Chromatin condensation during *Drosophila* spermiogenesis and decondensation after fertilization

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Sunil Jayaramaiah Raja

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Erstgutachter: Prof. Dr. Renate Renkawitz-Pohl
Zweitgutachter: Prof. Dr. Guntram Suske

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Table of contents

1. Summary

Chromatin condensation is a typical feature of sperm cells. During mammalian spermiogenesis, histones are first replaced by transition proteins and then by protamines, while little is known for *Drosophila*. Here I characterize three male specific genes in the fly genome, *Mst35Ba*, *Mst35Bb* and *Mst77F*. With eGFP fusion for these above mentioned proteins, I show here that *Mst35Ba* and *Mst35Bb* indeed encode for dProtA (*Drosophila* ProtamineA) and dProtB (*Drosophila* ProtamineB), respectively and show 94% identity to each other. *Drosophila* protamines are considerably larger than mammalian protamines, but, as in mammals, both protamines contain typical cysteine/arginine clusters. Ectopic expression of both dProtA and dProtB in the salivary gland cells localizes to the nucleus. Both the protamines binds to the polytene chromosomes without any specificity for the euchromatin or the heterochromatin reflecting the binding status of protamines in sperm nucleus. Unlike in mammals, *Drosophila protamine* genes are not haplo insufficient. Screening of Zuker mutant collection did not show any mutation in both *protamine* genes, which argues for functional redundancy.

Mst77F encode a spermatid specific linker histone-like protein. The expression pattern of Mst77F overlaps the pattern of protamines as a chromatin component. Mst77F shows significant similarity to mammalian HILS1 protein. The ProtamineA-eGFP, ProtamineB-eGFP and Mst77F-eGFP carrying *Drosophila* lines show that these proteins become the important chromosomal protein components at the canoe stage in elongating spermatids and stays in mature sperm nucleus and His2AvDGFP vanishes at the canoe stage. Thus, transition from histone based nucleosomal configuration to protamine based chromatin configuration takes place at the canoe stage of spermatid development. *Mst77F* mutants (*ms(3)nc3*) are characterized by small round nuclei and are male sterile. These data suggests, the major feature of chromatin condensation in *Drosophil*a spermatogenesis corrrespond to those in mammals.

During the canoe stage of spermatid development, histone H2AvD is degraded. Here I show a novel function of UbcD1 an E2-ubiquitin conjugating enzyme that is required for H2AvD degradation. E3 ligase Cullin-1 and Cullin-3 both are expressed during *Drosophila* spermatogenesis. Cullin-1 is expressed in all early germ cells and localizes to the fusomes and degraded during meiosis. In the later stages, Cullin-1 is expressed again and localizes to the perinuclear space at the canoe stage of spermatid differentiation. Cullin-3 is expressed in the elongated spermatids.

During early fertilization steps, the paternal pronucleus still contains protamines and Mst77F, but regains a nucleosomal conformation before zygote formation. In eggs laid by *sesame* mutant females, the paternal pronucleus remains in a protamine-based chromatin status, but Mst77F-eGFP is removed, suggesting that the *sesame* gene product (HIRA) is essential for removal of protamines while Mst77F removal is independent of Sesame.

2. Introduction

2.1 Why study "Spermatogenesis" ?

Spermatogenesis provides an opportunity to explore the maintenance of the stem cell identity, cell to cell interaction, regulation of mitotic divisions and execution of meiosis. The post meiotic pathways involved in the morphogenetic process such as chromatin remodelling, axoneme formation and mitochondrial elongation to build a mature spermatozoon makes this an excellent model system to study.

2.2 Structure and cytology of *Drosophila melanogaster* spermatogenesis

The adult *Drosophila melanogaster* testis is a coiled shaped organ. The stem cells present at the tip of the testis divide asymmetrically, amplify, undergo meiosis and differentiate to form a mature spermatozoon with a head and a long tail with an axoneme. As the stem cells differentiate into gonioblast, primary spermatocyte, haploid spermatid and mature spermatozoon, the cells of these respective stages traverse down the coil from the tip of the testis as clearly visible under the phase contrast microscope (Fig 2.1 A) (Fuller 1998). In contrast the stem cells in mammals are lined at the periphery of the testis and the cells proceeds towards the lumen as it differentiates into mature spermatozoa (Fig 2.1 B).

2.3 Germinal Proliferation center

The germinal proliferation center is present at the tip of the testis. It comprises of a group of somatic cells called hub cells (Fig 2.1 B). The stem cells (16-18 in larvae and 5-9 in adults) surround the specialized somatic hub cells. These stem cells are further encompassed by a set of cyst progenitor cells. As the stem cells divide asymmetrically one of the daughter cells close to hub cell retains the stem cell identity thus self renewing the stem cell population (Fig 2.1 B). The other daughter cell which displaces away from the parent stem cell

differentiates into gonial founder cell (gonioblast) (Fig 2.1 B). The cyst progenitor cells which surrounds the stem cell divide simultaneously in a similar fashion and two cyst cells wrap around the gonioblast (Fig 2.1 B). Each gonioblast undergoes four rounds of mitotic amplification with incomplete cytokinesis to 16 primary spermatocytes. Due to incomplete cytokinesis all the 16 primary spermatocytes remain interconnected by ring canal till the individualization stage thus making the cytoplasm homogeneous (Fuller 1998).

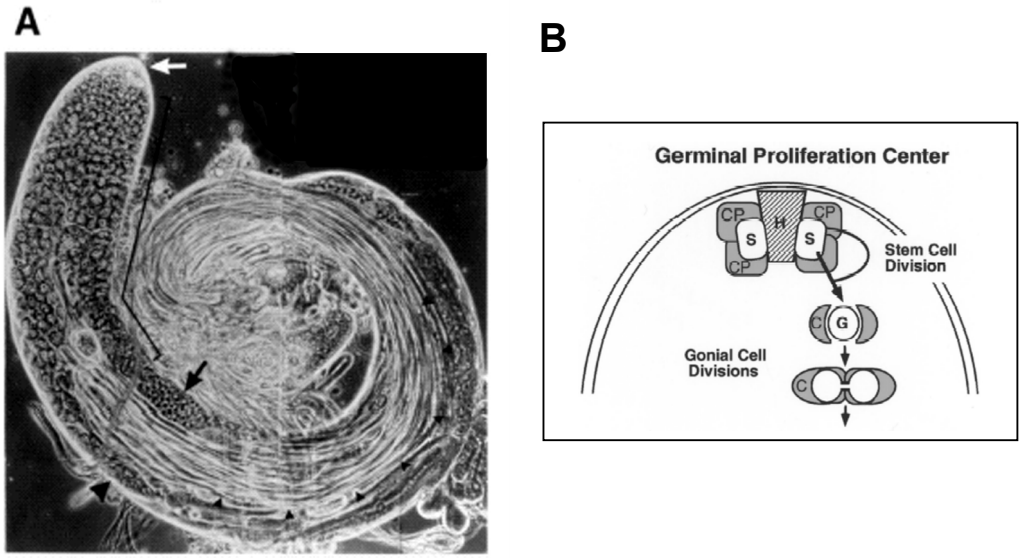


Fig 2.1. Whole testis of *Drosophila melanogaster*, a phase contrast microscopic picture. (A) Testis, coiled shape in structure. At the apical tip (white arrow) are present the male germ line stem cells surrounded by mitotically amplifying spermatogonial cells. Further towards the caudal end of the testis, the primary spermatocytes (bracket), the late primary spermatocytes (black arrow), elongated spermatids (large arrowhead). (B) Schematic representation of the germinal proliferation center, (H) denotes the somatic hub cells attached to the outer sheath of the testis, (S) stemcells surround the hub, (CP) cyst progenitor cells around the stem cells, (G) gonioblast are wrapped by the non dividing but differentiating pair of cyst cells (C).**(taken and modified from *Fuller 1993)***

2.4 Primary spermatocyte growth phase

Each interconnected spermatogonia ceases mitosis after the 4th round of mitotic amplification and then cells enter the phase of growth and gene expression in meiotic prophase I. The primary spermatocyte stage lasts for 3.5 days (Fuller, 1998) (Fig 2.2 B). During this phase the cells grow approximately 25 times in volume and undergo extensive gene expression. This switch from the mitotic cell division to cell growth and gene expression sets up the terminal differentiation program of meiosis and spermatid differentiation. Many genes are transcribed for the first time during this phase. Some genes

whose protein products are required in the post-meiotic stage are highly transcribed and the mRNAs are translationally repressed till the right time.

Each group of 16 interconnected primary spermatocytes enters the phase of meiotic cell divisions (Fig 2.2 e), giving rise to the bundles of 64 interconnected haploid spermatids at the end of meiosis. Majority of the transcription is terminated by the end of meiotic prophase. Since the main focus of this study is at the stage of spermatid differentiation and nuclear shaping, I would like to give an elaborate introduction to this part.

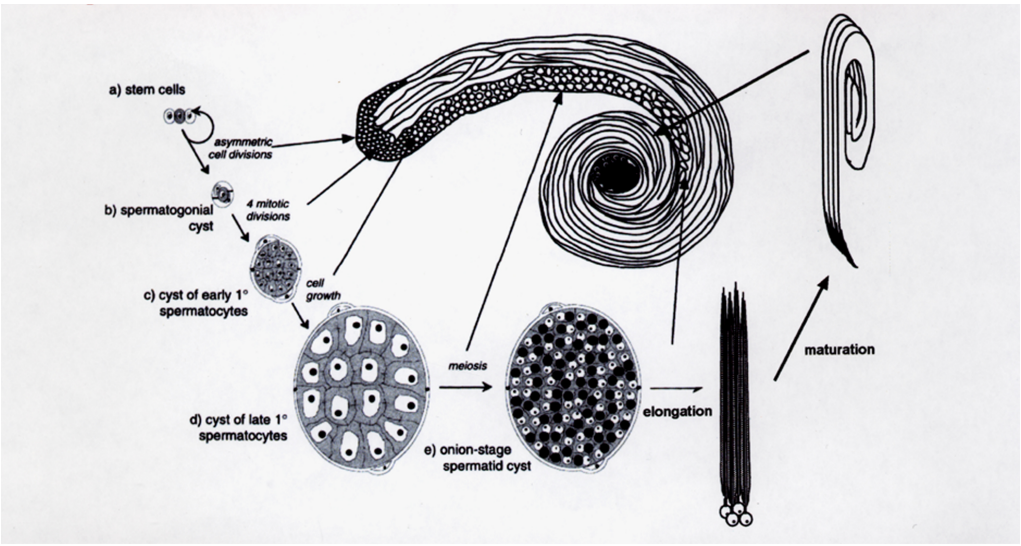


Fig 2.2. An overview of *Drosophila* spermatogenesis. Different stages of spermatogenesis in the testis coil are depicted with a magnified diagram indicated by the arrows. a) stem cells, b) spermatogonial cyst before mitotic division, c) cyst of early spermatocytes, d) cyst of late spermatocytes, e) onion stage spermatid cyst, each nucleus with an adjacent nebenkern (mitochondrial aggregate) and further each spermatid cysts elongate and individualize to form mature sperms (taken from Fuller, 1998)

2.4.1 Translational repression

Many of the gene products that are involved in the later stages of spermatid development are transcribed in the primary spermatocyte stage (Fig 2.3) (Renkawitz-Pohl *et al.,* 2005). Early transcription of these genes are crucial for spermiogenesis as the transcription ceases after the long meiotic prophase-II. These transcripts are translationally repressed till the elongated spermatid stage (Fig 2.3). The transcripts are relieved from the repression and the protein is made. The translation repression elements (TREs) responsible for the repression of these mRNAs are usually present at the 5‘UTR in case of *Drosophila.* But, in case of mammals, TREs are found to be generally present in the 3‘UTR region.

During the primary spermatocyte growth phase of meiotic prophase the cell size increases and the nucleus is highly transcriptionally active (Olivieri and Olivieri, 1965). During this time, many mRNAs are synthesized which are translationally repressed until the appropriate time during sperm morphogenesis when the encoded protein is required for spermatid differentiation. The transcription of these translationally repressed mRNAs underlies a distinct control, as it is dependent on the tissue specific $dTAF_{II}80$ homologue Cannonball (Can) in the transcription initiation complex (White-Cooper *et al.,* 1998; Hiller *et al.,* 2001). The analysis of cis regulatory elements which mediate translational repression has been pioneered by the analysis of the *Mst(3)CGP* gene family, which is proposed to encode proteins of the satellites which are homologous to the outer dense fibers in the flagellum of mammals. These mRNAs contain a translational control element of 12 nucleotides in the 5´untranslated region, named TCE, that acts in a position dependent manner (Kuhn *et al.,* 1988, Schäfer *et al.,* 1990, Kempe *et al.,* 1993). The tissue specific transcripts of the *dhod* and the *janus B* gene contain an element with sequence similarity to the TCE of the *Mst(3)CGP* gene family (Yanicostas and Lepesant, 1990; Yang *et al.,* 1995). During sperm morphogenesis the required proteins have to be synthesized in a coordinated timing. Therefore it might be expected that individual mRNAs contain distinct translational repression elements. Since the translational control is critical for spermiogenesis, several RNA binding proteins have been identified till date. Some of these proteins are made both in the female and the male germ line, whereas some are testis specific. The *boule* gene which is the homologue of the *Deleted In Azoospermia (DAZ),* is found to encode for an RNA binding protein (Eberhart *et al.,* 1996). Boule mutants show defects in the entry into meiosis due to the lack of Twine (Cdc25 protein). This was investigated with respect to the translational activation and it was found that Boule to be involved in the translational activation of *Twine* transcripts. Another RNA binding protein Rb97D is found to be associated with the *ks-1* fertility locus on the Y-chromosome and is limited to pre-meiotic stages. Mutations in Rb97D cause developmental arrest during spermiogenesis which shows defects, including arrest in the nuclear shaping (Heatwole and Haynes, 1996; Haynes *et al.,* 1997). Thus, the translational repression mechanism is an important feature required for normal spermiogenesis, which involves the repression of the transcripts in some cases and translational activation in the other cases as described above.

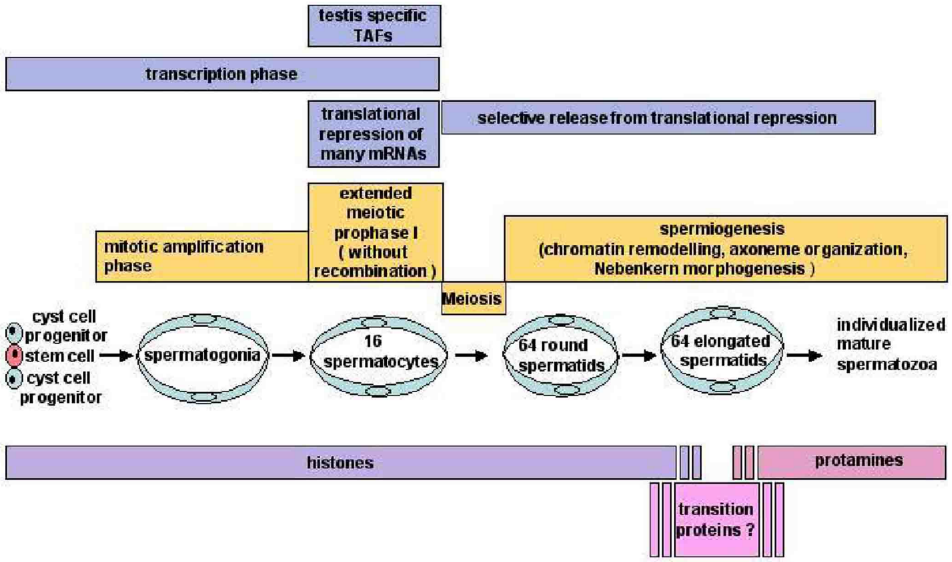


Fig 2.3 Major developmental and regulatory features during *Drosophila melanogaster* spermatogenesis. From left to right is shown the major features of transcriptional regulation phase, translational repression phase and the known chromatin remodelling aspects during sperm differentiation (taken from Renkawitz-Pohl *et al.,* 2005)

2.5 Spermatid Differentiation

After the completion of meiosis, the spermatids undergo extensive differentiation. The cytokinesis remains incomplete at this stage, now the bridges of the ring canals open up into multiple irregular connections. In mammals, persistence of this interconnection may be an important mechanism to make sure that all the individual spermatids have access to the entire diploid complement of the gene products but the transcription is ceased by this stage in flies. During this stage of spermatid differentiation the mitochondria begin to gather into a single mass and fuse.

2.5.1 Onion Stage spermatid

Individual mitochondria fuse to give rise to two gaint structures called onion-stage nebenkern (Fig 2.2). The mitochondria arranges itself into a densely packed round structure where the layers upon layers seems to be wrapped with mitochondrial membrane. When taken a cross section at this stage of spermatid differentiation, one can observe the structure similar to that of an onion and hence the name onion-stage spermatid (Fig. 2.2 e). In the next step, the

mitochondrial derivative changes from a 7 μm sphere to cylindrical shape of 1.8 mm length. The volume of the major and minor mitochondrial derivatives being the same it increases the total surface area during this transformation. Beside the mitochondrial structural modifications, there are many other organelles which at this phase undergo rapid morphological changes. The axoneme begins to grow and gets attached to the base of the nucleus, thus forming a kind of preconnecting adaptor between the prospective head and the flagellum. Golgi complexes fuse to form an acroblast at the apical side of the nucleus present opposite to the basal body. A dark round structure appears acentrically within the nucleus which is called the protein body (composed primarily of basic proteins) which gradually increase in size when it progresses to the next stage of differentiation. This dark spot serves as a useful marker for early spermatid cysts (Fuller 1993).

The nuclear size at this onion stage is directly proportional to its chromatin content. Any variation from the normal haploid content can be easily recognized by the size of the nucleus at this stage which would be easy to isolate the mutants for meiotic segregation defect.

2.5.2 Spermatid elongation and maturation

The dramatic change in shape from the onion stage to the elongated spermatids is particularly interesting (Fig 2.3). The mitochondrial derivatives (nebenkern) unwrap, split into two parts and elongate along with the growing axoneme. The chromatin condenses and the nuclear shaping takes place followed by the individualization and coiling of the mature spermatozoa.

2.5.3 Morphological changes in the mitochondrial derivative during the elongation stage

Nebenkern which is round in shape maintaining the constant distance between the layers, now begin to unfurl and the variation in this distance between the layers appear. The two gaint mitochondrial derivatives now aligns along the flagellum and give the shape of a leaf blade, which is clearly visible under phase contrast microscope (Fuller 1993). The layers at this stage are less in number and becomes thick relative to the onion stage. The cytoplasmic

inclusions containing ribosomes and other cytoplasmic components are particularly prominent at this stage. As the mitochondrial derivatives continue to elongate along the length, the flagellum becomes long and slender. The cytoplasmic inclusions gradually disappear. At this early elongation stage the spermatids assumes a parallel, bipartite mitochondrial derivatives, and round nucleus (Fuller 1993).

In the next stage of mitochondrial differentiation, the mitochondrial derivative which are roughly equal in size progress to be distinguished as minor mitochondrial derivative and major mitochondrial derivative. This is achieved by the gradual deposition of granules inside the major mitochondrial derivative. The amount of deposition of granules which gives a dense staining serves as the marker for the developmental age of spermatids.

The cytoplasmic microtubules run in parellel to the mitochondrial derivative. These cytoplasmic microtubules disappear before the individualization stage. It is speculated that these cytoplasmic microtubules might be required for the elongation of mitochondrial derivatives.

2.6 Chromatin condensation and nuclear shaping

Just prior to the deposition of the densely staining material in the major mitochondrial derivative is the onset of nuclear shaping. The nucleus which is round in shape at this stage now gradually transforms into a needle shaped nucleus. At first one side of the nucleus flattens and the other side still being curved gives a concave shaped appearance which is the intermediate stage of the nuclear shaping process. Extensive movement of perinuclear cytoplasm containing an array of microtubules occurs which further facilitates this process.

Nuclear shaping is a microtubule based event (Fuller 1993). In the wild-type situation, during the later part of flagellar elongation period, the nucleus becomes concave on one side and convex on the opposite side. The perinuclear microtubules start to accumulate both on the convex and on the concave side of the perinuclear space and later on forming a single row of laterally compacted microtubules on the convex surface. As the nuclear transformation proceeds, the chromatin becomes increasingly condensed along the inner side of the nuclear envelop opposite to the microtubules. Chromatin condensation continues as the dense chromatin accumulates with a net like appearance in the nucleoplasm and further on, the excess of the nucleoplasm is eliminated from the originally concave side of the nucleus in the form of vesicles (Fuller 1993). The chromatin continues to condense as the nuclear shaping

progress. This process of accumulation of perinuclear microtubules is required for the normal nuclear shaping. During chromatin condensation, the volume of the nucleus is reduced to over 200 fold.

2.6.1 Sperm nuclear basic proteins involved in chromatin condensation during spermiogenesis

Chromatin reorganization of the complete genome leading to compaction is an essential feature during spermiogenesis of many animals (Braun 2001, Sassone-Corsi 2002, Hecht 1998, Hennig 2003.) The switch from the nucleosomal to the condensed conformation in mammals is essential to get a more hydrodynamic sperm head, and also may protect the genome from physical and chemical damage. As this process leads to an extremely condensed state of the haploid genome in the sperm, a reorganization of the paternal genome in the male pronucleus after fertilization and before zygote formation is essential (Loppin and Karr 2005).

For mammals, it is known that the somatic set of histones are modified as these are in part replaced by specific variants during meiotic prophase. After meiosis, histones are replaced by major transition proteins TP1 and TP2 (Meistrich *et al.,* 2003), and subsequently by highly basic protamines to ensure the remodeling of chromatin to a typically highly condensed and transcriptionally silent state of mature sperm. These replacements leads to a shift from histone based nucleosomal conformation to a radically different conformation, resembling stacked doughnut structures, containing protamines as major chromatin condensing proteins and DNA (Fig 6.1). Some mammals have only one *protamine* gene (Dadoune 2003), while mice and humans have two genes encoding two different protamines, both of which are essential for fertility and are haploinsufficient (Cho *et al.,* 2001). Recently, HILS1 (spermatid-specific linker histone H1-like protein) was proposed to participate in chromatin remodelling in mouse and human spermiogenesis (Yan *et al.,* 2003, Iguchi *et al.,* 2004). The transition between histone removal and its replacement by protamines in mice and humans is characterized by small 6-10 kDa transition proteins acting as short term chromosomal proteins (Meistrich *et al.,* 2003). In mice, the transition proteins TP1 and TP2 are redundant in function. In fishes and birds, transition proteins are missing and protamines directly reorganize the chromatin. In annelids and echinoderms, the nucleosomal configuration is maintained in sperms (Wouters-Tyrou *et al.,* 1998, Lewis *et al.,* 2003) while protamine-like proteins have been described for mussels (Lewis *et al.,* 2002). These

protamine-like proteins lack the typical high cysteine content necessary for disulfide bridges (Chapman *et al.,* 2003). Therefore a doughnut-type chromatin structure as in mammals is unlikely to occur in mussels. Lewis and Ausió (Lewis *et al.,* 2002) propose that the protamine-like proteins in mussels belong to the histone H1 family. The sperm chromatin of mussels contain core histones and thus a nucleosomal configuration, but histone H1 is replaced by protamine-like molecules which organise the higher order structure of the chromatin.

For *Drosophila*, chromatin reorganization after meiosis has not been studied so far, at the molecular level. At the light microscopic level, the *Drosophila* spermatid nucleus is initially round after meiosis and then is shaped to a thin needle-like structure with highly condensed chromatin, so that the volume of the nucleus is condensed over 200 fold (Fuller, 1993). Whereas, in mammals, the volume of the nucleus is reduced over 20 fold (Braun, 2001). In the mature sperms of *Drosophila,* core histones are not detectable by immunohistology (Akhmanova *et al.,* 1997). There is histochemical evidence for presence of very basic proteins in sperm (Das *et al.,* 1964).

2.6.2 Don Juan as a candidate for basic chromosomal protein

Drosophila don juan gene is located in the right arm of the second chromosome at 84B position and has two exons and a promoter of approximately 450bps (Santel *et al.,* 1997). *don juan* is testis specifically expressed and encode a protein which is lysine rich and basic in nature with an approximate molecular weight of 29kDa. It contains a mitochondrial localization signal at the N-terminus after 17 amino acids and a long nuclear localization signal at the C-terminus (Santel *et al.,* 1998). Immediately in front of the mitochondrial localization signal is present a predicted protein cleavage site as shown in Fig 2.4 B. It also carries an unusual lysine rich DPCKKK sequence in the C-terminal region which is repeated eight times (Fig 2.4 B). The biological significance of this repeat along with the overall function of Don Juan is being investigated.

don juan is transcribed in the primary spermatocytes. The transcript is translationally repressed by an unknown RNA binding protein at the 5` UTR. The transcript is relieved from repression at the post-meiotic stage and is translated into a functional protein (Blümer *et al.,* 2002).

Besides these it is very important to get mutants for *don juan* to know the role of Don Juan during *Drosophila* spermatogenesis. Previous studies have shown that the peptide antibody staining overlaps with the chromatin staining in the nucleus. Moreover Don Juan shows considerable structural homology to histone-1 protein, so it could possibly bind to DNA. Because of its binding capacity it was initially isolated (Santel *et al.,* 1997). It is expressed during elongated spermatid stage (Santel *et al.,* 1998).

Fig. 2.4 (A) The *don juan* gene structure. (B) The predicted protein sequence. IRPHHI = mitochondrial localization signal. Green underscore = nuclear localization signal. Arrow = predicted protein cleavage site. (according to Santel *et al.,* 1997, 1998)

It is known that Don Juan localizes both to the nucleus and mitochondria. The antibody staining done in our lab in the previous work has given the evidence that Don Juan could play a role both in nucleus and mitochondria. The peptide antibody raised against the N-terminal amino acid sequence recognizes Don Juan mainly in the nucleus. The peptide antibody raised against the C-terminal recognizes Don Juan both in nucleus and in mitochondria (Santel *et al.,* 1998).

