

**Histones, histone modifications and the chromatin landscape
in sperm of the Fruit-fly, *Drosophila melanogaster* and its
embryological significance.**

By

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Abstract

During spermiogenesis, nucleosome-based DNA packaging is replaced by protamine toroids to generate the highly compacted nucleus of the mature sperm of many species including human, mouse and bovine; however, the sperm from these species also retain some nucleosomes. It has been suggested that the histones of sperm nucleosomes may play important roles in embryo development. In the fruit-fly, *Drosophila melanogaster* it had been thought that nucleosomes and their component histones are completely replaced by protamine, and that no residual histones are retained. In this study, three experimental models, (*Drosophila melanogaster*, *Mus musculus* and *Bos taurus*) were used to investigate i) the presence of histone protein and/or its modifications in mature sperm, ii) paternal DNA packaging by histone, and iii) the possible epigenetic roles of sperm histones in embryogenesis.

In this study, the presence of all the canonical histones of nucleosomal chromatin (H2A, H2B, H3 and H4) was confirmed in the mature sperm of *Drosophila melanogaster* by microscopic, biochemical and immunoprecipitation analysis. Evidence is also provided for the presence of post-translational modifications (acetylation and methylation) of the histones from fly, bovine and murine sperm. The molecular landscape of histones and histone modification (H3K27me3, H3K36me3 and H3K9me3) was investigated using ChIP-chip on a *Drosophila*-based, 2.4 million probe whole genome tiling array. Data analysis revealed that histones (and by extension, nucleosomes) are preferentially positioned within exons rather than introns and that they also mark intron/exon boundaries and gene promoters. However, nucleosomes are depleted upstream of Transcription Start Sites (TSS). Sperm chromatin also contains modified histones associated with gene regulation including repressive (H3K9me3, H3K27me3) and permissive (H3K36me3) marks.

Gene ontology analysis showed that the H3K27me3 and H3K36me3 are associated with gene sequences carrying distinctive embryological developmental process terms. Moreover, the *in silico* analysis indicated that the early zygote expresses genes are marked by H3K36me3 in sperm chromatin, while the late zygote expresses genes that are marked by H3K9me3 instead.

The same sequences are depleted of the H3K36me3 modification in sperm chromatin. These data suggest that modified sperm histones might play epigenetic roles in post fertilisation events such as formation and functioning of the male pronucleus and in early and late embryo development.

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List of abbreviations

Abbreviation	Meaning
A	Adenine
AgNO ₃	Silver Nitrate
AP	Alkaline Phosphatase
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium
BiTS-ChIP	a specific chromatin immunoprecipitation approach
BORIS	Brother Of Regulator of Imprinted Sites
BRDT	Testis specific double bromodomain containing protein
BSA	Bovine Serum Albumin
C	Cytosine
Ca ²⁺	Calcium ion
CaCl ₂	Calcium Chloride
CENP-A	Centromere protein A
CGH	Comparative Genome Hybridisation
CH ₃ COONa	Sodium acetate
ChIP	Chromatin Immunoprecipitation
ChIP-chip	Chromatin immune precipitation- microarray analysis
ChIP-seq	Chromatin immune precipitation- sequencing
Ct	threshold cycle
CTAB	Cetyltrimethylammonium Bromide
CTCF	CCCTC binding factor
CTCFL	CTCF like
Cy3	Cyanine 3
Cy5	Cyanine 5
DAPI	4,6-diamidino-2-phenylindole
DAVID	The Database for Annotation, Visualization and Integrated Discovery
di Me H3K4	di methyl histone H3 at lysine 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic
DNAase I	Deoxyribonuclease I
DNMA1	DNA methyltransferase A1

DNMA3a	DNA methyltransferase A3a
DNMA3b	DNA methyltransferase A3b
DNMA3I	DNA methyltransferase A3I
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
Dphb	DNA probe–hybridization buffer
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein Isothiocyanate
G	Guanine
GFP	Green Fluorescent Protein
GFP	Green Fluorescent Protein
GO	Gene Ontology
GPC	Germinal Proliferation Centre
GuHCl	Guanidine hydrochloride
h-HSH2B	Human Testis/Sperm-specific Histone 2B
H1	Histone 1
H1t	Testis-specific Histone 1
H2A	Histone 2 A
H2A L1	Histone 2 A Like 1
H2A L2	Histone 2 A Like 2
H2A L3	Histone 2 A Like 3
H2A.Bbd	H2A Barr body- deficient (H2A.Bbd)
H2A.v	Histone 2A.v
H2A.X	Histone 2A.X
H2A.Z	Histone 2A.Z
H2Av-RFP	Histone 2 vA tagged with red fluorescence protein
H2B	Histone 2B
H2BFWT	H2B histone family, member W, testis-specific
H2BL1	Histone 2 B Like 1
H2BL2	Histone 2 B Like 2
H3	Histone 3
H3.3A	Histone 3.3A

H3.3B	Histone 3.3B
H3K20me1	Histone3 mono methyl lysine 20
H3K27me1	Histone3 mono methyl lysine 27
H3K27me3	Histone3 tri methyl lysine 27
H3K36me3	Histone3 tri methyl lysine 36
H3K4me2	Histone 3 di methyl lysine 4
H3K4me3	Histone 3 tri methyl lysine 4
H3K9me2	Histone3 di methyl lysine 9
H3K9me3	Histone3 tri methyl lysine 9
H4	Histone 4
H4K12ac	Histone 4 acetyle lysine 12
H4K16	Histone 4 acetyle lysine 16
H4K20me1	Histone 4 mono methyl lysine 20
H4K8ac	Histone 4 acetyle lysine 8
H4r	Histon 4r
HAT	Histone acetyl transferase
HCl	Hydrochloric acid
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNA	heterogeneous nuclear RNA
Hox	Homeobox genes
IP	Immunoprecipitation
IVF	In Vitro Fertilization
JHDM2A	JmiC domain containing histone demethylase 2A
Kda	Kilo Dalton
LC-MS	Liquid Chromatography Mass Spectrometry
LiCl	Lithium Chloride
LSD1	Lysine demethylase
MAR	matrix attachment regions
Mg ⁺	Magnesium ion
MgCl ₂	Magnesium Chloride
mH2A	macroH2A
MNase	Micrococcal Nuclease

modENCODE	The Model Organism Encyclopedia Of DNA Elements
mono Ac	mono acetyl histone H4 at lysine 8
mono Ac H4K12	mono acetyl histone H4 at lysine 12
mono Me H3K4	mono methyl histone H3 at lysine 4
Mst77f	Male-specific transcript 77F
N	Normality
NaCl	Sodium Chloride
NaHCO3	Sodium bicarbonate
NaOH	Sodium Hydroxide
NP-40	Tergitol type NP-40
P1	Protamine 1
P2	Protamine 2
PBS	Phosphate Buffer Solution
PcG	polycomb group
PFA	Paraformaldehyde
Plzf	Promyelocytic leukemia zinc-finger
PMSF	PhenylMethylSulfonyl Floride
PMT	Photo MultiplierTube
Protamine- GFP	Protamine tagged with green fluorescent protein
qPCR	quantitative Polymerase Chain Reaction
REVIGO	Reduce, Visualize Gene Ontology
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RNAase	Ribonuclease
RNF8	Ring Finger protein 8
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Sodium Chloride Citrate
T	Thymine
TCA	Trichloroacetic acid
TE	Tris-EDTA
Tetramethylethylene diamine	TEMED

