2007). It is tempting to speculate that the remaining 4 uncharacterized MST-HMG-box proteins (MST33A, CG15510, CG30356 and CG42355) might also form part of sperm chromatin.

MST77F has been compared to the mammalian spermatid-specific histone H1-like protein HILS1, while MST35Ba and MST35Bb are commonly referred to as homologs of mammalian protamines 1 and 2 (Rathke et al., 2014). Protamines are fast-evolving, testes-specific, arginine-rich proteins that organize the paternal genome into sperm chromatin (Balhorn, 2007; Figure S1A). However, *Hmmsearch* failed to identify any protein sequences with similarity to HILS1 or protamines in *Drosophila*. Direct alignments of either MST77F with histone H1 or HILS1, or MST35Ba/Bb with protamines, failed to uncover any significant sequence conservation. Likewise, there were no protamine homologs among newly discovered testes-specific genes in the most recent *D. melanogaster* annotation (Brown et al., 2014). Thus, there is no evidence for the presence of protamine homologs in *Drosophila*. Instead, our analysis suggests that MST-HMG-box proteins form the basis of *Drosophila* sperm chromatin. Phylogenetic analysis revealed that the MST-HMG-box proteins emerged in the *Neoptera* taxon but is absent from mammals (Figure S1B). MST-HMG-box proteins are typically very basic, but, unlike protamines, they tend to be rich in lysine rather than arginine. In summary, the MST-HMG box characterizes a set of proteins that play a central role in the formation of *Drosophila* sperm chromatin. Among these, MST77F is of particular interest because it is essential for male fertility. However, the biochemical pathway that mediates its incorporation into sperm chromatin remained unknown.

tNAP Binds the Essential Sperm Chromatin Component MST77F

To identify potential chaperones for MST77F, we immunopurified MST77F from extracts prepared from dissected testes. Following extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, associated proteins were identified by mass spectrometry. In addition to MST77F itself and the closely related Mst77Y, encoded on the Y chromosome, we identified a testis-specific member of the NAP protein family, which we named tNAP (Figures 1B and S1C). Immunoprecipitation of tNAP followed by mass spectrometry confirmed the association between tNAP and MST77F. Although NAP1 and tNAP are structurally related, NAP1 was absent from both tNAP and MST77F IPs, emphasizing the specificity of the interaction (and of our antibodies). MST35Ba/Bb and their chaperone CAF1-p75 did not bind to tNAP or MST77F. Immunoblotting of immunopurified MST77F and tNAP confirmed their association (Figure 1C). Incubation of testes extract with recombinant glutathione S-transferase (GST)-MST77F also revealed the selective binding of tNAP, but not NAP1 (Figure 1D). Interestingly, previous genetic studies established that tNAP (also known as Hanabi) is essential for *Drosophila* spermiogenesis (Kimura, 2013). However, its molecular mode of action remained unclear. Our biochemical results now suggest that tNAP may play a role in the MST77F-dependent assembly of sperm chromatin.

Analysis of RNA-sequencing data (Brown et al., 2014) revealed the highly tissue-restricted expression of *tNap* compared to *Nap1*, which is broadly expressed throughout all tissues and developmental stages (Figure 1E). *tNap* mRNA was mainly present in the testes and male accessory glands but was also detected in third-instar larval (L3) imaginal discs and white prepupae (WPP) fat body. The expression pattern of the eight MST-HMG-box-containing genes and *tHMG1* and *tHMG2* is similar to that of tNAP and is mainly restricted to the testes. qRT-PCR analysis and immunoblotting showed that tNAP is expressed in adult males, but not in females or embryos (Figure 1F).