Moreover *don juan* mRNA is expressed in the primary spermatocyte stage where large number of genes are transcribed at this stage. The mRNA is subjected to translational repression at the primary spermatocyte stage. This translation repression is mediated by an unknown RNA binding protein which binds to the 5` UTR of *don juan* mRNA and does not give an access to the ribosomal proteins to translate the mRNA. The sequence responsible for this repression in mRNA has been well characterized by the previous research done in our lab

(Blümer *et al.,* 2002). This translational repression is relieved at the post-meiotic stage and the protein is made. However, the role of Don Juan in spermiogenesis is not clear.

2.6.3 Candidate genes for protamines and spermatid specific linker histone variants

It still remains an open question whether histones are replaced by protamine-like basic proteins in *Drosophila*. The analysis of the *Drosophila* genome sequence (Adams *et al.,* 2000, Celniker *et al.,* 2002.) revealed that the proteins encoded by two genes show similarity to mammalian protamines, for which the male-specific transcripts *Mst35Ba* and *Mst35Bb* have been found (Andrews *et al.,* 2000) and proposed previously to encode for protamine-like proteins (Ashburner *et al.,* 1999). Another male specifically transcribed gene, *Mst77F*, is a distant relative of histone H1/H5 (linker histone) family and has been proposed to play a role either as a transition protein or as replacement protein for compaction of the *Drosophila* sperm chromatin (Russell and Kaiser., 1993). The main focus of my thesis is to study the role of *Mst35Ba, Mst35Bb* and *Mst77F* genes during spermiogenesis.

2.7 Ubiquitin mediated protein degradation in *Drosophila*

The protein degradation pathway mediated by ubiquitination is highly conserved in all eukaryotic species. Ubiquitin (Ub) is a small peptide of 76 amino acids in length which is covalently conjugated to the substrates that are targeted for degradation. The E1 activating enzyme forms a high-energy thioester bond with Ub by consuming ATP and transfers Ub to E2 conjugating enzyme (Fig 2.5). Ub from Ub-E2 is transferred to the substrate recognized by E3 ligases and polyubiquitinates the substrate. Most of the known E3 ligases are either HECT domain or RING finger containing proteins. In the case of RING finger containing E3 ligases, the ligase interacts with both Ub-E2 and the substrate for ubiquitination (Fig 2.5). HECT domain containing E3 ligases first shifts the Ub from E2 to E3 and then covalently conjugates Ub to the substrate (Fig 2.5). Many RING ligases are in complexes, such as SCF, VBC and APC complexes, that contain the core module to bind Ub-E2 and an adaptor for substrate binding to polyubiquitinate. The polyubiquitinated chains are recognized by the 26S proteosome for subsequent proteolysis. Ubiquitination is reversible by deubiquitinating

(DUB) enzymes (Fig 2.5), which are thiol proteases that cleave polyubiquitin chains or ubiquitin conjugated proteins. The Ub degradation pathway can be regulated in the process of substrate recognition, Ub conjugation, deubiquitination and recruitment to the 26S proteosome.

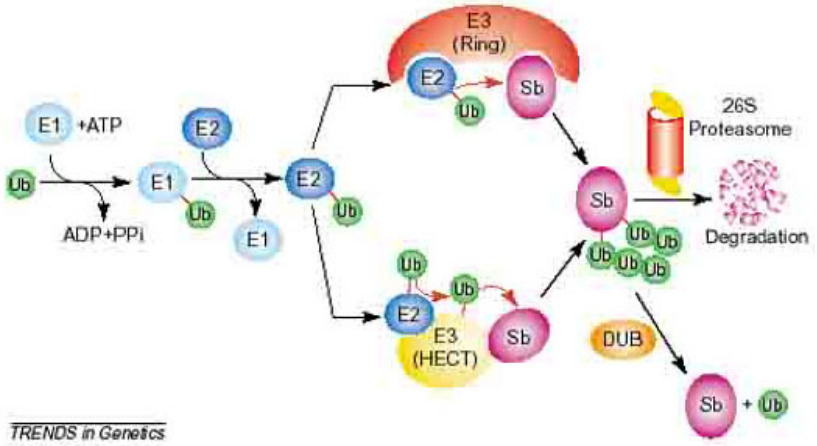


Fig. 2.5 Ubiquitin mediated protein degradation pathway. Ubiquitin (Ub) is covalently linked to E1 enzyme by using ATP and the Ub is transferred to E2 conjugating enzyme. Ub-E2 forms a complex with either RING finger or the HECT domain containing E3 ligase. RING fingure E3 ligase directly transfers the Ub to the substrate from Ub-E2, where as in case of HECT domain containing E3 ligase, the Ub is shifted to E3 first and then linked to the substrate. The substrate is polyubiquitinated and subjected to degradation by 26S proteosome. In some cases the deubiquitinating enzymes (DUB) removes the polyubiquitin chains from the substrates (taken from *Ou et al., 2003*).

2.7.1 SCF complex (Skp/Cullin/Roc1/F-box)

The SCF complex consists of four major components: Skp1, Cdc53/Cul1, Rbx1/Hrt1/Roc1 and an F-box protein. Cul1 is a scaffold protein, its C-terminus interacts with Roc1 which recruits Ub-E2, the N-terminus interacts with Skp1 which inturn binds to the conserved F-box domain of an F-box protein. The major role of F-box protein is substrate recognition. Usually one F-box protein recognizes several substrates for ubiquitination. The binding between the F-box protein and the substrate requires substrate phosphorylation.

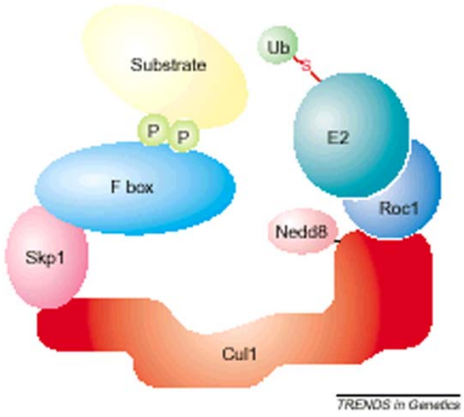


Fig. 2.6 SCF complex. Cullin-1 is a scaffold protein which binds to Roc1 at the C-terminus and an E2 conjugating enzyme bound to ubiquitin, the N-terminus binds to Skp1 with F-box protein. F-box protein recognizes the phosporylated substrate for ubiquitination (taken from *Ou et al., 2003*).

2.8 Individualization

Individualization is a process where in, from the bundle of 64 spermatids each spermatid detatches from the adjacent spermatid. The individualization complex (IC) is formed at the tip of the nucleus which is actin based, and the cystic bulge progresses along the entire length of the spermatids initiating from the head region and proceeds to the tip of the flagellum forming a waste bag and is eventually pinched off. During this process the nucleoplasm and the spermatid cytoplasm is expelled out and each spermatid is packed with its own plasma membrane thus completing individualization. The process of individualization is coupled to apoptosis like mechanism. The movement of IC is dependent on the activation of the caspases. This activation of the caspases leads to the degradation of the collected excess of the cytoplasm in the waste bag but, the sperm nucleus is protected from the caspase activity by an E2 ubiquitin conjugating enzyme dBruce (Arama *et al,* 2003).

Following individualization, the mature sperm is free to move and finds its way to the seminal vesicle.

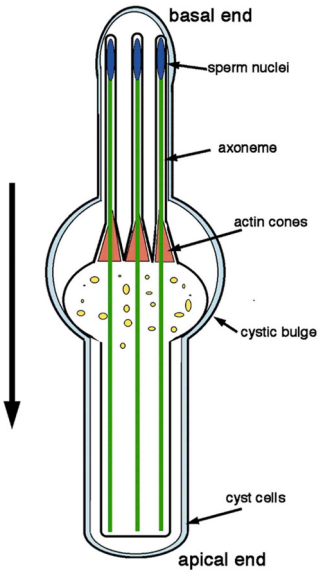


Fig. 2.7. An individualizing cyst. Individualization takes place at the cystic bulge. The membrane reorganization occurs as the actin cone (red triangle) moves down along the sperm axoneme from its head to the end of the tail. Arrow indicates the direction of movement. Cytoplasm and membranous organelles are squeezed out and accumulate in the cystic bulge as the actin cones move down the cyst. At the end of individualization all cytoplasm and organelles are discarded as a waste bag and 64 thin individual sperm are formed (taken from *Nagouchi et al.,2003*)

2.9 Fertilization in *Drosophila*

During spermiogenesis, following the meiotic divisions, the round spermatids gradually transforms into elongated spermatids aquiring a long flagellum with an axoneme and a needle shaped head region with an highly condensed chromatin as described above. Sperm bundles individualize and give rise to motile sperms. These mature sperms move to the seminal vesicle which are ready to fertilize the egg.

In *Drosophila*, fertilization occurs in the uterus, shortly after egg ovulation. The two different organs in the female genital tract, the spermatheca and the seminal receptacles store a relatively small number of sperms. Usually eggs are deposited shortly after fertilization. In *Drosophila*, the diploid zygote formation occurs very rapidly and it takes only about 17 mins, from the sperm entry to the end of the first zygotic division (Loppin and Karr 2005). The mature *Drosophila* oocyte is arrested in the metaphase of the first meiotic division. Ovulation is the signal for the female meiosis to resume and is concomitant with the sperm entry. Next, the second meiotic division continues giving rise to four haploid nuclei in the egg. One of the nuclei is determined to become the female pronucleus and the three remaining nucleus form the polarbodies. The polar bodies eventually fuse into a triploid nucleus and are arrested at a pseudometaphase state at the egg periphery. The polar body that appears as star like structures does not participate in the development of the embryo.

The mature sperm nucleus in *Drosophila* is extremly compacted with a needle shape structure as it enters the egg. The needle shape structure rapidly transforms into a round male

pronucleus. The nuclear basic proteins that are used for condensing DNA during spermiogenesis, are rapidly removed and the maternal histones are incorporated into the male pronucleus. This transformation of the male pronucleus from the needle shaped highly condensed form to a replication competent male pronucleus is absolutely criticle for the zygote formation. Following the resumption of the histone based nucleosomal conformation from the protamine based conformation, the competent male pronucleus fuses with the female pronucleus thus initiating the first mitotic division.

2.10 Questions to be addressed

The main interest in this thesis focuses on the chromatin reorganization during *Drosophila* spermiogenesis. The questions addressed are as follows:

1) What is the role of *don juan* in spermiogenesis?
2) Are *Mst35Ba* and *Mst35Bb* encoding protamines?
3) Is *Mst77F* encoding a spermatid specific linker histone variant or protamine?
4) When is the histone based nucleosomal configuration replaced by protamine based configuration?
5) Are protamine *mRNAs* translationally repressed like in mammals?
6) Are protamine genes haploinsufficient in *Drosophila* like in mammals?
7) Is Mst77F essential for male fertility?
8) Can protamine substitute for Mst77F?
9) What is the mechanism of histone degradation?
10) What is the role of HIRA (sesame) in removal of Protamines and Mst77F from the male pronucleus before zygote formation?

With the genetic accessibility in *Drosophila melanogaster* it is feasible to study the various interesting aspects of the molecular mechanisms involved in chromatin condensation process during the spermatid terminal differentiation. The exact molecular mechanisms underlying during this process, such as the histone displacement, degradation and incorporation of protamines togather with the role of linker histone variants are poorly understood. Moreover, after fertilization it is critical for the male pronucleus to regain the nucleosomal conformation by displacement and degradation of chromatin condensing proteins and incorporation of the maternal histones on to the chromatin in the male pronucleus. This study reports a few of these molecular mechanisms unraveled, with respect to the histone displacement, degradation, and incorporation of protamines during spermiogenesis and removal of protamines and linker histone varient Mst77F from the male pronucleus.

3. Materials

3.1 Instruments

Digital Camera	Polaroid
Gel Electrophoresis apparatus	University of Marburg
Fotomicroscope	Zeiss
Capillary extracting equipment	vertical pipette puller 720
Cool centrifuge	Heraeus Megafuge 1.0 R
Microinjector	5242 Hermle ZK 401, Eppendorf
Micromanipulator	Leitz
Photometer	Ultraspec, Pharmacia
PCR-Machine	Personal Cycler, Biometra
Table top centrifuge	Biofuge 13, Heraeus
UV-Transilluminator	Spektroline TS-302
Vediocamera and printer	Biotech-Fischer
Confocal Laser Scan Microscope	TCSSP2 Leica, Heidelberg
Magnetic stirrer	Variomag, H+P Labortechnik
pH-Meter	Ultrospec 3000, Pharmacia, Freiburg
Stereomicroscope	Stemi SV, Zeiss, Jena
Thermoblock	Driblock DB.2A, Techne
UV Crosslinker	UV Stratalinker™ 2400, Stratagene, La Jolla USA
Vaccum Centrifuge	Savant SVC 199 H
Vortex Machine	MAGV, Rabenau, Londorf
Water bath 37°C	Julabo U3

3.2 Chemicals and growth media

Acrylamide	Serva, Heidelberg
Adenosintriphosphat (ATP)	Roche, Mannheim
Agarose	GibcoBRL, Eggenstein
Ammoniumpersulfate (APS)	Merck, Darmstadt
Bacto-Agar	Difco, Eggenstein
Bacto-Trypton	Difco, Eggenstein
Borsäure	Fluka, Neu-Ulm

5-Bromo-4-chloro-3-indolyl-Phosphat (BCIP, X-Phosphat)	Boehringer, Mannheim
5-Bromo-4 chloro-3-indoxyl-β-D-thiogalactoside (X-Gal)	Roth, Karlsruhe
Bromphenolblue	Merck, Darmstadt
3,3'-Diaminobenzidintetrahydrochloride (DAB)	Sigma, Deisenhofen
Di-Natriumhydrogenphosphate	Merck, Darmstadt
Diethylether	Roth, Karlsruhe
Digoxygenin	Roche, Mannheim
1,4-Dithio-L-threitol (DTT)	Fluka, Neu-Ulm
Aceticacid	Fluka, Neu-Ulm
Ethanol	Roth, Karlsruhe
Ethidiumbromide	Sigma, Deisenhofen
Ethylendiaminotetraessigsäure (EDTA)	Roth, Karlsruhe
Formaldehyde	Merck, Darmstadt
Formamide	Merck, Darmstadt
Glucose	Merck, Darmstadt
Glutaraldehyde	Sigma, Deisenhofen
Glycine	Roth, Karlsruhe
Glycogen	Fluka, Neu-Ulm
Harnstoff	Roth, Karlsruhe
Heparin	Sigma, Deisenhofen
Heptane	Fluka, Neu-Ulm
Isopropanol	Roth, Karlsruhe
Calciumacetate	Merck, Darmstadt
Calciumhydroxide	Fluka, Neu-Ulm
Levamisol	Sigma, Deisenhofen
Magesiumchloride	Merck, Darmstadt
Magnesiumsulfate	
Methanol	Roth, Karlsruhe
MOPS (Morpholinopropansulfonsäure)	Roth, Karlsruhe
Sodiumacetate	Roth, Karlsruhe
Sodiumchloride	Roth, Karlsruhe
Sodiumdihydrogenphosphate	Merck, Darmstadt
Sodiumdodecylsulfate (SDS)	Roth, Karlsruhe
Sodiumhydroxide	Fluka, Neu-Ulm
4-Nitrotetrazoliumchloride (NBT)	Roche, Mannheim
N,N'-Methylenbisacrylamide	Serva, Heidelberg
Octylphenolpolyethylenglycolether (Triton X-100)	Serva, Heidelberg
Piperazin-N,N'-bis [2-ethanolsulfonsäure] (PIPES)	Sigma, Deisenhofen
Phenol	Merck, Darmstadt
Polyoxyethylensorbitanmonolaurat (Tween 20)	Sigma, Deisenhofen
Propionsäure	Sigma, Deisenhofen
Ribonucleotide	Roche, Mannheim
Bovineserumalbumin (BSA)	Roth, Karlsruhe
Salzsäure	Roth, Karlsruhe
N,N,N',N'-Tetramethylendiamine	Sigma, Deisenhofen

(TEMED)	
Thioglycolsäure	Sigma, Deisenhofen
Wasserstoffperoxid	Merck, Darmstadt

3.3 Antibodies and Antiserum

Anti-Digoxygenin-Fab-Fragment	Roche, Mannheim
Anti-GFP Polyclonal made in Rabbit 1:300	Abcam, Cambridge
Anti-myc Monoclonal made in mouse 1:100	Roche, Mannheim
Anti-Rabbit IgG, Cyanine3-conjugated Dilution 1:100	Dianova, Hamburg
Anti-Mouse IgG, Cyanine3-conjugated Dilution 1:100	Dianova, Hamburg
Anti-Mouse IgG, biotinylated Normal Goat serum	Vector Laboratories, Vector Laboratories, Burlinghame
Anti-Cullin-1 antibody (Polyclonal antibody made in rabbit)	Gift from Fillipova

3.4 Molecular biological reagents and kits

DIG-DNA-Labeling-Kit	Roche, Mannheim
Elutip-D	Schleicher & Schüll, Dassel
GFX™ PCR DNA and Gel Purification Kit	Pharmacia, Freiburg
Mass Ruler DNA-Ladder, Mix	MBI Fermentas, St. Leon-Roth
Mass Ruler DNA-Ladder, Low Range	MBI Fermentas, St. Leon-Roth
Oligotex® mRNA Mini Kit	Qiagen, Hilden
OneStep RT-PCR Kit	Qiagen, Hilden
RNeasy RNA Kit	Qiagen, Hilden
TOPO TA Cloning-Kit	Invitrogen, Groningen
Tyramide Amplification Signal (TSA)	New England Nuclear Life
System Fluorescein	Science Products

Vektastain Elite ABC Standard Kit	Vector Laboratories, Burlingame, USA
Zero Blunt TOPO PCR Cloning Kit	Invitrogen, Karlsruhe
Jetstar Plasmid kit 2.0	genomed, Bad Oeynhausen

3.5 Enzymes

Desoxyribonuclease I	Roche, Mannheim
Klenow Polymerase	Amersham, Braunschweig
Lysozyme	Serva, Heidelberg
Proteinase K	Roche, Mannheim
Restriction endonucleases	Amersham, Braunschweig Roche, Mannheim
RNaseA	Roche, Mannhein
Taq DNA Polymerase	Qiagen, Hilden, Roche, Mannheim
T4-DNA-Ligase	Roche, Mannheim

3.6 Other Materials

Bleach	Colgate Palmolive Gmbh, Hamburg
Hybond N-Membrane	Schleicher & Schüll, Dassel
Whatmanpaper (Blotting-Papier GB 2000)	Schleicher and Schüll, Dassel

3.7 Plasmids

pBluescript IIKS^{+}	Klonierungsvektor, Stratagene, Heidelberg
pChabΔSal	P-Element mediated transformation vector for *Drosophila melanogaster* (Thummel *et al.,* 1988)
pCR II-Topo	Cloningvector, Invitrogen, Karlsruhe
pCR-Blunt	Cloningvector, Invitrogen, Karlsruhe

pπ25.7wc	Helperplasmid for P-Element-Transformation encoding P-Element-Transposase, (Karess and Rubin 1984).
pUAST	Transformation vector containing 5 GAL4 binding sites before the multiple cloning site (Brand und Perrimon, 1993)

3.8 Fly stocks

white	w^1,+ laboratory breed of the working group Renkawitz- Pohl, Marburg
CSTM	*w*; CyO/Sp; Sb/Ubx* Multiple balancer used for the localization of the P-element.
Sgs 58 AB	GAL4-Driver-Line, under the control of *Sgs4* regulatory element, obtained from A. Hoffmann, Berlin. (unpublished)
Boule^1/TM3	recessive male sterile mutant with the P-Element-insertion in *boule* (Eberhart *et al.,* 1996)
Rb79D^1	*ry^{506} P{PZ}Rb97D^1/TM3, ryRK Sb^1 Ser^1* recessive male sterile mutant with the P-element insertion in *Rb97D* (Heatwole and Haynes 1996) ; from Susan Haynes
can^{12}/TM3	*can^{12}/TM3.Sb^1* recessive male sterile mutant with the P-Element-insertion in *cannonball* (Hiller *et al.,* 2001)
H2AvD-GFP	*w^{1118};P{His2Av$^{T:Avic\GFP-S65T}$}/TM3,Sb^1* Construct: A 4.1kb BglII genomic fragment containing the *His2Av* gene tagged at the 3' end of the open reading frame (ORF) with *T:Avic\GFP^{S65T}*. The last two codons of the *His2Av* ORF have been removed and replaced with an additional 5 codons before the 5' end of the *T:Avic\GFP^{S65T}* ORF. (*Clarkson and Saint,* 1999)
sesame (ssm^{185b})	*ssm^{185b} /FM7c* is an EMS induced mutant allele with a maternal effect mutation in the gene HIRA (*Loppin et al.,* 2003)

Bloomington, Line 0011768	*cn[1] P{PZ}cbx[05704]/CyO; ry[506]* recessive male sterile mutant with the P-Element-insertion in *crossbronx* (Castrillon *et al.,* 1993)
Bloomington, Line 10219	*w[1118]; P{w[+mC]=lacW}l(3)L2100[L2100]/TM3,Ser[1]* Insertion in coding region of gene *CG 1965* (Homozygous lethal and this P-Element does not mobilise)
EP3393	*w[1118]; P{w[+mC]=lacW}l(3)L3393[L3393]/TM3,Ser[1]* Insertion in promoter region of gene *Alhambra* (Homozygous lethal)
Balancer used for local hop	*Dr/Tm3,Sb[1],tld[17] or Tm3,Ser[1]*
Pka-R1[c06969]	*w[1118];PBac{PB}Pka-R1[c06969]/TM6B,Tb[1]* is a piggyBac insertion in the promoter region of *Mst77F* (Thibault *et al.,* 2004)
ms(3)nc3	*ms(3)nc3, red[1], e[1]/TM3, Sb[1]* is a second-site non complementation (nc) mutation that was isolated in an EMS screen to identify the interacting proteins involved in microtubule function in *Drosophila* (Fuller *et al*., 1989).
Bloomington, Line 0003127	*Df(3L)ri-79c/TM3, Sb[1]* with the breakpoints 077B-C;077F-78A
Bloomington, Line 0002052	*Df(3L)rdgC-co2, th[1] st[1] in[1] kni[ri-1] p[p]/TM6C, cu[1] Sb[1] Tb[1] ca[1]* with the breakpoints 077A01;077D01
Bloomington, Line 0005878	*Df(3L)ri-XT1, ru[1] st[1] e[1] ca[1]/TM3, Ser[1]* with the breakpoints 077E02-04;078A02-04
Bloomington, Line 0007828	*w[1118];Df(2L)Exel8033,P+PBac{XP5.WH5} Exel8033/ CyO* with the breakpoints 35B1;35B8
Bloomington, Line 0007742	*w[1118]; Df(3R)Exel6275, P{XP-U}Exel6275/TM6B, Tb[1]* with the breakpoints 88D1;88D7
FRT Cullin1	*FRT[42] Cul1[EX]/Cyo* a deletion that removes sequences encoding Cullin-1 from aa 1 to 90. recombined on FRT chromosome (Ou *et al.,* 2002)
FRT Nedd8	*Nedd8[AN015]FRT[40A]*/Cyo encode a C-terminus-truncated protein by a nonsense mutation at aa 49 recombined on FRT chromosome (Ou *et al.,* 2002)

FRT Cullin3(guftagu)	*Cul3*^{gft2} *FRT*^{40A}*/Cyo* EMS induced allele, functions like genetic null, recombined on FRT chromosome (Ou *et al.,* 2002)
eff^{8}	*ry*^{506} *P{PZ}eff*^{8}*/TM3, ry*^{RK} *Sb*^{1} *Ser*^{1} recessive male sterile allele, with a P-Element insertion in the gene effete encoding UbcD1 E2-ubiquitin conjugating enzyme.

3.9 Bacterial strain used for transformation and culture (*Escherichia coli*)

DH5α	*supE*44, Δ*lacU*169 (Φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1

3.10 Liquid Culture

LB-Medium (LuriaBertani)

1	% (w/v) Bactotrypton
0.5	% (w/v) Yeast extract
85.5	mM NaCl

3.11 Synthetic oligonucleotide

Synthetic Oligonucleotides were used as primers for the sequence-specific DNA amplification in PCR reactions. They were referred by the company MWG-Biotech (Ebersberg).