TH1	Testis Histone 1
TH2	Testis Histone 2
TH2B	Testis-Specific Histone 2B
TH3	Testis Histone 3
Tpl ^{94D}	Transition protein-like 94D
TPs	Transition Proteins
tri Me H3K4	Tri Methyl Histone H3 at Lysine 4
Tris-HCl	Tris- Hydrochloric acid
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
V	Volt
WGA	whole Genome Amplification
ZF	Zinc finger
ZGA	Zygote genome activation

Chapter 1: Introduction

Successful fertilization and embryo development depend on the molecular quality of maternal and paternal gametes, including their Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA) and proteins. While extra-genomic oocyte molecules (proteins and RNAs) are well documented as important factors for embryo development (Stitzel and Seydoux,2007), the information about an equivalent sperm contribution in embryo development is relatively unknown.

It was believed that the oocyte provides the zygote with all developmental requirements. However, during fertilization, in addition to haploid sets of chromosomes, the sperm provides the zygote with molecules and chemical modifications that may affect embryogenesis such as the centrosome, which has a significant role in euploidy maintenance (Manandhar *et al.*,2005). Moreover, sperm chromatin is imprinted in particular sites, which is compatible with the maternal genome (Wu *et al.*,2006). Furthermore, the sperm contains a small amount of RNA, which may contribute to fertilization and embryo development (Ostermeier *et al.*,2005).

Generally, paternal gametes are highly specialized cells, and in most metazoan organisms, the sperm nucleus becomes highly compacted as protamine replaces histone (Dadoune,2003). This special genome packaging is an important marker of sperm quality for fertilization and embryo development. At the present time there is a clear evidence that abnormal chromatin condensation leads to sperm infertility (Spano *et al.*,2000).

The chromatin of the paternal gamete undergoes dramatic changes in its organization, and most histone protein is replaced by highly basic protamines (Gatewood *et al.*,1987). Recent studies have indicated, however, that this unique packaging of sperm chromatin by histone and protamine transfers a significant epigenetic message to the zygote, and the most important elements of this message are DNA methylation and histone modifications (Hammoud *et al.*,2009; Hammoud, *et al.*,2009). In this introduction, the chromatin organization by the nucleoproteins in the mature sperm (mainly in mammals), in addition to the possible epigenetic roles of sperm in embryo development are highlighted.

1.1 Nucleosome

The nucleosome is the basic unit of DNA packaging, mainly consists of histones protein, these proteins are basic proteins present in the nucleus of somatic cells, which package chromatin into nucleosomes (figure1-1) (Purves,1997). These proteins are classified into five main types namely, histone 1 (H1), histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4). The four canonical histones (H2A, H2B, H3 and H4) exist in pairs and form the octomeric basic DNA binding complex called a nucleosome $[(H2A, H2B)_2(H3, H4)_2]$. The nucleosomes fold eukaryotic cell DNA about 10.000 times to fit inside the cell (Razin *et al.*,2007), this packaging occurs in two steps, firstly the DNA is organised in small fibre called 30nm fibre, then this fibre folds into giant loop (50-200 kbp)(Razin *et al.*,2007).

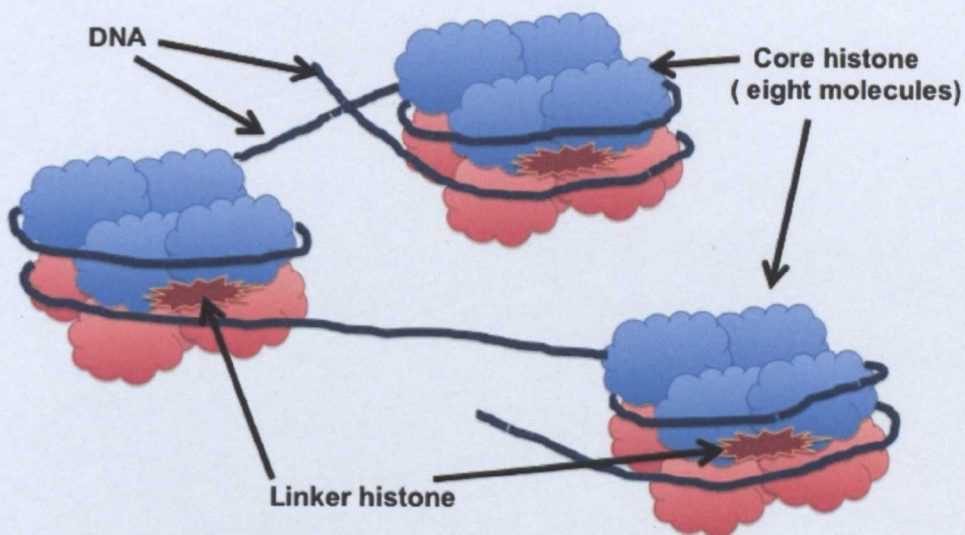


Figure 1.1 Nucleosomes structure in somatic cell.

Each of five main histones has some variant forms. These variants might be classified into heteromorphous and homomorphous based on the amino acid differences from main histone type (Ausio *et al.*,2001; West and Bonner,1980). While heteromorphous group are characterised by large changes in the amino acids, (such as H2A.X, H2A.Z, macroH2A (mH2A), and centromeric protein A (CENP-A), H2A Barr body- deficient (H2A.Bbd)), in the homomorphous group the changes include a few number of the amino acid Such as H2A.1, H2A.2, H3.1, H3.2 and H3.3(Ausio,2006; Ausio *et al.*,2001; West and Bonner,1980).