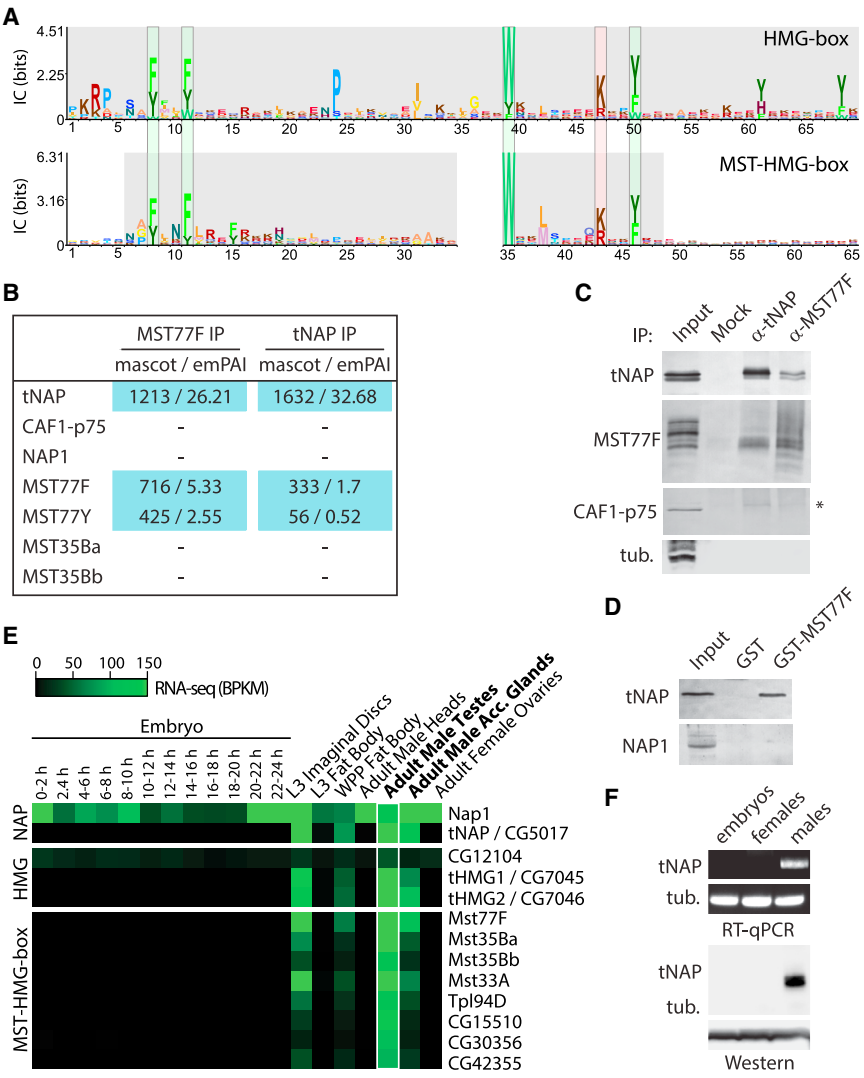

Figure 1. tNAP Binds the *Drosophila* MST-HMG-Box Sperm Chromatin Protein MST77F

(A) An iterative search with *jackhmmer* revealed the MST-HMG box, resembling a truncated form of the canonical HMG box (pfam: PF00505) and DUF1074 (pfam: PF06382), in *Drosophila* sperm chromatin proteins. The height of each amino acid in the motif logo corresponds to information content (IC). See also Figures S1A and S1B.

(B) MST77F and tNAP were immunopurified from *Drosophila* testes extract and analyzed by mass spectrometry. Mascot and emPAI scores are indicated.

(C) Co-immunoprecipitations (coIPs) from testes extracts with either pre-immune serum (mock) or antibodies against tNAP or MST77F. Bound proteins were resolved by SDS-PAGE and detected by immunoblotting. A total of 10% of input is loaded. *, a background band.

(D) Binding of tNAP and NAP1 to either GST or GST-tagged MST77F analyzed by immunoblotting.

(E) Heatmap of RNA-seq expression profiles of mRNAs encoding NAP1, tNAP, HMG-box transition proteins and MST-HMG-box proteins, expressed as bases per kilobase of gene model per million mapped (BPKM). Gene expression data from Brown et al. (2014).

(F) tNAP is a male-specific protein. Top: qRT-PCR analysis of *tNap* in mRNA isolated from embryos, adult females, or males. Bottom: western blotting analysis. Tubulin serves as a loading control.

See also Figure S1.

We conclude that tNAP, a testis-specific paralog of the somatic histone chaperone NAP1, selectively binds MST77F.

tNAP Is a MST77F Chaperone that Is Required for Sperm Chromatin Assembly

Visualization of tNAP in adult testes by immunofluorescence (IF) revealed a broad distribution during spermatid development, with a strong staining of the flagella (Figure 2A). To deplete tNAP during spermatogenesis, we used the C135 GAL4 enhancer trap line to drive expression of the GAL4 transcriptional activator in the male reproductive tract (Brand and Perrimon, 1993; Hrdlicka et al., 2002). GAL4, in turn, activates a upstream activating sequence (UAS)-controlled transgene expressing double-stranded RNA (dsRNA) directed against *tNAP* mRNA (*C135 > tNAP^{RNAi}*). Indeed, in *C135 > tNAP^{RNAi}* testes, tNAP is no longer detectable, confirming the specificity of our antibodies (Figures 2A and S2A). As noted by Kimura (2013), NAP1 has a strikingly different distribution and localizes to the apical tip of sperm cells, separate from chromatin (Figure 2B).

To compare their functions in spermatogenesis, we used RNAi-mediated depletion of tNAP and MST77F. DAPI staining of DNA revealed that loss of either tNAP or MST77F leads to strikingly similar defects in spermatogenesis, which include the loss of sperm clustering, malformed nuclei, and the absence of mature sperm (Figure 2C). These observations support the notion that the physical interaction between tNAP and MST77F is functionally relevant. To explore this possibility further, we monitored the transition from nucleosomal to sperm chromatin using antibodies raised against core histones, MST77F and MST35Ba/Bb (Doyen et al., 2013). During post-meiotic spermiogenesis, the sperm nucleus undergoes extensive reshaping, transforming from a round shape to a needle shape with a dramatic reduction of nuclear volume. Histones are removed from the paternal genome during the early canoe stage and then replaced by MST77 and MST35Ba/Bb at late canoe (Figures 2D–2F and S2B). In spite of the malformed nuclei, removal of the core histones at early canoe appeared normal in both *C135 > tNAP^{RNAi}* and *C135 > MST77F^{RNAi}* males. Previously, tNAP was proposed to be involved in histone removal, based on defective eviction of ectopically expressed fluorescently labeled H2Av in *tNap/Hanabi* mutant males (Kimura, 2013). The discrepancy between these observations and our

