# Transition from a nucleosome-based to a protamine-based chromatin configuration during spermiogenesis in *Drosophila*

**Christina Rathke[1], Willy M. Baarends[2], Sunil Jayaramaiah-Raja[1,*], Marek Bartkuhn[3], Rainer Renkawitz[3] and Renate Renkawitz-Pohl[1,‡]**

[1]Philipps-Universität Marburg, Fachbereich Biologie, Entwicklungsbiologie, 35043 Marburg, Germany
[2]University Medical Center Rotterdam, Department of Reproduction and Development, Erasmus MC, 3000 DR Rotterdam, Netherlands
[3]Justus Liebig-Universität, Institut für Genetik, 35390 Giessen, Germany

*Present address: EMBL, Heidelberg, Germany
‡Author for correspondence (e-mail: renkawit@staff.uni-marburg.de)

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## Summary

**In higher organisms, the chromatin of sperm is organised in a highly condensed protamine-based structure. In pre-meiotic stages and shortly after meiosis, histones carry multiple modifications. Here, we focus on post-meiotic stages and show that also after meiosis, histone H3 shows a high overall methylation of K9 and K27 and we hypothesise that these modifications ensure maintenance of transcriptional silencing in the haploid genome. Furthermore, we show that histones are lost during the early canoe stage and that just before this stage, hyper-acetylation of histone H4 and mono-ubiquitylation of histone H2A occurs. We believe that these histone modifications within the histone-based chromatin architecture may lead to better access of enzymes and chromatin remodellers. This notion is supported by the presence of the architectural protein CTCF, numerous DNA breaks, SUMO, UbcD6 and high content of ubiquitin, as well as testes-specific nuclear proteasomes at this time. Moreover, we report the first transition protein-like chromosomal protein, Tpl$^{94D}$, to be found in *Drosophila*. We propose that Tpl$^{94D}$ – an HMG box protein – and the numerous DNA breaks facilitate chromatin unwinding as a prelude to protamine and Mst77F deposition. Finally, we show that histone modifications and removal are independent of protamine synthesis.**

Key words: *Drosophila*, Histone acetylation, Histone methylation, Histone ubiquitylation, Transition protein, Protamine, CTCF, DNA breaks, Rad6, UbcD6, RNA polymerase II

## Introduction

The switch from a nucleosome- to a protamine-based chromatin structure is a characteristic feature of sperm maturation in many vertebrates including humans. First, testis-specific linker histones appear (Catena et al., 2006; Martianov et al., 2005; Tanaka et al., 2005; Yan et al., 2003), and then histones are replaced by transition proteins (major types: TP1 and TP2), which in turn are replaced by protamines, leading to condensed chromatin with a doughnut structure (reviewed by Braun, 2001; Kimmins and Sassone-Corsi, 2005; Sassone-Corsi, 2002). Recently, we have shown that *Drosophila* sperm also contain protamines as well as Mst77F, representing at least one further chromatin component, whereas transition protein-like chromatin components have not been found in *Drosophila* so far (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). In *Drosophila*, GFP-tagged versions of the histone variant H2AvD are degraded during the canoe stage of spermatid development when protamines and Mst77F are starting to accumulate (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). This raises the question of whether the histones and histone variants are depleted from the chromosomes simultaneously. However, to date, little is known about the mechanism of histone displacement and degradation, and it is also unclear whether protamine synthesis is a signal for histone degradation. Multiple histone modifications have been analysed that mark transcriptionally active and inactive chromatin either on the level of euchromatin versus heterochromatin or on the level of individual genes (Lachner et al., 2003; Peterson and Laniel, 2004; Shilatifard, 2006). In *Drosophila* spermatogenesis, transcription nearly ceases with the entry into meiotic division (for review see Renkawitz-Pohl et al., 2005), which is long before the protamine-based chromatin status is established. Histone modifications in the haploid phase might play a fundamental role in keeping the genome largely transcriptionally inactive. In addition, we also expected that histones to be displaced from the chromatin would be marked by characteristic modifications, as is known to be the case for histone H4 that is acetylated in mammals. Furthermore, phosphorylation and ubiquitylation were also suggested to be important during this stage (reviewed by Braun, 2001). Here, we show for *Drosophila* that histones carry multiple modifications, which also characterise the transcriptionally highly active spermatocyte stage. Interestingly, we found de novo H2A mono-ubiquitylation and a tremendous increase in H4 acetylation shortly before histone degradation in the spermatid nucleus. This finding might indicate an opening of the chromatin which is accompanied by the expression of a transition protein such as chromatin

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component ($Tpl^{94D}$), the presence of CTCF and DNA breaks. We furthermore show that histone removal is independent of the presence of protamines. Both this histone removal and protamine accumulation are essential for transmission of the male genome to the oocyte, and therefore of fundamental importance for the persistence of species.

## Results

### Core histones and their variants are removed simultaneously from the DNA of spermatid nuclei prior to protamine accumulation

In *Drosophila melanogaster*, sperm morphogenesis, i.e. from meiosis until sperm individualisation, lasts 3.5 days. After meiosis, the nucleus initially is round and then gradually changes its shape accompanied by reorganisation of the chromatin during the canoe stage (Jayaramaiah-Raja and Renkawitz-Pohl, 2005), resulting in sperm containing a slim nucleus in which the nuclear volume is decreased by a factor of 200 (for review see Fuller, 1993; Renkawitz-Pohl et al., 2005).

In the work reported here we concentrated on post-meiotic sperm morphogenesis with particular focus on chromatin reorganisation from the nucleosomal- to the protamine-based structure, which is a dramatic switch. Previously, we have reported that the histone variant H2AvD-GFP vanishes at the canoe stage while protamines begin to accumulate simultaneously (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). To analyse the timing of histone removal and protamine accumulation we brought *protamine-eGFP* and *H2AvD-RFP* (Clarkson and Saint, 1999) into one genetic background to enable a study in the same individual. We found that H2AvD-RFP disappeared before protamine-eGFP accumulation took place (data not shown). We then went on to immunostain testes of protamine-eGFP flies with an antibody recognising all histones. This antibody was raised against total histones of humans and detects all core histones and the linker histone H1 in mammals. As – in contrast to core histones – H1 is not well conserved between mammals and *Drosophila*, we presumably detect solely core histones with this antibody. Our findings show that core histones (Fig. 1A) are detectable up to the canoe stage whereas protamines start to be synthesised at the canoe stage (Fig. 1B) but with no apparent positional overlap. As the canoe stage is quite long, we defined the early canoe stage by the start of histone removal, and the late canoe stage by the start of protamine accumulation (see Fig. 1A-E, second and third columns). With histone H3.3, a further replacement variant is expressed in the testis and disappears in post-meiotic stages together with the bulk of histones (Akhmanova et al., 1997). To reinvestigate this, we used an H3.3-Flag carrying *Drosophila* strain (Loppin, 2005). Histone H3-Flag (data not shown) and its variant H3.3-Flag (Fig. 1C) were observed to vanish along with the bulk of histones during the early canoe stage. Within the genus *Drosophila*, the timing of histone degradation is a well conserved feature, as our analysis of distantly related *Drosophila* species (*D. hydei*, *D. mojavensis*, *D. pseudoobsura* and *D. virilis*) revealed a comparable timing of the removal of histones at the canoe stage (data not shown).