1.1.1 Histone modification

Histones are characterised by covalent modifications, which increase the diversity level of nucleosome particles, these modifications are mainly located on the N- terminal region of histone amino acid chain (Razin *et al.*,2007), in addition to the various residues (Martin and Zhang,2007; Tan *et al.*,2011). There are many chemical modifications characterised on histones such as histone methylation, acetylation, phosphorylation, ubiquitination, crotonylation, propionylation, butyrylation, formylation, sumoylation, citrullination, proline isomerisation, and ADP ribosylation (Martin and Zhang,2007; Ruthenburg *et al.*,2007; Tan *et al.*,2011), these modifications are distributed at 130 sites on histones (Tan *et al.*,2011).

Here the four common main types will be described. First, histone methylation: is a modification that occurs by the addition of one, two or three methyl groups on a particular residue of histone protein, and the function of histone methylation varies based on the number of methyl groups and their position. Many studies have indicated that the methylation of H3 and H4 on K (lysine) residue leads to gene activation and/or repression in eukaryotic cells (Lachner and Jenuwein,2002). Second, histone acetylation: refers to the addition of an acetyl group to lysine residues in nucleosomal histone. This reaction is regulated by transferase enzymes known as Histone acetyl transferase (HAT). Histone deacetylation is the opposite reaction, which refers to the removal of acetyl groups by Histone Deacetylase (HDAC). Histone acetylation and deacetylation are fundamental regulatory mechanisms controlling gene transcription activity by changing the chromatin organization. While the addition of acetyl groups opens up closed (condensed) chromatin and promotes the activity of polymerase II, the deacetylation leads to gene silencing (Jenuwein and Allis,2001). Third, Histone phosphorylation: refers to the addition of phosphate to histone protein, and it was detected in H3 (Prigent and Dimitrov,2003), H2A.X (Rogakou *et al.*,1998), H2A (Zhang *et al.*,2004), and H2B (Cheung,2003). Generally, histone phosphorylation is associated with gene transcription activation (Berger,2002; Xing *et al.*,2008), in addition to DNA repair (Galleani *et al.*,2009), chromatin condensation (Nowak and Corces,2004) and apoptosis (Stucki,2009). Fourth, histone ubiquitination is the modification of histone protein that occur by addition of a small regulatory protein called

ubiquitin. This modification plays a fundamental role in many cellular activities, mainly in protein degradation, in addition to cell cycle regulation, repair of damaged, stress responses, and epigenetics (Baarends *et al.*,1999).

1.2 Spermatogenesis

1.2.1 Spermatogenesis in mammals

Spermatogenesis in mammals is defined as a series of steps of paternal germ cell proliferation followed by development and differentiation to produce mature motile sperm. These processes occur within the seminiferous tubules in the testis (Eddy,1994). Generally, the seminiferous tubules support waves of germ cells at different stages of differentiation, with the most mature germ cells located near to the lumen or centre of the tubules, while the less mature cells lie close to the basement membrane (Figure 1-2).

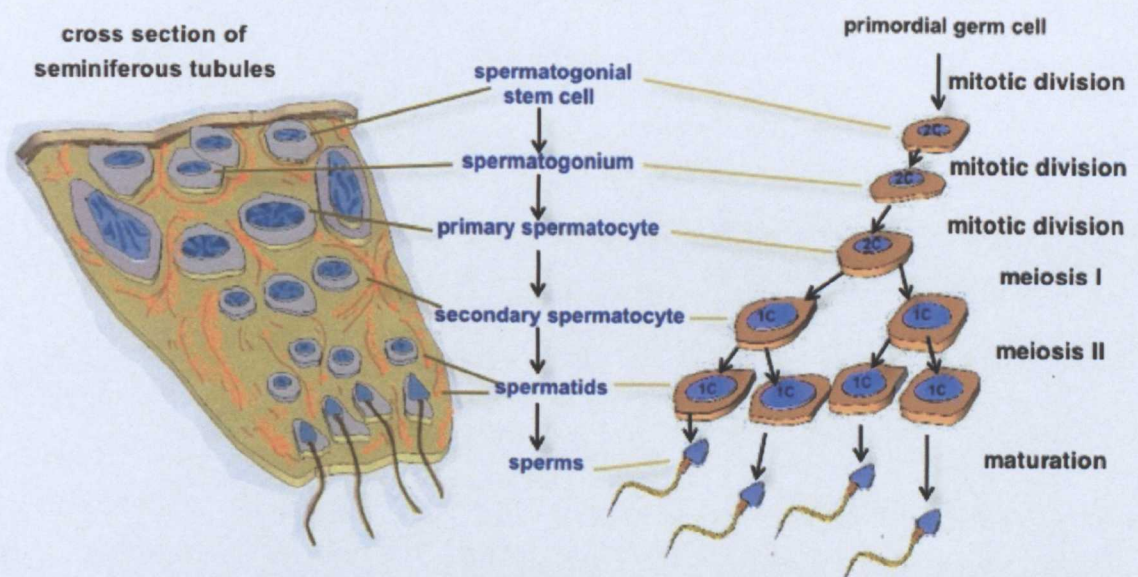


Figure 1-2 Spermatogenesis in mammals.

The development of paternal germ cells is mostly classified into three stages: spermatogonial proliferation, spermatocyte meiosis, and spermiogenesis (Sharpe,1994). Spermatogenesis starts by mitotic division of stem cells called spermatogonia located on the basement membrane of seminiferous tubules. There are two types of spermatogonia, one is undifferentiated and proliferates, mitotically to maintain the pool of spermatogonia; the other type is differentiated spermatogonia type B (de Rooij,2001). Spermatogonia type B undergo many mitotic divisions to produce pre-leptotene spermatocytes, which commit to meiosis I (leptotene, zygotene and pachytene), followed by meiosis II. By the completion of meiosis II, each spermatocyte will (potentially) generate four

haploid spermatids. Subsequently, the round spermatids go through spermiogenesis, during which they undergo dramatic changes in their morphological and molecular structure, including chromatin condensation, decrease of cytoplasmic volume, development of sperm flagella and the acrosome (de Kretser *et al.*,1998; de Kretser,1988). The chromatin of the nucleus is re-packaged and organized into a far more compact form, that is reduced about 20 times in comparison with typical somatic cells (Ward and Coffey,1991).

The mature sperms are then released into the lumen of the seminiferous tubules, and transported to the epididymis where the sperm undergo the final maturation changes to achieve fertilization capacity; these processes are called spermiation (Eddy,1994). The most important spermiation step is the formation of disulphide bounds between protamine molecules to increase chromatin compacting (Katz,1983).