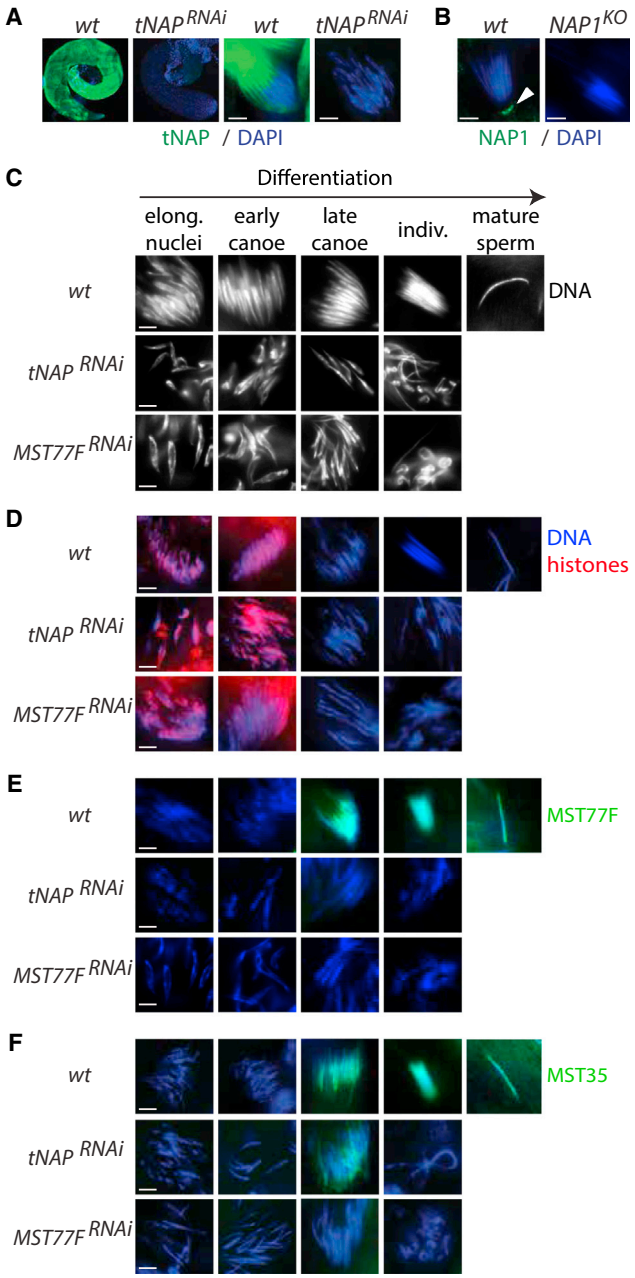

Figure 2. tNAP Is Required for the Formation of MST-HMG-Box-Protein-Based Chromatin during Spermiogenesis

(A) IF of tNAP in testis of WT or *C135* > *tNAP*^{RNAi} males. DNA is visualized by DAPI staining. See also Figure S2A.

(B) IF of NAP1 (arrowhead) localized at the apical tip of sperm cells in WT males. NAP1 is not detectable in *NAP1*^{KO1} males.

(C) Depletion of tNAP (*tNAP*^{RNAi}) or MST77F (*MST77F*^{RNAi}) leads to malformed sperm nuclei and loss of sperm clustering. Post-meiotic stages shown: elongating (elong.) nuclei, early canoe, late canoe, individualization (indiv.), and mature sperm. In *C135* > *tNAP*^{RNAi} and *C135* > *MST77F*^{RNAi} males spermatid maturation is disrupted and no mature sperm was observed. DNA is visualized by DAPI staining. Scale bars represent 5 μm.

(D) Histone removal at early canoe appears normal following depletion of tNAP or MST77F, as visualized by IF. See also Figure S2B.

(E) MST77F deposition requires tNAP.

(F) MST35Ba/Bb incorporation is abrogated upon depletion of either tNAP or MST77F.

See also Figure S2.

results might be due to differences between H2Av and the canonical core histones or the result of differences in experimental setup. Based on our monitoring of endogenous histones, we suggest that tNAP is not required for core histone removal. However, in the absence of tNAP, MST77F is no longer loaded onto the paternal DNA (Figure 2E). MST77F, in turn, is required for the stable incorporation of MST35Ba and MST35Bb into sperm chromatin (Figure 2F). These results suggest that MST77F is a key component in the hierarchical assembly of sperm chromatin.

In summary, tNAP binds Mst77F and mediates its deposition onto the paternal genome but is itself not part of sperm chromatin. Loss of either MST77F or tNAP leads to strikingly similar defects in sperm chromatin assembly, nuclear shaping, and sperm maturation. Thus, whereas NAP is a histone chaperone in somatic cells, tNAP is a MST77F chaperone during spermatogenesis.

ISWI Acts Outside Its Somatic Assemblages in the Male Germline and Is Required for Sperm Chromatin Assembly

Nucleosomal chromatin assembly or reorganization involves the joined action of histone chaperones and ATP-dependent chromatin remodelers (Ito et al., 1997; Moshkin et al., 2002). Thus far, no remodeler has been implicated in the assembly of non-nucleosomal sperm chromatin. To explore their potential involvement, we monitored the binding of different remodelers to the paternal genome during spermatogenesis (Figure S3A). We found that ISWI coats the paternal genome after histone removal, precisely during the deposition of MST77F and MST35Ba/Bb at late canoe (Figure 3A). Once assemblage of MST-HMG-box proteins onto the DNA is completed, ISWI dissociates. As ISWI covers the paternal genome concurrently with the deposition of sperm chromatin proteins, we analyzed spermatogenesis in *Iswi*^{1} mutant males or following RNAi-directed depletion of ISWI in the reproductive tract. The *Iswi*^{1} mutation creates a stop codon leading to a predicted protein product lacking the C-terminal 78 amino acids (Deuring et al., 2000). Although the viability of *Iswi*^{1} homozygous animals is impaired, we obtained adults, establishing it as a hypomorphic allele. IF revealed substantially reduced levels of ISWI protein in homozygous *Iswi*^{1} males, confirming these animals are hypomorphs. In spite of its low abundance, the chromatin dynamics of ISWI appeared normal in *Iswi*^{1} males. In *C135* > *ISWI*^{RNAi} males, however, there was no detectable ISWI and spermatid maturation was severely disrupted, suggesting that ISWI is essential for spermatogenesis (Figure 3A).