1) myc BamHI	5‘-GGATTCTTTAAAGCTATGGAG-3‘
2) myc-Stop+SpeI	5‘-ACTAGTAGGCTCGAGAGGCC-3‘
3) DJSCFMSp	5‘-ACTAGTATGTTTAAGAGAACC-3‘
4) DJStop+tra+Sp	5‘-ATAGTATATTTATTTACTAGT-3‘
5) DJ-stop+Sp	5‘-ACTAGTCTTTTTCTTGCATTT-3‘
6) myc start+frame dj	5‘-ACTAGTTTTAAAGCTATGGAG-3‘
7) myc+stop+Spe	5‘-ACTAGTTCACTATAGTTCTAG-3‘
9)DJSTOP+TRA+SP	5‘-ACTAGTACCATGACCGTCCTGAC-3’
10) Myc-XbaI	5‘-GGGGTCTAGAGAGGTCGCCCAAGCT-3‘
11) Dj (-) stop + BamHI	5‘-GGGGGGATCCCTTTTTCTTGCATTTGTC-3‘
12) Myc+stop+XbaI	5‘-GGGGTCTAGATCACTAGAGGTCGCCCAAGC-3‘
13) MYC-XHOI	5‘-GGGGCTCGAGGTCGCCCAAGCT-3‘
14) DJ-XHOI	5‘-GGGGCTCGAGATGTTTAAGAGAACC-3‘
15) DJ-KPNI	5‘-GGGGGGTACCACCATGACCGTCCTGAC-3‘
16) MYC-ECORI	5‘-GGGGGAATTCATTTAGGTGACACTATA-3‘
17) SPEI-DJ-ATG	5‘-GGGGACTAGTCTCGAAATGTTTAAGAGAACCGC-3‘

18) DJstop-SpeI	5‘-GGCGACTAGTCCGCTTGGGGTTCATGCTC-3‘
21) 35Ba(-)Stop+BamHI	5‘-GGCGGATCCGTATTGCTGGCAAATCCGTCGGCG-3‘
22) 35Bb(-)Stop+BamHI	5‘-GGCGGATCCGTATTACTTGCAAATCCGTCGGCG-3‘
23) F-35Ba-BamHI	5‘-GGTGGGATCCGAACCGCCACTTAGGCTCCCACAC-3‘
24) F-35Bb,Ba-ATG	5‘-GGCGGATCCATGAGTTCAAATAATGTAAAT-3‘
25) F-35Bb-BamHI	5‘-GGTGGGATCCGTCTACTCTCCCTGTGTGCGTGCC-3‘
26) Re-35Ba-XbaI	5‘-GAGGTCTAGATTCGAAACAAGACCGCTTCAGCTT-3‘
27) Re-35Bb-XbaI	5‘-GAGGTCTAGATTTGTATGGTATGTTGTTCTGTA-3‘
28) Re35Ba,BbProPstI	5‘-GGGGCTGCAGCGTAGAAAATTTTTACAAACTCTG-3‘
29) Dj(+)Stop-speI	5‘-GGGGACTAGTTCACTACTTTTTCTTGCATTTGTC-3‘
30) Bb(-)Stop+BamHI	5‘-GGCGGATCCCTTGCAAATCCGTCGGCGCTTGTG-3‘
31) Fr-Bb-intron-I	5‘-GGCGCTCGAGTTAAGTGAAATAAAACGAATTAC-3‘
32) Ex1Int1RevDj	5‘-GGGGGGATCCTCTGTAAAGTAGTAT-3‘
33) Int1-dj-BamHI	5‘-GGATCCATCTGTAAAGTAGTATTAACAGTAACAG-3‘
34) 35Ba-Pr-F-EcoI	5‘-GAATTCGATTCTAGGATGTTGGAATAGCG-3‘
48) Ba-rev-NcoI	5‘-GCCGCCATGGATTGCTGGCAAATCCGTCGGC-3‘
49) Baba-pr-KpnI	5‘-GGGGGGTACCACAAGCGCCGACGGATTTGC-3‘
50) Bebe-Pr-KpnI	5‘-GGGGGGTACCGTCTACTCTCCCTGTGTGCGT-3‘
51) Bebe-Rev-NcoI	5‘-GCCGCCATGGTGCAAATCCGTCGGCGCTTGTGG-3‘
52) DJ(-)Stop-NcoI	5‘-GCCGCCATGGTTTTCTTGCATTTGTC-3‘
53) Ba-Pro-KpnI-new	5‘-GGGGTTACCATTTTCATTGATTTGATACCATTCG-3‘
54) Bb-sq-pr-1	5‘-GCCGTATACCCCTTATACCG-3‘
55) Bb-sq-pr-2	5‘-CCCACGCCTTGTAATGACGTT-3‘
56) Gfp-sq-pr	5‘-GATGCCGTTCTTCTGCTTGT-3‘
57) Ba-sq-pr-5	5‘-GGCGTAGGTTTTGCCGTATAC-3‘
58) Ba-sq-pr-6	5‘-GGCAGCAAGCGCGGGCAATTA-3‘
59) Ba-sq-pr-7	5‘-CCTGCCCTCGTTGGCTTTTAA-3‘
60) CG4480-Fw-EcoRI	5‘-GGGGAATTCCACAGGAGAATCACCCAGGGACCA-3‘
61) Rev-CG4480-NcoI	5‘-GCGGCCATGGAGTTACGACCGCCCAGGGCATAGA-3‘
62) CG4480(1)FW-EcoRI	5‘-GGGGAATTCCACAGGTGCCACAAACAGTTAAAT-3‘
83) Bam-(Ba)-F-EST	5‘-GGGGGATCCTTCCAAAAGTCTTATTTTTATGCTCT-3‘
84) Pst(Ba)-F-Pro	5‘-GGGCTGCAGATTTTCATTGATTTGATACCATTCG-3‘
85) Bam(Ba)Rev-Pro	5‘-GGGGGATCCACGAACACTTGACAAACACATGGTT-3‘
86) Bb297Eco-1-F	5‘-GGGGAATTCGCCACACCGAGTTTGTAAGCC-3‘
87) Bb297Bam-1-R	5‘-GGGGGATCCTACCAAGTTACAACTAATGTTTAA-3‘
88) Bb229Eco-1-F	5‘-GGGGAATTCAAAAATTGTTTCACATAATTTATGCC-3‘
89) Mst77f-F-E1-250	5‘-GGGGAATTCCGCGTTACTCAGCTAGTCGGA-3‘
90) Mst77f-R-Xb	5‘-GGGTCTAGACATCGAGCACTTGGGCTTGGA-3‘
91) eGFP-F-Xb	5‘-GGGTCTAGAATGGTGAGCAAGGGCGAGGAG-3‘
92) eGFP-R-Not	5‘-GGGGCGGCCGCTTTACTTGTACAGCTCGTCCA-3‘
95) Bam_rev_Bb_6	5‘-GGCGGATCCGTACCAAGTTACAACTAATGTTT-3‘
96) EcoRI_Bb_Fw_6	5‘-GGCGGAATTCCGTCGTTTCGATCAAATCTAAC-3‘

3.12 Sequencing DNA

Sequencing was done by the company Seqlab GmbH, Goettingen for all the DNA samples.

4. Methods

I *Drosophila melanogaster* culture

Drosophila melanogaster stocks were maintained at 25°C all the time unless otherwise stated. The stocks were raised and maintained on yeast/glucose/maize medium (15 L ddH2O, 150 g Bacto-Agar (DIFCO), 1.1 kg D-glucose (Sigma), 700 g dried yeast (Derwitt), 1kg organic maize (UHURU, Oxford), 380 ml 10% Nipagen (Sigma) in ethanol (Hayman) (w/v) and 45 ml propionic acid (Sigma) following the standard conditions as decribed in Roberts, 1998. Flies were anaesthetized with CO_2 or Di-ethylether and examined under the microscope.

4.1 P-element mediated germ line transformation in *Drosophila melanogaster* *(Ruby & Spradling, 1982; Spradling & Ruby, 1982)*

4.1.1 Collection of embryos

About 200 to 300 *white* flies of 3-5 days old were collected and transferred to the apple juice agar plates coated with yeast to stimulate egg laying. The embryos for injection were collected every 30 min at 18°C with 1-2 rounds of precollection on apple juice agar plates. The eggs were transferred with a brush carefully into a fine-mesh metal filter, washed with 0,7 % NaCl solution. The embryos were then bleached with 1:1 ratio of Bleaching solution (Colgate Palmoliv, Hamburg) and water for approximately 1 minute to dechorionate. After thorough rinsing with 0,7 % NaCl the embryos were transferred with a brush onto a rectangular apple juice agar block and oriented in such a manner which would facilitate the injection. The lined up embryos were attached now onto the glass slide with adhesive (10 ml heptane desolved adhesive for 10 cm adhesive tape) by giving a slight pressure on the embryos. The embryos were then dried a little bit depending upon air humidity and ambience temperature for 8 to 12 minutes in a dessicator over silicagel, in order to reduce the internal

pressure of the embryos. Afterwards the embryos were laminated with mineral oil, in order to prevent further drying.

4.1.2 Microinjection of Embryos

10 × Injection Buffer	1	mM $NaHPO_4$ (pH 7,4)
	50	mM KCl

10 µg of DNA, 10x injection buffer (2.5 µl) and 2.5 µl of helper plasmid pπ25.7wc (0,5 µg/µl concentration) the volume was made up to 25 µl with ddH_2O and centrifuged (13000 rpm, 30 min and 4°C) in order to sediment the disturbing floating particles. 1µl of this DNA mixture was filled from the rear end of the injection needle with the help of Borosilica glass capillary of internal diameter 1.2 mm. The liquid flowed up into the needle through the capillary action. This capillary was connected with the pressure system of the micro injection equipment and was fastened to the micromanipulator. To open the capillary the front part of the capillary was broken off under the microscope (20x objective) by touching to the edge of the glass slide. After introducing the needle to the posterior end of the embryos the DNA solution was injected. Embryos, in which the formation of the pole cells had already taken place and in which the germ cells were inaccessible for transformation, the injection needle was passed through the whole embryo and thus killed to prevent the emergence of non transgenic flies. After the injection few more drops of mineral oil was added over the embryos and the glass slide was transferred onto apple juice agar petriplate. The injected embryos were incubated at 25°C for approximately 24 hours. The embryos which survived the trauma of microinjection developed into larvae and crawled around in the petriplate. The larvaes were collected with the help of a needle and transferred into fly bottles with breading medium. Each larvaes which developed into adult flies were collected.

4.1.3 Selection of transformed flies (*Klemenz et al. 1987*)

The selection of transformed flies is made possible by the use of the *white*$^+$ gene (red eye color) as dominant selection marker. Since the insertion of the P-element-construct takes

place in the germ line of the injected embryos, the insertion event can be observed only in the G1-Generation. The injected animals (G0-generation) which eclosed were immediately crossed with *white* flies. In the G1-generation the transformed individuals can be recognized by the appearance of eye color ranging from orange to red. Each transgenic animal was crossed now again with *white* flies and the developing heterozygote descendants were further-crossed among themselves. The homozygote descendants, which are to be usually recognized by the darker eye color, were then further-bred for the establishment of a stable transgenic line.

II Preparation and Analysis of DNA

4.2 Production of chemically competent bacteria (*Escherichia coli*)

(Sambrook et al., 1989)

SOB-Medium	2	% (w/v) Bactotrypton
	0,5	% (w/v) Yeast extract
	10	mM NaCl
	2,5	mM KCl
	10	mM $MgCl_2$
	10	mM $MgSO_4$

RF1	10 mM RbCl 50 mM $MnCl_2$ 30 mM KCl 10 mM $CaCl_2$ 15 % (v/v) Glycerin	RF2	10 mM MOPS (pH 6,8) 10 mM RbCl 75 mM $CaCl_2$ 15 % (v/v) Glycerin

250 ml SOB medium was innoculated with 2.5 ml of fresh *E. coli*-culture (1:100) and incubated at 37°C with continuous shaking till it reached up to a OD600 of 0.5-0.6. After 15 minutes of incubation on ice the cells were centrifuged at (4000 rpm, 4° C, 10 min), and the pellet was resuspended in 80 ml cold RF1-buffer and placed again on ice for 15 minutes. The cells were centrifuged to pellet (4000 rpm, 4° C, 10 min) and resuspended in 20 ml RF2-buffer and incubated on ice for 15 minutes. The cell suspension was finally aliquoted into 200 µl each and quick frozen in liquid nitrogen. The cells were used immediately for transformation if needed and the remaining aliquots were frozen at -80°C for future use.

4.3 Transformation of chemically competent bacteria *(Sambrook et al, 1989)*

200 µl competent cells were thawed on ice for 10-15 minutes, 1-10 µl plasmid DNA solution or ligation mixture was added and incubated on ice for 30 minutes. The cells were heat shocked in 42°C waterbath for 60-90 seconds and immediately placed on ice for 5 min. In the mean time LB medium was warmed to room temperature. 900 µl of LB medium was added to the ice cold competent cell mixture and incubated at 37°C for 1 hour to gain antibiotic resistance. The incubated mixture is spun for 1 minute at 5000 rpm. Pour out majority of the supernatant and dissolve the pellet with the left out supernatant slowly by vortexing. Plate the entire cell mixture on LB selection plate (ampicillin 100 mg/ml, Kanamycin 50 mg/ml, IPTG, X-Gal) depending on the type of antibiotic resistant gene used in the construct. Dry the plates and incubate in 37°C overnight.

4.4 Preparation of plasmid DNA from *E. coli*

Mini preparation of plasmid DNA (alkaline lysis method) *(Birnboim & Doly, 1979)*

S1-Buffer	50 mM Tris/HCl (pH 8,0) 10 mM EDTA 100 µg RNase A/ml	S2-Buffer	200 mM NaOH 1 % (w/v) SDS
		S3-Buffer	2,6 M KAc/Aceticacid (pH 5,2)

3 ml LB medium (+ antibiotic) is innoculated with a single colony of bacteria and incubated at 37°C with continuous shaking for 6-7 hours. Centrifuge and pellet down the cells at 13000 rpm for 30 seconds and discard the supernatant, do the same for the remaining amount of the incubated broth. Add 250 µ1 of S1-buffer and resuspend the pellet by vortexing briefly. Add 250 µl of S2-Buffer to lyse the cells, incubate at room temperature for 5-10 minutes. Add 250 µl of S3-Buffer for neutralization and immediately mix gently, the cell membrane as well as the genomic DNA gets precipitated by giving curd like appearance. Centrifuge at room temperature for 10-15 minutes at 13000 rpm. The curd separates from the supernatant. The supernatant is transferred to another eppendorf tube. Add 0.5V of isopropanol to the supernatant and centrifuge at 13000 rpm for 30 minutes at 4°C. Decant the supernatant and add 500µl of 70% ethanol and incubate for 15 minutes at room temperature

for the salt to dissolve. Centrifuge for 15 minutes at room temperature and slowly decant the supernatant. Vacuum dry the pellet and dissolve the DNA in 10-30 µl of ddH_2O or TE-buffer depending on the concentration required.

4.5 Midi preparation of plasmid DNA (Genomed jet star, according to the manufacturers instruction given in the manual)

To prepare the large amount of plasmid DNA which is pure and free of all the materials such as SDS and salts Midi preparation was done. The incubated 50 ml bacterial culture was transferred into 50 ml Falcon tubes and centrifuged at 4000 rpm for 10 minutes. The pellet was resuspended in 4 ml of E1-buffer and the cells are finally lysed by addition of 4ml E2-buffer with a gentel mix. This mixture was incubated for 5-10 minutes and 4ml of E3-Buffer is added for neutralization. The mixture was centrifuged at 6000 rpm at room temperature for 20 minutes. The curd separates from the supernatant containing plasmid DNA. Slowly the supernatant was transferred to the pre- equilibriated anion exchange column with E4-Buffer. The solution was let to pass through the column by gravity flow. When all the solution was passed, the column was washed twice with E5-buffer, 10 ml each. the solution was let to pass through the column completely. Finally the plasmid DNA was eluted by adding 5 ml E6-buffer. The eluted DNA was mixed with 0.7 V isopropanol. Centrifuge at 4°C , 6000 rpm for 15 minutes. The supernatant was decanted, washed with 70% ethanol and centrifuged again as stated above. The supernatant was decanted and the DNA pellet is vacuum dried. The DNA was dissolved in required amount of ddH_2O for the future use.

4.6 Preparation of genomic DNA from *Drosophila* *(Pirotta, 1986; Steller & Pirotta, 1986)*

Extraction Buffer	100 mM Tris/HCl (pH 9,0) 100 mM EDTA 1 % (w/v) SDS

10 anesthetised flies were homogenized in 100 ml extraction buffer with a pestle in a glass homogeniser. Whole extract was transferred into a 1.5 ml micro reaction container and

incubated for 30 minutes at 65°C water bath. After addition of 14 µl of 8 M KAc the mixture was incubated on ice for 30 minutes and centrifuged at 13000 rpm at 4°C for 15 minutes. The supernatant was transferred to a new reaction container which contained genomic DNA and 0.5 V of isopropanol was added. This mixture was centrifuged for 30 minutes at 4°C and 13000 rpm. The pellet was washed twice with 70% ethanol, centrifuge and pellet was air dried. Finally the genomic DNA was dissolved in 20-100µl of ddH_2O and stored at –20°C for future use.

4.7 Agarose gel electrophoresis *(Sambrook et al., 1989)*

1 × TAE-Buffer	200	mM Tris-Acetat (pH 7,7)
	10	mM EDTA
10 × Loading buffer	0,5	% (w/v) Xylencyanol
	0,5	% (w/v) Bromphenolblau
	40	% (v/v) Glycerin in 1 × TAE

DNA molecules were separated on the basis of size by agarose gel electrophoresis.The horizontal gels of the size 6 cm x 9 cm or 8 cm x 12 cm were used. For proper separation based on the size of the DNA to be analyzed the agarose concentration of 0.8 – 2% (w/v) in 1x TAE buffer was used to which Ethidiumbromide (0.5µg/µl) was added. The DNA samples to be loaded onto the gel were mixed with 1/10 V of loading buffer. The gel was run at 60-100 V. Subsequently the gel was documented with UV light and the picture was printed out.

4.8 Isolation of DNA from agarose gels *(GFX PCR, DNA and gel purification kit, Amersham Pharmacia Biotech)*

The desired fragment was cut with a sharp scalpel under the UV light from the agarose gel and transferred to 1.5 µl micro reaction tube. To every 100 mg of piece of gel 100 µl of Capture buffer was added and incubated at 60°C for 15 mins with brief vortexing for every 2-3 min in order to dissolve the agarose. After the agarose was dissolved completely, the solution was transferred to GFX column was kept for 2 mins and centrifuged at 13000 rpm for

30 sec. The column was washed with 500 µl of Wash buffer and dried. 20-30 µl of ddH_2O was added to elute the DNA from the column by centrifuging at 13000 rpm for 1 min.

4.9 Estimation of DNA concentration using Spectrophotometer

Nucleic acids can be quantified due to their maximum absorption at the wavelength of $\lambda = 260$ nm using spectrophotometer. From the absorption (optical density = OD) the nucleic acid concentration can be computed in µg/ml using a quartz cuvette with the thickness of 1 cm considering the following parameters.

double stranded DNA	$OD_{260} \times 50 \times$ Dilution factor
single stranded DNA	$OD_{260} \times 33 \times$ Dilution factor
RNA	$OD_{260} \times 37 \times$ Dilution factor

4.10 Enzymatic manipulation of nucleotides

4.10.1 Digestion of DNA with the help of Restriction endonuclease

The following formula is generally used to determine the units of restriction enzyme required to cleave the DNA with a particular concentration.

$$\text{Required Units/µg DNA} = \frac{48{,}5 \text{ kb } (= \text{bp } \lambda) \times \text{Number of restriction sites in target DNA}}{\text{get-DNA (bp)} \times \text{Number of restriction sites in } \lambda\text{-DNA}}$$

Restriction endonuclease digestion were performed using the enzymes and buffer from Roche, (Mannheim) or Amersham Pharmacia Biotec, (Freiburg) according to the given instruction in the manual. Reactions were generally performed in the total volume of 30 µl. For some enzymes (EcoRI) which show the star activity the total volume of 50 µl was taken to prevent star activity. Double digestions were done with suitable buffers which are compatible with both the chosen enzymes. All digestions were done at 37°C for 2 hours.

4.10.2 Dephosphorylation of the 5` ends to prevent religation of the vector

To prevent the linearized vector from religation in the future ligation reactions, the 5` end of the DNA was dephosphorylated. The DNA to be dephosphorylated was subjected to alkaline phosphatase treatment, where 0.5 Units of alkaline phosphatase was used in 1/10 V of the reaction buffer and incubated at 37°C for 30 min. The DNA was GFX column purified later.

4.10.3 Ligation of DNA fragments

The ligation of DNA fragments requires the presence of compatible ends. 100-150 ng of vector DNA were used together with a three to fivefold molar excess of insert depending upon the insert size with 1/10 V of ligation buffer and 2 units of T4-DNA ligase in a final reaction volume of 15-20 µl. The ligation mixture was incubated at 16°C overnight.

4.10.4 Polymerase chain reaction (PCR) *(Saiki et al, 1988; Qiagen, Stratagene according to the manufacturers manual)*

The amplification of specific DNA fragments is facilitated by polymerase chain reaction. The synthetically manufactured oligonucleotides serves as specific primers to amplify the DNA of interest. The thermostable polymerase plays a major role in the synthesis of complementary DNA from the template. By the cyclic denaturation and renaturation the DNA region of interest amplifies exponentially. The reaction was done in a total volume of 50 µl by adding the following reaction components.

For pfu DNA polymerase

x µl Template-DNA (5-100 ng)
1.5 µl Sense-Primer (100 pmol/µl)
1.5 µl Antisense-Primer (100 pmol/µl)
1.5 µl dNTP-Mix (10 mM dNTP)
5.0 µl 10 × *pfu* polymerase buffer
1.0 µl of 50 mM $MgSo_4$
1.0 µl *pfu*-DNA-Polymerase (5 U/µl)
Make up the volume to 50 µl with ddH_2O

For Vent polymerase

x µl Template-DNA (5-100 ng)
1.0 µl Sense-Primer (25 pmol/µl)
1.0 µl Antisense-Primer (25 pmol/µl)
1.0 µl dNTP-Mix (10 mM dNTP)
5.0 µl 10 × Thermo polymerase buffer
3.0 µl of 50mM $MgSo_4$
1.0 µl Vent-DNA-Polymerase (5 U/µl)
Make up the volume to 50 µl with ddH_2O

For Taq polymerase

x µl Template-DNA (5-50 ng)
1 µl Sense-Primer (25 pmol/µl)
1 µl Antisense-Primer (25 pmol/µl)
1 µl dNTP-Mix (10 mM pro dNTP)
5 µl 10 × PCR-Puffer
10 µl 5 × Q-Solution (optional)
0,5 µl *Taq*-DNA-Polymerase (5 U/µl)
Make up the volume to 50 µl with ddH_2O

The following program was set for the PCR in the thermocycler.

Step 1. Denaturation	95 °C	300 s
Step 2. Denaturation	94 °C	10 s
Step 3. Primer anneling	40-60 °C	60 s
Step 4. Polymerization	72 °C	30-180 s
Step 5. Polymerization	72 °C	300 s

After completion of the DNA synthesis in step 4 the reaction was taken up again to step 2. The cycles were repeated 30-35 times. After the reactions were complete it was stored at 4°C in the final step.

4.11 P-Element jumpout

Flies with the P-element to be jumped out were crossed to the flies carrying transposase source w^+*; Sp/CyO;ry* ^{506}*Sb{P ry*$^+$*Δ2-3}/Ubx* as described in (Greenspan, 1997). Jumpouts were detected through the disappearance of *white*$^+$ in *white* mutant background.

4.11.1 P-Element Local hop

Flies with the P-element are crossed to the the flies expressing transposase w^{+}; *Sp/CyO;ry* ^{506}*Sb{P ry*$^{+}$*Δ2-3}/Ubx* , the local hops were detected through change in the eye color ranging from light yellow to deep red which indicates the hop of P-element into euchromatic regions in case of deep red color and light yellow in case of partially heterochromatic region.

4.11.2 Location of the site of integration of P-element in each local hop lines

Plasmid rescue cloning from genomic DNA of *Drosophila* *(Pirotta, 1986)*

P-elements which are used for the local hop experiment still contain the bacterial plasmid portion. This facilitates to locate the site of integration of P-element in genomic DNA of *Drosophila* by finding the flanking sequence at the P-element ends with the help of plasmid rescue cloning from genomic DNA. About 5µg of genomic DNA of the P-element insertion line is incubated with 10-20 units of a suitable restriction enzyme in a total volume of 200 µl with 1 µl of RNase (100 µg/ml) at 37°C for 2-3 hours. Heat inactivate the enzyme at 65°C for 20 min. Add 1/10 V of 3M NaAc (pH 4.8) incubate on ice for few mins ,add 2.5 V of ethanol and centrifuge at 13000 rpm at 4°C for 30 min. Wash with 70 % ethanol and vaccum dry the pellet. Add required amount of ddH_2O and resuspend the DNA pellet. Take the entire amount for ligation, with 40 µl of ligation buffer and 2 units of T4-DNA Ligase and make up the volume to 400 µl with ddH_2O. Incubate the ligation mixture overnight at 16°C. Ethanol precipitate the DNA and use for transformation into competent cells. Plate the cells, select the colonies, make mini preps and sequence with suitable primers.

III. Histological methods

4.12 mRNA in situ hybridization on adult testis

4.12.1 Preparation of Dig labelled DNA probes

200 ng of template DNA was dissolved in 11 µl distilled water. Subsequently, the DNA was denatured for 10 minutes at 100° C and immediately frozen in liquid nitrogen. The eppendorf tubes were thawed for 30 seconds, centrifuged at 13,000 rpm for 30 seconds and the following components were added for the reaction mixture: 6µl hexanucleotide-mix, 2 µl DIG-labelled nucleotide-mix and1 µl of Klenow-enzyme (labelling grade). The mixture was incubated overnight at 25°C with blank as control. DIG labelled nucleotides are incorporated as the polymerization takes place thus marking the synthesized DNA with the DIG label.

4.12.2 Testing the DIG labelled DNA probe

10 × DIG I–Buffer	1 M Tris/HCl 1,5 M NaCl, pH 7,5; (autoclaved)
DIG II–Buffer	DIG I–Buffer 1 % Blocking Reagent (w/v) Heated for 1 hr at 70°C
DIG III–Buffer	100 mM Tris/HCl 100 mM NaCl 50 mM $MgCl_2$, pH 9,5 (autoclaved)
Staining solution	10 µl NBT 10 µl X-Phosphat 2 ml DIG III-Buffer

1:10, 1:100 and 1:1000 and 1:10000 dilutions of the probe are set. Every dilutions are spotted on a small piece of Hybond N membrane and is UV crosslinked. UV crosslinks the DNA covalently to the membrane so that the further washes can be performed as follows. The membrane is washed for 1 minute with DIG-I-buffer and then incubate on shaker for 30 minutes with DIG-II-buffer, in order to reduce nonspecificity. In the meantime dilute the anti-

body with DIG-I-buffer (1:5000). The anti-body (anti- Digoxygenin AP Fab fragment (0.75 units/1 ml) is directed against the Digoxygenin marking of the probe DNA. After the wash with DIG-II-buffer, incubate the filter for at least 30 minutes in the diluted antibody solution. Wash the membrane twice in DIG-I-buffer every 5 minutes, wash with DIG-III-buffer for 2 min for equilibration, wash and add the staining solution and incubate in dark without shaking at RT till the color appears. The color reaction is based on the transformation of a colorless solution to a blue precipitation, which is released by an alkaline phosphatase coupled to the anti-body. The coloring can be stopped by rinsing the membrane with tap water. If the color of the spots are strong enough (with the 1:10000 dilution), the probe can be used for hybridization.

4.12.3 Fixation of adult testis

Approximately 20-30 testis are dissected from the adult flies in PBS (see 4.7.1) and transferred in a 1.5 ml Eppendorf tube with 100 µl 4 % formaldehyde in PBS, 10 µl DMSO (facilitates permiablization) and 300 µl heptane. Incubate for 25 min at RT on the shaker. Discard the solution and incubate twice for 10 min with 300 ml methanol and 300 ml heptane on the shaker. Rinse the testis twice with methanol. At this stage testis can be stored in methanol at –20°C.