1.2.2 Spermatogenesis in *Drosophila melanogaster*

Drosophila melanogaster is a well known experimental model for studying the genetic changes and the molecular reactions of cellular proliferation and maturation during spermatogenesis (Fuller,1993; Fuller,1998). In insects, as in mammals, male germ cell production occurs in the testis. Although the structure of *Drosophila melanogaster* testis is different from that of mammals, the main steps of sperm production (cell proliferation, meiosis, differentiation) in *Drosophila melanogaster* are similar to those in mammals. *Drosophila* has two testes (Figure 1-3) (Miller,1950), each one has a tubular, coiled, long shape measuring about 2.0 mm in length and 0.1 mm in diameter. Each testis has two ends, while the apical one is closed and free, the distal end is attached to the seminal vesicle, the place where the sperm are stored after maturation (Lindsley and Tokuyasu,1980).

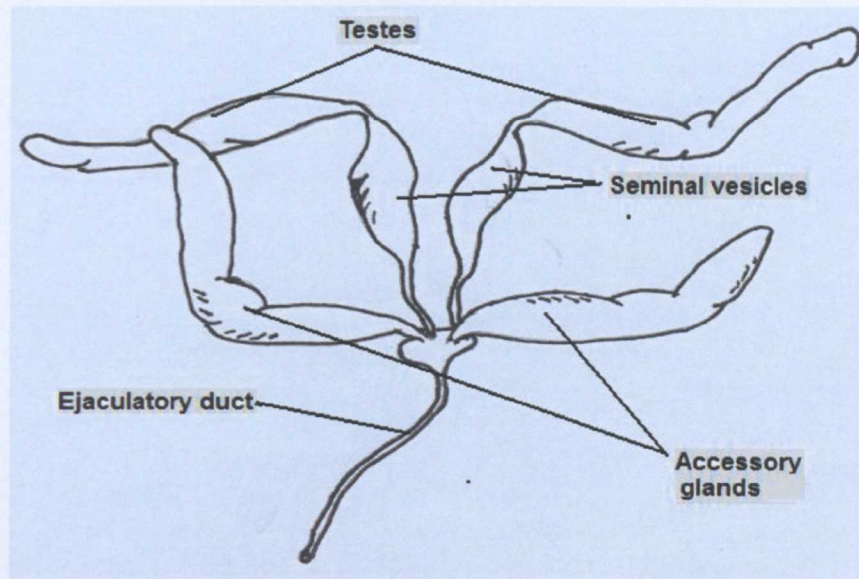


Figure 1-3 Dorsal view of male reproductive system in *Drosophila melanogaster*.

The morphological changes and the development of *Drosophila melanogaster* germ cells during spermatogenesis were described clearly in many early studies (Stanley *et al.*, 1972; Tates, 1971; Tokuyasu *et al.*, 1972).

Spermatogenesis in *Drosophila* starts in the apical part of the testis, specifically in a group of cells known as the Germinal Proliferation Centre (GPC) (figure 1-4). This GPC consists of three kinds of cells (Hardy *et al.*, 1979): 1) apical cells in the centre, 2) stem cells arranged in germ lines localized around the apical cells and 3) a pair of cyst progenitor cells surrounding the germ line stem cells (Aboim 1945; Lindsley and Tokuyasu, 1980). While the apical cells and cyst progenitor cells are produced from somatic cells, the stem cells are germ cells (Aboim 1945).

Sperm production starts when one of the germ line stem cells divides mitotically, and produces two cells: the first cell stays as a stem cell and remains in contact with the germ line stem cells, while the second cell migrates away from the germ line stem cells and gives rise to a primary spermatogonium (Hardy *et al.*, 1979). In turn, this primary spermatogonium gives rise to a cyst of secondary spermatogonial cells by mitotic division, after which these cells divide in the same pattern and produce a cyst of sixteen primary spermatocytes. Generally there are four mitotic divisions from primary spermatogonium to primary spermatocyte stage (Liebrich, 1984).

The primary spermatocyte stage is a critical step in *Drosophila melanogaster* spermatogenesis, because in this stage the genetic programme of germ cells changes from cellular proliferation into cellular growth and gene expression, which lead to meiosis and spermatid maturation (Fuller,1993). Within ninety hours, the total length of the primary spermatocyte stage, most of the genes are transcribed, and as result of this the volume of these cells increases about twenty fold (Lindsley and Tokuyasu,1980). In the next step, the primary spermatocyte enters into meiosis I and produces a secondary spermatocyte, then the germ cells undergo meiosis II. At the end of meiosis the germ cells are arranged in a cyst of 64 spermatids connected by cytoplasmic bridges (Laughran LJ *et al.*,1976; Resamussen,1973). In many respects, the testis in *Drosophila* is equivalent to a single seminiferous tubule in mammals.

By the end of meiosis II, the germ cells start spermiogenesis. In this stage the haploid spermatids undergo dramatic changes in their molecular structure and morphology, which lead to the production of mature sperm. In general, spermiogenesis can be classified into three distinct phases. The first is known as the pre-elongation spermatid phase, which is microscopically characterized by the presence of a small spherical nucleus and mitochondria arranged in crescent at one pole of the cell (Tates,1971). The second phase is known as the onion stage of spermatid development, which is marked by many morphological changes, the most clear being the fusion of the mitochondria into two giant mitochondria, which are interleaved and form a spherical shape called Nebenkern (Tates,1971). Spermatid elongation and maturation is the third phase of spermiogenesis, in which the germ cells undergo dramatic changes in their form. These include opening and splitting of the Nebenkern (mitochondrial structure) into two parts, the flagellar axonemes, which are gathered together and elongate to form the sperm tail (Lindsley and Tokuyasu,1980).