We compared ISWI protein in extracts prepared from either 0- to 12-hr-old embryos or testes dissected from *wild-type* (*wt*) males. Immunoblotting revealed a clear difference in the migration of embryonic and testicular ISWI (Figure 3B). ISWI isolated from embryos migrates at its expected size of ~130 kDa. In testis extract, however, anti-ISWI antibodies detected a doublet that migrates slightly slower than embryonic ISWI and a band

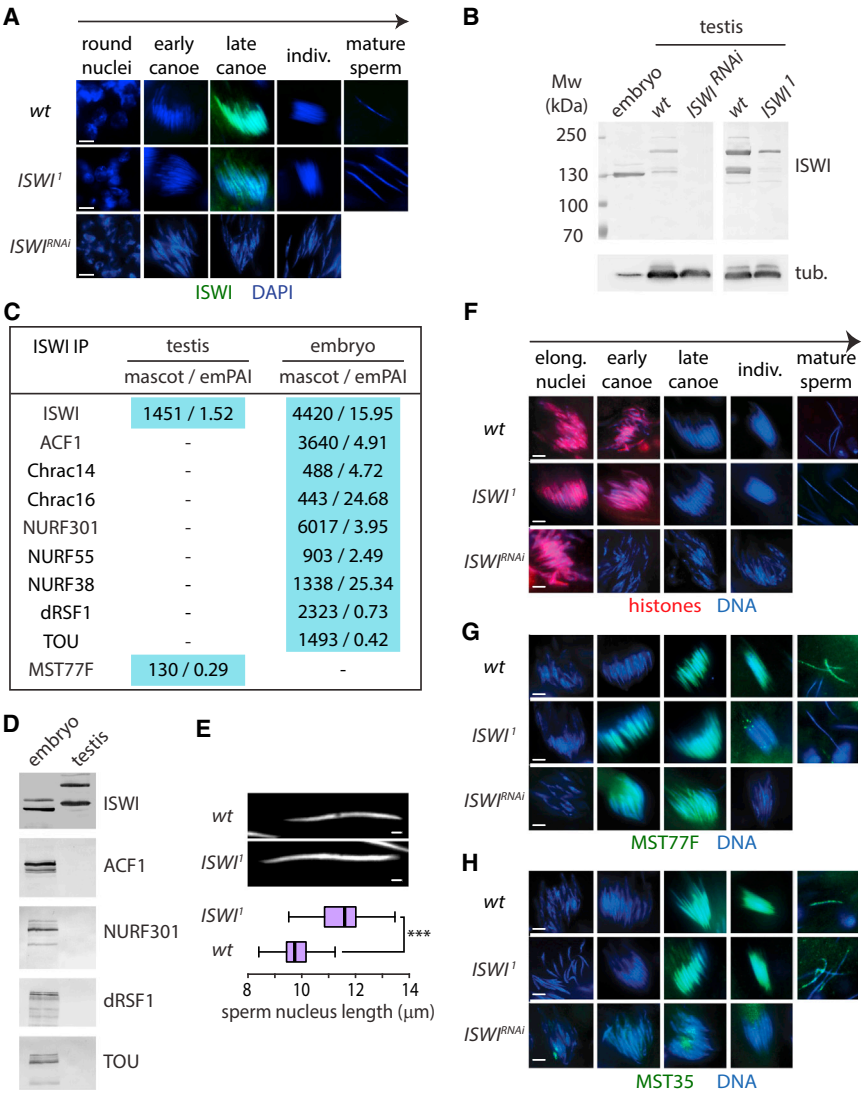

Figure 3. ISWI Is Required for Sperm Chromatin Formation

(A) Chromatin dynamics of ISWI during spermatogenesis in WT, homozygous *Iswi*^{1} and *C135* > *ISWI*^{RNAi} males, visualized by IF. Scale bars represent 5 μm.

(B) Immunoblotting of ISWI in embryo- or testes extracts.

(C) Mass spectrometric analysis of ISWI and associated proteins immunopurified from either testes- or embryo extracts.

(D) Immunoblotting analysis of ISWI and partner proteins in embryo and testes extracts. See also Figure S3E.

(E) Median boxplot depicts the length of mature sperm nuclei, visualized by DAPI staining, of WT males (n = 95) and homozygous *Iswi*^{1} males (n = 168). Boxes span from lower (first) to upper (third) quartile and whiskers show 5% to 95% range. *ISWI*^{1} sperm nuclei are on average ~2 μm longer than those in WT animals. ***, p ≈ 0, derived from Student's t test. Scale bar represents 1 μm.

(F–H) Histone removal (F) and the deposition of MST77F (G) and MST35 (H) in WT, *C135* > *ISWI*^{RNAi}, or homozygous *Iswi*^{1} males was monitored by IF. Scale bars represent 5 μm. See also Figures S3F–S3I.