4.12.4 Pre hybridization of testis

Testis are washed thrice for 5 min each in PBT. PBT is discarded and testis are incubated with proteinase K with an end-concentration of 150 ng/ml in PBT for 3.5 to 4 min at RT on shaker. Testis are then washed twice for 1.5 min each in 100 µl glycin (stock solution 20 mg/ml) in 900 µl PBT (to stop the proteinase reaction). The testis are washed twice for 5 min each in PBT, and for 20 min in 1ml PBS-formaldehyde (4%), and washed five times for 5 min each in PBT. The testis are then washed for 10 min in 1ml PBT/hybridyzation solution (1:1) ratio, washed thrice for10 min each in 1ml HS. The testis are then incubated for 1 hour at 45°C in a heat block for pre hybridization.

4.12.5 Hybridization and staining

With alkaline phosphatase

Hybridization solution (HS)	50 ml Formamide 25 ml 20 × SSC
0,1 ml Heparine (50 mg/ ml)	
	0,1 ml 10% Tween 20 pH 6,4 up to 100 ml with distilled water
Staining buffer	100 mM NaCl 50 mM $MgCl_2$ 100 mM Tris/HCl (pH 9,5) 1 mM Levamisol

After 1 hr of incubation with the hybridization solution (HS), the HS is discarded. The probe-DNA (app: 8 µl) in HS (72 µl) which is already preheated to 100°C for 5 min. The heated probe is immediately added to the pre hybridized testes. Incubate overnight at 45°C in heat block. Wash four times for 5 min each in preheated HS at 45°C. Wash thrice for 20 min in PBT/HS (1:1) at RT. Wash five times for 10 min each in PBT. Add the anti-DIG-Fab Fragment alkaline phosphatase conjugated antibody with 1:2000 dilution in PBT. Incubate overnight at 4°C on shaker. Wash five times for 10 min each in PBT, wash thrice for 5 min each in staining solution buffer, the testis are transferred onto a glasblock (rinse out of eppendorf tube) and start the reaction by adding 3.5 µl NBT and 3.5 µl X-Phosphate (BCIP) per ml staining solution buffer. Keep in dark until specific staining is visible (can take a few hours). Wait till the staining becomes intense and wash with PBS twice for 10 min each. Transfer the testis samples on to a slide with a drop of PBS. Drain out the PBS from the sides of the slide using a pipettman and add glycerol on the samples, spread the samples evenly on the slide before carefully putting the cover slip without air bubble.

4.13 Antibody staining on adult *Drosophila* testis squash preparations

Disect *Drosophila* testis in PBS and transfer the testis into a drop of PBS on the polylysine coated slide (app 4-5 testis per slide). Cover the slide with the cover slip and using a kimwip or other blotting paper drain the PBS from the sides of the cover slip by viewing under the dissection microscope. Drain the solution until the testis is properly squashed with the contents spilled out of the testis sheath. Quick freeze the slide in the liquid nitrogen and

flip off the cover slip using a razor blade. Immediately dip the slide and store it in the coupling jar containing 95% pre frozen ethanol at –20°C. The slides can be stored at this step until all the slides are prepared.

Remove the slides from ethanol and air dry the slides partially. Add approximately 60 µl of 4 % PBS-paraformaldhyde onto each slide and incubate for 7 mins at RT. Tip off the solution from the slide and dip the slides in a coupling jar containing PBS for 5 mins. Shift the slides to the coupling jar containing PBTD (PBS + 0.3 % Triton-X-100 + 0.3 % sodium deoxycholate (DOC)) for 30 mins (for better permiablization). Shift the slides to the second jar containing PBTD for another 30 min. Transfer the slides into the jar containing BBT (PBS + 0.1 % Triton-X-100 + 3 % BSA) for 30 mins. In the mean time, prepare the antibody diluted in BBT. Remove each slide from the jar, wipe off the excess solution from the sides of the slide and place all the slides in a tray (care should be taken that the slides do not dry at this time). Add 60 µl of antibody solution onto each slide and cover all the slides with the cover slip (always use 24 X 36 mm cover slips for the best coverage) and incubate overnight in an airtight humid chamber at 4°C. Next, carefully remove the cover slip from the slides without disturbing the squash and wash the slides 4 times for 15 min each in the coupling jar containing BBT. In the meantime, make the dilution of the secondary antibody in BBT with 2 % normal serum (if using fluorescent secondary antibody care should be taken that all the further steps is processed in dark). After the final wash, remove the slides from the jar and wipe out the sides of the slides to remove the excess solution (care should be taken that the slides do not dry at this time). Add 60 µl of antibody solution onto each slide and cover all the slides with the cover slip and incubate in dark at RT for 1hr and 15 min. Next, carefully remove the cover slip from the slides without disturbing the squash and dip the slides in the coupling jar containing BBT for 15 min in dark. In the meantime, make the Hoechst counter staining solution (8 µl in 1ml BBT). Carefully remove the slides from the jar, wipe out the sides of the slides to remove the excess solution and add 60 µl of Hoechst on each slide, cover with the cover slip preventing the air bubble and incubate in dark for 13-15 min at RT. Carefully remove the cover slip from the slides without disturbing the squash and dip the slides in the coupling jar containing BBT and wash in dark 3 times for 15 min each. Remove each slide from the jar, wipe off the excess solution from the sides of the slide and place all the slides in a tray and cover the slides with an opaque tray so that the slides completely air dries in dark (normally takes 5 min for drying). Add a drop of Fluoromount-G on the samples and cover it with the cover slip preventing air bubbles. The slides have to be always stored at 4°C or –20°C in dark.

4.14 X-Gal Staining for *Drosophila* testis

Dissect the males holding the abdomen of males with one forceps, hold the animal at about 3/4th the distance from the head near the posterior with another forceps and pull the posterior end so that it rips apart exposing the internal organs. Some times you also get the whole posterior portion. In such cases you can open it with the forceps a little bit more to find the testis. Hold the cuticle with one forceps and the center of the testis coil with another forceps and slowly pull apart so that the testis coil gets separated from the other organs. After disecting about 5-6 animals transfer the testis to the mesh collector containing PBS with a container at the bottom (easy to handle for further fixing and washing without loosing the testis). Dip the mesh in container with Fixer solution (make sure the testis sink to the bottom of the mesh). Leave it at room temperature for 15 min. Discard the Fixer and transfer the mesh into the same container with PBS solution. Wash twice for 3-4 mins each. Dip the mesh into the container with (5 ml staining solution + 100 µl X-gal solution{20 µlX-Gal/ml staining solution} per warm to 37°C for fast staining) Make sure the testis sink to the bottom for better staining. Incubate at room temperature in dark till the bright staining appears. Check once in every 15-30 min for the staining under the microscope (appearance of staining is easy to visualize with a white background). Once the staining appears bright enough, stop the reaction by dipping the mesh in PBS solution and wash (3 times) for 5 min each. With the mesh still in PBS solution looking under the microscope, transfer the testis onto a slide containing 20µl of mounting media (glycerol). Arrange the testis on the slide so that the do not overlap. Cover the slide with the cover slip without air bubbles and the sample is ready to look for further details under the microscope.

4.15 Analysis of the embryonic phenotype

Wild-type virgin females were mated with ProtamineB-eGFP and ProtamineA-eGFP males separately, ssm^{185b}/ssm^{185b} virgin females were mated with ProtamineB-eGFP, ProtamineA-eGFP and Mst77f-eGFP containing male flies separately and the embryos were collected every 15-30 min after egg deposition and stored at 4°C up to 2 h before fixation. Egg collection and fixation were done essentially as described (Loppin *et al.,* 2000). After

fixation, the embryos were incubated for 1 h in a 2 mg/ml RNaseA solution at 37°C, rinsed with PBS-Triton-X 0.1 % and was stained with Propidium iodide (PI) 5 µg/ml for 30 min, or with 8 µl Hoechst in 1 ml of PBS-Triton-X 0.1%. Washed with PBS-Triton-X 0.1% twice for 15 min each and mounted with Fluoromount medium (Southern Biotech). Photomicrographs of PI stained male and female pronuclei were made with a Leitz confocal microscope and processed with Adobe Photoshop 6.0.

4.16 Fertility test

Young adult males were placed individually with three wild-type virgin females in separate vials at 25°C, and vials were scored for offspring after a week.

4.17 Immunostaining of Polytene Chromosomes

Immunostaining of the polytene chromosomes were done as described by Zink *et al.,* Salivary glands were dissected from third instar larve in PBS. The fat bodies surrounding the glands were carefully removed. Glands were transferred on to a siliconized cover slip containing (50 µl tritonX-100, 400 µl PBS, 50 µl 37% p-formaldehyde) for 30 seconds. The glands were then shifted to another siliconized cover slip containing 40 µl of (50 µl 37 % p-formaldehyde, 200 µl H_2O, 250 µl 100 % acetic acid) and left for 2-3 min. The cover slip was carefully lifted with a polylysine coated slide, gently tap the cover slip using the eraser end of the pencil and squashed by pressing slide onto blotting paper. The squashes were examined under the phase contrast microscope, good squashes were stored in 95% ethanol. The slides were then blocked for 1 hr using blocking solution (3 % BSA, 0.2 % NP40, 0.2 % Tween 20 and 10 % non fat dry milk in PBS). To each slide 60 µl of anti-GFP (Abcam 6556 polyclonal antibody raised in rabbit) diluted 1:300 in blocking solution was added and incubated overnight at 4° C in a humid chamber. Slides were washed with washing solution WS-I (PBS containing 300mM NaCl, 0.2 % Tween 20, 0.2 % NP40) and WS-II (PBS containing 400 mM NaCl, 0.2 % Tween 20, 0.2 % NP40) for 15 min each, rinsed with blocking solution and incubated with anti rabbit-FITC diluted 1:200 with 2 % normal goat serum in blocking solution for 45 min at RT. The slides were washed with WS-I and WS-II, counter stained with

1 μg/ml of Hoechst for 10 min. The slides were washed with PBS twice for 10 min each, air dried and mounted with the flourocent mounting medium (Flouromount-G). Pictures were taken under the 65X objective and processed with a red pseudocolor for Hoechst staining using Adobe Photoshop 6.0 (Adobe Systems).

5. Results

I. Functional analysis of Don Juan during spermiogenesis

Don Juan GFP shows the localization both in the nucleus and in the mitochondria at the canoe stage of spermatid differentiation. The role of Don Juan may not be involved in the mitochondrial aggregation nor in the mitochondrial elongation process, since Don Juan protein is made in post-mitochondrial elongation stage. The protein stays in the mature sperm flagellum, which could possibly have a function in sperm motility. In order to study the function of Don Juan during spermiogenesis generation of mutant and analysis of mutant phenotype is essential. An EMS mutagenesis screen that was performed in our lab to obtain a mutation in *don juan* was unsuccessful as there was no hit in this gene (Leonie Hempel, Ph.D Thesis 2004). The inefficiency in obtaining a mutant for *don juan* may be because of the functional redundancy with another gene present at the immediate downstream of *don juan* named *don juan-like*. Don juan-like shows 42% similarity at the protein level to Don Juan, which is also expressed in the same fashion as Don Juan (Hempel et al., 2005).

5.1 Premature expression of Don Juan leads to male sterility

As a further tool to investigate the function of Don Juan, I chose to express Don Juan prematurely in the primary spermatocyte stage. For this, the *β2-promoter* and leader which is solely expressed in the male germ line and confers high level of transcription and translation level during meiotic prophase (Santel *et al.,* 2000) was chosen and *β2-promoter-don juan-eGFP* construct was made. The *β2 tubulin* regulatory region comprising the region -511 to +156 was amplified by PCR using primers with linked *EcoRI* and *BamHI* restriction sites. The PCR fragment was inserted into the transformation vector AUG-β-Gal (Thummel *et al.,* 1988). For construction of the transgene *β2-promoter-dj-eGFP,* a 743 bp fragment with linked *BamHI* and *XbaI* sites comprising the *eGFP* open reading frame was inserted in the opened vector *β2 tubulin*-AUG-β-Gal replacing the *ADH-lacZ* gene. The *dj* open reading frame was amplified by PCR using primers with linked *BamHI* sites. The obtained fragment

was inserted in the *BamHI* opened vector and transgenic flies bearing *β2-promoter-dj-eGFP* were generated.

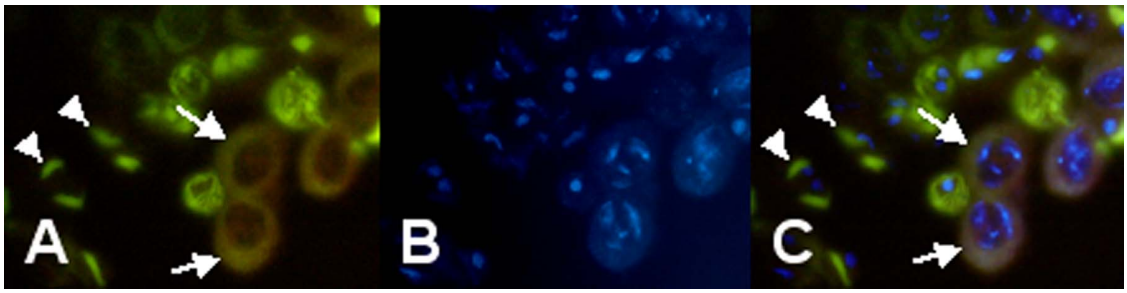


Fig. 5.1 Premature expression of Don Juan in the primary spermatocyte stage. (A) eGFP fluorescence on squashed testes could be observed in the cytoplasm of primary spermatocytes (arrows) and in the nebenkern of early post-meiotic spermatid stages (arrowheads). (B) Chromatin staining with Hoechst. (C) Merged picture.

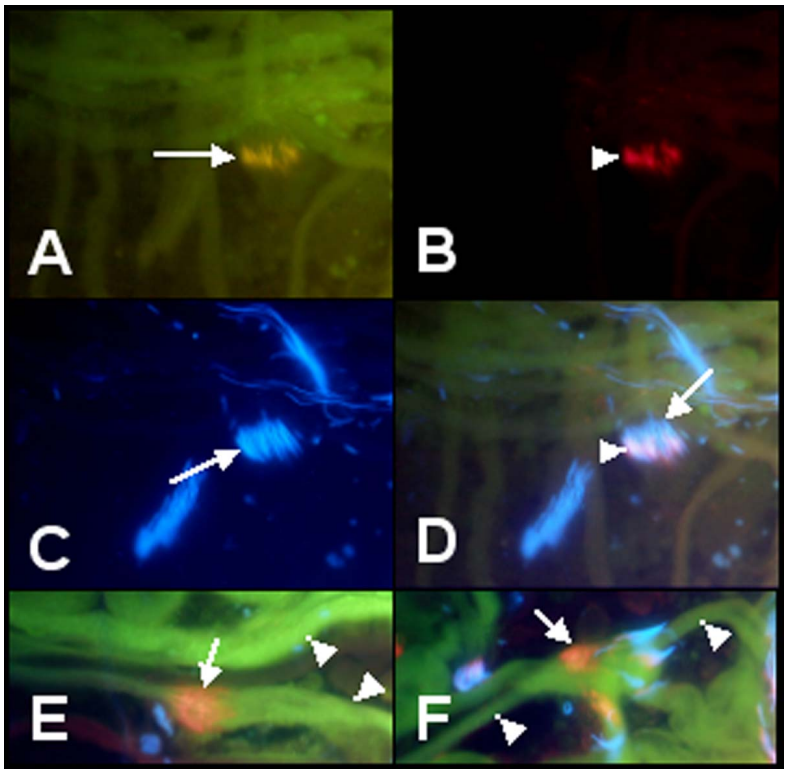


Fig. 5.2. **Individualization complex (IC) is formed but, fails to move further when Don Juan is prematurely expressed.** (A-D) Squash preparations of dissected testes of flies expressing DJ-eGFP prematurely. (A) By the eGFP auto fluorescence we could follow the expression of DJ-eGFP which is localized during nuclear shaping in the spermatid heads (arrow). (B) The testes squash is stained with rhodamin-phalloidin to visualize actin fibers (arrowhead), Hoechst to visualize nuclei (C, arrow) or double exposed for both (D) showing only the assembly of the actin cones (arrowhead) around each spermatid nucleus (arrow) but no progression of the individualization complex. (E and F) Squash

preparations of dissected testes of flies bearing a construct for the expression of DJ-eGFP under the control of the natural *dj* promoter. DJ-eGFP localizes along the flagella of elongated spermatids (E, arrowheads). The testes squash is stained with rhodamin-phalloidin (red) to visualize actin fibers (arrows) and Hoechst (blue) to stain the nuclei, respectively (arrowhead). The progression of the actin cones are visible (arrow).

This allowed to follow the expression of this fusion gene in the male germ line. The prematurely expressed DJ-eGFP is localized in the cytoplasm of primary spermatocytes (Fig. 5.1 B,C,D, arrows), in the nebenkern of early spermatids (Fig. 5.1 B,C,D, arrowheads) and along the flagella of elongating spermatids. Flies expressing DJ-eGFP prematurely were sterile due to the failure to individualize the spermatids. No individualized sperm in these testes were observed, so, I analyzed whether the individualization complex (IC) is assembled at all. In testes of flies expressing DJ-eGFP prematurely, actin assembles initially around each spermatid nucleus in a cone shape (Fig. 5.2 A,B,C,D). But the actin cones do not progress from the nuclear region to the end of the tail in contrast to that observed in testes of wild-type flies (Fig. 5.2 E,F). Thus, the premature expression of Don Juan in the primary spermatocyte stage interferes with the normal function of the mitochondrial derivatives leading to male sterility.

In order to study the role of Don Juan in spermiogenesis, it is very important to look at the mutant phenotype of *don juan.* There are no EMS nor P-element mutant available for *don juan* yet. In order to obtain mutant for Don Juan, a P-element local hop was performed. There existed two independent fly lines in which a P-element was inserted close to *don juan*. In the line designated EP 2100 the P-element was inserted over 6kb 3` from *don juan*. The other line EP 3393 the P-element was inserted over 35kb 3` from *don juan*. The homozygous flies of these P-element insertion were lethal. Since, there were no other viable P-insertions available, these two fly lines were chosen for the P-element local hop experiment.

5.2 P-element local hop

5.2.1 Is the P-element insertion indeed responsible for the lethality?

To start with, the original P-insertion lines had to be first eliminated from any secondary mutations to clarify whether the lethality is caused by the P insertion or by another mutation in the genome. This was done by crossing the original balanced P-insertion lines for 4-5 generations with (w^-) flies. The crossing over occurs between the homologous

chromosomes and if any secondary mutations present will be eliminated by recombination. The flies with the P-element can be easily tracked with the help of the eye color they exhibit due to the presence of the *white* gene in the P-element they carry. Thus, it was confirmed that the lethality is indeed due to the P-insertion. The virgin females from the final cross were crossed to the balancer males to balance and maintain the stable P-insertion line. These balanced P-insertion virgin females were crossed with the males expressing the transposase Sb^{1},*(P Δ2-3)/Tm3,*Ser^{1}). The males with P-element in trans to the transposase were selected in the next generation by the mosaics seen in the eyes (due to the transposase being expressed ubiquitously, excision of P-element in the somatic cells, and the clone of cells emerging from these cells leads to a mosaic eye) and crossed to virgin females with appropriate marker over the balancer for the respective chromosome. The flies that emerge out of this cross can be of three types.

1) The flies with change in the eye color when compared with the eye color of the original P-insertion flies (Indication of the P-element local hop).

2) The flies with white eye color (Indication of the P-element Jump out).

3) The flies with the same eye color as that of the original P-insertion line (no P-element local hop nor jump out).

Each fly with the change in eye color were crossed to the balancer virgin females to establish the stable stock of each line.

In $P\{w^{+mC}=lacW\}l(3)L2100^{L2100}$ fly line mosaic eyes were observed in the second generation, which gave the clear indication that the transposase source (F11) was active. For this line however no local hops were identified as there was no change in the eye color. In contrast, the local hop experiment was succesful with $P\{w^{+mC}=lacW\}l(3)L3393^{L3393}$ line as shown in Table 1, leading to 62 new lines.

Finally the flies with change in eye color were crossed with the balancer flies to maintain the stocks. These flies were lethal because of the lesion created at the original insertion point at the time of the P-element hop. To eliminate the lethal lesion, these lines were again crossed several times with w^{-}. Flies that allowed recombination leading to the elimination of the lethal lesion. After these crossings, out of these lines, three lines were viable (1B7, 5B3 and 2B22). One line 1B7 showed male sterility. This stock was used for genomic DNA isolation and checked for the insertion site by inverse PCR. The insertion was found to be in the gene CG8036 which is predicted to encode for a trans-ketolase enzyme. However, this experiment was unsuccessful in obtaining a mutation in *don juan*.

Table 1. Table of number of local hops and jump outs from every respective cross

EP 3393 (*P{w^{+mC}=lacW}l(3)L3393^{L3393})*

Insertion series	Jumpout males	Jumpout females	Localhop males	Localhop females	Total Jumpout Vs Localhop
1B	6	3	10	4	9:14
2B	1	2	13	5	3:18
3B	4	6	-	-	10:0
4B	12	5	3	2	17:5
5B	-	-	3	2	0:5
6B	19	23	5	7	42:12
7B	-	1	1	-	1:1
8B	2	5	3	2	7:5
9B	-	-	1	-	0:1
10B	1	1	-	-	2:0
11B	-	-	1	-	0:1
12B	-	-	-	-	0:0
Total	45	46	40	22	91:62

*Total number of 91 jumpouts and 62 local hops were obtained from mobilization of EP3393.

II. *Drosophila* Protamines and Mst77F replace histones during chromatin condensation in late spermatids

5.3 In *Drosophila*, two putative *protamine* genes and *Mst 77F* encode proteins of the sperm chromatin

The genes encoding the two predicted protamines of *D. melanogaster* encoding *Drosophila* ProtamineA (dProtA) and *Drosophila* ProtamineB (dProtB) were characterized. These genes, *Mst35Ba* and *Mst35Bb,* are present at cytological position 35B6 and 35B6-7 respectively on the chromosome arm 2L. These two genes are arranged in tandem and both consist of three exons (Fig. 5.5 A). The 5´UTR, coding region and the 3´UTR of these genes are highly identical (data not shown) as they probably arose from a recent gene duplication. The encoded protamines have over 94% identity to each other (Fig. 5.4 A and B). With the predicted molecular weight and pI of 16.9 kD and 10.63 for dProtA, and 16.6 kD and 10.24 for dProtB respectively, they exhibit a very basic character.

A remarkable feature of protamines is their ability to form intermolecular disulfide bridges, which is reflected by the conserved cysteine residues within mammalian protamines (Chapman *et al.,* 2003). The dProtA and dProtB are of 146 aa and 144 aa respectively, and thus longer than even the human and mouse Protamine-2 which is of 102 aa and 107 aa respectively (Fig. 5.3 B). Both *Drosophila* protamines contain 10 cysteines each and show significant similarity, particularly with respect to a high cysteine, lysine and arginine content to mammalian protamines (Fig. 5.3 A, B and Table-2). Human and mouse Protamine-1 aligns to the N-terminal half of the *Drosophila* protamines (from aa 27-68), four cysteine residues are conserved and regularly spaced (Fig. 5.3 A, asterisks). On the other hand, Protamine-2 of human and mouse show relatively high similarity to the C-terminal half of the *Drosophila* protamines with four cysteines in this region which are conserved and regularly spaced (Fig. 5.3 B, asterisks) whereas one cysteine (Fig. 5.3 B first asterisk) shares also with the mouse and human Protamine-1 (Fig 5.3 A last asterisk).

Mst77F is present at the cytological position 77F on the chromosome arm 3L and lies within the large intron of *PKA-R1*. *Mst77F* is also male specifically transcribed and the encoded protein has been proposed to be a linker histone H1/H5 type, which could also play a role of transition protein or a protamine (Russell and Kaiser, 1993). The predicted gene

structure is as shown in Figure 5.5 B. We observed that the Mst77F protein shares a significant similarity to the HILS1 protein of mouse (Fig. 5.3 C), and human HILS1 (Fig 5.4), where the percent of cysteine, lysine and arginine are similar to that of mHILS1 and hHILS1 (Table-2). HILS proteins have recently been described as a component of the mammalian sperm nucleus (Yan *et al.,* 2003). The *Drosophila Mst77F* encode for a protein of 215 aa with a molecular weight of 24.5 kD with a pI of 9.86. mHILS1 is of 170 aa and show 39% similarity over Mst77F (Fig. 5.3 C). Mst77F contains 10 cysteine residues as do *Drosophila* protamines (Table-2) and mHILS1 contains 8 cysteine residues of which 4 residues are conserved (Fig. 5.3 C, asterisks).

As there are considerable differences between the mammalian protamines as well as between the mammalian HILS1 proteins and the presumptive *Drosophila* homologue, Mst77F, additional experiments are essential to clarify if these proteins are indeed involved in the condensation of sperm chromatin.

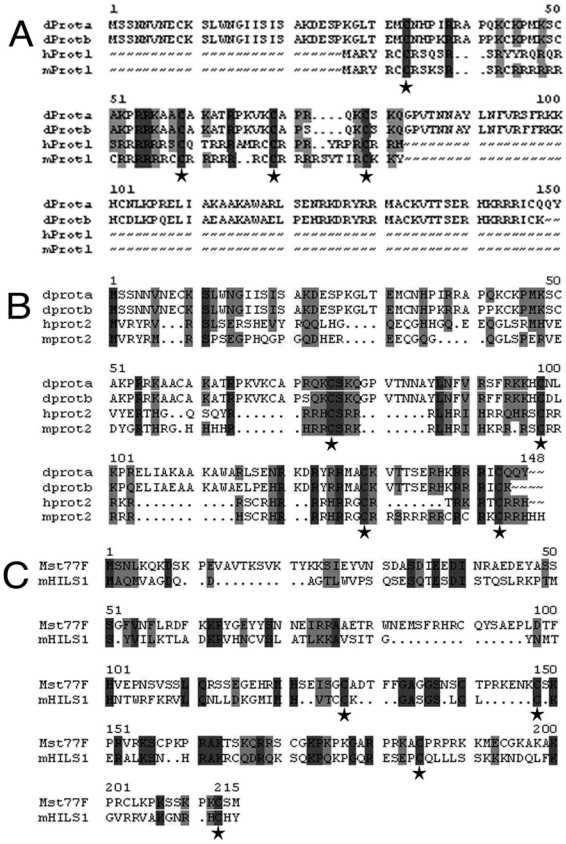


Fig. 5.3. Sequence alignment of Protamines and Mst77F.
Black boxes show identity, gray box shows similarity and conserved cysteines are marked by asterisks.
A) Alignment of *Drosophila* ProtamineA and B (dProtA and dProtB) with human Protamine-1 (hProt1) (Strausberg *et al.,* 2002, Tanaka *et al.,* 2003) and mouse Protamine-1 (mProt1) (Strausberg *et al.,* 2002, Cho *et al*., 2001). B) Alignment of *Drosophila* ProtamineA and B (dProtA dProtB) with human Protamine-2 (hProt2) (Strausberg *et al.,* 2002, Tanaka *et al.,* 2003) and mouse Protamine-2 (mProt2) (Strausberg *et al.,* 2002, Cho *et al*., 2001). C) Alignment of *Drosophila* Mst77F with mouse spermatid-specific linker histone H1-like protein (mHILS1).