### A transition protein-like chromatin component ($Tpl^{94D}$) is transiently expressed during the removal of histones and early stages of protamine synthesis

We observed a relatively sudden switch between the presence of histones and of protamine-eGFP, when analysing the histone distribution in transgenic flies carrying *protamine-eGFP* (Fig. 1A,B). This sudden switch is difficult to explain without an intermediate stage. Therefore, we speculated that a functional homologue of at least one of the transition proteins must be present in *Drosophila*; however, sequence homology searches failed to reveal the presence of any mammalian-type transition proteins. The testis-specific and basic proteins Don Juan and Don Juan-like are transiently present in the spermatid nucleus (Hempel et al., 2006; Santel et al., 1998). Since these two proteins disappear along with core histones (data not shown), it is unlikely that they are the true homologues of mammalian transition proteins. However, we identified in FlyBase five predicted genes (CG4691, CG15510, CG32308, CG31861, CG31281) encoding very basic proteins, which are expressed in testes. For all of them we generated corresponding *eGFP* fusion genes and analysed their expression patterns (C.R. and R.R.-P., unpublished). With this approach, we identified only one new fusion protein (CG31281-eGFP), which is transiently expressed exactly at the time when the switch between histone- and protamine-based chromatin configuration occurs (Fig. 1E; compare with D for histone distribution in the same sample). We named this gene *transition protein-like*$^{94D}$ (*tpl*$^{94D}$), because of its expression pattern and its location on the polytene chromosomes (FlyBase). We then investigated the distribution of $Tpl^{94D}$-eGFP with special focus on the canoe stage, where chromatin remodelling takes place. $Tpl^{94D}$-eGFP is first detectable at the early canoe stage, shortly before histones disappear (Fig. 1A-E, 2nd column) and most strongly expressed during the late canoe stage, when protamines are clearly visible and histones are completely gone (Fig. 1A-E, 3rd column). When protamines are fully expressed and the chromatin appears completely condensed, $Tpl^{94D}$-eGFP is no longer detectable (Fig. 1A-E, column 4). $Tpl^{94D}$ is a 164 aa protein with a predicted molecular mass of approximately 18.8 kDa and a pI (isoelectric point) of 11.4. $Tpl^{94D}$ shows no sequence similarity to the transition proteins of mice, but like these it has a high arginine (11.6 %) and serine (13.4 %) content. $Tpl^{94D}$ contains a high mobility group (HMG)-box (underlined in Fig. 1F), which suggests that $Tpl^{94D}$ is a component of the chromatin. The cell-type-specific expression in the testis shows that $Tpl^{94D}$ is a chromatin component during the switch from histone- to protamine-based configuration. We suggest that this abundant high mobility group protein plays a key role in disrupting nucleosomal structure as has been suggested for recombination and transcription (Travers, 2003). Since the presence of histones and protamines is absolutely exclusive, and since $Tpl^{94D}$ expression is characteristic for the time of chromatin switch, we think that $Tpl^{94D}$ is a good candidate for a functional homologue of mammalian transition proteins.

We next addressed the question of whether $Tpl^{94D}$ is expressed exclusively in the testes, as are transition proteins in mammals. We performed RT-PCR experiments using RNA from adult males without testes, from testes, from adult females, embryos and larvae. We see that the *tpl*$^{94D}$ RNA is restricted to the testes in adult flies (Fig. 1G). We observe *tpl*$^{94D}$ RNA in larvae but not in embryos, which reflects the transcription in the spermatocytes of larval testes. The testes-specific expression further supports its functional role as a transition protein.

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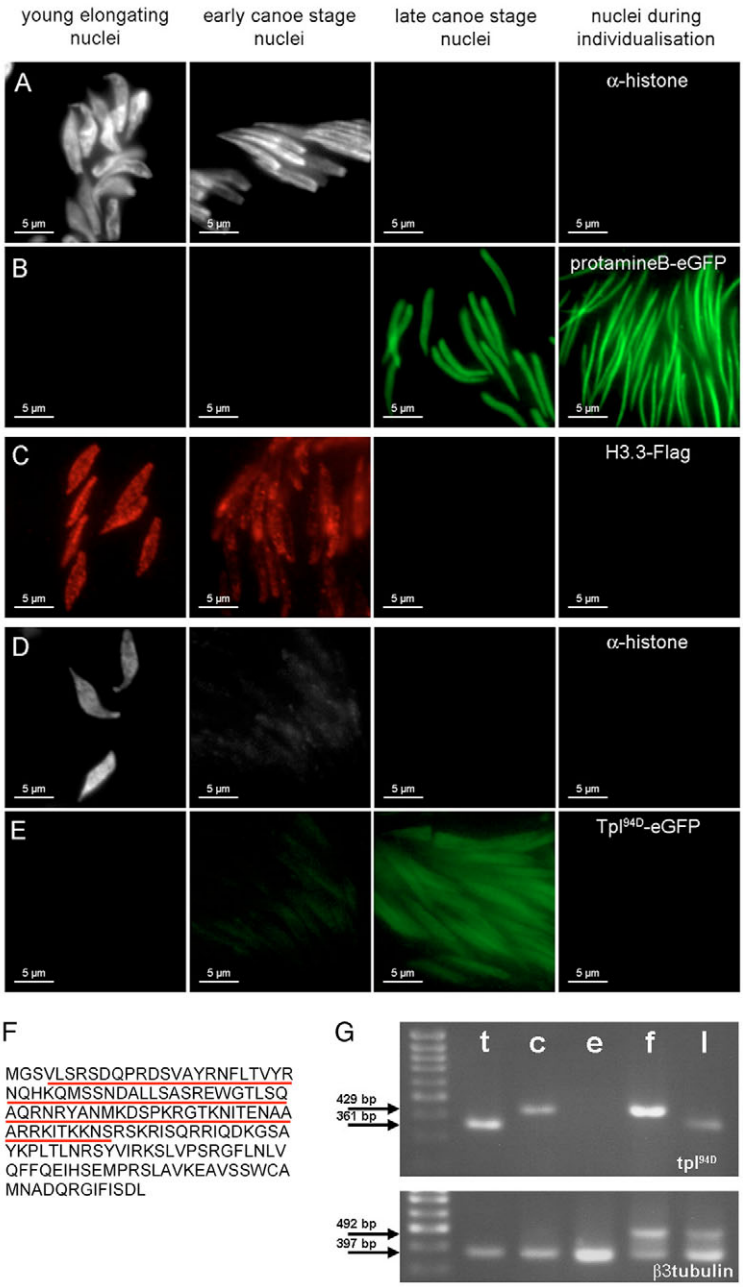


**Fig. 1** (A-E) Tpl$^{94D}$ is transiently expressed during the switch between histones and protamines. (A,D) Anti-core histone antibody staining of transgenic flies (A) expressing protamine-eGFP and (D) bearing *tpl$^{94D}$-eGFP* to compare histone and protamine expression in the same fly tissue. Core histones are detectable in young elongating nuclei and in early canoe stage spermatids but not in later stages. (B) Protamine-eGFP expression starts at the late canoe stage when histones have already gone and stays in the individualising spermatids. (C) Anti-Flag antibody staining of flies expressing histone H3.3-Flag. Histone H3.3 is detectable in young elongating nuclei and in early canoe stage spermatids and shows the same pattern as the core histones (A,D). (E) Expression of Tpl$^{94D}$-eGFP starts in the early canoe stage and becomes highest in late canoe stage spermatids. In individualising sperm, it is not detectable (see D for histone staining of the identical slide). Bars, 5 µm. (F) Tpl$^{94D}$ is a HMG protein. The deduced 164 amino acid protein sequence of the *tpl$^{94D}$* transcript is shown. The N-terminal HMG domain is underlined in red and lasts from amino acid 4 to 84. The HMG box was determined using the Ensembl genome browser (www.ensembl.org). (G) *tpl$^{94D}$* expression is testes specific. RT-PCR of *tpl$^{94D}$* from wild-type testes (t), carcass males (m), embryos (e), adult females (f) and larvae (l). The *tpl$^{94D}$*-specific primers amplified a 361 bp cDNA fragment from the open reading frame of *tpl$^{94D}$* in testes and in larvae. In carcass males and in adult females a 429 bp DNA fragment was amplified, whereas in embryos no fragment was detectable. A 397 bp cDNA fragment of the *β3-tubulin* gene was amplified as control. The cDNA *β3-tubulin* fragment was visible in all samples, whereas DNA contamination shown by the 492 bp fragment was visible only in adult females and larvae.

## Spermatid nuclei contain high levels of ubiquitin during the histone to protamine transition

It is well known that proteins are marked by ubiquitin for degradation in proteasomes (for a review, see von Mikecz, 2006; Nandi et al., 2006). At the stages relevant for histone degradation, proteasomes are detectable in spermatid nuclei and include subunits expressed specifically in the testis (Ma et al., 2002). Thus, we first applied an antibody against ubiquitin in general, to start the analysis of protein degradation. We observed that, in post-meiotic stages of spermatogenesis, ubiquitin is very abundant in the nuclei at the early canoe stage (Fig. 2, row A, arrowhead) and detectable up to the late canoe stage of chromatin reorganisation (Fig. 2A, double arrow). We also observed the testis-specific nuclear proteasome subunit Prosα3T-eGFP and the corresponding somatic type Prosα3-eGFP during the early and late canoe stage in the nucleus (Fig. 2B,C, arrowhead and double arrow). Prosα3T-eGFP stays in the individualising spermatids, while the corresponding somatic type, Prosα3-eGFP, vanishes during the late canoe stage [Prosα-eGFP lines see Ma et al. (Ma et al., 2002)]. This stage-specific abundance of ubiquitin and proteasomes corresponds to the time of histone and Tpl$^{94D}$ degradation. As we observed ubiquitin in the nucleus during histone degradation, we next investigated male sterile mutants with mutations in ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Homozygous mutants arrest spermatogenesis during spermatid development. In testes of homozygous mutants, we observed that histones disappear at the appropriate time in *crossbronx*, *dbruce* and *effete*[8] (E2) and *purity of essence* (E3) mutants (Table 1). Therefore, we conclude that histone removal neither depends on these ubiquitin-conjugating enzymes nor on this ubiquitin ligase, although they are required for male fertility.