See also Figure S3.

migrating at ~180 kDa. This suggests that ISWI in testes might be encoded by alternative transcripts or subjected to extensive post-translational processing. Analysis of *Iswi* mRNA transcripts in the testes did not reveal alternative splicing or differential promoter utilization (Figure S3B). Thus, the divergent gel mobility of ISWI from testes is most likely due to post-translational modifications. We note that the migration of most chromatin factors we analyzed in testis extracts differs from that of their somatic counterparts (Doyen et al., 2013; unpublished data). In testis extracts from *C135* > *ISWI*^{RNAi} males, ISWI was no longer detectable by immunoblotting. This observation agrees well with our IF results and demonstrates the specificity of our antibodies. Similar to our IF results, immunoblotting of testes extracts from homozygous *Iswi*^{1} males revealed substantially lower levels of all ISWI protein isoforms. To identify potential associated proteins, we used mass spectrometry to analyze ISWI immunopurified from either testes- or embryo extracts (Figure 3C). Notably, MST77F was present in the ISWI immunoprecipitated from testes, suggesting a functional connection. None of its known partners in somatic cells were present in ISWI purified from testes extract, although they were readily identified in IPs of embryonic ISWI. We note that the ISWI peptide identification agreed with the mRNA analysis and is consistent with the absence of alternative forms of ISWI in testes (Figure S3C). Immunoblotting and mRNA expression analysis revealed that ISWI is robustly expressed in the testes, whereas its somatic partners ACF1, NURF301, dRSF1, and TOU were either undetectable or present at negligibly low levels (Figures 3D and S3D). As expected, immunoblotting confirmed the binding of embryonic ISWI to its known partner proteins (Figure S3E). These results suggest that in testes ISWI acts alone, outside of its normal assemblages. Likewise, in the male germline, the CAF1 subunits function separate from the canonical CAF1 complex (Doyen et al., 2013).

Next, we tested the effect of ISWI depletion on male fertility and spermatogenesis. Mating with WT females revealed that *C135* > *ISWI*^{RNAi} males are completely sterile and that the fecundity of *Iswi*^{1} homozygous males is reduced significantly (Figure S3F). We did not detect mature sperm in *C135* > *ISWI*^{RNAi} animals, whereas sperm cells of homozygous *Iswi*^{1} males did not fully compact their chromatin and have enlarged nuclei (Figure 3E). Histone removal was normal in *Iswi*^{1} males but occurred prematurely in the majority of spermatids in *C135* > *ISWI*^{RNAi} males (Figure 3F). We note, however, that in *C135* > *ISWI*^{RNAi}

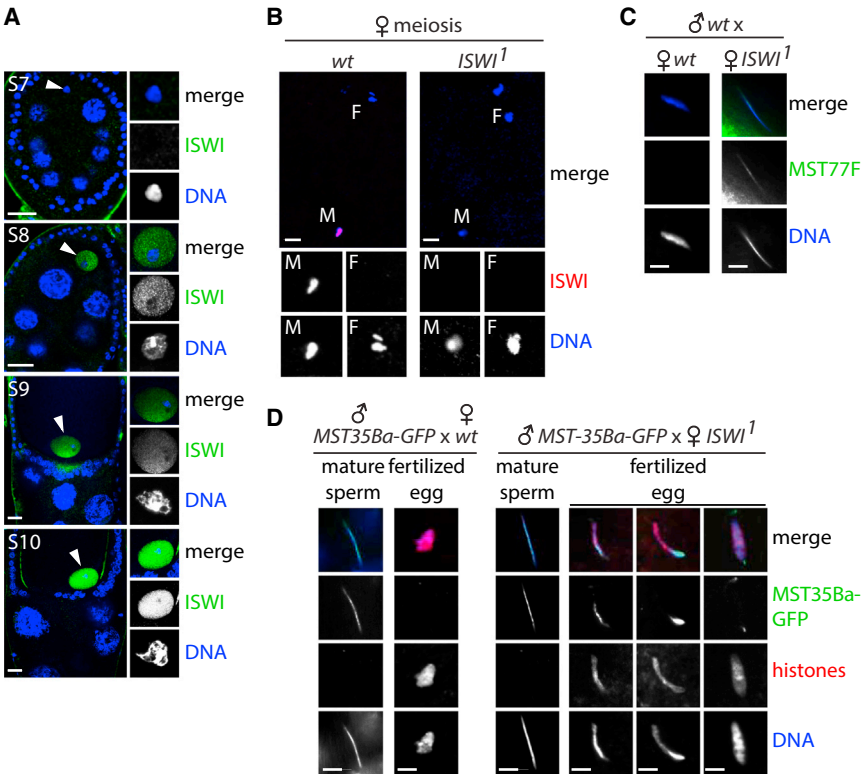

Figure 4. ISWI Promotes Rapid Repackaging of the Paternal Genome at Fertilization

(A) Selective deposition of maternal ISWI into the oocyte nucleus (arrowhead) during egg chamber formation. ISWI was monitored by IF in ovarioles. Stages of egg chamber formation are indicated (S7–S10). Scale bar represents 20 μm.

(B) IF of ISWI in WT fertilized eggs or in fertilized eggs from homozygous *Iswi*^{1} females crossed with WT males. ISWI selectively binds the male pronucleus (M) early after sperm entry at female meiosis. In fertilized eggs from homozygous *Iswi*^{1} females, ISWI is not detectable. The polar body (PM) is indicated. Scale bar represents 10 μm.

(C) MST77F retention on the male pronucleus in the absence of ISWI. MST77F coating the paternal DNA was only detected in embryos from *Iswi*^{1} females. Embryos were collected after 20 min of egg-laying.

(D) Males expressing MST35Ba-GFP were crossed with either WT or homozygous *Iswi*^{1} females. Embryos were analyzed by IF to monitor the presence of MST35Ba-GFP and histones. In embryos from WT females, we did not detect MST35Ba-GFP on the paternal genome, reflecting its rapid removal. In a substantial portion of embryos from homozygous *Iswi*^{1} females, MST35Ba-GFP removal is delayed.