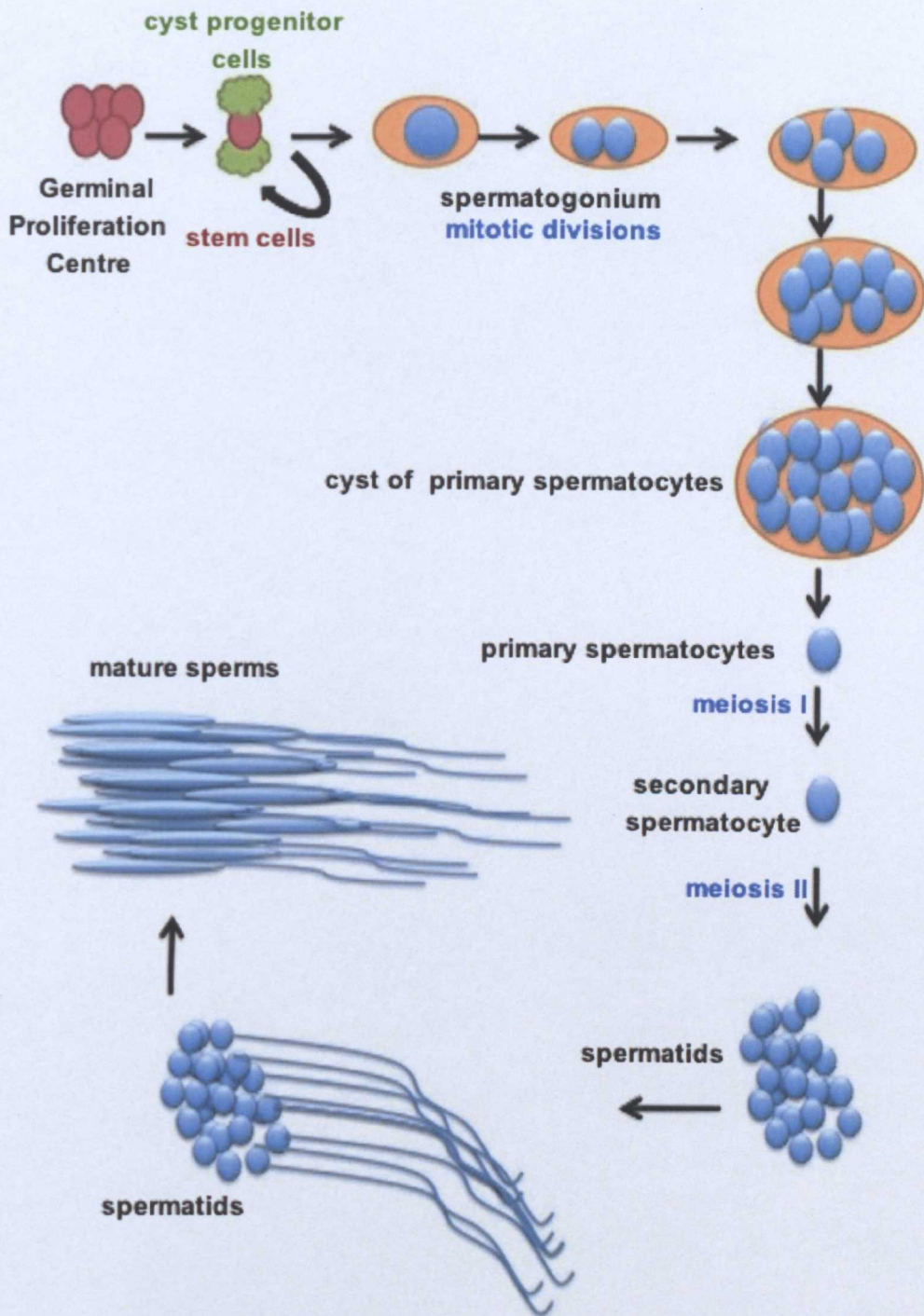


Figure 1-4 Spermatogenesis in *Drosophila melanogaster*.

The most remarkable feature during this elongation is the nuclear shaping and condensation. The spherical nucleus is subjected to a series of morphological changes as a result of chromatin condensation so that, by the end of spermatid maturation, the nucleus becomes a long, thin needle shape, and the nuclear volume has decreased about 20 times (Tates, 1971). After completion of spermatid nucleus condensation, the final process of spermatid maturation starts, named sperm individualization and coiling. During this step the spermatid loses its cytoplasmic connections and the spermatid components are removed

into a waste bag at the posterior part of the spermatid bundle (Tokuyasu, K.T. *et al.*,1972; Tokuyasu, K.T.; *et al.*,1972). After that, the sperm coils and the mature sperm are released into the lumen of the testis, then stored in the seminal vesicle (Lindsley and Tokuyasu,1980) .

1.3 Histone modification during spermatogenesis

During spermatogenesis, paternal germ cells undergo dramatic changes in epigenetic information to produce uniquely 'modified' germ cells (Seki *et al.*,2005; Surani,2001). Nucleosome histones are the target of chemical modifications, including acetylation, methylation, phosphorylation and ubiquitination which may work together or alone to influence gene activation or/and inactivation (Douglas,2010).

1.3.1 Histone methylation

The role of histone methylation in the development of paternal germ line is of particular interest. One study suggested that histone methylation has a significant role in the maintenance of spermatogonial stem cells by repressing promyelocytic leukemia zinc-finger (Plzf) gene (Payne and Braun,2006). The results of this study indicated that transcriptional activity of Plzf correlates with the absence of Histone3 mono methyl lysine 27 (H3K27me1) and Histone 4 mono methyl lysine 20 (H4K20me1). In addition, it was noted that the methylation levels of H3K27 and H3K9 are very low in early spermatogenesis compared with post meiotic stage, suggesting that the high level of histone methylation in later stages may have an epigenetic role in genome silencing (Payan and Braun, 2006). Glaser and his group (Glaser *et al.*,2009) studied the role of H3K4 methyltransferase on H3K4 methylation in spermatogenesis and noted that the inactivation of methyltransferase by tamoxifen led to an absence of spermatocytes and loss of spermatogenesis, suggesting that H3K4 methylation might be required for spermatogonial differentiation. In addition, another study found that sex chromosomes are occupied by Histone di methyl lysine 9 (H3K9me2). This finding suggested that this histone modification might play an important role in X and Y chromosome inactivation (Khalil *et al.*,2004).

The development of paternal germ cells is regulated by the establishment and removal of histone methylation, which varies during spermatogenesis and any disturbance of the timing of these actions leads to abnormal development in

germ cells. In *Caenorhabditis elegans*, the failure to remove dimethylation from H3K4 by Lysine demethylase (LSD1) in primordial germ cells resulted in an accumulation of H3K4me2 and abnormal gene expression during spermatogenesis, and this abnormal epigenetic information was transferred to the next generation (Katz *et al.*,2009). Similarly, demethylation of H3K9me2/1 by a specific enzyme known as demethylase JHDM2A (JmiC domain containing histone demethylase 2A) is critical step in the expression of transition nuclear proteins and protamine1 in spermiogenesis, and the disruption of JHDM2A activity resulted in abnormal chromatin packaging and infertile sperm in mouse (Okada *et al.*,2007).