## Lysine methylation of histone H3 as well as arginine methylation of histone H4 marks the histone to protamine transition in spermatid nuclei

A general histone code is established whereby certain modifications indicate transcriptionally active chromatin whereas others indicate transcriptionally inactive regions of the genome. At the spermatocyte stage, histones carry multiple modifications (Table 2). We also observed all these

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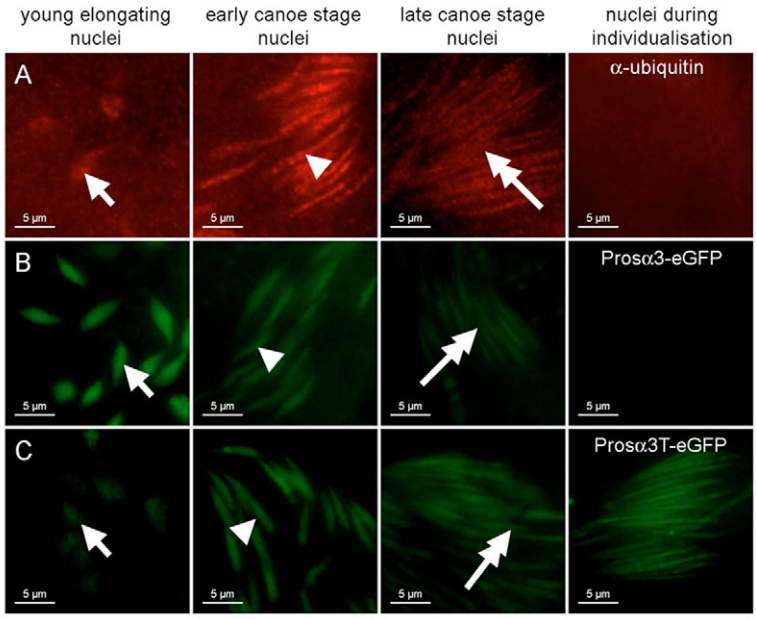


**Fig. 2.** Ubiquitin and proteasomes accumulate in the nuclei during spermiogenesis. (A) Anti-ubiquitin antibody staining on testes squashes of wild-type flies. Ubiquitin is detectable in young elongating nuclei (arrow) as well as in canoe stage nuclei (arrowhead and double arrow) with the highest expression in early canoe stage nuclei. (B) Expression of the proteasome subunit Prosα3-eGFP is very strong in young elongating nuclei (arrow), decreases during the canoe stage (arrowhead and double arrow) and is no longer detectable after the canoe stage. (C) Expression of the proteasome subunit Prosα3T-eGFP (isoform of Prosα3-eGFP) starts in young elongating nuclei (arrow) and stays in the nucleus during all later stages (arrowhead and double arrow). Bars, 5 μm.

modifications in post-meiotic stages (Table 2). Methylation of lysines 9 and 27 of histone H3 indicate repression, whereas methylation of lysine 4 of histone H3 indicates activation. Histone lysines can be mono-, di- or trimethylated (Lachner et al., 2003). Post-meiotic germ cells of *Drosophila* are virtually completely transcriptionally silent (for a review, see Renkawitz-Pohl et al., 2005); thus, histone methylation on lysine residues K9 or K27 might be expected in post-meiotic stages. We analysed mono-, di- and trimethylation of H3K9 as well as di- and trimethylation of H3K27 and found these modifications to be present from the nebenkern stage immediately after meiosis (data not shown) until the removal of histones (Table 2). Therefore, we conclude that the post-meiotic stages of male germ cells contain multiple histone methylations characteristic of transcriptionally inactive chromatin, in agreement with their transcriptionally silent status. At the same stages we also observed that N-terminal tails of core histones contain modifications which otherwise mark transcriptionally active chromatin, such as H3K4 or H4R3 methylation. At first glance, this appears surprising, but previously it was shown that trimethylation of H3K4 can also function in active gene repression (Shi et al., 2006). But it is also possible that H3K4 and H4R3 methylation at this stage might indicate overall opening of the chromatin as a prelude for histone removal. As a representative example, we show nuclear distribution of H3K4 trimethylation during the transition between histones and the protamine deposition in flies expressing protamine-eGFP (Fig. 3). The DNA staining in Fig. 3 shows the nuclei of three spermatid bundles with 64 synchronously developing spermatids each. Only the nuclei of the left bundle accumulated protamine-eGFP (Fig. 3C). This bundle cannot be visualised with antibodies recognising H3K4 trimethylation, but the middle and right bundles can (Fig. 3B). The level of H3K4 trimethylation during the different stages of chromatin reorganisation can be compared in the overlay presented in Fig. 3D, where the level of H3K4 decreases in early canoe stage spermatid nuclei (middle bundle) compared to young elongating spermatid nuclei with very high H3K4 methylation levels (right bundle), the stage directly before the canoe stage. The high levels of H3K4 and H4R3 methylation (see Table 2) as well as other modifications (see next subsection) shortly before histone degradation might be relevant for opening of the chromatin.

**Table 1. Investigation of histone removal, using an anti-core histone antibody on testes of flies with a mutation in ubiquitin-conjugating enzymes or ligases**

| Mutant gene | Male sterile | Predicted function | Histone removal disturbed |
|---|---|---|---|
| *crossbronx* (*cbx*$^{05704}$)* | +‡ | Ubiquitin-conjugating enzyme | – |
| *dbruce*$^{E81}$† | +† | Ubiquitin-conjugating enzyme | – |
| *effette*(*eff*$^{6}$) (allel of *UbcD1*)* | +§ | Ubiquitin-conjugating enzyme | – |
| *purity of essence* (*poe*$^{01659}$)* | +¶ | Ubiquitin ligase activity | – |

*Castrillon et al., 1993; †Arama et al., 2003; ‡Fabrizio et al., 1998; §Cenci et al., 1997; ¶Spradling et al., 1999.

### At the early canoe stage, hyper-acetylation of histone H4 and ubiquitylation of histone H2A precedes histone removal and protamine deposition

For mammals, hyper-acetylation of histone H4 is thought to cause loss of contact between histones and DNA. Therefore, to visualise protamine accumulation, histone removal and H4 acetylation in the same spermatids, we performed double immunostainings on testes squashes of a transgenic *Drosophila* line carrying *protamine-eGFP* (Fig. 4A-C). Indeed, we found a dramatic increase in H4 acetylation during the canoe stage immediately before histones are removed and degraded (see Fig. 4B for acetylated H4, and Fig. 4A for histones). In round spermatids (Fig. 4E, arrow) and young elongating spermatids

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**Table 2. Methylation of histones in different stages of spermiogenesis**

| | Spermatocytes* | Young elongating nuclei | Early canoe stage nuclei | Late canoe stage nuclei until individualised sperm | Removed in *protΔ38.1* mutants |
|---|---|---|---|---|---|
| Anti-H3K4 monomethyl | +++ | +++ | ++ | – | yes |
| Anti-H3K4 dimethyl (Abcam) | +++ | +++ | ++ | – | yes |
| Anti-H3K4 dimethyl (Upstate) | +++ | +++ | ++ | – | yes |
| Anti-H3K4 trimethyl (Abcam) | +++ | +++ | ++ | – | yes |
| Anti-H3K4 trimethyl (Upstate) | +++ | +++ | ++ | – | yes |
| Anti-H3K9 monomethyl | +++ | +++ | ++ | – | yes |
| Anti-H3K9 dimethyl (Abcam) | +++ | + | + | – | yes |
| Anti-H3K9 dimethyl (Upstate) | +++ | +++ | ++ | – | yes |
| Anti-H3K9 trimethyl (Abcam) | +++ | + | + | – | yes |
| Anti-H3K9 trimethyl (Upstate) | +++ | + | + | – | yes |
| Anti-H3-K27 dimethyl | +++ | +++ | ++ | – | yes |
| Anti-H3-K27 trimethyl | +++ | +++ | ++ | – | yes |
| Anti-H4-R3 methylation | +++ | +++ | +++ | – | yes |

This table is based on immunostainings of testes squashes and represents a subjective measurement of staining intensity during the different stages of spermiogenesis. Note therefore that the degree of staining intensity for different types of stainings is not comparable. *As the spermatocyte stage lasts 3.5 days an average of staining intensity is shown here. +++, highest amounts; ++, medium amounts; +, lowest amounts of detectable signals; –, no detectable signals. The same modifications were investigated in *protamine* loss-of-function mutants.