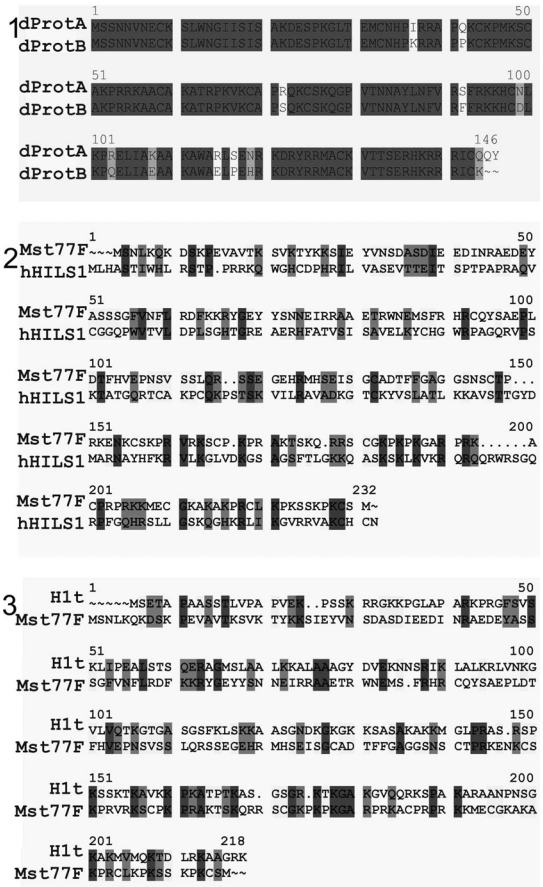


Fig. 5.4 Sequence alignment
1) Alignment of *Drosophila* ProtamineA and ProtamineB (dProtA and dProtB).
2) Alignment of Mst77F with human HILS1 (hHILS1) protein.
3) Alignment of Mst77F with mouse H1t (mH1t) protein.

TABLE 2. Chromatin condensing proteins of mouse, human and fly

Protein(aa)	Protamines, % (number of residue)			Protein(aa)	Spermatid specific linker histone varients and transition proteins % (number of residue)		
	Cysteine	Lysine	Arginine		Cysteine	lysine	Arginine
dProta(146)	6.8%(10)	14.4%(21)	12.3%(18)	Mst77F(215)	4.7%(10)	13.5%(29)	9.8%(21)
dProtb(144)	6.9%(10)	15.3%(22)	10.4%(15)	mHILS1(170)	4.7%(8)	11.2%(19)	7.1%(12)
mProt1(51)	17.6%(9)	5.9%(3)	54.9%(28)	hHILS1(231)	3.5%(8)	10.0%(23)	9.5%(22)
hProt1(51)	11.8%(6)	0.0%(0)	47.1%(24)	mH1t(208)	0.0%(0)	19.2%(40)	7.2%(15)
mProt2(107)	6.5%(7)	2.8%(3)	35.5%(38)	mTP1(55)	0.0%(0)	18.2%(10)	20.0%(11)
hProt2(102)	4.9%(5)	2.0%(2)	32.4%(33)	mTP2(117)	6.0%(5.1)	9.4%(11)	13.7%(16)

5.4 *Mst35Ba*, *Mst35Bb* and *Mst77F* are transcribed in the male germ line from the primary spermatocyte stage onward.

To check whether *Mst35Ba*, *Mst35Bb* and *Mst77F* are expressed in the male germ line, whole mount *in situ* hybridizations on testes of adult flies were performed. The results showed that indeed *Mst35Ba* (Fig. 5.5 C1), *Mst35Bb* (Fig. 5.5 C2) and *Mst77F* (Fig. 5.5 C4) transcripts are detectable in the cytoplasm from the primary spermatocyte stage onward, while

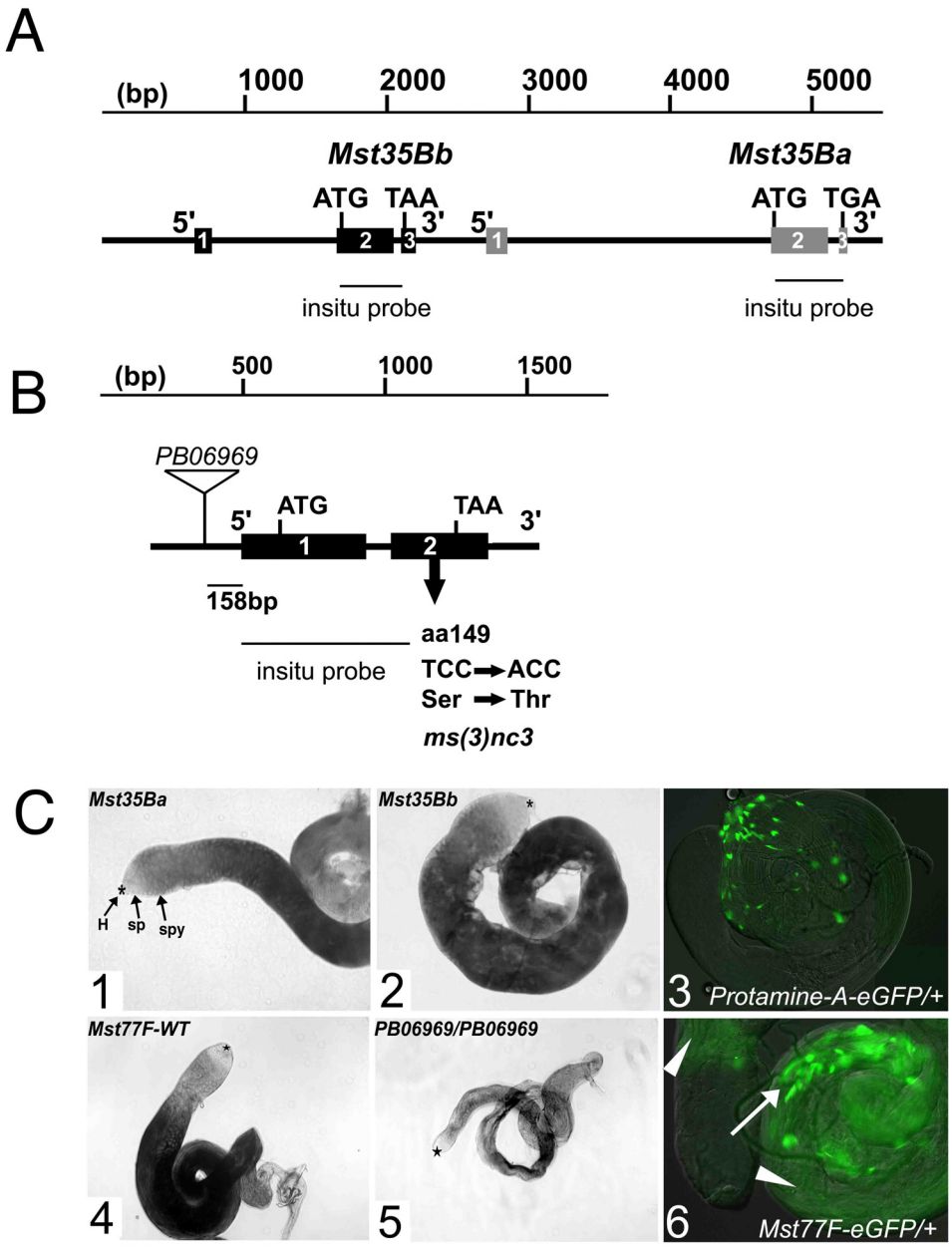


Fig. 5.5. Molecular genetic analysis of *Drosophila Protamines* and *Mst77F* genes.
A) Genomic organization of the two *protamine* genes. The map illustrates the position of *Mst35Bb* (thick lines black) and *Mst35Ba* (thick lines grey) exons and introns (thin lines). The predicted ORF for *Mst35Bb* (ATG within Exon-2, TAA in Exon-3) encode a *Drosophila* protamineB (dProtB) of 144 aa. The *Mst35Ba* gene is located 448bp downstream of *Mst35Bb*. The predicted ORF for *Mst35Ba* (ATG within Exon-2, TGA within Exon-3) encode the *Drosophila* ProtamineA (dProtA) of 146 aa.
B) Genomic organization of *Mst77F* gene. (thick lines black): Exons and (thin lines): Intron. The predicted ORF for *Mst77F* (ATG within Exon-1, TAA in Exon-2) encode for a *Drosophila* linker histone varient of 215 aa. *PB06969* (*PBac{PB}Pka-R1*^{c06969}) is a P-element insertion in the promoter of *Mst77F* at 158bp upstream (45) of presumptive transcription start site. *ms(3)nc3* is a missense mutation in codon 149 from TCC to ACC which encode for threonine instead of serine.
C) *In situ* hybridization for (1) *Mst35Ba*, (2) *Mst35Bb*, (4) *Mst77F* in wild-type flies and (5) *in situ* for *Mst77F* in *PB06969/ PB06969* shows the reduction in transcript level of *Mst77F*. In all cases the whole testis are shown with the apical tip. The hub (H) denoted by asterisks and spermatogonial cells (sp) are free of staining. The dark blue staining seen in the cytoplasm of early primary spermatocyte (spy) stage onwards till the late elongated spermatids indicates the presence of the transcripts *Mst35Ba*, *Mst35Bb* and *Mst77F*. (3 and 6) eGFP fluorescence in the transgenic flies expressing ProtamineA-eGFP (3) and Mst77F-eGFP (6) fusion genes in the spermatid nucleus indicating the translation repression of the transcript until the late spermatid stage. A pattern of Mst77F-eGFP is also seen in the flagellum (arrowheads) and nucleus (arrow).

stem cells, spermatogonia and the testis sheath are free of transcript. As discussed below these *Mst35Ba* (Fig. 5.5 C3), *Mst35Bb* and *Mst77F* (Fig. 5.5 C6) mRNAs are translationally repressed until the elongated spermatid stage.

5.5 Identification of the minimal promoter and the Translational Repression Element (TRE) for *Mst35Bb*

It is a unique and characteristic feature that the post-meiotic stages lack transcription nearly completely in *Drosophila*, while in mammals a short post-meiotic transcriptional phase exists (for review see Fuller, 1993, Schäfer *et al.,* 1995, Renkawitz-Pohl *et al.,* 2005). During the primary spermatocyte growth phase of meiotic prophase the cell size increases and the nucleus is highly transcriptionally active (Olivieri and Olivieri, 1965). Also the *protamine* genes encode a translationally repressed testis specific mRNA.

Drosophila protamine mRNAs are transcribed at the primary spermatocyte stage, which is in contrast to the situation seen in mammals, where *protamine* mRNAs are synthesized at the round spermatid stage (Steger, 1999). Translational repression of mammalian *protamine-1* mRNA is controlled by the 3'UTR (Zhong *et al.,* 2001, Zhong *et al.,* 1999). The *Drosophila* ProtamineA-eGFP and ProtamineB-eGFP constructs do not contain

the 3'UTR of the respective *protamine* genes. Nevertheless, the transgenic flies carrying these constructs still show repression of translation (Fig. 5.5 C1,2 and 3). So, in *Drosophila*, the region responsible for the translational repression is most likely in the 5'UTR.

Mst35Ba and *Mst35Bb* promoter sequence and 5'UTR sequences show very high identity. Due to this reason we chose one of the *protamine* genes to analyze the minimal

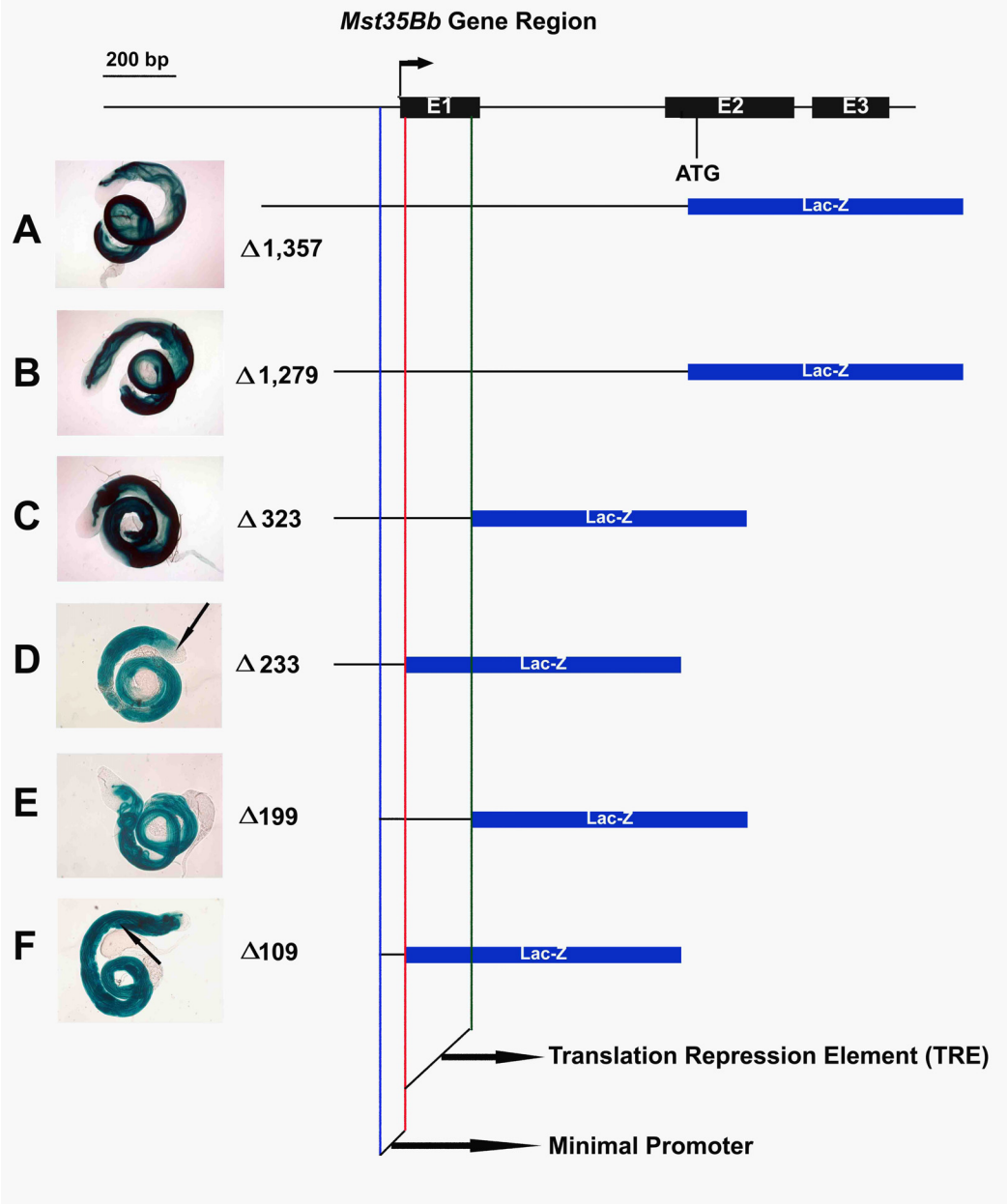


Fig. 5.6. Minimal promoter and the translational repression element (TRE) for *Mst35Bb*. (A) 1,357bp upstream of ATG, and (B) 1,279bp upstream of ATG and (C) 323bp containing the promoter and 5'UTR in the exon-1 of Mst35Bb fused to *lac-Z* reporter gene show complete expression and translational repression. (D) Containing 233bp upstream of exon-1, with the putative transcription start site from exon-1 showing premature translation in primary spermatocytes (arrow), (E)199bp containing the minimal promoter and 5'UTR in the exon-1 is sufficient for the transcription and the

translation control. (F) 109bp upstream of exon-1, with the putative transcription start site from exon-1 shows the loss of translational repression (arrow).

promoter and the translational repression region. Gradually deleting the 5′ upstream region of the *Mst35Bb* gene, the size of this regulatory region were analyzed (Fig 5.6). Generating deletion mutants in combination with the *lacZ*-reporter gene and establishing transgenic *Drosophila* lines carrying the deletion constructs it was revealed that Δ1,357, Δ1279, Δ323 and Δ199 (Fig. 5.6 A, B, C and E respectively) still allow a correct cell type specific transcriptional and translational regulation of the *Mst35Bb* reporter gene construct. The β-Galactosidase enzyme activity was visualized by a histochemical reaction using the chromogenic substrate X-Gal. The β-Galactosidase activity solely appears in elongated spermatid bundles, while stem cells and spermatogonia at the testis tip and also the following spermatocytes as well as meiotic and early post-meiotic stages which are located in the inner curve of the testis are free of staining. Whereas, the translation repression is lost in the Δ233 and Δ109 deletion constructs (Fig. 5.6 D and F arrows) but still retains the minimal promoter sequence of 109bp sufficient enough for transcription of *Mst35Bb*, which is clearly evident in Δ109 (Fig. 5.6 F). Thus the reporter gene expression reflects the expression of *Mst35Bb* in space and time, which shows that the 5′ UTR is responsible for translational repression.

5.6 Loss of histones during nuclear shaping with the simultaneous accumulation of Protamine A, Protamine B and Mst77F in the sperm head

During spermiogenesis, the round nuclei dramatically changes its shape from a spherical structure (Fig. 5.7 A and 5.9 F) into a hook like structure forming at the tip of the nucleus as a result of onset of nuclear shaping (Fig. 5.7 A and 5.9 G). The nuclei start to flatten on one side and appear thereafter canoe or banana shaped with the densely stained chromatin interspersed with lightly stained chromatin showing a “net like” appearance when stained with Hoechst, as a result of onset of chromatin condensation process (Fig 5.7 A and 5.10H). Furthermore, as the condensation proceeds, eventually the densely stained chromatin appears to be evenly distributed, the nuclei cluster together (Fig. 5.7 A and 5.9 I) and leads into a thin needle shaped nucleus just prior to individualization (Fig 5.7 A and 5.9 J). Each sperm from the cluster individualize synchronously with a fully condensed chromatin

compacted in a thin needle shaped nucleus (Fig 5.7 A and 5.9 K). During this process the volume of the nucleus is reduced approximatly 200-fold (Fuller, 1993). To analyze the fate of histones during spermiogenesis an existing His2AvDGFP fly line were analyzed (Clarkson and Saint, 1999). His2AvDGFP fluorescence in these fly lines was retained in all the early

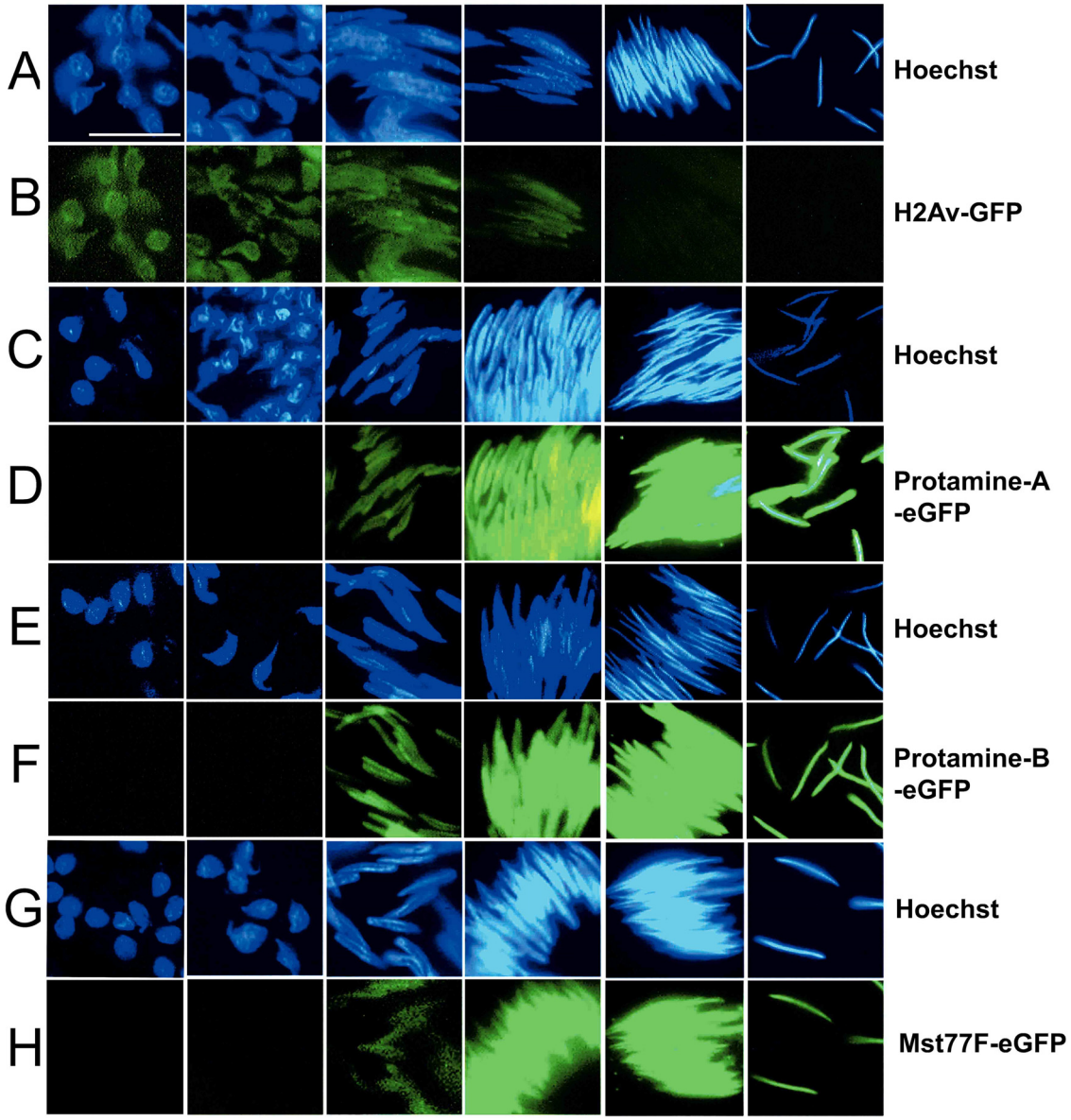


Fig. 5.7. Loss of Histone His2AvDGFP and appearance of ProtamineB-eGFP, ProtamineA-eGFP and Mst77F-eGFP during nuclear shaping and chromatin condensation.
(A, C, E, G) Hoechst counter staining for the chromatin (blue) visualises the transformation from round nuclei via a canoe shaped nuclei to the typical needle shaped nucleus of individualized mature sperms of the respective GFP fusions (from left to right).
(B) Histone His2AvDGFP pattern, showing the gradual loss of His2AvD from the canoe stage onwards to late spermatid stage.
Appearance of ProtamineA-eGFP (D), ProtamineB-eGFP (F) and Mst77F-eGFP (H) from the canoe stage. Intensity of fluorescence increases indicative of accumulation of these proteins in the nucleus as chromatin condensation continues and persists in the individualized sperm.
The scale bar represent 20µm (see A).

sperm nuclei up to canoe stage indicative of the presence of histone based nucleosomal conformation. During the canoe stage, the His2AvDGFP begins to fade and is no longer detectable in the needle shaped nuclei of elongated spermatids (Fig. 5.7 B from left to right).

To analyze whether core histones are replaced by dProtA, dProtB and Mst77F, the transgenic lines bearing eGFP fusion to dProtA, dProtB and Mst77F were established, by adding eGFP in frame to the putative protamine coding regions. The cytoplasmic eGFP was fused at the C-terminus since it is known from mammals that the protamines are processed at the N-terminus (Chapman *et al.,* 2003). To comprehend the complete coding region including the introns, 665bp upstream of the predicted 5'UTR for *Mst35Bb* and 280bp upstream of the predicted 5'UTR of *Mst35Ba* and the complete coding region was chosen, and fused eGFP within the last exon. The polyadenylation signal was supplied by the transformation vector *p*ChabΔSal. Using these constucts several independent transgenic fly lines were established. These respective genes are transcribed much earlier in the primary spermatocytes (Fig. 5.5 C1, 5.5 C2 and 5.5 C4). The proteins are made after several days in the elongated spermatid stage (Fig. 5.5 C3 and 5.5 C6 respectively). Thus *protamine* mRNAs and *Mst77F* mRNAs are translationally repressed for several days. The Mst77F-eGFP is also clearly seen transiently in the flagellum of the sperm bundles (Fig. 5.5 C6). These data reflects the pattern of expression and localization of these proteins in the *Drosophila* male germ line.

The pattern of these fusion proteins (green) were analyzed by testis squash preparation and counter stained with Hoechst for DNA (blue). All the three fusion proteins, ProtamineA-eGFP, ProtamineB-eGFP and Mst77F-eGFP show the appearance of these proteins at the canoe stage, the fluorescence became brighter in the later steps and stayed in the mature sperm nucleus (Fig 5.7 D, 5.7 F and 5.7 H respectively). The comparison of DNA distribution to eGFP fused to protamines and Mst77F distribution in the nucleus revealed that both protamines and Mst77F start to colocalize with DNA at the canoe stage of nuclear shaping (Fig. 5.7 C to H). At this stage His2AvDGFP fluorescence begins to diminish (Fig. 5.7 B). Protamines and Mst77F persists in individualized sperms until after fertilization.