See also Figure S4.

males, the distinction between different stages of spermiogenesis is somewhat blurred. In both *Iswi*^{1}- and *C135* > *ISWI*^{RNAi} males, MST77F binds sperm DNA prematurely at early canoe (Figure 3G). Pertinently, in both *Iswi*^{1}- and *C135* > *ISWI*^{RNAi} males, MST77F is not stably associated with the paternal genome and is removed by individualization. MST35Ba/Bb deposition appeared normal in *Iswi*^{1} males but was completely disrupted in *C135* > *ISWI*^{RNAi} males (Figure 3F). The failure to stably incorporate MST77F or MST35Ba/Bb in *Iswi*^{1}- or *C135* > *ISWI*^{RNAi} animals was not due to their absence (Figure S3G). As the DNA binding of CAF1-p75 and MST35Ba/Bb is mutually dependent (Doyen et al., 2013), it was not surprising that CAF1-p75 also failed to associate stably with the in sperm chromatin *C135* > *ISWI*^{RNAi} males (Figure S3H). These results suggest that ISWI acts after the removal of histones and deposition of MST77F to mediate the formation of sperm chromatin.

ISWI Enables Repackaging of the Paternal Genome after Fertilization

After the fertilizing sperm nucleus has entered the egg cytoplasm, sperm chromatin proteins must be replaced with maternally provided histones. Given its role in MST-HMG-box protein chromatin assembly, we wondered whether ISWI might also function during its reversal following sperm entry into the oocyte. IF microscopy revealed a striking accumulation of ISWI in the maturing oocyte nucleus (Figure 4A). ISWI fills the whole oocyte nuclear volume rather than binding the maternal chromosomes, which form a highly compacted structure called the karyosome. Mating with WT males revealed that homozygous *Iswi*^{1} females lay eggs but are completely sterile, suggesting a role for ISWI during the early stages of fertilization.

After passage of the egg through the oviduct, the four female nuclei, which were arrested at metaphase of meiosis II, start the final phases of meiosis. One nucleus forms the female pronucleus (F), whereas the remaining three will become the polar body (PB). Upon entry in the oocyte, the sperm nucleus undergoes a process of rapid chromatin de-condensation and transforms into a round male pronucleus (M). IF microscopy revealed that ISWI binds the paternal chromatin very early after sperm entry (Figures 4B, S4A, and S4B). We failed to detect ISWI in embryos from homozygous *Iswi*^{1} females. Although we never observed retention of sperm chromatin in embryos from WT mothers, in a significant portion of embryos from *Iswi*^{1} females, MST77F and MST35Ba remained associated with the paternal DNA (Figures 4C, 4D, and S4C). These results suggest that maternal ISWI promotes the rapid repackaging of the paternal genome after fertilization.

Following histone deposition, the male pronucleus continues to de-condense while the two pronuclei migrate toward each other, appose, and then undergo replication and mitosis. Strikingly, the majority of embryos from homozygous *Iswi*^{1} females displayed a wide range of chromatin and mitotic defects (Figures 5A and S5A). In embryos from *Iswi*^{1} mothers, the male pronucleus remained abnormally condensed (Figure 5B). Nevertheless, core histones were deposited efficiently onto the condensed paternal genome in the absence of ISWI. Even more surprising, acetylated histone H4 (H4ac), a classic mark of open chromatin, was readily detected in