(data not shown), we also observed mono-ubiquitylated histone H2A, which we solely find in these very early post-meiotic stages (Fig. 4, row E) and in late primary spermatocytes (data not shown). This is in clear contrast to H4 acetylation, which we also observed in early canoe stage nuclei (Fig. 4B) and in all pre-meiotic stages (data not shown). At these early post-meiotic stages, protamine-eGFP is not yet synthesised (Fig. 4C,F). Thus, we conclude that histone H4 hyper-acetylation and H2A mono-ubiquitylation shortly after meiosis and histone H4 hyper-acetylation at the canoe stage are indicative of the switch in chromatin architecture.

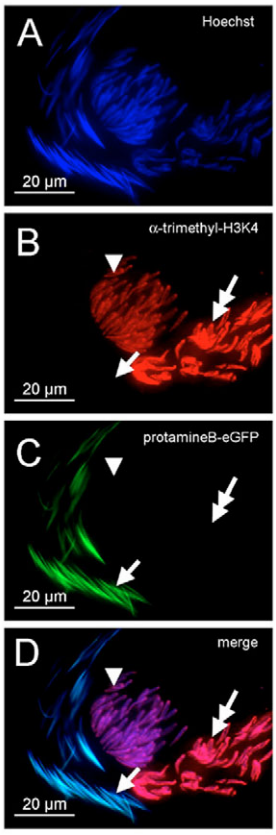


**Fig. 3.** H3K4 methylation is removed during the early canoe stage. (A) Hoechst staining to visualise chromatin. (B) Anti-methylated H3K4 antibody staining on squashed testes of protamine-eGFP expressing flies. A high level of H3K4 trimethylation is detectable in spermatids with young elongating nuclei (double arrow). During the early canoe stage, the signal decreases (arrowhead) and vanishes completely from the late canoe stage (not shown) onwards (arrow). (C) There is no protamine-eGFP expression in young elongating nuclei and early canoe stage spermatids, whereas high expression is observed in individualising spermatids (arrow). (D) Merged images. Bars, 20 μm.

## The histone to protamine transition is accompanied by DNA breaks and a high level of UbcD6 and SUMO

It is difficult to imagine how the chromatin with the core histone octamers surrounded by DNA can suddenly lose the nucleosomal structure all at once in the majority of the chromatin. In mammalian male germ cells, DNA breaks occur during chromatin remodelling (Laberge and Boissonneault, 2005). Local nicks or breaks in the DNA might facilitate the opening of the chromatin structure and thus lead to the removal of histones and the incorporation of $Tpl^{94D}$, protamines and Mst77F. To investigate this, we performed immuno- and TUNEL staining on testes squashes of *protamine-eGFP* transgenic flies, thereby allowing comparison of histone removal, protamine deposition and DNA breaks in male germ cells within one testis tube (Fig. 5A-C). At the early canoe stage when histones start to vanish (Fig. 5A, arrow), numerous nicks are present in the DNA (Fig. 5C, arrow). When histones are removed and protamine-eGFP starts to accumulate at the late canoe stage, the TUNEL signal is very strong, indicating numerous nicks in the DNA (see Fig. 5 column 2, arrowhead). Nicks are no longer detectable after the late canoe stage, an example is shown for the individualisation stages when protamine-eGFP is fully expressed (Fig. 5, column 3, double arrow in B). These observations indicate that chromatin remodelling is facilitated by local opening of the chromatin due to DNA nicks. Furthermore, we checked whether SUMO and UbcD6, the *Drosophila* homolog of Rad6 in yeast and HR6A and HR6B in mice, are expressed. Rad6 has multiple functions including involvement in postreplication repair (Baarends et al., 2003). HR6B knock-out mice show malformed spermatid nuclei indicating a role in post-meiotic stages in the nucleus (Roest et al., 1996). In addition, a high level of SUMO is characteristic of DNA repair (Branzei et al., 2006). Indeed, both SUMO and UbcD6 (detected with anti-HR6A/B, recognising HR6A and HR6B in mice, directed against the conserved N terminus) are highly expressed at the same time when DNA nicks are detected (Fig. 5C-E).

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### The late canoe stage is characterised by CTCF and active RNA polymerase II

The switch between a nucleosome-based chromatin configuration and a protamine-based structure is a specialised form of chromatin remodelling in the male germline. The mammalian zinc finger protein CTCF is involved in many epigenetic processes (Ohlsson et al., 2001). Furthermore, paralogous variant of CTCF which is testis-specifically

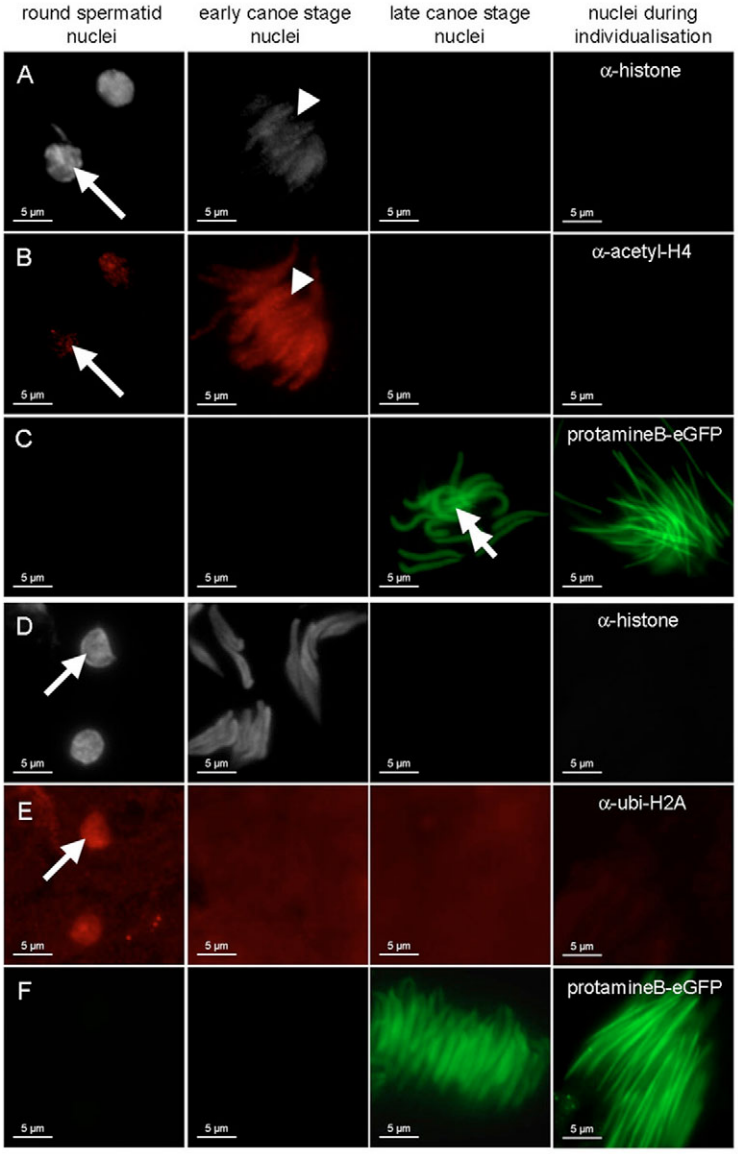


**Fig. 4.** Histone H4 hyper-acetylation and de novo H2A ubiquitylation characterise the stages before histone removal. (A-C) Double immunostaining of core histones and acetylated histone H4 on squashed testes of *protamine-eGFP* flies. (D-F) Double immunostaining of core histones and mono-ubiquitylated histone H2A on squashed testes of *protamine-eGFP* flies. (A,D) Histones are abundant in round spermatids (arrows), whereas the staining decreases in early canoe stage spermatids (arrowhead in A). In later stages when protamines are present (double arrow in C), histones are no longer detectable. (B) Nuclei of round spermatids show a faint staining of acetylated histone H4 (arrow). In early canoe stage spermatids, acetylation of histone H4 increases (arrowhead) and has vanished completely from the late canoe stage onwards. (C,F) Protamine-eGFP expression starts in late canoe stage spermatids (double arrow in C). (E) Mono-ubiquitylated histone H2A is detectable in spermatids with round nuclei (arrow). Mono-ubiquitylated histone H2A is no longer detectable during the canoe stage and in later stages when protamines are present (double arrow in C). Bars, 5 μm.