1.3.2 Histone acetylation

Many studies have recorded that histone acetylation and deacetylation result in dramatic changes in the development of the paternal germ cell of many animal species (Candido and Dixon,1972; Christensen *et al.*,1984; Grimes and Henderson,1984; Grimes,1975; Kurtz *et al.*,2007; Oliva and Mezquita,1982). By using immunocytochemistry, Hazzouri and his group (Hazzouri *et al.*,2000a) found that the acetylation signal clearly marks the H2B, H3 and H4 during spermatogonial and preleptotene spermatocyte stages in mouse testis, but the signal disappears in leptotene, pachytene spermatocytes and most round spermatid stages. The acetylation then re-appears and marks all histones (H2A, H2B, H3 and H4) again in the steps 9-11 of elongating spermatids. Subsequently, the acetylation signal is dramatically decreased in condensed spermatids. Histone acetylation during chromatin condensation in spermiogenesis plays a significant role in histone protamine replacement (Hazzouri *et al.*,2000a; Nair,2008).

The establishment and removal of histone acetylation has significant roles in normal spermatogenesis and sperm fertility. Blocking of acetylation removal by using inhibitor trichostatin-A to inactivate HDAC in murine spermatogonia results in loss of spermatocyte (Fenic *et al.*,2008). In an immunocytochemical comparison of normal and abnormal human spermatogenesis, results showed additional abnormal positive signal in spermatocytes of the abnormal spermatogenesis. The result indicated that the absence of normal histone acetylation in elongating spermatids which led to abnormal chromatin packaging and infertile sperm (Sonnack *et al.*,2002). The study suggested that

premature histone acetylation might lead to abnormal histone protamine replacement resulting in infertility.

1.3.3 Histone phosphorylation

In spermatogenesis, Histone 2A.X (H2A.X) phosphorylation has been detected in mouse pachytene cells, and is probably associated with meiotic sex chromosome inactivation (Turner *et al.*,2004). Recently, phosphorylation has been detected in Testis-specific Histone 1 (H1t) in mouse elongating spermatids, and it is thought that this might participate in histone replacement and chromatin condensation (Rose *et al.*,2008; Sarg,2009). Moreover, by using liquid chromatography mass spectrometry (LC-MS), Testis-specific Histone 2B (TH2B) phosphorylation has been detected in different stages of spermatogenesis (spermatogonia, spermatocytes and round spermatids), suggesting that this modification may have epigenetic regulation during spermatogenic cells development (Lu,2009).

1.3.4 Histone ubiquitination

This modification has been characterized during spermatogenesis in many vertebrates (Agell *et al.*,1983; Baarends *et al.*,1999; Chen *et al.*,1998; Nickel *et al.*,1987). For example H2A ubiquitination was found in late stages of primary spermatocytes and elongating spermatids in rooster and mouse (Agell *et al.*,1983; Baarends *et al.*,1999). Another study showed that H3, Testis histone 3 (TH3), H2A, H2A.Z and H2B were poly and mono ubiquitinated in rat elongating spermatids (Chen *et al.*,1998). These results support the hypothesis that ubiquitination takes part in histone replacement and chromatin condensation during spermiogenesis (Govin *et al.*,2004; Liu *et al.*,2005).

1.3.5 Histone crotonylation

This modification has been described recently in male germ cells of mammals (Montellier *et al.*,2012; Tan *et al.*,2011). This modification marks testis specific genes on sex chromosomes immediately after meiosis (Tan *et al.*,2011). Moreover, crotonylation was found associated with active autosomes genes after meiosis (Montellier *et al.*,2012). These studies suggested that this modified histone maintain gene activity during spermiogenesis (Montellier *et al.*,2012; Tan *et al.*,2011).

1.4 Histone- Protamine replacement during spermiogenesis

In the majority of species including mammals, histone replacement is a remarkable event in chromatin condensation during sperm maturation. In the round spermatid stage the chromatin is bound by somatic histones, but in later stages this histone is replaced by transition proteins (TP), then by protamines (Meistrich,1989; Meistrich *et al.*,1978) after these process sperm genome folding in the nucleus will change from 100.000 times (somatic cell) (Razin *et al.*,2007) to 10^6 fold (Miller *et al.*,2009). Although the exact mechanism of histone removal is still unclear, it has been classified into three phases (Gaucher *et al.*,2010). These phases are: i) nucleosome instability by addition of or replacement by testis histone variants, ii) hyperacetylation of histone 4 and iii) direct histone replacement by transition proteins (TPs) and protamines. In addition to somatic histone, many histone variants are only found in spermatogenic cells such as Human Testis/Sperm-specific Histone 2B (h-HSH2B) (Li *et al.*,2005), and H2B histone family, member W, testis-specific (H2BFWT) (Boulard *et al.*,2006). Histone 2 A Like 1 (H2AL1), Histone 2 A Like2 (H2AL2), and Histone 2 B Like 1 (H2BL1) are found in mouse sperm (Govin *et al.*,2007). The above studies have mentioned that the nucleosomes containing testis-specific histone variants are less stable than somatic nucleosomes. Moreover, similar results have been reported by a recent study on H1t , the result indicated that H1t nucleosomes have weak binding with the DNA (Godde and Ura,2009). Generally, it has been proposed that the incorporation of testis-specific histone variants in the chromatin is important step in histone removal and chromatin condensation (Gaucher *et al.*,2010).

During spermatid elongation, a wave of histone hyper-acetylation was observed in many metazoan species (Govin *et al.*,2004; Rathke *et al.*,2007). However this phenomenon has not been recognized in species whose sperm have open chromatin and retain complete histones. *In vitro* studies indicated that histone acetylation leads to changes in nucleosome characteristics that facilitate histone removal (Benson *et al.*,2006; Shogren-Knaak *et al.*,2006). This acetylation gradually decreases during histone removal (Hazzouri *et al.*,2000a; Rathke *et al.*,2007). In addition, the direction of DNA condensation is similar to the direction of acetylation: from the anterior to the posterior part of the nucleus (Hazzouri *et al.*,2000a) . Therefore, it has been postulated that histone hyper-

acetylation is a key feature in histone replacement (Govin *et al.*,2004). The exact mechanism of histone acetylation and how this modification works in histone removal is still unclear. One study noted that histone deacetylase enzymes were dramatically degraded in spermatids and it was supposed that this degradation might produce an environment which promotes histone acetylation and chromatin remodelling process (Caron *et al.*,2003). It has been suggested that histone acetylation during spermiogenesis triggers a chain of chemical reactions which leads to histone removal. Recent studies found that bromodomain proteins recognise and bind hyperacetylated nucleosomes and that these proteins might mediate this chain of events (Govin *et al.*,2004; Zeng,2002). In support of this it was noted that BRDT protein (testis specific double bromodomain containing protein) can activate chromatin condensation after histone acetylation was induced using trichostatin A (Pivot-Pajot,2003). This study concluded that bromodomain protein activity depends on histone hyperacetylation (Pivot-Pajot,2003).