As mentioned earlier, the role of the *Mst77F* gene product was not clear. Interestingly, Mst77F-eGFP pattern was not restricted to the nucleus alone but we could also see a clear eGFP pattern in the flagellum of the sperm bundles from canoe stage onwards (Fig. 5.7 C6, 5.9 B and 5.9 C). Just prior to individualization, Mst77F-eGFP was no longer seen in the sperm flagellum but still remained in the nucleus (Fig. 5.9 B). One or two additional copies of

Protamine-A-eGFP, Protamine-B-eGFP and Mst77F-eGFP does not dusturb the normal process of spermatogenesis.

5.7 Ectopic overexpression of protamines in salivary gland cells

Clearly, the Protamines-eGFP and Mst77F-eGFP fluorescence appeared to overlap with the DNA staining. The resolution of structures within the extremely compact sperm nucleus, however, is not sufficient to identify the chromosomes. Moreover, to check if there are specific regions on the chromosomes, where the protamines bind, both *Mst35Ba* and *Mst35Bb* were ectopically expressed in the salivary gland cells under the control of Sgs-GAL4 driver (Fig. 5.8). These results showed that there are no specific regions on the chromosomes where ProtamineA-eGFP and ProtamineB-eGFP binds to and thus covers the whole chromosome (also enters the nucleolus). Furthermore, with this ectopic expression in the salivary gland cells we could not see condensation of the polytene chromosome may be because these chromosomes are still with the histone based conformation. This further insinuates that histone displacement is critical for chromatin condensation by protamines.

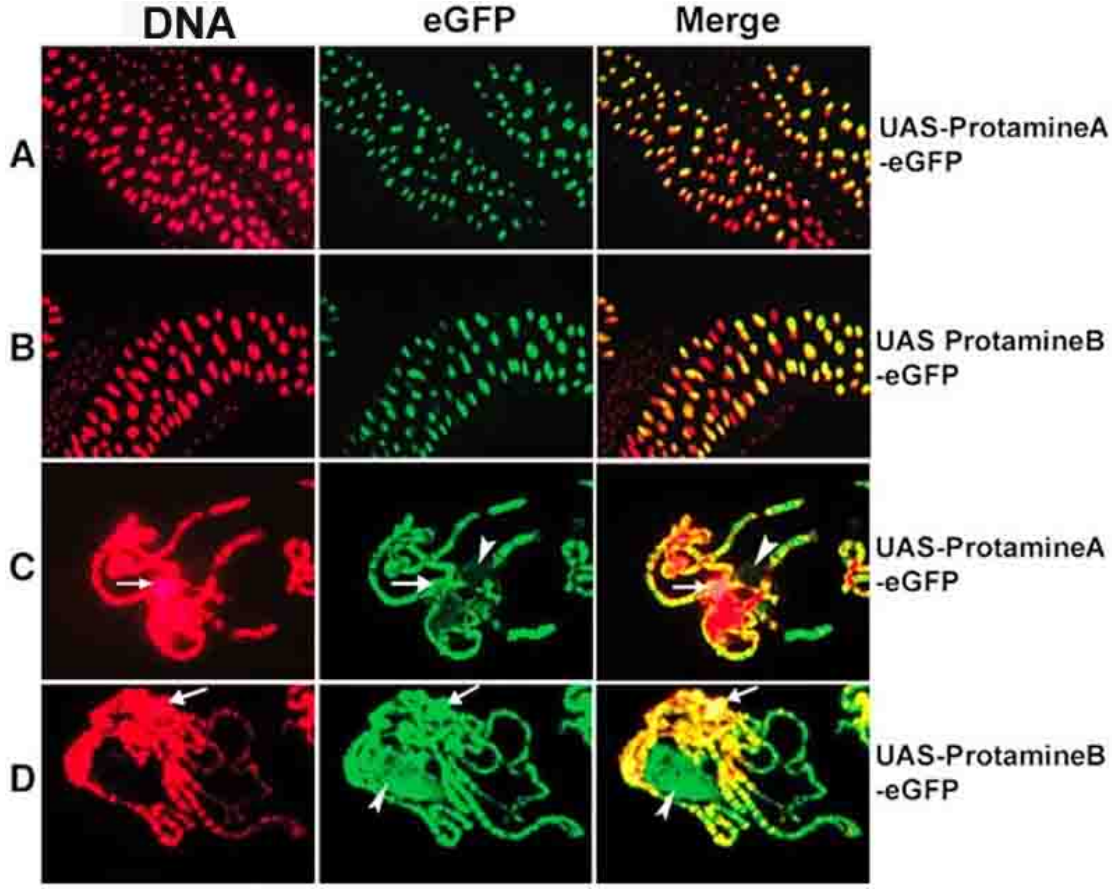


Fig. 5.8. Ectopic expression of Protamine A-eGFP and Protamine B-eGFP in salivary glands and on polytene chromosomes
DNA (red), Protamine-eGFP (green) and Merge (yellow).
Using the Sgs-GAL4 driver line (a gift from A. Hofmann) UAS-ProtamineA-eGFP and UAS-ProtamineB-eGFP were selectively expressed in the salivary glands.
(A) ProtamineA-eGFP and (B) ProtamineB-eGFP localize within the nucleus, (red) shows the chromatin staining which overlaps with the Protamine-eGFP in the merge.
(C, D) Squash preparations of salivary glands were analyzed histochemically and counter stained with Hoechst for chromatin. Both ProtamineA-eGFP and ProtamineB-eGFP binds all over the chromatin and enters the nucleolus also (arrowheads). The chromocenter (arrows).

5.8 Protamine genes are not haplo insufficient in *Drosophila*

In order to investigate whether all three basic sperm proteins are essential for male fertility in flies further genetic analysis were performed. For mice (Cho *et al.,* 2001), it was shown that loss of Protamine-1 or Protamine-2 is haplo insufficient and causes male sterility. The analysis of a haploid situation is *Drosophila* for the *Mst35Ba* and *Mst35Bb* genes with the deficiency *Df(2L)Exel8033/+* was done. These flies were male fertile (Table-3) and showed normal spermatogenesis. The deficiency *Df(2L)Exel8033* is defined by the breakpoints 35B1;35B8 which deletes over 35 genes including *Mst35Ba* and *Mst35Bb.* As this deficiency is not viable in a homozygous situation, it was not possible to check for complete removal of both *Mst35Ba* and *Mst35Bb*. So far, no mutants for these genes are available. Dan Lindsley had screened the male sterile Zuker mutant collection (Koundakjian *et al.,* 2004, Wakimoto *et al.,* 2004), where 31 male sterile mutant lines were mapped to the region 035B2 to 035D7 using the large deficiency *Df(2L)A48*. I tested these lines against a smaller deficiency *Df(2L)Exel8033* (breakpoints 35B1;35B8) and several lines mapping to this region were isolated.

Two mutant lines which are allelic to each other showed chromatin condensation defects (with normal nuclear shaping) from the canoe stage onwards. *Mst35Ba* and *Mst35Bb* were isolated and sequenced from these homozygous mutants and was found that neither of the protamine genes were mutated (data not shown). It might be that these small reading frames of *Mst35Ba* and *Mst35Bb* where either not mutated in this large screen or alternatively dProtA and dProtB which are 94% identical may be functionally redundant so that mutation in one is rescued by the other.

5.9 Mst77F is essential for male fertility

Besides the nuclear localization, Mst77F-eGFP also shows a transient pattern in the flagella of canoe stage spermatids to a stage just before individualization (Fig. 5.9 B and 5.9 C). In order to elucidate the function of Mst77F, the two existing mutants *ms(3)nc3* and *PBac{PB}Pka-R1^{c06969}* were analyzed. *ms(3)nc3* is a second-site non complementation (nc) mutation that was isolated in an EMS screen to identify the interacting proteins of the β2 tubulin isotype involved in microtubule function in *Drosophila* (Fuller *et al.,* 1989). *ms(3)nc3* fails to complement class I alleles at the *betaTub85D* locus on the chromosome arm 3R (Fuller, 1986). *betaTub85D*encode the β2 tubulin isotype which is a component of the axoneme and in all other microtubules from the primary spermatocyte phase onward (Fuller, 1993). The *ms(3)nc3* mutation was mapped to the cytological region 77E-77F on the chromosome arm 3L (Fuller, 1986) in the region where *Mst77F* is located. In order to confirm this mapping, *ms(3)nc3* in trans-heterozygotic situation with *Df(3L)ri-79c* (77B7-77F1) was tested and these males were sterile (Table-3). *PBac{PB}Pka-R1^{c06969}* is a Piggy bac P-element insertion (Thibault *et al.,* 2004) in the promoter region of *Mst77F* at 158bp upstream of the presumptive transcription start site. These homozygous *PBac{PB}Pka-R1^{c06969}/PBac{PB}Pka-R1^{c06969}* males and *PBac{PB}Pka-R1^{c06969}/Df(3L)ri-79c* trans-heterozygotes were viable and fully fertile (Table-3). But, *PBac{PB}Pka-R1^{c06969} /ms(3)nc3* trans-heterozygotes were sterile (Table-3). Since the P-element insertion was in the promoter region of *Mst77F* the level of transcript made was checked by performing whole mount *in situ* hybridization on the *PBac{PB}Pka-R1^{c06969}/PBac{PB}Pka-R1^{c06969}* homozygous testis and in comparision with the wild-type testis. Homozygous mutant testis clearly showed a reduced level of transcript to that of wild-type testis (Fig. 5.5 C4 and 5.5 C3 respectively). This led to infer that *ms(3)nc3* is a mutation in *Mst77F* and this mutation is dosage dependent. Furthermore, the *Mst77F* gene in *ms(3)nc3*/ *Df(3L)ri-79c* trans-heterozygotes was PCR amplified, cloned into the *TOPO* vector and 5 independent clones were sequenced in comparison with the wild-type *Mst77F*. All the clones from *ms(3)nc3*/ *Df(3L)ri-79c* revealed a single missense mutation from T→A transition, causing the substitution of threonine instead of serine at position 149 (Fig. 5.5 B).

As *ms(3)nc3* was not investigated concerning nuclear condensation, *ms(3)nc3*/*Df(3L)ri-79c* and *PBac{PB}Pka-R1^{c06969}/ms(3)nc3* were analyzed to exclude any further mutation on the *ms(3)nc3* carrying chromosome that contribute to the phenotype. In an

overview, nuclei of spermatids from these males are mainly scattered and are round or ellipsoid (Fig. 5.9 D), some spermatid nuclei begin to elongate but far less than in wild-type spermatids (Fig. 5.9 A). In the wild-type situation, at higher magnification, the chromatin distribution can be observed very well. First, the chromatin is visible as a broad network

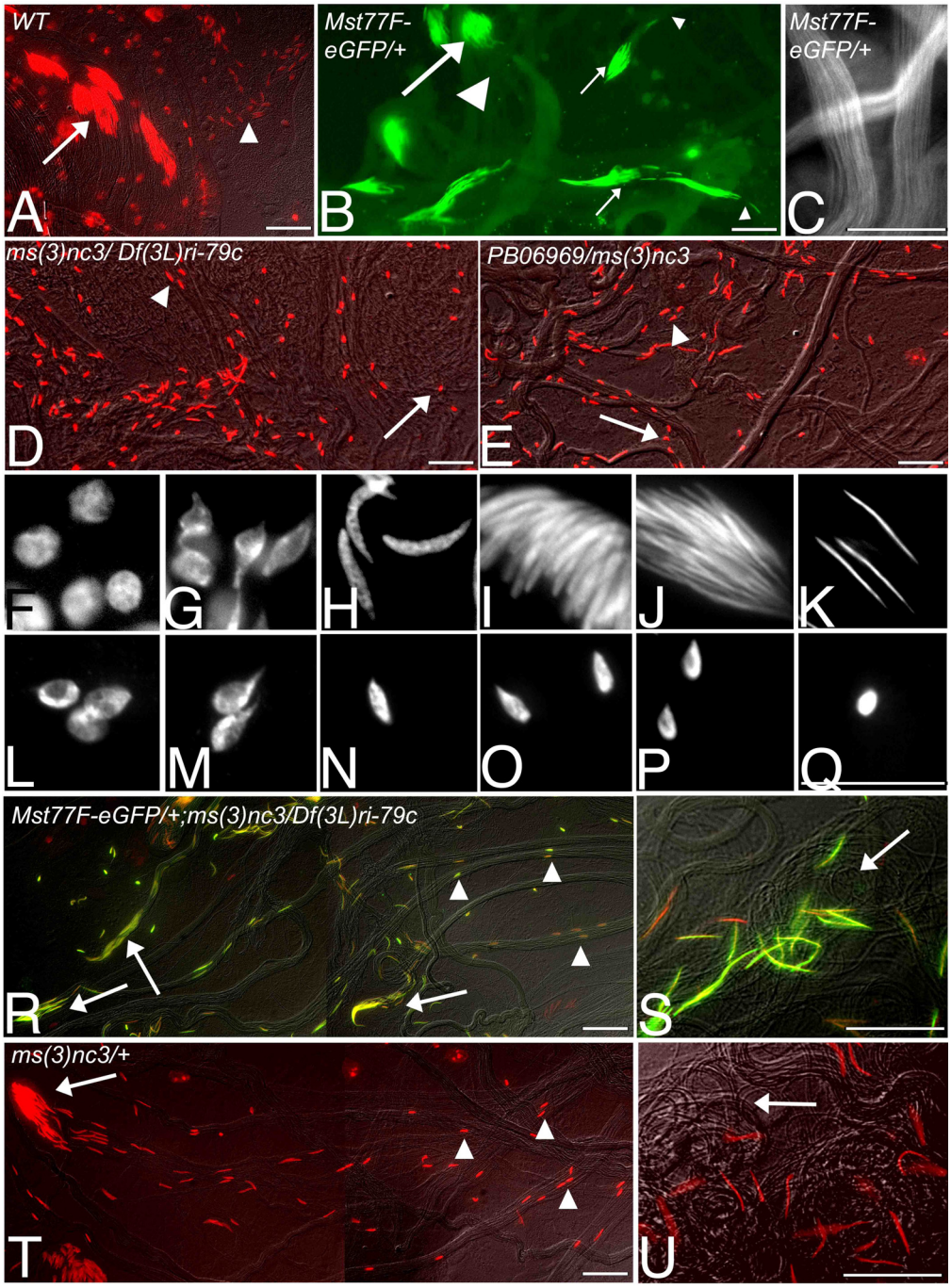


Fig. 5.9. Defective spermatid nuclei in *ms(3)nc3* and rescue of mutant phenotype by *Mst77F-eGFP* transgene.
Testis squash preparations: Hoechst (red), Mst77F-eGFP (green) and Merge (yellow)
A) WT Hoechst staining. Uncondensed early spermatid nuclei undergo nuclear shaping showing the light staining (arrowhead) and condensing spermatid nuclei are clustered, show the bright staining (arrow).

B) Mst77F-eGFP transgenic flies. Condensing spermatid nuclei clustered (large arrow), Mst77F-eGFP in the flagellum (large arrowhead), condensed nuclei at the onset of individualization (small arrows), disappearance of Mst77F-eGFP during individualization (small arrowheads).
C) Higher magnification of sperm flagellar bundles showing Mst77F-eGFP (gray scale) in the flagellum.
D) *ms(3)nc3/Df(3L)ri-79c* , Spermatid nuclei scattered all through the sperm bundles showing the tid nuclear phenotype. Defective sperm nuclei during the process of chromatin condensation (arrowhead), Tid nuclei showing the bright staining representative of condensed chromatin (arrow).
E) *PBac{PB}Pka-R1^{c06969}/ms(3)nc3* . Defective sperm nuclei during the process of chromatin condensation (arrowhead), tid nuclei showing the bright staining representative of condensed chromatin (arrow).
(F-K): Nuclear shaping and chromatin condensation in wild-type situation (Hoechst staining in gray scale), (L-Q): *ms(3)nc3/Df(3L)ri-79c* (Hoechst staining in gray scale). The scale bar 20μm shown in Q.
(R and S) rescue of *ms(3)nc3/Df(3L)ri-79c* by Mst77F-eGFP expression.
(R): Normal spermatid nuclei clustered (arrows). Few tid shaped nuclei scattered (arrowheads). (S): Individualized sperms in rescued testis.
(T and U): *ms(3)nc3/+*
(T): Normal spermatid nuclei clustered (arrow), few tid shaped nuclei scattered (arrowheads). (U) Sperms individualize.
The scale bars represent 20μm.

TABLE 3. Fertility and Phenotype

Genotype	Fertility	Tid nuclear phenotype	individualised sperms
+/+	Fertile	-	+++
ms(3)nc3/+	Fertile	+	++
ms(3)nc3/Df(3L)ri-79c	Sterile	+++	-
PB06969/PB069069	Fertile	-	+++
PB069069/Df(3L)ri-79c	Fertile	-	+++
PB069069/ms(3)nc3	Sterile	++	-
Mst77F-eGFP/+; ms(3)nc3/Df(3L)ri-79c	Fertile	+	++
Protamine-B-eGFP/+; ms(3)nc3/Df(3L)ri-79c	Sterile	+++	-
Df(2L)Exel8033/+	Fertile	-	+++

PB06969 is PBac{PB}Pka-R1^{c06969}

within the round nucleus (Fig. 5.9 F), then the chromatin accumulates at one side of the nucleus (Fig. 5.9 G). The nucleus flattens and the chromatin is localized in spots (net like)

(Fig. 5.9 H). In later stages, the condensing chromatin is visible all over the nucleus (Fig. 5.9 I-K). In spermatids of *ms(3)nc3*/*Df(3L)ri-79c* testis, the early spermatid nuclei appear to be normal (Fig. 5.9 L and M), but the accumulation of the chromatin in spots (net like) were observed, though the nuclear shaping does not proceed (Fig. 5.9 N and 5.9 O). Later the nuclei are smaller and ellipsoid but no further development was seen (Fig. 5.9 P and Q). *PBac{PB}Pka-R1*^{c06969}*/ms(3)nc3* males showed a slightly less severe phenotype (Fig. 5.9 E) as compared to *ms(3)nc3*/*Df(3L)ri-79c* with no individualized sperms leading to male sterility (Table-3).

5.10 Mst77F-eGFP rescues ms(3)nc3 mutants

In order to clarify if Mst77F-eGFP could rescue the phenotype caused by *ms(3)nc3*/*Df(3L)ri-79c,* the transgenic flies carrying *Mst77F-eGFP* on the second chromosome were used. In these males the *Mst77F-eGFP* transgene indeed clearly can restore chromatin condensation, the nuclei are clustered like in the wild-type situation (Fig. 5.9 R for an overview) and spermatids individualize to mature sperm (Fig. 5.9 S). Furthermore, these males are fertile (Table-3). But, these males showed a few tid shaped nuclei scattered through the sperm bundles (Fig. 5.9 R). To address this question the spermatid development of *ms(3)nc3/+* males were checked and these males though fertile also showed a few tid nuclei scattered (Fig. 5.9 T) and individualized sperms (Fig. 5.9 U) comparable to that of *ms(3)nc3* rescue by *Mst77F-eGFP*. These results indicate that ms(3)nc3 is indeed a mutation in *Mst77F* and incorporation of the mutant Mst77F protein might act as a poison to the complex.

5.11 ProtamineB-eGFP cannot replace Mst77F

It was proposed that *Mst77F* mRNA might encode a protamine (Russell and Kaiser, 1993), which might be supported by the fact that the Mst77F protein contains many cysteines. Therefore, it was important to know whether Mst77F might be functionally redundant to protamines. One copy of ProtamineB-eGFP were brought into *ms(3)nc3*/*Df(3L)ri-79c* genetic background and spermiogenesis was analyzed. Clearly, spermiogenesis was not improved in this situation (Fig. 5.10 A compare to Fig. 5.9 D). This concludes that Mst77F cannot be replaced by increasing the amount of ProtamineB. Furthermore, it was checked whether

ProtamineB-eGFP deposition was normal in these mutants and this clearly is the case (Fig. 5.10 A).

The detailed comparison of nuclear stages of spermatids from wild-type and mutant flies revealed that ProtamineB-eGFP is made at the identical stages as that of wild-type (Fig. 5.10 J to M compare to Fig. 5.10 D to G). Furthermore, the accumulation of ProtamineB-eGFP on the dense chromatin concentrated on one side of the nucleus was observed (Fig. 5.10 L) and in the final stage, nuclei became round and the accumulation of ProtamineB-eGFP was seen in the two opposite sides with a small gap of light staining in the middle (Fig. 5.10 M). When compared the size of the round shaped uncondensed nuclei in Fig. 5.10 H with Fig. 5.10 M taken with the same 100X magnification, it is clear that the nuclear size is reduced which is an indication of chromatin condensation and the removal of nucleoplasm, but, it is unclear if the chromatin condensation is complete with Mst77F mutant protein in *ms(3)nc3*.

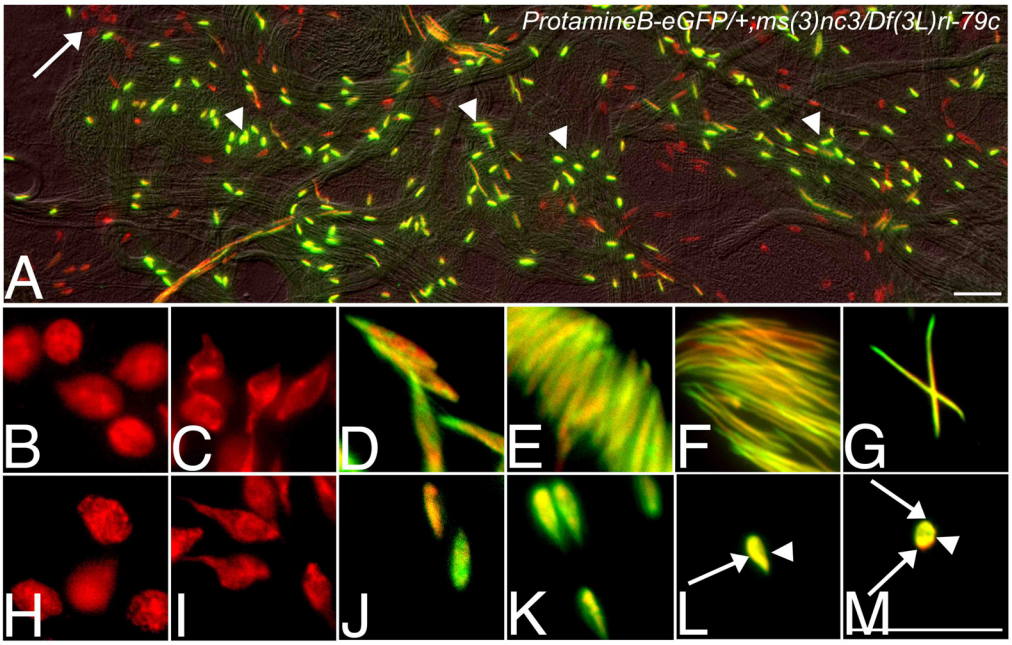


Fig. 5.10. Protamines in *Mst77F* mutant.
Testis squash preparation: Hoechst (red). ProtamineB-eGFP (green) and Merge (yellow).
A) ProtamineB-eGFP in *ms(3)nc3/Df(3L)ri-79c* males. Uncondensed early spermatid nuclei undergoing nuclear shaping (arrow), condensing spermatid nuclei showing scattered tid nuclear phenotype (arrowheads).
(B-G) ProtamineB-eGFP transgenic flies showing the wild-type pattern.
(H-M) *ms(3)nc3/Df(3L)ri-79c* flies showing ProtamineB-eGFP pattern. Dense chromatin (arrows in L and M), a small gap at the center showing a faint eGFP fluorescence (arrowheads in L and M).
The scale bars represent 20μm.

5.12 Protamine deposition is independent of nuclear shaping

It is now known that *Mst35Ba* and *Mst35Bb* are transcribed during early primary spermatocyte stage (Fig. 5.5 C1 and 5.5 C2), and that the transcripts are translationally repressed till the post-meiotic stage, when the repression is relieved and the protein is made (Fig. 5.5 C3). Rb97D is a RNA binding protein which is associated with the ks-1 fertility locus on the Y-chromosome and limited to pre-meiotic stages (Heatwole and Haynes, 1996). As the *protamine* mRNAs are localized in cytoplasm of spermatocytes (Fig. 5.5 C1 and 5.5 C2) it is unlikely that Rb97D binds to *protamine* mRNA. In the context of chromatin remodeling, the $Rb97D^{1}$ mutant is of interest with respect to the question whether nuclear shaping and protamine deposition to the chromatin are independent processes or not.

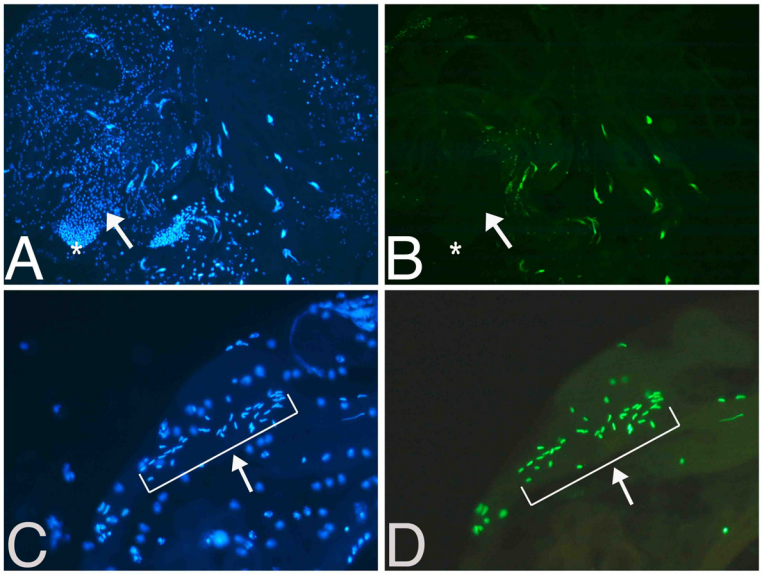


Fig. 5.11. Protamine deposition in *Rb97D*^{1} mutants
Squash preparation of testis with ProtamineB-eGFP in the $Rb97D^{1}$ mutant. Testis are stained with Hoechst (blue) in (A and C) and ProtamineB-eGFP (green) in (B and D). The ProtamineB-eGFP protein is made in the later stages of elongated spermatids (arrowheads) as in the wild-type, but not in the primary spermatocyte stage (arrow). (C-D) At higher magnification, a spermatide bundle of $Rb97D^{1}$ mutants (arrow and bracket) depicts the normal accumulation of ProtamineB-eGFP (indicative of normal chromatin condensation) despite the defective nuclear shaping.
The scale bars represent 20μm.