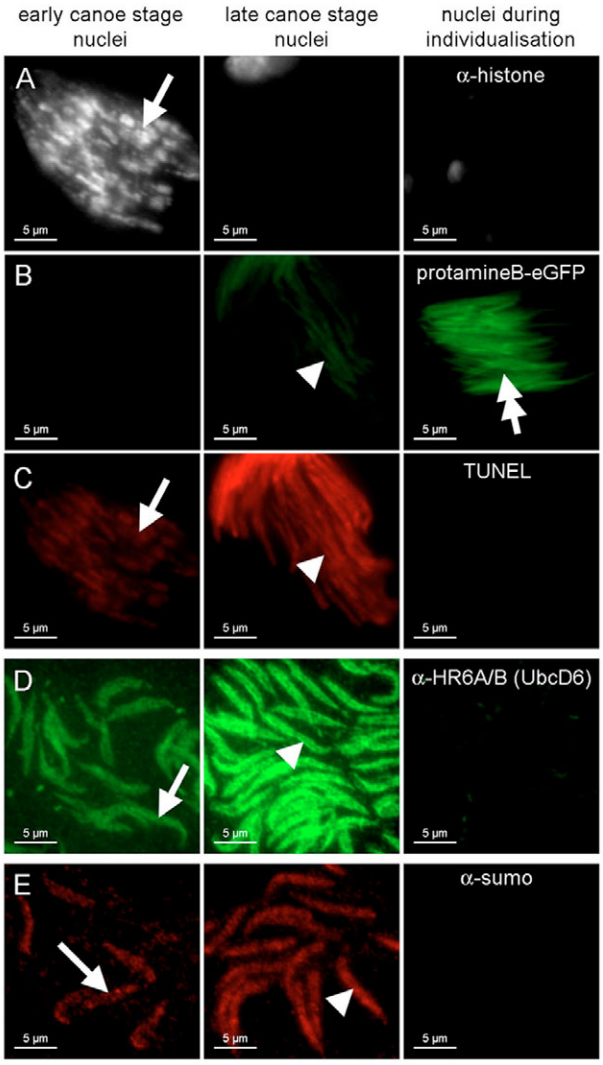


**Fig. 5.** DNA breaks, UbcD6 and SUMO are abundant at the late canoe stage. (A-C) Anti-core histone antibody staining together with TUNEL staining on testes squashes of *protamine-eGFP* flies to visualise DNA breaks in comparison to histone deposition and protamine incorporation during the canoe stage in the same animal. (A) Anti-histone staining is detectable in early canoe stage spermatids (arrow), but not in any later stages. (B) Protamine-eGFP expression is detectable in late canoe stage (arrowhead) and individualising (double arrow) spermatids. (C) DNA breaks marked by TUNEL staining are detectable only at a low level in early canoe stage spermatids (arrow) reaching their highest level in late canoe stage spermatids (arrowhead). During individualisation, DNA breaks are no longer detectable. (D) Anti-HR6A/B antibody staining is detectable in early (arrow) and late (arrowhead) canoe stage spermatid nuclei, but not in later stages. (E) Anti-SUMO staining is detectable in early (arrow) and late (arrowhead) canoe stage spermatid nuclei, but not in later stages. Bars, 5 μm.

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expressed, called BORIS, is exclusively expressed in the mammalian male germline. The function of BORIS in this context is still not clear (Loukinov et al., 2002). Recently, we identified CTCF in *Drosophila* and showed that *Drosophila*, in contrast to mammals, contains only one CTCF gene (Moon et al., 2005). We therefore asked whether *Drosophila* CTCF is also expressed in the testes, and performed immunostaining and anti-histone staining on testes of transgenic flies expressing protamine-eGFP. We observed CTCF expression during pre-meiotic and meiotic stages at the chromosomes (data not shown) as has been shown for mitotic cell division in mammalian cell culture (Burke et al., 2005). Shortly after meiosis, CTCF is visible in young elongating nuclei, where it co-localises with the chromatin as indicated by the histone distribution (Fig. 6, column 1). CTCF is also present in the early and late canoe stage spermatid heads. At the early canoe stage, CTCF is very diffusely distributed in comparison to histones (Fig. 6, column 2). CTCF does not co-localise with the chromatin which starts to condense at one side of the nucleus (Fig. 6A and C, arrow). This diffuse distribution is still visible at the late canoe stage (Fig. 6, column 3) when protamine-eGFP starts to be deposited to the chromatin. CTCF is no longer detectable after the canoe stage (Fig. 6C). The earlier chromatin-associated CTCF localisation might indicate a very early role in chromatin reorganisation at the switch between the nebenkern and canoe stage. Furthermore, CTCF might be associated primarily with the chromatin, which is not yet condensing during these stages [for a scheme of chromatin distribution based on EM studies, see Fuller (Fuller, 1993)]. The late canoe stage is the only post-meiotic stage where we find distinct regions of RNA polymerase II with an antibody directed against a phosphorylated subunit of active polymerase, which is indicative of transcription (Fig. 6D, double arrow). At this precise stage, only a very small set of genes is thought to be transcribed (H. White-Cooper, University of Oxford, UK, personal communication). Also in *D. hydei* we detected CTCF expression during chromatin reorganisation in the nucleus (data not shown).

### Histones are removed and degraded independently of the deposition of protamines

We asked whether histone and CTCF degradation is dependent on protamine synthesis. To this end, we used a fly strain recently generated by FLP/FRT technology. This strain harbours a deletion (*protΔ38.1*) spanning the protamine genes, *Mst35Ba* and *Mst35Bb*, as well as three other ORFs from the genome (CG15279; CG4480; CG15278). Furthermore CG33309 is destroyed by one of the FRT sites. Mutant males of these strains show severely reduced fertility (S.J.-R. and R.R.-P., unpublished).

Unlike mammals, fly protamine mutants are not haploinsufficient. CTCF is removed in protamine loss-of-function mutants, as observed in wild type. Histone is not present in this mutant or in transheterozygous *Mst77F* mutants (*ms(3)nc3* over *Df(3L)ri-79c*; data not shown). All the investigated histone modifications such as diverse lysine and arginine methylation (Table 2), H4 acetylation or H2A mono-ubiquitylation (data not shown) also occur in protamine loss-of-function mutants (*protΔ38.1*) as in wild-type flies. SUMO and ubiquitin also vanished in the mutant as in wild-type, and active RNA polymerase II was observed at the same time as in wild type (data not shown). We therefore conclude that histone and CTCF removal during the late canoe stage are independent of protamine synthesis involving *Mst35Ba* and