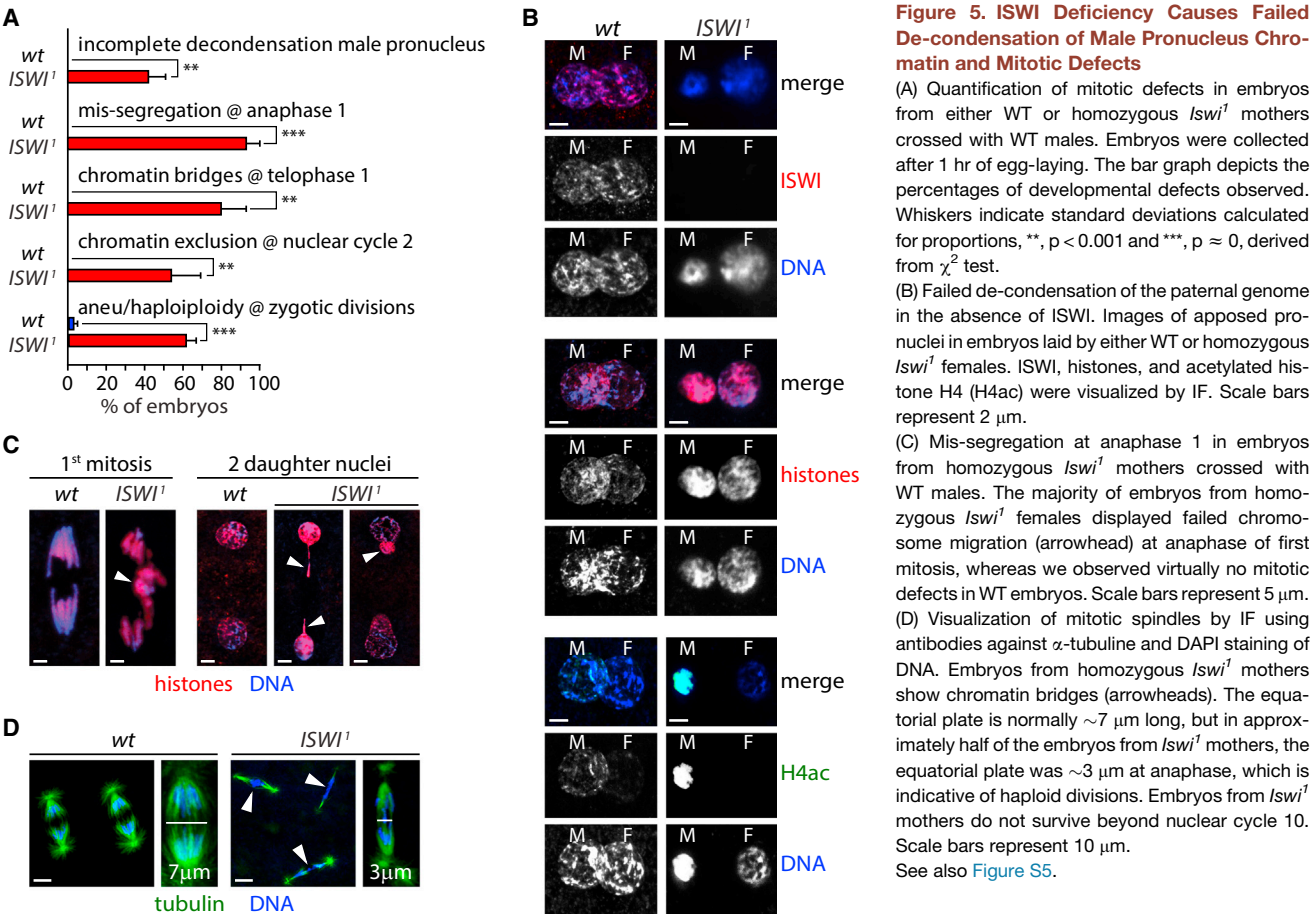

Figure 5. ISWI Deficiency Causes Failed De-condensation of Male Pronucleus Chromatin and Mitotic Defects

(A) Quantification of mitotic defects in embryos from either WT or homozygous *Iswi*^{1} mothers crossed with WT males. Embryos were collected after 1 hr of egg-laying. The bar graph depicts the percentages of developmental defects observed. Whiskers indicate standard deviations calculated for proportions, **, $p < 0.001$ and ***, $p \approx 0$, derived from χ^2 test.

(B) Failed de-condensation of the paternal genome in the absence of ISWI. Images of apposed pronuclei in embryos laid by either WT or homozygous *Iswi*^{1} females. ISWI, histones, and acetylated histone H4 (H4ac) were visualized by IF. Scale bars represent 2 µm.

(C) Mis-segregation at anaphase 1 in embryos from homozygous *Iswi*^{1} mothers crossed with WT males. The majority of embryos from homozygous *Iswi*^{1} females displayed failed chromosome migration (arrowhead) at anaphase of first mitosis, whereas we observed virtually no mitotic defects in WT embryos. Scale bars represent 5 µm.

(D) Visualization of mitotic spindles by IF using antibodies against α-tubuline and DAPI staining of DNA. Embryos from homozygous *Iswi*^{1} mothers show chromatin bridges (arrowheads). The equatorial plate is normally ~7 µm long, but in approximately half of the embryos from *Iswi*^{1} mothers, the equatorial plate was ~3 µm at anaphase, which is indicative of haploid divisions. Embryos from *Iswi*^{1} mothers do not survive beyond nuclear cycle 10. Scale bars represent 10 µm.

See also Figure S5.

the compacted male pronucleus of *Iswi*^{1} embryos. These observations suggest that ISWI is not required for the recruitment of histones to the male pronucleus but is crucial for the formation of an open chromatin structure after histone deposition.

The failed de-condensation of the male pronucleus is followed by a series of mitotic defects. At the end of the first telophase and at the two-nuclei stage, the majority of ISWI-deficient embryos showed chromatin bridges and chromatin exclusion (Figure 5C). In the majority of syncytial blastoderm embryos from homozygous *Iswi*^{1} mothers, we observed lagging of chromatin at the metaphase plate, chromatin bridges, and loss of synchronization of the nuclear cycles (Figure S5B). Although the formation of bipolar mitotic spindles appeared normal, the equatorial plate was shortened in embryos from *Iswi*^{1} mothers (Figure 5D). In WT embryos, the equatorial plate is typically ~7 µm long at anaphase. However, in over half of the embryos from *Iswi*^{1} females, the equatorial plate is only ~3 µm, which is highly suggestive of haploid divisions. Embryos from homozygous *Iswi*^{1} mothers undergo only a limited number of mitotic cycles and do not survive beyond nuclear division 10. These results suggest that the abnormally condensed paternal chromatin in *Iswi*^{1} embryos fails to participate properly in mitosis and is not incorporated productively into daughter nuclei.

DISCUSSION

We uncovered a structural motif, the MST-HMG box, shared between *Drosophila* sperm chromatin components, including MST77F, TPL94D, MST35Ba, and MST35Bb. As none of these proteins bear any structural relationship to either histones or vertebrate protamines, MST-HMG-box-based chromatin represents a unique way of genome packaging. We suggest that other, still-uncharacterized MST-HMG-box proteins are likely candidate sperm chromatin components. We identified a testis-specific member of the NAP protein family, tNAP, as a chaperone that is essential for the deposition of MST77F onto the paternal genome. Like MST77F, tNAP is absolutely required for the formation of sperm chromatin, nuclear shaping, and male fertility. MST77F, in turn, enables the stable incorporation of MST35Ba/Bb into sperm chromatin. However, neither MST35Ba and MST35Bb nor their assembly factor CAF1-p75 are involved in the chromatin association of MST77F (Doyen et al., 2013). These results suggest a hierarchical model for sperm chromatin assembly, with MST77F as a key component. Thus, whereas NAP1 is a histone chaperone that mediates the assembly of nucleosomes in somatic cells, the closely related tNAP is a Mst77F chaperone that mediates the assembly of non-nucleosomal sperm chromatin. Curiously, during spermatogenesis, NAP1 has no direct chromosomal function.