Histone ubiquitination is another modification that marks H2A and H2B in elongating spermatids and it has been suggested that this modification may mediate the removal of nucleosomes (Baarends *et al.*,1999; Chen *et al.*,1998). Recently, Ring Finger protein (RNF8) dependent histone ubiquitination was shown to be capable of activating H4K16 acetylation, which might mediate the initial step of histone removal during spermiogenesis (Lu *et al.*,2010). In this study, it was shown that H2A ubiquitination significantly decreased in elongating spermatids of RNF8 deficient mice, in addition to absence of H4K16 acetylation and decrease in male fertility. This suggests that RNF8 might play an important role in post meiotic chromatin packaging and sperm quality (Lu *et al.*,2010).

1.5 Mature sperm

There is a wide diversity of sperm phenotype in the animal kingdom (Figure 1-5) (modified from(Poccia,1986)), and microscopic analysis has revealed that each species has a particular sperm form. This gives the impression that these sperm differ considerably in the molecular structure of their nuclei. In general, the paternal gamete nucleus consists of chromatin and proteins. Interestingly, sperm vary in chromatin condensation pattern, histone types and variants, protamine types and the histone-protamine ratio, and from these differences the sperm gets its own (species) identity.

1.5.1 Chromatin organization

Unlike somatic cell DNA, the majority of sperm have condensed DNA in crystalline-like form (Dadoune *et al.*,2004). This chromatin condensation varies between species. For example, in mouse sperm the chromatin is six times more compacted than in their somatic cells (Pogany *et al.*,1981). Many patterns have been suggested for chromatin organization in sperm. However, in the common pattern, protamine binds with the DNA strand in the minor groove, then this DNA-protamine complex fits into the major groove of an adjacent DNA strand. So by this binding DNA, strands are packaged side by side linearly and horizontally within the sperm nucleus (Balhorn,1982).

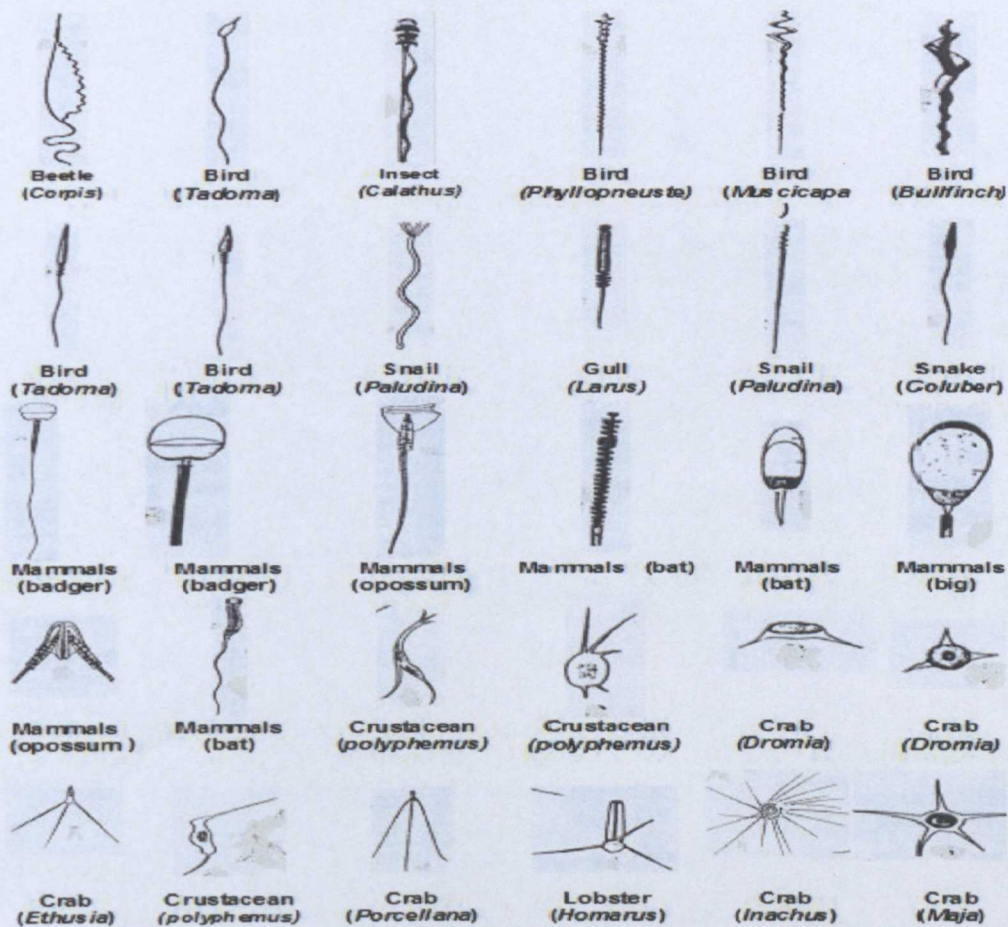


Figure1-5 Various forms of metazoan sperms. modified from(Poccia,1986).