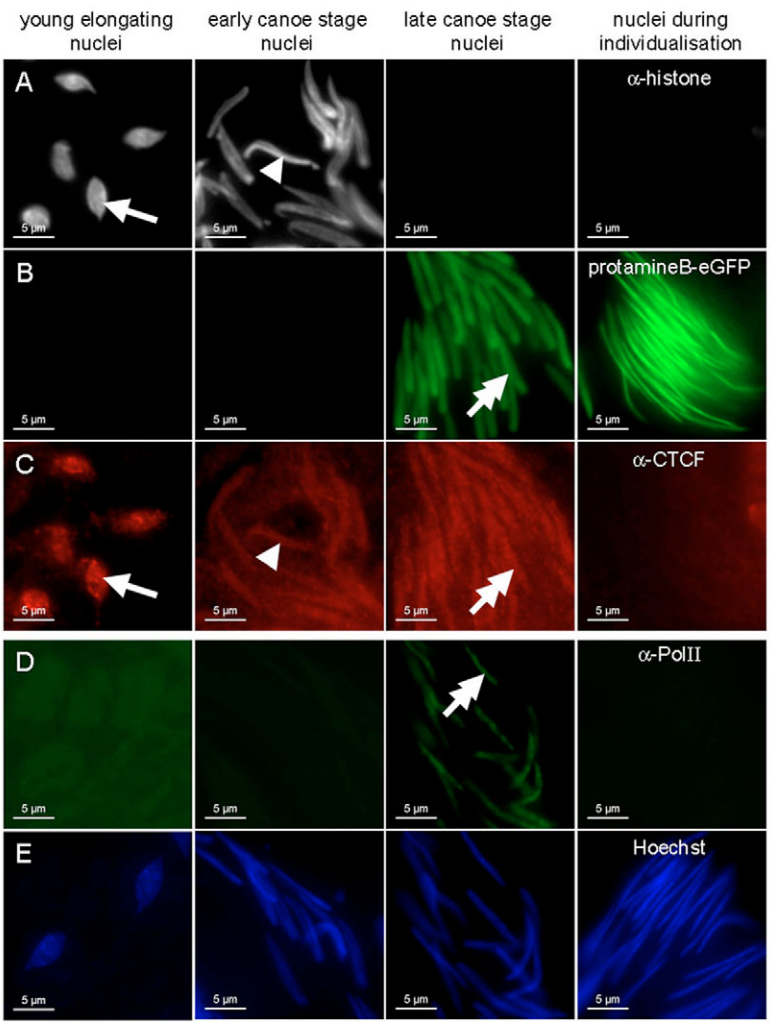


**Fig. 6.** CTCF, a zinc finger DNA binding protein, and RNA polymerase II are expressed when protamines start to accumulate at the late canoe stage. (A-C) Anti-CTCF (C) and anti-histone antibody (A) staining on testes squashes of flies expressing protamine-eGFP (B). (A) Core histones are detectable in young elongating nuclei (arrow) and in early canoe stage spermatids (arrowhead), but not in later stages. (B) Protamine-eGFP expression starts in late canoe stage spermatids (double arrow). (C) Anti-CTCF is detectable in young elongating nuclei (arrow) and early canoe stage spermatids (arrowhead). During the late canoe stage, expression of CTCF overlaps with that of protamine-eGFP (double arrow), whereas CTCF is no longer visible in individualising spermatids. (D) Active RNA polymerase II is detectable by antibody staining in late canoe stage nuclei (double arrow), but not in any other post-meiotic stage. (E) Hoechst staining of the same cells as in D. Bars, 5 μm.

**Table 3. Investigation of histone removal in different mutant backgrounds using an anti-core histone antibody**

| Mutant gene | Male sterile | Predicted function according to FlyBase | Histone removal disturbed |
|---|---|---|---|
| *belle* ($bel^{cap\text{-}1}$)* | +‡ | Spermatid development | – |
| *bellwether* ($blw^{1}$)* | +† | ATP binding | – |
| *betel* ($bet^{1}$)* | +‡ | Spermatid development | – |
| *blanks* ($bln^{1}$)* | +‡ | Spermatid individualisation | – |
| *emmenthal* ($emm^{1}$)* | +‡ | Spermatid development | – |
| *extra sexcombs* ($esc^{1}$)§ | +§ | DNA-binding; histone methylation | – |
| *gelded* ($Bsg^{gel}$)* | +* | Spermatid development | – |
| *gilgamesh* ($gish^{04895}$)* | +‡ | Kinase activity | – |
| *gorp* ($gor^{1}$)* | Semi-sterile* | Spermatid development | – |
| *goulash* ($goul^{1}$)* | +* | Spermatid development | – |
| *modulo* ($mod^{07570}$)* | Semi-sterile* | Spermatid development | – |
| *ms(2)29F*$^{07717}$* | +‡ | n.d. | – |
| *ms(2)42D*$^{09967}$* | Semi-sterile* | n.d. | – |
| *ms(3)72D*$^{03957}$* | +‡ | Spermatid development | – |
| *ms(3)80*$^{03817}$* | +‡ | Spermatid development | – |
| *ms(3)85D*$^{03565}$* | +‡ | Spermatid development | – |
| *ms(3)98B*$^{06302}$* | +‡ | Spermatid development | – |
| *mulet* ($mlt^{1}$)* | +* | Sperm individualisation | – |
| *peanuts* ($pea^{1}$)* | +§ | Spermatid development | – |
| *seedless* ($sdl^{05090}$)* | +‡ | Spermatid development | – |
| *thousand points of light* ($tho^{1}$)* | +‡ | Spermatid development | – |
| *trail mix* ($tmx^{1}$)* | Semi-sterile* | Spermatid development | – |

*Castrillon et al., 1993; †Jacobs et al., 1998; ‡Spradling et al., 1999; §Lindsley and Zimm, 1992.

*Mst35Bb*. Furthermore, we analysed the fate of histones in different known male sterile mutants (Table 3). Again, none of the mutants showed signs of impaired histone removal. In summary, we conclude that histone degradation and CTCF removal are regulated independently of the synthesis of protamines.

## Discussion

### Histones disappear, transition protein-like proteins appear transiently and protamines are deposited to the chromatin

Sperm morphogenesis is characterised by an impressive degree of changes in cell architecture based on stored, translationally repressed mRNAs that are recruited at the appropriate time to the polysomes (for a review, see Renkawitz-Pohl et al., 2005). Among these are mRNAs that encode $Tpl^{94D}$ and protamines. A dramatic switch in structure from the nucleosomal- to the protamine-based structure of chromatin takes place, and this remarkable chromatin reorganisation of the complete genome is a typical feature depending on stored mRNAs, e.g. for protamine synthesis (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). This process ultimately leads to an extremely condensed state of the haploid genome in the sperm, which is essential for male fertility in mammals. Here, we focused on the switch between a nucleosomal- and a protamine-based chromatin reorganisation. We observed that the major steps in chromatin organisation take place in the canoe stage of spermatid development (summarised in Fig. 7). For the first time, we identified a candidate for a transition protein in *Drosophila*. The corresponding gene $tpl^{94D}$ (CG31281) encodes a predicted basic high mobility group (HMG) protein of 18.8 kDa. In transgenic flies, $Tpl^{94D}$-eGFP fusion proteins are expressed solely during the switch between histones and protamines, as is typical for mammalian transition proteins. Since we observe a highly similar chain of events to those reported in mammals, we consider the *Drosophila* system an excellent choice to study the mechanism of chromatin remodelling during male germ cell development.

### Histone degradation correlates with high level of ubiquitin and proteasomes in the nucleus

Generally, we observe that the bulk of histones, including their diverse modifications in the N-terminal tail, appear to be removed during the canoe stage. Furthermore, we show that the nucleus accumulates ubiquitin at the early canoe stage, when mono-ubiquitylation of histone H2A is no longer detectable (compare Fig. 2A and Fig. 4E). Therefore, taking into account the known presence of proteasomes in the nucleus at this stage of chromatin reorganisation (Ma et al., 2002) and the overlap of expression shown here, we hypothesise that this ubiquitylation is targeting histones for degradation. Here, we investigated several mutants having mutations in ubiquitin-conjugating enzymes or ubiquitin ligases, exhibiting arrested spermiogenesis during spermatid development and that are male sterile. However, in all investigated mutants, histone removal is indistinguishable from that of wild-type flies.

### Histones carry multiple modifications before being removed from the chromatin

We found many histone modifications after meiosis (see Table 2 and Figs 2-4) and have categorised them into three classes.

(1) Histone modifications that persist from pre-meiotic stages and keep the genome silent. The vast majority of the genome is transcriptionally silent in post-meiotic stages. This is accompanied by multiple histone modifications that persist from pre-meiotic stages and indicate silencing such as H3K9 and H3K27 methylation. These modifications do not change significantly during post-meiotic stages, which is in agreement with our hypothesis that these modifications predominantly play a role in maintaining transcriptional silencing. Previously, phosphorylation of histones have been analysed during

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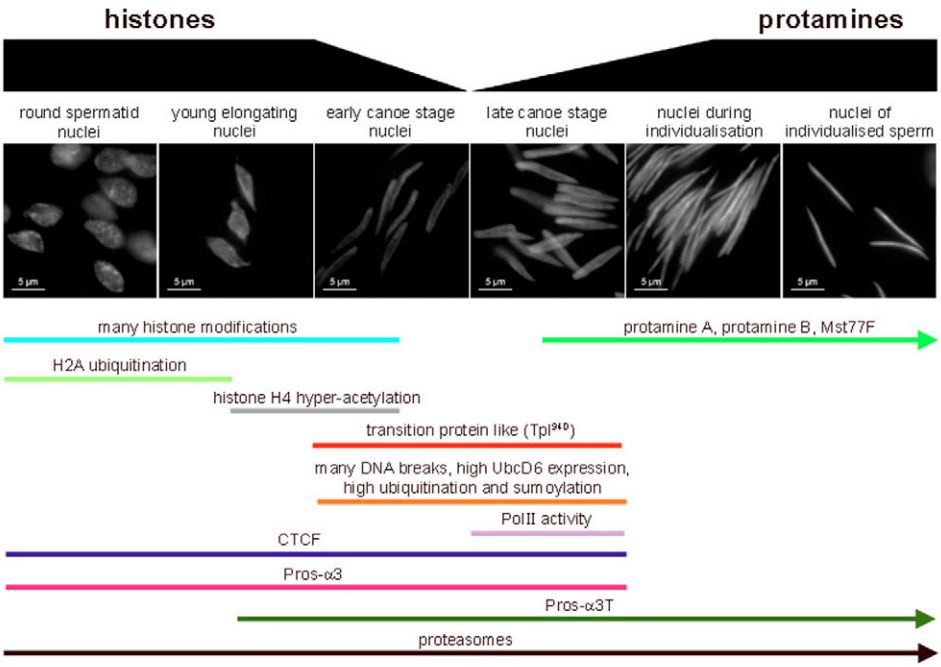