Therefore, the expression of eGFP-tagged version of ProtamineB in Rb97D mutants were analyzed. Mutation in *Rb97D* cause developmental arrest during spermiogenesis (Karsch-Mizrachi and Haynes, 1993). In the wild-type, protamines just start to accumulate in the nuclei at the canoe stage (Fig. 5.11 A and B). In $Rb97D^{1}$ mutants, DNA staining shows that the nuclei start to elongate, and some reach the canoe stage of nuclear shaping (Fig. 5.11 C), but, in contrast to the wild-type, the $Rb97D^{1}$ mutant nuclei at the canoe stage contain

already high amount of protamines but, the nuclear shaping dose not proceed further (Fig. 5.11 D). These observations led to the conclusion that synchronous advancement in nuclear shaping is not essential for protamine deposition but is an independent process.

III. Histone degradation

5.13 Transcriptional silencing and histone H2AvD degradation

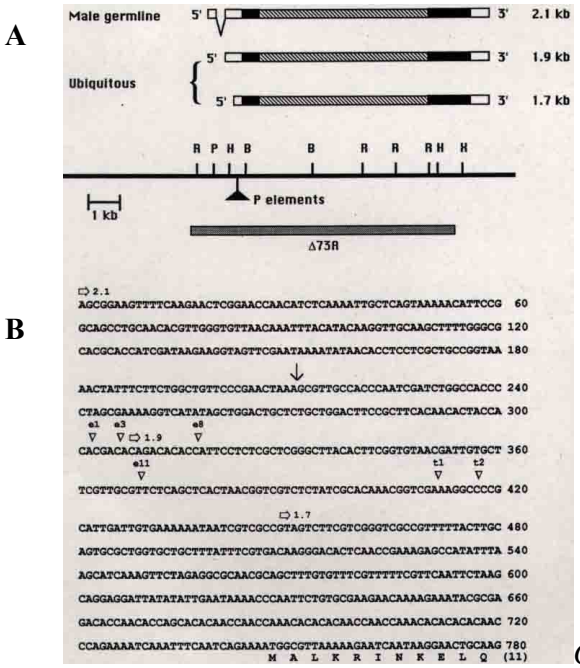
During the later stages of the *Drosophila* sperm terminal differentiation, the transcription ceases almost completely during the meiotic prophase II (Fuller, 1993). Histones persists in the spermatid nuclei till the elongated spermatid stage and begins to degrade at the canoe stage. The study of the mechanism of the global transcription shut down and the mechanism of histone degradation is a very interesting aspect to be addressed during spermiogenesis. In mammals, for example, in mice, it was initially thought that the transition proteins that appear as the intermediate proteins at the step of nucleosomal transition from histones to protamines during spermiogenesis could possibly play a role in the transcription shut down. Recent studies by Zhao *et al.,* (2004) have shown that mice lacking both TP1 and TP2 show normal transcriptional repression, histone displacement, nuclear shaping and protamine deposition. But, these mice show the loss of genomic integrity with large number of double strand DNA breaks, leading to male sterility. Though the study by Zhao *et al.,* show that transition proteins are not essential for transcription cessation. The mechanism of transcription cessation still remains unclear. The existence of transition proteins in *Drosophila* is still a question. In *Drosophila*, there is a long phase between transcription shut off (at meiotic prophase-II) and the histone degradation (at canoe stage of elongated spermatids) which is provocative to speculate that these two processes may be independent of each other.

5.14 Screening of male sterile mutants for histone degradation.

In order to check for the candidate proteins that degrade histones in late spermatids, male sterile mutants generated by Castrillon *et al., 1993* were screened. In this screen H2AvD-GFP was visualized in various independent mutant background. Around 36 mutants were tested and I found that eff^{8} mutant, a P-element insertion in *effete (UbcD1)* gene (Fig. 5.12) showed the persistence of histones during the late spermatids.

5.15 UbcD1 (Effete) is required for spermatogenesis

effete encode for an E2 ubiquitin conjugating enzyme UbcD1. UbcD1 is also required outside of spermatogenesis. Several homozygous mutants that have been isolated for this gene are embryonic lethal. The *UbcD1* gene gives rise to multiple transcripts (Cenci *et al.,* 1997) The longest transcript 2.1kb is specific to the male germ line (Fig. 5.12), two smaller transcripts 1.9 and 1.7kb are broadly expressed outside spermatogenesis. All three *UbcD1* transcripts encode the same protein product (Fig. 5.12). eff^{s8} is the P-element insertion in the *UbcD1* 5' UTR of the 1.9kb transcript and is a recessive male sterile mutant. eff^{s8}/eff^{s8} males show mitotic defects in the early male germ cells but no meiotic defects. The phenotype observed was frequent telomere-telomere attachments of the homologous chromosomes (Cenci *et al.,* 1997). Hence, one of the targets of the UbcD1 ubiquitination is proposed to be the telomere-associated polypeptide that may be required to maintain proper chromosomal orientation during interphase.



Cenci et al., 1997

Fig. 5.12. Map of *UbcD1,* indicating the location of the transcripts and the P-element hits for this gene.
A) Structure of *UbcD1* gene and its transcripts. The longest 2.1kb is specific for the male germ line. 1.9kb is also expressed in the male germ line where, both 1.9kb and 1.7kb transcripts are broadly expressed. Open areas of the transcript represent the 5'UTR, solid areas are the protein coding regions of the exons and the hatched areas are the long introns.
B) Nucleotide sequence of the presumptive 5'UTR region of *UbcD1* mRNA. The downward arrow indicates the 66bp intron in this transcriptional unit for the male germ line specific transcript (2.1kb). The P-element insertion eff^{s8} (e8) is in the 5'UTR of the 1.9kb transcript which may also affect 2.1kb

transcript since this region is shared. All the inverted triangles represent the sites of respective P-element insertions in *UbcD1* gene (taken from *Cenci et al., 1997*).

5.16 A novel function of UbcD1 during spermiogenesis

As shown by Cenci *et al.,* UbcD1 is required for the normal telomere behavior in the early male germ cells. The phenotype exhibited during the late elongated spermatid differentiation was not analyzed in eff^{8}/eff^{8} males. In the late spermatids of eff^{8}/eff^{8} the normal individualization fails, leading to the scattered sperm heads when visualized with the Hoechst staining for DNA and the head of the post-canoe stage spermatids appears thicker when compared to the wild-type.

In order to check for the fate of histones in the late spermatids of eff^{8}/eff^{8} testis, the histone H2AvD-GFP was visualized in the eff^{8}/eff^{8} mutants. When compared to the wild-type situation, where the H2AvD-GFP is degraded at the canoe stage of spermatid development (Fig. 5.13), in the eff^{8}/eff^{8} mutants, it was observed that H2AvD-GFP was retained in the post-canoe stage spermatids (Fig. 5.13) with the scattered spermatid heads throughout the spermatid bundles. The same results were obtained with $eff^{8}/Df(3R)Exel6275$ transheterozygotes (Fig. 5.14), the deficiency *Df(3R)Exel6275* deletes 23 other genes including *UbcD1* having the breakpoints 88D1;88D7. This clearly suggests that, besides the role of UbcD1 in maintaining the normal telomere behavior in the early germ cells, UbcD1 function is required for the histone degradation during spermiogenesis. Further experiments are needed to elucidate whether the effect of UbcD1 in histone degradation during spermiogenesis is direct or indirect.

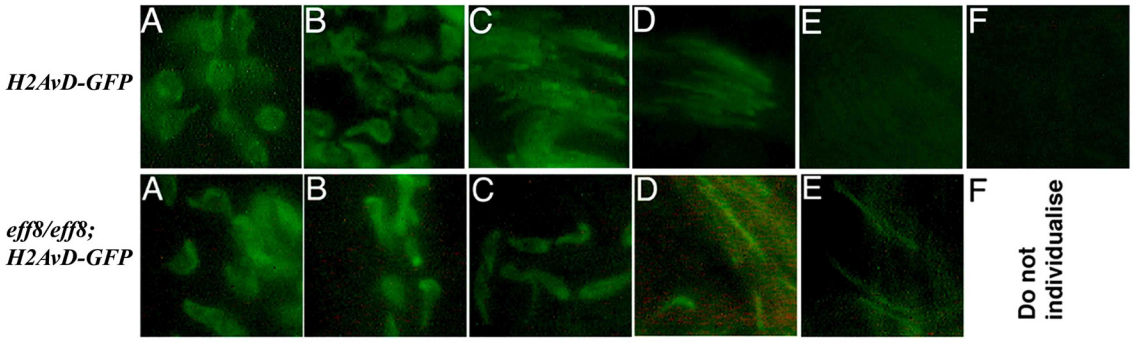


Fig. 5.13. H2AvD-GFP is not degraded in eff^{8}/eff^{8} mutants. The upper pannel shows the wild-type situation with the gradual degradation of H2AvD-GFP from the canoe stage onwards. The lower pannel is the eff^{8}/eff^{8} mutants showing the clear retention of H2AvD-GFP even in the post-canoe stages, and the sperm bundles do not individualize.

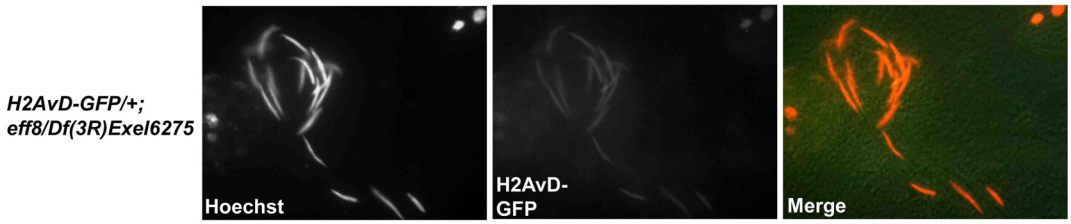


Fig 5.14. H2AvD-GFP is not degraded in the *eff*^{8}*/Df(3R)Exel6275.* Left dipicting the Hoechst staining for DNA in the gray scale which shows the late condensing chromatin. In the middle is shown the H2AvD-GFP clearly retained still at this stage. Right, showing the merge.

5.17 Ring finger E3 ligase Cullin-1 and Cullin-3 are expressed during *Drosophila* spermatogenesis

The recruitment of Ub-E2 enzyme to ubiquitinate the substrate is dependent on E3 ligase complex (see introduction). Furthermore, in order to get an insight into the pattern of expression of RING finger E3 ligase proteins, the following experiments were done.

5.18 Pattern of expression of Cullin-1 during *Drosophila* spermatogenesis

Antibody stainings using anti-Cul-1 (a gift from Fillipova, antibody raised in rabbit), was performed on the wild-type testis squash preparations. These stainings showed that indeed Cullin-1 is expressed during *Drosophila* spermatogenesis. A clear pattern of staining was seen in all early germ cells, where Cullin-1 was localized to the spectrosomes of the gonioblast cells (Fig. 5.16A), the fusomes of spermatogonia (Fig. 5.15 A), primary spermatocytes (Fig. 5.15 A) and early meiotic cells (Fig. 5.15 A). Cullin-1 disappeared after the meiotic divisions, and reappeared at the canoe stage of spermatid development (Fig. 5.15). At this stage a peculiar localization of Cullin-1 was seen, where Cullin-1 localizes to the tip of the spermatid head at the canoe stage in the perinuclear space, but not in the nucleus and disappeares when the nuclear condensation is complete (Fig. 5.15). Further experiments are required where, by performing the mosaic clones for Cullin-1 in the male germ line and looking for the fate of Histone H2AvD-GFP in these clones, it would be possible to infer if Cullin-1 is the E3 ligase used for Histone degradation.

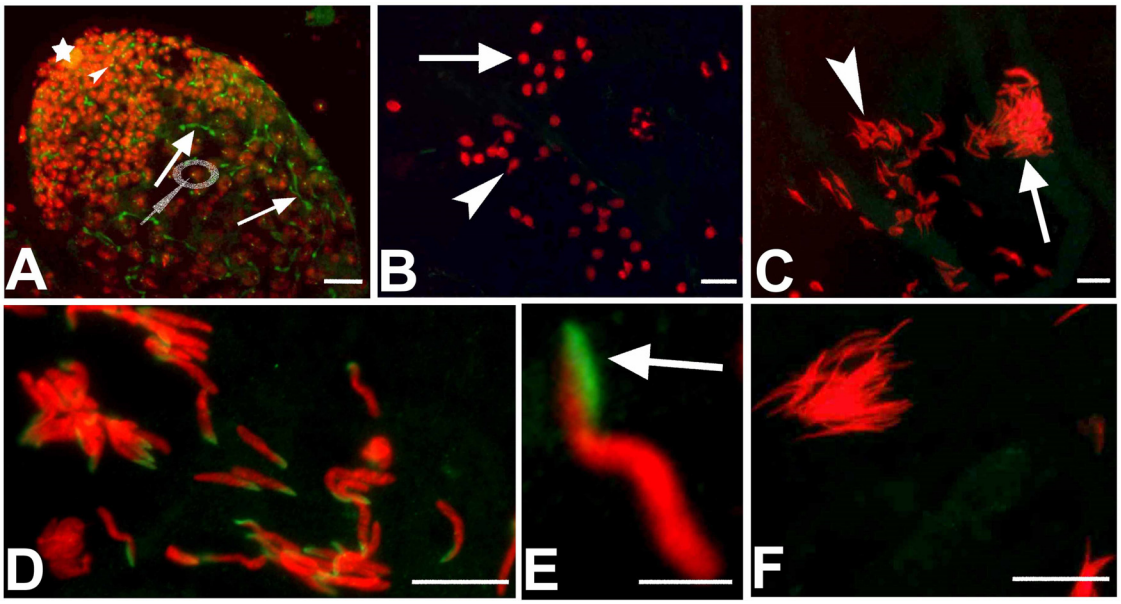


Fig. 5.15. Pattern of Cullin-1 expression during *Drosophila* spermatogenesis. Cullin-1 (Green) and DNA (Red). A) The apical tip of the testis, with Hub indicated by asterisks, Cullin-1 staining is seen in the stem cells and the gonioblast cells with a dot like appearance (arrowhead) indicating the spectrosomes. In the late spermatogonial cells and the primary spermatocytes Cullin-1 is associated with the fusomes (arrows), and a dot like staining is also seen in all the primary spermatocyte nuclei (transparent circle with an arrow indicating the staining in the nucleus). A similar pattern is seen till the meiotic stages. Cullin-1 disappeares during the late meiotic stages.
B) No Cullin-1 staining is seen in the early elongated round head spermatids (arrow), and also when the spermatid nuclei have begun to shape (arrowhead).
C) Spermatid nuclei undergoing shaping (arrowhead) where still Cullin-1 staining is absent. Reappearance of Cullin-1 at the early canoe stage of nuclear shaping (arrow) starts to accumulate at the spermatid head.
D) A clear pattern of Cullin-1 seen at the tip of the canoe stage spermatid head which does not overlap with the DNA staining.
E) An higher magnification of the canoe stage spermatid head with a side on view, dipicting the localization of Cullin-1 in the perinuclear space, particularly to the tip of the perinuclear space.
F) When the chromatin is fully condensed with the spermatid heads spread, indicative of individualization complex has already passed through head region and Cullin-1 is no more seen.

The scale bars in A,B,C,D and F represent 20µm. The scale bar in E represents 5µm

5.19 Cullin-3 (*guftagu*) is expressed in the elongated spermatids during *Drosophila* spermiogenesis

To check whether the E3 Ligase Cullin-3 is expressed during *Drosophila* spermatogenesis, an enhancer trap line *P{PZ}gft*^{06430} for Cullin-3 was used. Flies with this P-insertion are homozygous lethal at the larval stage of development. A β-galactosidase staining was performed on the testis of the *P{PZ}gft*^{06430} /+ heterozygous testis. A specific staining

was observed in the elongated spermatids (Fig. 5.16 A,B and C). All the early germ cells are free of staining (Fig. 5.16 A) indicating that Cullin-3 is expressed in the later stages of the spermatid development. The staining was detected in these spermatid nuclei because of the Lac-Z with the nuclear localization signal (*NLS-Lac-Z*) integrated in the P-element. Thus this

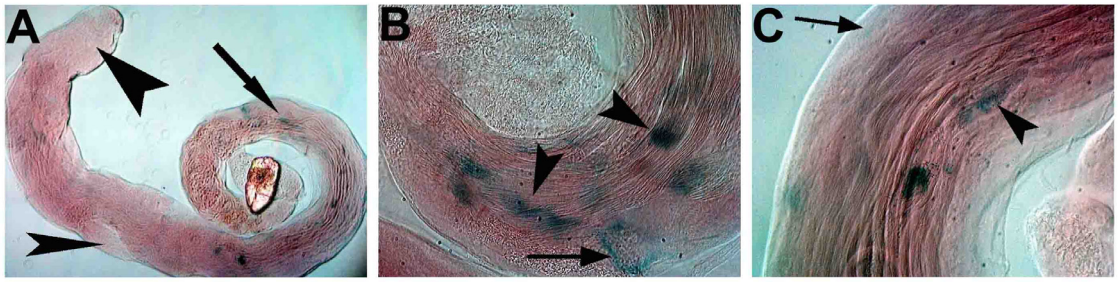


Fig. 5.16. β-galactosidase staining of the enhancer trap line *P{PZ}gft^{06430}* for Cullin-3. A) Whole tesis showing (arrowheads) all the early germ cells are free of staining, a pattern of staining (arrow) in the elongated spermatid heads (because of NLS-Lac-Z). B) (arrow) elongated spermatids with round spermatid heads and (arrowheads) late spermatid heads with needle shaped nuclei stained. C) (arrow) late primary spermatocytes at the periphery shows no staining, (arrowhead) elongated spermatids with the round head shows the β-galactosidase staining.

led to infer that Cullin-3 is expressed in the elongated spermatids, but the localization of Cullin-3 is still a question as there are no antibodies available for Cullin-3. Further clonal analysis of Cullin-3 during spermatogenesis or generation of transgenic flies with *UAS-Cullin-3-RNAi* driven under the control *β2-Gal4* would be useful to elucidate the function of Cullin-3 during spermatogenesis. These experiments when done with the *H2AvD-GFP* background would provide the information if Cullin-3 is required for histone degradation or not.

IV Removal of Protamines and Mst77F after fertilization

5.20 ProtamineA and ProtamineB removal from the male pronucleus depends on the maternally supplied Sesame protein

Protamines are the typical chromatin organizing proteins in the mature sperm. After fertilization but before zygote formation, the chromatin of the male pronucleus has to be remodelled to obtain the nucleosomal conformation. Transgenic flies with eGFP-tagged versions of protamines were used for the analysis of protamine removal from the male pronucleus.

In eggs laid by wild-type females, the needle shaped male pronucleus is visible by eGFP-tagged ProtamineA (Fig. 5.17 A). No sign of ProtamineA-eGFP is visible in the first mitotic division after zygote formation (Fig. 5.17 B). A comparable result is found for ProtamineB-eGFP (Fig. 5.17 C and D).

It is likely that maternally supplied proteins induce the chromatin reorganization of the male pronucleus. Indeed, a maternal effect mutant, *sesame*, has been described, in which the male pronucleus is not able to form a zygote with the female pronucleus, probably caused by a defect in chromatin reorganization of the male pronucleus. In *sesame* mutants, the male pronucleus looses the needle like shape and becomes spherical like in the wild-type, but the male pronucleus is not competent enough to fuse with the female pronucleus because maternal histones are not incorporated into the male pronucleus (Loppin *et al.,* 2001). This immediately raises the question whether the male pronucleus remains in the protamine-based chromatin structure in *sesame* mutants. Homozygous virgin *sesame* mutant female flies were collected and mated with the transgenic males carrying *protamine-eGFP* fusion genes separately. In eggs laid by homozygous *sesame* mutants, DNA staining visualizes the male pronucleus (m) and female pronucleus (f) dividing after fertilization with ProtamineA-eGFP sperm (Fig. 5.17 G). The male pronucleus had transformed from its needle like to a round shape but retained ProtamineA-eGFP (Fig. 5.17 H). With ProtamineB-eGFP we observe essentially the same situation. The DNA staining shows the enlarged spherical male pronucleus (m) and the haploid female pronucleus (f) dividing (Fig. 5.17 I). The male pronucleus still contains the ProtamineB-eGFP (Fig. 5.17 J). With these observations it was

clear that Sesame function is required for removal of both dProtA and dProtB from the male pronucleus for the successful restoration of a nucleosomal chromatin configuration.

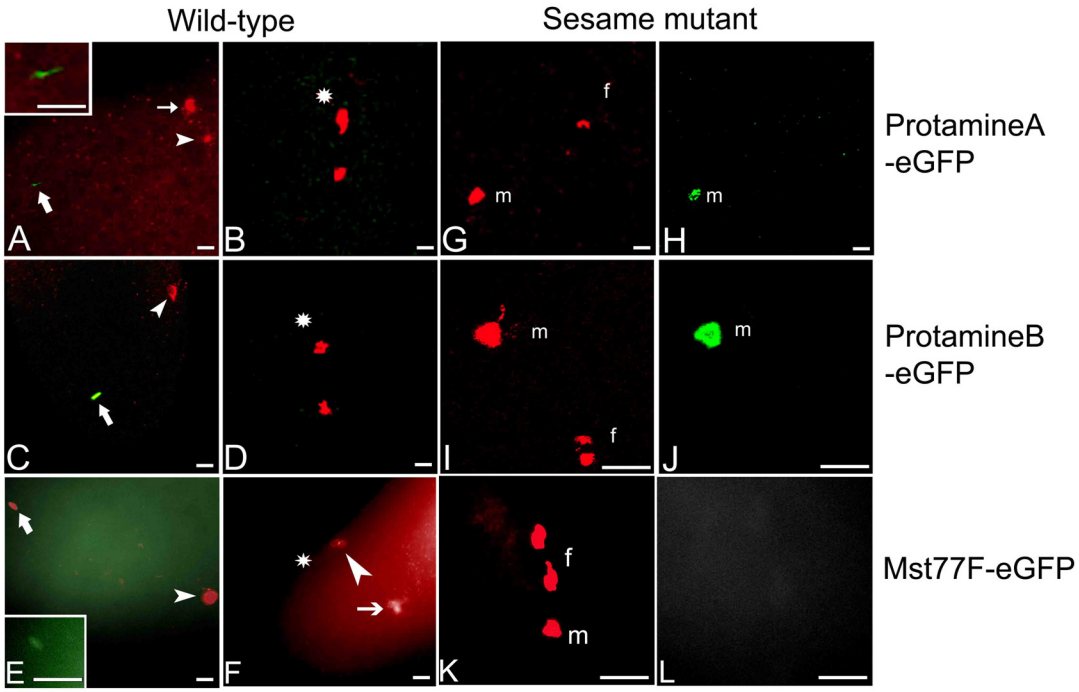


Fig. 5.17. Loss of both protamines in the male pronucleus is dependent on the maternally encoded protein Sesame, while loss of Mst77F is independent of Sesame.
Propidium iodide (red), eGFP fusion proteins (green) and merge (yellow).
(A-F) Wild-type eggs and (G-L) *sesame* mutant eggs.
(A) Fertilization with ProtamineA-eGFP sperm, thin needle shaped sperm nucleus containing ProtamineA-eGFP (thick arrow). The female pronucleus (arrowhead) and the polarbody (thin arrow). Higher magnification of the sperm head in the egg depicted at the top left corner.
(B) After first mitotic division (star) the Protamine-A-eGFP is no longer present.
(C) Fertilization with a Protamine-B-eGFP sperm, needle shaped sperm nucleus which has already begun the transformation to round shaped male pronucleus (thick arrow). The female pronucleus (arrowhead).
(D) After the first mitotic division the ProtamineB-eGFP is no more present.
(E) The sperm nucleus (arrow) contains Mst77F-eGFP (see magnification without merge), the female pronucleus (arrowhead).
(F) male and female pronucleus undergoing fusion (arrowhead), the male pronucleus has already lost Mst77F-eGFP, polarbody (thin arrow).
(G-L) In the *sesame* mutant eggs the protamines are not degraded in the male pronucleus.
(G) ssm^{185b}/ssm^{185b} oocyte fertilized with ProtamineA-eGFP sperm. PI staining (red) showing the male pronucleus (m) and the female pronucleus (f) undergoing the first haploid division.
(H) In the male pronucleus (m) ProtamineA-eGFP persists.
(I) ssm^{185b}/ssm^{185b} oocyte fertilized with ProtamineB-eGFP sperm. PI staining (red) showing the male pronucleus (m) and female pronucleus (f) undergoing the first haploid division.
(J) In the male pronucleus (m) ProtamineB-eGFP persists.
(K) ssm^{185b}/ssm^{185b} oocyte fertilized with Mst77F-eGFP sperm. PI staining (red) showing the male pronucleus (m) and the female pronucleus (f) undergoing the first haploid division.
(L) In the male pronucleus Mst77F-eGFP is no more present. The scale bars represent 20µm.

5.21 Removal of Mst77F from the male pronucleus is independent of Sesame

In the wild-type situation Mst77F is seen in the nucleus of sperm until shortly after fertilization (Fig. 5.17 E), but, is removed from the male pronucleus before zygote formation (Fig. 5.17 F). To check whether this process also depends on maternally supplied Sesame, it was analyzed by fertilizing eggs from homozygous *sesame* mutants with Mst77F-eGFP containing sperm. Clearly, in Sesame mutant eggs, Mst77F-eGFP is removed from the male pronucleus. No zygote is formed and the haploid female nucleus starts to divide (Fig. 5.17 K and L). This clearly illustrated that Mst77F removal from the male pronucleus is independent of Sesame function.