ISWI is the defining DNA translocase subunit of an important class of ATP-dependent chromatin remodelers (Toto et al., 2014; Clapier and Cairns, 2009). As the motor subunit of different remodeler complexes, ISWI mediates not only transcription-factor-directed chromatin disruption but also the assembly of periodic nucleosome arrays. Here, we showed that the activities of ISWI are not restricted to the regulation of nucleosome-based chromatin. In the male germline, ISWI is a free protein and not part of any of the canonical ISWI complexes. ISWI is not required for histone removal during spermatogenesis, but it plays a crucial role in the stable incorporation of MST77F and MST35Ba/Bb into sperm chromatin. At fertilization, maternal ISWI selectively targets the paternal genome and promotes the rapid removal of sperm chromatin proteins. Following histone deposition onto the paternal genome, ISWI is crucial for the formation of a de-condensed nucleosomal chromatin structure. In the absence of ISWI, acetylated histone H4 accumulates at the male pronucleus, which nevertheless remains abnormally compacted. Thus, in spite of the buildup of a typical mark of open chromatin, actual chromatin de-compaction still requires ISWI.

The molecular basis of ISWI activity in repackaging of the paternal genome remains unclear. In particular, an indirect effect due to a transcriptional function of ISWI cannot be excluded. However, during spermatogenesis transcription ceases after completion of meiosis long before the late canoe stage, when ISWI-dependent sperm chromatin assembly occurs. At fertilization, the defective de-condensation of paternal chromatin caused by loss of maternal ISWI occurs prior to the onset of zygotic transcription. Suggestively, both in spermatogenesis and at fertilization, ISWI coats the paternal genome during chromatin reorganization. Based on these observations, we favor the hypothesis that ISWI plays a direct role in the (dis)assembly of sperm chromatin and in the further de-compaction of the paternal genome after histone deposition. The function of ISWI in the paternal pronucleus is reminiscent of its role in the reprogramming of somatic nuclei after nuclear transplantation. ISWI mediates chromatin de-condensation and the release of general transcription factor TBP from somatic nuclei incubated with *Xenopus* oocyte extracts (Kikyo et al., 2000). Thus, ISWI drives genome-scale chromatin reprogramming in somatic nuclei after nuclear transplantation, during spermatogenesis, and in the paternal pronucleus.

In summary, this study shows that tNAP is an essential chaperone for the pivotal sperm chromatin component MST77F. In addition, the remodeler ISWI mediates bi-directional genome-scale transitions between nucleosomal and MST-HMG-box-protein-based chromatin in the germline. In somatic cells, the histone chaperone NAP1 and the ISWI-containing ACF complex cooperate during the assembly of nucleosomal arrays (Ito et al., 1997). Thus, ISWI and NAP-family proteins appear to be specifically adapted to team up and mediate assembly of structurally unrelated forms of chromatin.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis

Details of the computational analysis of the MST-HMG-box motif are outlined in the Supplemental Experimental Procedures. Briefly, the MST-HMG-box motif was detected in a search for MST77F homologs in 12 *Drosophila* species with the accelerated profile HMM search algorithm (Eddy, 2011). Next, we constructed an initial HMM for the MST-HMG box, which was refined by additional homology searches for MST-HMG-box-containing proteins in 43 Ensembl annotated metazoan genomes. In total, we identified 101 proteins in 25 *Neoptera* species harboring 132 MST-HMG boxes, from which we build the final HMM used for phylogenetic analysis with the phyML Maximum Likelihood algorithm.

Drosophila Husbandry

For details on the lines used, see the Supplemental Experimental Procedures. All crosses were carried out at 25°C, and embryos used for imaging developed at 28°C. For the fertility tests ten females were crossed with ten 4-day-old males. The average number of offspring from four independent experiments was determined.

Biochemical Procedures

Details on antibodies can be found in the Supplemental Experimental Procedures. Extract preparations, immunoblotting analysis of testes and embryo extracts, co-immunoprecipitations (coIPs), and GST pull-downs were performed as described previously (Doyen et al., 2013; Moshkin et al., 2009). Testes were dissected from 4-day-old males, collected in an Eppendorf tube, and resuspended ~1:1 in HEMG/150 buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, and 150 mM KCl containing protease inhibitors 1 mM PMSF, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF], and 1 μM Pepstatin) using a small pestle. Following removal of debris by centrifugation, the extract was used.

Immunofluorescence

For details on imaging, see the Supplemental Experimental Procedures. Briefly, testes and ovaries were dissected from 4-day-old males or 6-day-old females and fixated with 4% formaldehyde followed by washes with TBST (50 mM Tris-HCl [pH 8], 150 mM NaCl, and 0.1% Triton X-100). Eggs or early embryos were collected after 20, 30, or 60 min of egg-laying on yeasted plates by rinsing with TBST followed by dechorionated in bleach, washing, and fixation with a heptane/methanol mixture (1:1). Embryos were washed with methanol and stored at −20°C until use. Prior to analysis, embryos were washed three times for 15 min in TBST. For IF imaging, embryos, testes, or ovaries were incubated with primary antibodies, followed by incubation with secondary Alexa Fluor antibodies (Invitrogen). Material was mounted in Vectashield containing DAPI.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.010.

AUTHOR CONTRIBUTIONS

C.M.D. Y.M.M., and C.P.V. designed the research, analyzed the results, and wrote the manuscript. C.M.D. performed the genetic experiments, G.E.C. the biochemistry, Y.M.M. the bioinformatics, and O.V. assisted with the ISWI coIPs. K.B. and J.A.D. performed the proteomics.

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