**Fig. 7.** Key chromatin remodelling events in *Drosophila* spermiogenesis. Hoechst stainings visualise the morphogenesis of the spermatid and/or sperm nuclei in the order of events after meiosis. Below is a scheme of histone degradation and Tpl$^{94D}$, Mst77F and protamine deposition in comparison to general and specific histone modifications, ubiquitylation, SUMOylation, DNA breaks, expression of UbcD6 and CTCF, active RNA polymerase II and the presence of proteasomes in the nucleus.

spermatogenesis. Phosphorylated histone H4S1 and H3S10 are present during meiotic divisions. H3S10 phosphorylation is hardly detectable after meiosis, whereas phosphorylation of H4S1 persists until chromatin compaction starts (Krishnamoorthy et al., 2006).

(2) Histone modifications that persist from pre-meiotic stages and characterise transcriptionally active chromatin. The primary spermatocyte phase is characterised by a high level of transcriptional activity of housekeeping genes. In addition, genes are transcribed that are needed for the subsequent steps in spermatogenesis, as the majority of transcription ceases once meiotic division starts. Here, we investigated H4 acetylation and H3K4 and H4R3 methylation of histones (Fig. 4; Table 2). These histone modifications, which are indicative of transcriptional activity, persist until histone degradation.

(3) Increasing or de novo appearance of histone modifications that decrease the affinity between histones and DNA as a prelude to histone removal. It might be that H4 hyper-acetylation, as postulated for mammals (reviewed by Braun 2001; Meistrich et al., 1992) and/or other secondary modifications of histones are the first step towards histone removal. The fact that these modifications are conserved between mammals and flies adds support to this hypothesis. Indeed, histone H4 acetylation is very pronounced at the canoe stage and we see de novo mono-ubiquitylation of histone H2A in round spermatids. Both types of histone modifications we propose to be necessary for opening the chromatin and decreasing the contact between DNA and histones. The fact that histone H2A mono-ubiquitylation vanishes before the early canoe stage, thus before the hyper-acetylation of histone H4, leads us to think about a stepwise remodelling of the chromatin. Here, we propose that these histone modifications open the chromatin, so that enzymes and regulators have access to histone-based chromatin and can induce and prepare the reorganisation of the genome in the male germline.

It remains to be clarified whether and how these histone modifications influence the topology of the chromatin as a prelude to histone removal as well as for Tpl$^{94D}$, Mst77F and protamine deposition. A functional approach based on analysis of mutants of histone-modifying enzymes is difficult, as all characterised histone-modifying enzymes are already active during *Drosophila* development or at least in spermatogonia and spermatocytes. Therefore a tissue-specific knock-out mutant would most probably exhibit arrest of spermatogenesis before meiosis, rendering it useless for our purposes.

At the first glance, it might seem surprising that histones and all their modifications are removed. Instead of specifically reverting the differentially modified histones to their unmodified state, they are removed together with all histones. This might allow the paternal genome to form nucleosomes with unmodified histones after fertilisation and before zygote formation. Thus, the paternal genome starts embryogenesis with a nucleosomal chromatin lacking histone modifications.

### The switch in chromatin organisation is characterised by DNA breaks

Our data show that most of the histones are removed between the early and late canoe stage; such a process requires a loosening of contact between the histones and DNA, which in turn requires an unwinding of the chromatin structure. We propose that this unwinding process is facilitated by DNA nicks as they were widespread at this stage of chromatin reorganisation. Finally, Tpl$^{94D}$, UbcD6 and SUMO were also observed to accumulate in the chromatin during this process. DNA breaks, Tpl$^{94D}$, UbcD6 and SUMO were no longer detectable when protamines were fully expressed. Thus, we propose that all these proteins and the DNA breaks act together in an unknown manner to allow chromatin remodelling.

### CTCF, a zinc finger protein which might act as a chromatin organiser during the early canoe stage

We observed that the CTCF protein is present during pre-meiotic stages in the nucleus and stays associated with the chromosomes during meiosis. After meiosis, however, we

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detected strong localisation to the nucleus during the transition from round spermatid nuclei to the early canoe stage of spermiogenesis. We speculate that CTCF might set borders in the chromatin for the histone modifications, which are characteristic of the canoe stage, such as acetylation and ubiquitylation. CTCF is visible for longer than histones and disappears together with active RNA polymerase II. CTCF might maintain chromatin accessibility to RNA polymerase II as a few genes are known to be transcribed at this time (H. White-Cooper, personal communication). In addition, transient occurrence of RNA polymerase II at the late canoe stage might require CTCF to insulate active genes from inactive ones. This idea needs to be tested in tissue-specific CTCF loss-of-function mutants; such mutants are, however, currently unavailable. CTCF expression in comparison to other features of chromatin remodelling is shown in Fig. 7.

### Histone removal is independent of protamine and Mst77F deposition

We addressed the question of whether histone removal is dependent on a signal that monitors the start of protamine and *Mst77F* mRNA translation. Both histone modification and degradation are indistinguishable from the wild-type in loss-of-function mutants of *Mst35Ba* and *Mst35Bb*, the genes encoding protamine A and B, respectively. Also in *nc3* mutants of Mst77F, histone removal is not disturbed. We conclude that N-terminal tail modification of histones and histone degradation, on the one hand, and protamine deposition, on the other, are controlled by different pathways in the cell.

### Many features of chromatin reorganisation are shared between *Drosophila* and mammalian spermiogenesis

In mammals, it is well known that after meiosis the nucleosomal conformation is lost. This is accompanied by the appearance of testis-specific linker histones (Catena et al., 2006; Martianov et al., 2005; Tanaka et al., 2005; Yan et al., 2003). So far, no linker histone variants have been identified in *Drosophila*, but variants of H2A (H2AvD) and H3 (H3.3) are known (Akhmanova et al., 1997; Swaminathan et al., 2005). In mammals, histones are hyper-acetylated before being displaced from the DNA, and phosphorylation and ubiquitylation have also been proposed to occur (reviewed by Braun, 2001). For *Drosophila*, we clearly show H2A mono-ubiquitylation and a strong increase in H4 acetylation shortly before histone removal and degradation. In mammals, histones are replaced first by transition proteins (major types: TP1 and TP2). In the investigation reported here we identified the high mobility group protein Tpl$^{94D}$, a first probable candidate for a functional homologue of mammalian transition proteins. In mammals, transition proteins are subsequently replaced by protamines leading to chromatin with a doughnut structure (reviewed by Braun, 2001; Kimmins and Sassone-Corsi, 2005; Sassone-Corsi, 2002). In *Drosophila*, we have recently shown that the sperm nucleus also contains protamines. Protamines A and B are encoded by two closely related protamine genes, *Mst35Ba* and *Mst35Bb*. In addition, the identification of Mst77F shows that sperm nuclei contain at least one further abundant chromatin component (Jayaramaiah-Raja and Renkawitz-Pohl, 2005). Moreover, in human sperm several new putative protamines have been identified by 2D gel electrophoresis and protein sequencing (Yoshii et al., 2005). In mammals, this chromatin reorganisation is essential for male fertility (reviewed by Kimmins and Sassone-Corsi, 2005). Male flies carrying the deletion *prot*Δ*38.1*, where both protamines as well as three additional ORFs are removed, show severely reduced fertility (S.J.-R. and R.R.-Pohl, unpublished).

In summary, we propose a step-by-step scheme for chromatin reorganisation (Fig. 7). First, histone modifications lead to subsequent histone removal and degradation. Second, the exposed chromatin becomes nicked, resulting in DNA breaks. Finally, Tpl$^{94D}$ deposition constitutes an intermediate stage that triggers subsequent protamine-based chromatin organisation.