6. Discussion

6.1 Role of Don Juan during *Drosophila* spermatogenesis

In order to study the role of Don Juan during *Drosophila* spermatogenesis, two different approaches were chosen. 1) To obtain a mutation in *don juan*, a P-element local hop was performed. 2) Don Juan was prematurely expressed under the control of *β2-promoter*. The first approach to obtain a mutation in *don juan* by P-element local hop was unsuccessful. This experiment resulted in several different new P-insertion lines, which were screened to find for the mutation in *don juan* and there was no hit in this gene. The second approach pursued by prematurely expressing Don Juan in the primary spermatocyte stage led to male sterility. This premature expression did not show any defects at the primary spermatocyte stage, but in later stages of spermatid differentiation, the individualization complex (IC) failed to move (Fig. 5.2). This failure of the movement of IC led to the male sterility. The reason that led to the IC movement failure is unknown. Since, the functional study of Don Juan during spermatogenesis was unsuccessful, my major focus of research was shifted to the chromatin condensation aspects in the late spermatids.

6.2 Chromatin condensation during *Drosophila* spermiogenesis

According to the evolution of species, three situations can be observed, 1) Persistence of the somatic histones in the mature sperms or the replacement of somatic histones by the sperm specific histones as seen in the case of echinodermates and annelides. 2) In case of birds and fishes, somatic histones are replaced by highly basic protamines. 3) In mammals, it is known that the somatic histones are replaced first by a set of intermediate proteins called transition proteins and later by protamines (Wouters-Tyrou *et al.,* 1998). The proteins involved in chromatin condensation in case of *Drosophila* were unknown. The chromatin condensation leads to the final compaction of the nucleus over 200 fold in the mature sperm (Fuller, 1993). This study involves in addressing the specific aspect of the question about the nuclear basic proteins in *Drosophila* during this chromatin condensation process. This study

began with the analysis of two male specific genes *Mst35Ba* and *Mst35Bb* which encode proteins that showed significant similarity to the mammalian protamines.

6.3 *Mst35Ba, Mst35Bb* and *Mst77F* are transcribed at primary spermatocyte stage and are translationally repressed till the elongated spermatid stage

protamine mRNAs are transcribed at the primary spermatocyte stage, which is in contrast to the situation seen in mammals where *protamine* mRNAs are synthesized at the round spermatid stage (Tanaka *et al.,* 2003). Translational repression of mammalian *protamine-1* mRNA is controlled by the 3'UTR (Zhong *et al.,* 2001, Yu *et al.,* 2002). The *Drosophila* ProtamineA-eGFP and ProtamineB-eGFP constructs do not contain the 3'UTR of the respective *protamine* genes. Nevertheless, the transgenic flies carrying these constructs still show repression of translation. So, in *Drosophila*, the region responsible for the translational repression is most likely in the 5'UTR, though the functional redundancy for the translational repression in the 3'UTR cannot be excluded. Deletion constructs of *Mst35Bb* and *Mst77F* 5'UTR fused to reporter *lacZ* showed the translational repression element is indeed present in the 5'UTR (data not shown). This holds true also for the mRNA of *Mst77F-eGFP* fusion gene, as is the case for all mRNAs investigated concerning translational repression so far in male germ line of *Drosophila* (Renkawitz-Pohl *et al.,* 2005). In contrast to mammalian spermatogenesis (Sassone-Corsi, 2002), in *Drosophila*, transcription ceases already with the entry into meiotic divisions (Fuller, 1993). Since, the protamines are made in the elongated spermatids, the transcriptional silencing in *Drosophila* spermatogenesis seems to be independent of protamines.

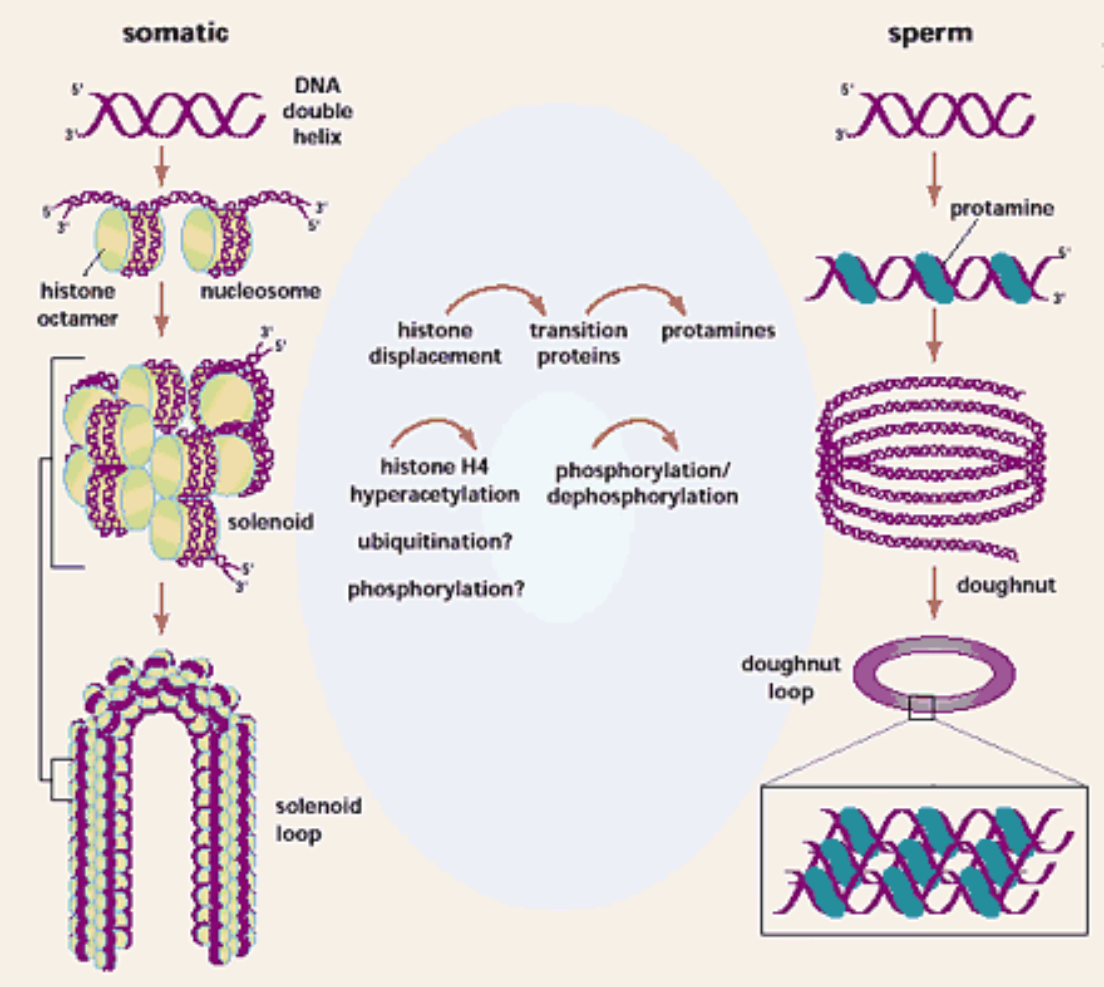
6.4 Comparison of mammalian and *Drosophila* protamines

When primary amino acid sequences of *Drosophila* protamines are compared to mammalian protamines, it is quite evident that *Drosophila* protamines are relatively large. The dProtA and dProtB are over 94% identical to each other. This could explain that both the protamines may be functionally redundant. Human and mouse Protamine-1 aligns with the N-

terminal of both *Drosophila* protamines (Fig. 5.4 A) and Protamine-2 aligns more to the C-terminal (Fig. 5.4 B). It is possible that the *Drosophila* protamines undergo post-translational cleavage at the N-terminus, as is known for mammals (Lewis *et al.,* 2003). The cytoplasmic eGFP fused at the C-terminus shows clear nuclear localization, indicating that the tagged protamine is functionally intact. *Drosophila* protamines each contain 10 cysteine residues at identical positions, while, over 4 of 10 cysteines at N-terminal and the C-terminal are conserved with human and mouse Protamine-1 and Protamine-2 respectively (Fig. 5.4 A and 5.4 B asterisks). With nine cysteines, the content is highest in Protamine-1 of mice. Inter or intra molecular disulfide bridges can be formed between the cysteine-rich protamines to condense the DNA. For mice (Cho *et al.,* 2001) it is shown that mutation in *protamine-1* or *protamine-2* is haplo insufficient and causes male sterility. We analyzed a haploid situation for the *Mst35Ba* and *Mst35Bb* genes in trans to the deficiency (*Df(2L)Exel8033/*+), these flies are male fertile (Table-3) and show normal spermatogenesis (data not shown). The high amount of identity that both dProtA and dProtB exhibits can contribute to the functional redundancy.

6.5 Histone displacement and incorporation of Protamines and Mst77F during *Drosophila* spermiogenesis

Chromatin reorganization is an essential feature during spermiogenesis. The functional significance of chromatin compaction during spermiogenesis is still unknown. The main explanation seems to be that compaction of the sperm nucleus is an essential factor for its mobility as well as for the penetration of sperm into the egg and genomic stability. In mammals, somatic histones are in part replaced by spermatid-specific variants during meiotic prophase (Drabent *et al.,* 1996), later by major transition proteins TP1 and TP2 (Meistrich *et al.,* 2003), and subsequently by highly basic protamines to ensure the remodeling of chromatin to a typically highly condensed and transcriptionally silent state of mature sperm. These replacements leads to a shift from histone based nucleosomal conformation to a radically different conformation, resembling stacked doughnut structures, containing major chromatin condensing proteins and DNA in the nucleus (Fig 6.1).



Braun, 2001

Fig. 6.1 Packaging chromatin. A model of chromatin packaging in somatic cells (left) and mammalian sperm (right). In somatic cells, the DNA is wound twice around histone octamers to form nucleosomes, which are then coiled into solenoids. The solenoids are attached at intervals to the nuclear matrix at their bases and form DNA loop domains. In the sperm nucleus, protamines replace the histones and the protamine-DNA complex is coiled into a doughnut shape. Inset shows the tight compacting of protamine-DNA strands. Presumably, displacement of the histones is facilitated by post-translational modifications of the proteins, in the form of histone H4 acetylation, ubiquitination and phosphorylation. Phosphorylation and dephosphorylation of the transition proteins facilitate their displacement before protamines bind (taken from *Braun, 2001*).

In *Drosophila*, so far no proteins have been identified that are involved in the packaging of the genome in the mature sperm nucleus. One observation that Histone3.3 variant and the somatic H3 isoform in *Drosophila* are vanishing at the time of chromatin condensation, supports the view of histone displacement (Akhmanova *et al.,* 1997, Hennig, 2003), but, it was still a question whether it is the real absence of histones at this stage in *Drosophila* or the antibodies are not accessible to the mature sperm due to the tight packaging of the chromatin (Hennig, 2003). To circumvent this problem, we chose the GFP fusion approach, made use of the existing *His2AvDGFP* (Clarkson and Saint., 1999) and generated

Protamine-eGFP and *Mst77F-eGFP* fusion transgenic flies in order to analyze the situation in *Drosophila*. Our results clearly show that histone His2AvD is lost from the spermatid nuclei, at the time of appearance of protamines and Mst77F during later stages of spermatid differentiation. The exact molecular mechanisms underlying the histone displacement, degradation and the incorporation of protamines onto the chromatin are poorly understood (Braun, 2001). For mammals, evidence has been obtained that histone H2A is ubiquitinated in mouse spermatids around the developmental time period when histones are removed from the chromatin (Baarends *et al.,* 1999, Baarends *et al.,* 2000). The mammalian HR6B ubiquitin-conjugating enzyme is the homologue of yeast RAD6, and both can ubiquitinate histones *in vitro* (Robzyk *et al.,* 2000). The nuclear uH2A staining in elongating spermatids of *HR6B* knockout mice was different from the staining observed in the wild-type testis due to the aberrant shape of the spermatid nuclei in the knockout testis. However the remaining elongating spermatids in *HR6B* knockout mice still seemed to contain a considerable amount of uH2A (Baarends *et al.,* 1999). The ubiquitination of H2A during sperm terminal differentiation may not be directly dependent on HR6B.

Nothing was known during spermiogenesis in *Drosophila* so far with respect to histone modifications, the mechanism of histone displacement, degradation and incorporation of protamines. In flies as well as in mammals, many questions remain unanswered about these underlying mechanisms of chromatin remodelling during spermiogenesis, which needs to be addressed. For an entree into the study of this mechanism see chapter 6.8.

6.6 In *Drosophila*, mammalian HILS1 related protein Mst77F is coexpressed with protamines

In mammals, transition proteins act as intermediates in the histone-to-protamine transition (Meistrich *et al.,* 2003). In mice, the onset of HILS1 and transition proteins TP1 and TP2 (major forms) overlaps with the pattern of Protamine-1 and later with Protamine-2 but, is no more present in the mature sperm. It was recently shown that, mice lacking both TP1 and TP2 show normal transcriptional repression, histone displacement, nuclear shaping and protamine deposition, but, shows the loss of genomic integrity with large number of DNA breaks, leading to male sterility (Zhao *et al.,* 2004). In *Drosophila,* we now know that histones are displaced with synchronous accumulation of protamines and Mst77F. Mst77F, a distant relative of histone H1/H5 (linker histone) family has been proposed to play a role

either as a transition protein or as a protamine for compaction of the *Drosophila* sperm chromatin (Russell and Kaiser, 1993). Mst77F shows highest similarity to HILS1 with respect to the cysteines and basic amino acid content (Fig. 5.3 C and Table-2), but not to mouse TP1, TP2 or H1t (Table-3). Moreover, our results show that the pattern of expression of Mst77F in the nucleus is similar to that of mHILS1 in the nucleus, with the exception that Mst77F is also transiently detected in the flagella and persists in mature sperm nuclei unlike mHILS1. In mammalian mature sperm nuclei it is only the protamines that are the condensing proteins which persists. This again raises the question whether Mst77F could also play a role of protamines? But, one additional copy of dProtB (dProtA and dProtB showing 94% identity may be functionally redundant), does not rescue the *ms(3)nc3* phenotype indicating that the role of Mst77F may be completely or partially different from that of protamines in the nucleus. However, a null mutation for *Mst77F* is required to answer this question with respect to chromatin condensation. In *ms(3)nc3* mutants, the chromatin condensation with the normal protamines continue to take place. When we take a closer look at the deposition of ProtamineB-eGFP in *ms(3)nc3*/ *Df(3L)ri-79c* trans-heterozygotes, it revealed that the condensed chromatin in the tid shaped nuclei (Fig. 5.10 M) is concentrated at the two opposite ends with a lightly stained chromatin spaced in the center. So, the chromatin condensation takes place, but may not be complete with the incorporation of the mutant Mst77F protein. The high degree of chromatin compaction or condensation seen in *Drosophila* mature sperm when compared to that of mouse and human sperm possibly could be the result of persistence of Mst77F in the mature sperm nuclei.

6.7 Role of Mst77F in nuclear shaping

ms(3)nc3 is a second-site non complementation (nc) mutation that was isolated in an EMS screen to identify interacting proteins involved in microtubule function in *Drosophila* (Fuller *et al.,* 1989). *ms(3)nc3* fails to complement class I alleles at the *β2 tubulin* locus on the chromosome arm 3R but, enhances the β2 tubulin phenotype (Fuller, 1986). β2 tubulin is an isotype in the axoneme and in all other microtubules from the primary spermatocyte phase onward (Fuller, 1993). We have shown that, *ms(3)nc3* is a single missense mutation from T→A transition, causing the substitution of threonine instead of serine at aa position 149 (Fig. 5.3 B). Mst77F shows a pattern of expression similar to protamines in the nucleus (Fig 5.7 H and 5.9 B), and was also seen in the flagella till the individualization stage (Fig. 5.9 B and C).

Since *ms(3)nc3* fails to complement class I alleles at the *β2 tubulin* locus (Fuller, 1986), it is possible that Mst77F has a dual role to play as a chromatin condensing protein in the nucleus and for the normal nuclear shaping, where the latter is dependent on perinuclear microtubules. Nuclear shaping is a microtubule based event (Fuller, 1993). In the wild-type situation, during the later part of flagellar elongation period, the nucleus becomes concave on one side and convex on the opposite side. The perinuclear microtubules start to accumulate both on the convex and on the concave side of the perinuclear space and later on forming a single row of laterally compacted microtubules on the convex surface (Fuller, 1993). As the nuclear transformation proceeds, the chromatin becomes increasingly condensed along the inner side of the nuclear envelope opposite to the microtubules. Chromatin condensation continues as the dense chromatin accumulates with a net like appearance in the nucleoplasm and further on, the excess of the nucleoplasm is eliminated from the originally concave side of the nucleus in the form of vesicles (Fuller, 1993). This process of accumulation of perinuclear microtubules is required for the normal nuclear shaping. The mutation in *ms(3)nc3* which is a serine to threonine exchange at the C-terminal cysteine rich domain leads to a tid shaped nuclear phenotype, where the nucleus fails to shape into a thin shaped nuclei form. Similar defective nuclear shaping is seen with the few homozygous and heteroallelic combinations of class I alleles of β2 tubulin (Fackenthal *et al.,* 1995). The incorporation of the defective subunit encoded by *ms(3)nc3* may interfere with the function of the resulting complex. The sea urchin histone H1 is known to stablize the sperm flagellar microtubules (Multigner *et al.,* 1992). The transient appearence of Mst77F-eGFP in the flagellum of the sperm bundles and the heteroallelic combinations of class I alleles of β2 tubulin with ms(3)nc3 enhances the β2 tubulin phenotype. These data suggests the involvement of a Mst77F (a linker histone varient) in the microtubule dynamics during the nuclear shaping and may be required to stabalize both the periplasmic and cytoplasmic microtubules during the sperm terminal differentiation in *Drosophila*.

6.8 Histone degradation during *Drosophila* spermiogenesis.

The P-element mutation *eff*^{s} was isolated by the large scale P-element mutagenesis screen performed by Castrillon *et al.,* to mutate the genes involved in spermatogenesis (Castrillon *et al.,* 1993). Moreover, the *effete* gene is a hot spot for the P-element integration because there are several number of P-element insertions (Fig. 5.12) that were isolated in this

gene by different groups. It was shown by Cenci *et al.,* that *effete* which encode for an E2 conjugating enzyme UbcD1 is required for the normal telomere behavior during mitosis and meiosis. UbcD1 function is also required outside spermatogenesis as there are few other mutants which are lethal. The mutant eff^{s} shows defects specifically during spermatogenesis, as these males are sterile. The sterility is caused by the improper telomere behavior during mitosis (Cenci *et al.,* 1997).

eff^{s} males also showed abnormal spermatid differentiation where the post-canoe shaped nuclei appeared to be thicker when compared to that of wild-type. When H2AvDGFP was visualized in these mutants, it was revealed that the H2AvDGFP are not degraded in the post-canoe stage spermatid nuclei (Fig 5.13 and 5.14). So, UbcD1 function besides retaining the normal telomere behavior is also required for histone degradation. However, it is important to check if the histones H2AvD during this stage are polyubiquitinated. This would shed a light if the degradation of histones are indeed by ubiquitination or not.

Furthermore, to know if the other components of the nuclear ubiquitin ligases were expressed during spermatogenesis, additional experiments were done. The first candidate to be checked was the ring finger E3 ligases as these ligases are known to have nuclear targets. The antibody staining against Cullin-1 showed that Cullin-1 is expressed in all the early germ cells and localizes to spectrosomes and fusomes up to meiotic stages (Fig. 5.15 A). Later on it disappears as it is no more present in the round head stage of elongated spermatids (Fig. 5.15 B), and reappears at the canoe stage where it specifically localizes to the tip of the perinuclear space of the canoe shaped heads (Fig. 5.15 D and E). Cullin-1 is later again degraded as the spermatid differentiation proceeds to the next stage where the chromatin is already condensed (Fig. 5.15 F).

The reappearance of Cullin-1 at the canoe stage in the perinuclear space is particularly interesting. We know that the nuclear shaping is a microtubule based event (Fuller, 1993). The appearance of the protofilaments with the singlet microtubules in the perinuclear space is the basis for the nuclear shaping process. At the canoe stage, the nuclear shaping is almost complete. It is important to stop the accumulation of the tubulin dimers onto the protofilament when the nuclear shaping has reached its maximum. Since, Cullin-1 specifically localizes to the tip of nuclear head in the perinuclear space, it is provocative to speculate that may be Cullin-1 is required to stop the growth of the protofilament at the canoe stage. One of the mutation in the region 35B2 that was isolated in the screen for the protamine mutants showed the phenotype where the nuclear shaping continues and forms a lariat head shape structure. In this region of 35B2 is present a gene *CG3473* which is predicted to encode for an E2

conjugating enzyme and the EST information indicate that this gene is testis specifically expressed (my database analysis and ongoing experiment). When taken into account the localization of Cullin-1 in the perinuclear space of the canoe stage spermatids and the testis specific E2 conjugating enzyme being present at the 35B2 locus mutation probably could contribute to the lariat head phenotype, it could be that Cullin-1 and *CG3473* play a synergistic role in stopping the extension of the microtubule structures when the nuclear shaping is complete.

The second possibility could be that the histones are displaced from the chromatin and exported out to the perinuclear space where it is ubiquitinated and degraded in the vicinity of the nucleus itself.

Another question that is interesting to address is about the expression of Cullin-1 itself. We know that the transcription ceases in the meiotic prophase-II (Fuller, 1993). But, we see that the Cullin-1 reappears at the canoe stage of spermatid development. How? It is unlikely that the transcript is made at the canoe stage, since the transcription ceases much earlier at the meiotic prophase-II. It is possible that one of the transcript isoforms could have a translational repression element, or the same transcript that is made in the earlier stage is translationally repressed from the meiotic stage onwards. The latter aspect is possible because one of the RNA binding protein TSR localizes to the nucleus during the primary spermatocyte stage and is exported out during the meiotic stages. Performing antibody staining for Cullin-1 in TSR mutants would show if the RNA binding protein TSR is required to translationally repress *Cullin-1 mRNA* up to canoe stage.

Also Cullin-3 is expressed during spermiogenesis as shown by the enhancer trap line *P{PZ}gft*^{06430}. Since the lacZ staining was observed particularly in elongated spermatids, it could be that *cullin-3* transcript like *cullin-1* is also translationally repressed. The localization and mosaic analysis or RNAi for *Cullin-3* would give some information about its function during spermiogenesis.

6.9 Sesame (HIRA) is essential to remove protamines from the male pronucleus but not for shape changes of the nucleus

After the first steps in the fertilization process, the male gamete is still in the highly compact protamine-based chromatin structure. In a wild-type egg, the paternal pronucleus changes the shape from the needle-like to a spherical structure. Furthermore, the male pronucleus acquires a nucleosome-based structure before zygote formation, and thus is transformed into a replication competent male pronucleus. *sesame* is a maternal effect mutation in *HIRA* and had been mapped to 7C1 (Loppin *et al.,* 2003). In *Drosophila*, HIRA is expressed in the female germ line and a high level of *HIRA* mRNA is deposited in the egg (Kirov *et al.,* 1998) Human HIRA is shown to bind to histone H2B and H4 (Lorain *et al.,* 1998). The WD repeats present at the N-terminal part of HIRA could probably function as a part of multi protein complex (Lorain *et al.,* 1998). Xenopus HIRA proteins are also known in promoting chromatin assembly that is independent of DNA synthesis *in vitro* (Ray-Gallet *et al.,* 2002). Loppin *et al*., analyzed the corrosponding maternal effect mutant *sesame*, in which the sperm fertilize the egg but no zygote is formed. Although the shape change of the nucleus to the spherical structure occurs in these mutants, maternal histones are not incorporated into the male pronucleus (Loppin *et al.,* 2001) which strengthens the function of HIRA in binding to the core histones. Here we show, that both *Drosophila* protamines are not removed from the male pronucleus in *sesame* mutants. This let us propose that the transport and incorporation of histones onto the chromatin in some manner is coupled to the removal of protamines in which HIRA could play an important role in the multi protein complex involved in this chromatin reconstitution process. We also show here that Mst77F removal from the male pronucleus in contrast to protamines is independent of HIRA.

During spermiogenesis, chromatin reorganization of the complete genome is an essential feature for male fertility. This process leads to an extremely condensed state of the haploid genome in the sperm and requires a reorganization of the paternal genome in the male pronucleus during fertilization and before zygote formation. With the characterization of the chromatin condensing proteins in *Drosophila*, it would be possible to gain more insight into the mechanisms of sperm chromatin reorganization during spermiogenesis and fertilization.

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Abbrivations

aa	Amino acid
A.dest.	Aqua bidestilled
APS	Ammoniumpersulfate
bp	Basepairs
dNTPs	Deoxyribonucleotide Phosphates
ddNTP	Didesoxyribonucleotide
cDNA	Complementary DNA
CIP	Alkaline Phosphatase from calf intestine
DNA	Desoxyribonuclic acid
DNase	Deoxyribonuclease
DMSO	Dimethylsulfoxide
kb	Kilobase
kDa	Kilodalton
M	Molar
OD	Optical Density
mRNA	messenger-RNA,
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pI	Iso electric point
PI	Propidium Iodide
U	Unit (Enzyme activity)
w/v	weight per volume
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
TritonX-100	Octylphenolpolyethylenglycolether
Tween 20	Polyyoxyethylensorbitanmonolaurate
Rpm	Rotations per Minute
UTR	Untranslated Region
UV	Ultraviolet light

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I declare that I myself wrote this study and carried out the experimental work described in it, without using any other sources and aids than those that are stated.

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Publications

Jayaramaiah Raja S and Renkawitz-Pohl. R.,

Drosophila Protamines and Mst77F replace histones during chromatin condensation in late spermatids and role of Sesame in the removal of these proteins from the male pronucleus.

Mol Cell Biol. 2005. (in press)

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In *Drosophila*, *don juan* and *don juan like* encode proteins of the sperm nucleus and the flagellum and both are regulated at the transcriptional level by the TAFII80 Cannonball while translational repression is achieved by distinct elements.

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Jayaramaiah Raja S Bridlin Barckmann and Renkawitz-Pohl. R.

Transcriptional regulation and translational repression of *Drosophila protamine* and *Mst77F* mRNAs during spermatogenesis.

(*Manuscript in preparation*)

Jayaramaiah Raja S and Renkawitz-Pohl. R.

Molecular mechanisms of nuclear shaping and histone degradation during *Drosophila* spermiogenesis.

(*Manuscript in preparation*)

Presentations

October 2004 — Germ Cell meeting, Cold Spring Harbor, New York.

Drosophila Protamines A and B replace histones during chromatin condensation in late spermatids and Sesame is required to remove Protamines from the male pronucleus (Poster presentation).