Since many features concerning spermiogenesis are conserved between *Drosophila* and mammals, we propose that *Drosophila* is an ideal system to gain further insight into the mechanism of chromatin reorganisation in spermatid nuclei, a process that is crucial for male fertility.

## Materials and Methods

### *Drosophila* strains and culture

*Drosophila* flies were maintained on standard medium at 25°C. *w* was used as the wild-type strain. Visible markers and chromosome balancers are as described in FlyBase unless otherwise specified. *bel*$^{cap\text{-}1}$, *bet*$^{1}$, *bln*$^{1}$, *blw*$^{1}$, *Bsg*$^{gel}$, *cbx*$^{05704}$, *Df(3L)ri-79c*, *eff*$^{8}$, *emm*$^{1}$, *esc*$^{1}$, *gish*$^{04895}$, *gor*$^{1}$, *goul*$^{1}$, *mlt*$^{1}$, *mod*$^{07570}$, *ms(2)29F*$^{07717}$, *ms(2)42D*$^{09967}$, *ms(3)72D*$^{03957}$, *ms(3)80*$^{03817}$, *ms(3)85D*$^{03565}$, *ms(3)98B*$^{06302}$, *pea*$^{1}$, *poe*$^{01659}$, *sdl*$^{05090a}$, *tho*$^{1}$ and *tmx*$^{1}$ were obtained from the Bloomington Stock Center. *protamine-eGFP* and *prot*Δ*38.1* fly lines were generated by S.J.-R. (Jayaramaiah-Raja and Renkawitz-Pohl, 2005) (S.J.-R., unpublished). Prosα3-eGFP and Prosα3T-eGFP were kindly provided by J. Belote (Ma et al., 2002). *ms(3)nc3* was obtained from M. T. Fuller (Fuller, 1986). H3-Flag and H3.3-Flag fly lines were provided by B. Loppin (Loppin et al., 2005), and H2Av-RFP flies by C. Wenzl (Clarkson and Saint, 1999). *dbruce*$^{E81}$ flies were obtained from H. Steller (Arama et al., 2003), the *Drosophila hydei* strain were from M. Schäfer (University Kassel), and *D. pseudoobscura*, *D. mojevensis* and *D. virilis* strains from U. Homberg (University Marburg).

### RT-PCR of *tpl*$^{94D}$

Total RNA was prepared using TRIzol® (Invitrogen) from 80 testes, 40 carcass of males, 40 adult females, embryos (7-24 hours) and 40 larvae. We used the OneStep RT-PCR Kit (Qiagen) to amplify a 361 bp cDNA fragment from the open reading frame of *tpl*$^{94D}$. The chosen primer pair spans an intron of 68 bp to distinguish between RNA and DNA. As a further control for DNA contamination the same RT-PCR was performed using primers for the *β3-tubulin* gene spanning an intron as well. *β3-tubulin* is expressed in embryos, larvae, adult males and females and the testis. The same conditions were always used for the *tpl*$^{94D}$ and the *β3-tubulin* primers in RT-PCRs.

### Cloning of the *tpl*$^{94D}$*-eGFP* construct

To analyse the expression pattern of *tpl*$^{94D}$, the open reading frame (ORF) together with 446 bp upstream of the ATG start codon was cloned and fused with *eGFP*. Therefore, the ORF together with the promoter and 5′ UTR was amplified by PCR using genomic DNA and primers with linked *Kpn*I and *Xba*I restriction sites. The PCR fragment was inserted into *p*KS$^{+}$*eGFP* in frame with the *eGFP*. This clone was digested with *Kpn*I and *Not*I and the resulting *tpl*$^{94D}$*-eGFP* fragment subcloned into the germline transformation vector *p*ChabΔsal, which was opened with *Kpn*I and *Not*I (Thummel et al., 1988). Prior to the last step, *p*ChabΔsal was digested with *Xba*I to cut out the *lacZ*, and then religated.

### P-element mediated germline transformation

Transgenic fly strains were established by injection of DNA as described by Michiels et al. (Michiels et al., 1993).

### Antibodies and immunofluorescence staining

Hoechst staining was used to visualise chromatin. All antibodies were used in immunofluorescence stainings of squashed testes carried out essentially as described in Hime et al., 1996. Squashed testes treated with fluorescent antibodies were embedded in Fluoromount-G (Southern Biotech, Birmingham, AL, USA). To follow the fate of the histones, an anti-histone antibody from Chemicon (1:1200) was used. This antibody recognises H1 and all core histones of human, mouse, bovine and hamster. To study lysine methylation of histones during spermatogenesis, the following antibodies were used: anti-monomethyl-histone H3(K4), (Upstate; 1:1000; species cross-reactivity: human); anti-dimethyl-histone

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H3(K4), (Upstate; 1:500; species cross-reactivity: human and *Tetrahymena*); anti-dimethyl-histone H3(K4), (Abcam; 1:1000; species cross-reactivity: all mammals and a wide range of other species including *D. melanogaster*); anti-trimethyl-histone H3(K4), (Upstate; 1:1000; species cross-reactivity: human; also recognises to a lesser extent dimethylated K4); anti-trimethyl-histone H3(K4), (Abcam; 1:500; species cross-reactivity: all mammals and a wide range of other species including *D. melanogaster*; weak reactivity to dimethylated K4); anti-monomethyl-histone H3(K9), (Upstate; 1:250; species cross-reactivity: human); anti-dimethyl-histone H3(K9), (Upstate; 1:50; species cross-reactivity: human, fission yeast and chicken); anti-dimethyl-histone H3(K9), (Abcam; 1:200; species cross-reactivity: all mammals; recognises also di- and trimethylated K27); anti-trimethyl-histone H3(K9), (Upstate; 1:100; species cross-reactivity: human – also recognises to a lesser extent dimethylated K9); anti-trimethyl-histone H3(K9), (Abcam; 1:50), (species cross-reactivity: all mammals and a wide range of other species including *D. melanogaster*; cross reacts with trimethylated K27); anti-dimethyl-histone H3 (K27), (Upstate; 1:50; species cross-reactivity: human); anti-trimethyl-histone H3(K27), (Abcam; 1:100; species cross-reactivity: all mammals and a wide range of other species including *D. melanogaster*; cross reacts in western blot analysis to a lesser extent with trimethylated K9). To study arginine methylation of histones, anti-methyl-histone H4 (R3), (Upstate; 1:100); species cross-reactivity: human) was used (a gift from U. M. Bauer). To study acetylation of histones, anti-acetyl-histone H4 (Upstate; 1:300, a gift from M. Eilers) was used. This antibody recognises acetylated K5, K8, K12 and K16 of human and *Tetrahymena*. To study ubiquitylation and SUMOylation during the histone to protamine exchange, anti-ubiquitin (DAKO; 1:10; species cross-reactivity: human), anti-ubiquitylated-histone H2A (IgM, Upstate; 1:100; species cross-reactivity: human, monkey, rat, mouse and frog) and anti-SUMO-1 C-term (Abgent; 1:400; species cross-reactivity: *Drosophila*, human; a gift from G. Suske) was used. Active RNA polymerase II was detected with anti-RNA polymerase II (H14) (IgM, Babco; 1:50; a gift from M. Eilers; species cross-reactivity: yeast to human). UbcD6 was detected with anti-HR6A/B (1:100; recognises HR6A and HR6B in mice, directed against the conserved N terminus) (Baarends et al., 2005). Anti-CTCF antibody (C-term; 1:200) was created by Moon et al. (Moon et al., 2005). To visualise the Flag-tag, an anti-Flag antibody was used (Cell Signaling; 1:500). To visualise IgG antibodies, anti-rabbit Cy3-conjugated (Dianova; 1:100) and anti-mouse Cy5-conjugated (Dianova; 1:100) antibodies were used. To visualise IgM antibodies, a FITC-labelled goat anti-mouse IgM (Sigma; 1:100) or a Cy3-conjugated donkey anti-mouse IgM (Dianova, 1:100) was used.

### Fluorescence microscopy

Immunofluorescence and eGFP samples were examined using a Zeiss Apotome microscope equipped with appropriate fluorescence filters. Images were individually recorded and processed with Adobe Photoshop 7.0.

### TUNEL stainings

For TUNEL stainings the 'In Situ Cell Death Detection Kit, TMR Red' from Roche was used. The stainings were performed on squashed testes together with antibody stainings. After incubation with the secondary antibody, the slides were air dried and then incubated with 5 μl enzyme solution and 45 μl labelling solution for 1.5 hours at 37°C in a humid chamber in the dark. Stainings were analysed by fluorescence microscopy.

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