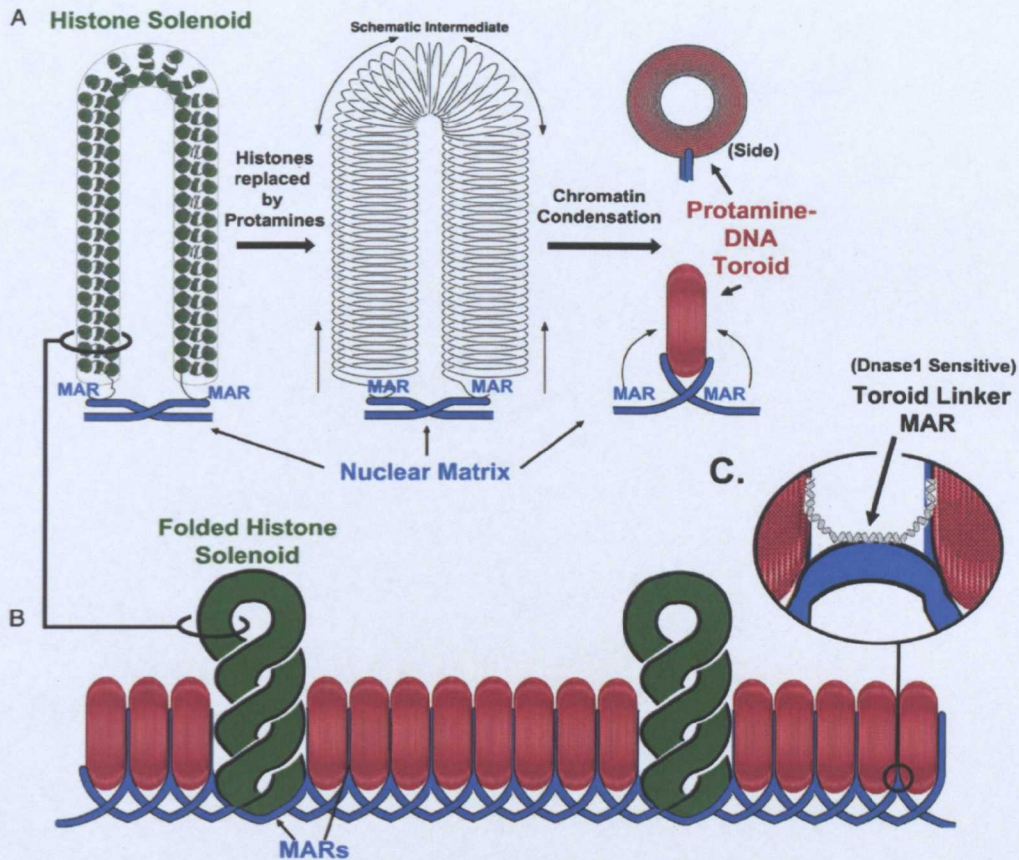


Figure 1-6 DNA packaging in human sperm (Ward,2010), with authorisation from the publisher.

Many studies have suggested that the DNA-protamine complex is organized into a basic unit that has a toroidal shape (doughnut) (figure 1-6). Each single toroid consists of around 50 kilobases of DNA and represents one DNA loop domain. These toroids are bound together by uncoiled DNA segments known as toroidal linker regions, in these regions the DNA loop domain binds to sperm nuclear matrix by particular sequences named matrix attachment regions (MAR) (Hud *et al.*,1993; McCarthy and Ward,1999; Sotolongo *et al.*,2003; Ward,1993; 2010).

Previous studies showed that chromatin organization in sperm takes place at four levels. Firstly, sperm chromosomes are arranged into DNA-loop domains anchored in the nuclear annulus at their bases. These DNA-loop domains are attached to the nuclear matrix by matrix attachment regions. The DNA is then arranged into doughnut structures as result of protamine binding. The final step is the spatial arrangement of packaged chromosomes when the chromosomes are arranged in a non-random pattern (Dadoune *et al.*,2004; Sotolongo *et al.*,2003; Ward,1993). In the final level of chromosome positioning the

centromeres are located in the interior of the nucleus, while the telomeres are located closer to the periphery (Zalensky *et al.*,1995) .

1.5.2 Sperm nucleus proteins

Proteomic analysis of whole sperm protein has been studied in many species such as *Drosophila melanogaster* (Dorus *et al.*,2006; Karr,2007), human (Baker *et al.*,2007; de Mateo *et al.*,2007; Martinez-Heredia *et al.*,2006), and bovine (Peddinti *et al.*,2008). All these studies indicated that the sperm cell contains a huge number of proteins. In human sperm about 1000 spots were detected by two-dimensional electrophoresis and LC-MS/MS (Baker *et al.*,2007; Martinez-Heredia *et al.*,2006). LC-MS/MS analysis indicated that about 403 proteins are located in the nucleus (de Mateo *et al.*,2011). Here two main proteins in the sperm nucleus, protamine and histone, will be discussed. The first is the predominant and more enriched nucleoprotein in the sperm nucleus, and the second is the largest family of nucleoproteins in human sperm, both of them play important roles in chromatin packaging and repackaging during late spermiogenesis, mature sperm and paternal pronucleus formation.

1.5.2.1 Protamine

Protamine is a family of small basic proteins (rich in arginine), that are produced in late stages of spermiogenesis, and which bind to chromatin. Two protamines have been described in mammals, protamine1 (P1), and protamine 2 (P2), reviewed in (Balhorn,2007; Dadoune,2003). P1 consists of 49 or 50 amino acids and packages all mammalian sperm chromatin .The protein has three domains with the central domain including the P1-DNA binding site with 3-11 arginine residues and two short peptides on both sides containing cysteine residues (Balhorn,2007). P2 is present in many mammals and is slightly larger than protamine P1 with about sixty three amino acids in mouse (Chauviere *et al.*,1992). Unlike P1, P2 contains a zinc atom (Bench *et al.*,2000). Both protamine types undergo disulphide bond formation as post-translation modification during the final step of sperm maturation in the epididymis (Vilfan *et al.*,2004).

1.5.2.1.1 Function of sperm protamine

Accumulation of protamine within the paternal nucleus and its binding with DNA leads to the formation of a highly compacted compartment of the spermatozoan genome, and as result of this the DNA loses its transcriptional activity

(Belokopytova *et al.*,1993). Many functions have been suggested for protamines, but the most acceptable function is that the condensed nucleus enables the production of an inactive hydrodynamic chromatin that protects the DNA prior to oocyte fertilization (Belokopytova *et al.*,1993; Oliva and Mezquita,1982). However, previous investigations of human sperm indicated that an abnormal P1/P2 ratio is associated with infertility, and any changes of this ratio lead to abnormal embryo development (Aoki *et al.*,2006a; Aoki *et al.*,2006b; Oliva,2006). Although a role for protamine in fertilization and embryo development is controversial (Kuretake *et al.*,1996), the result of intracytoplasmic injection of abnormal sperm lacking P2 in *in vitro* fertilization (IVF) indicated that the oocytes fertilized normally and the embryos developed typically, but embryo implantation rates were markedly reduced (Aoki *et al.*,2006b). As a consequence of this finding, many researchers have suggested that the protamine might have an epigenetic effect on the sperm genome (Oliva,2006). It seems that protamine has complex functions, so a comparative study between protamine-DNA binding sites among different species, with different chromatin condensation patterns, might help to understand the effect protamine has on the paternal *and* zygotic genomes.

1.5.2.2 Histones

In spite of the bulk replacement of histones by protamine in late spermiogenesis, many species still contain some histones in their mature sperm. For example, core histones were detected in marsupial mammals and mouse sperm (Pittoggi *et al.*,1999; Soon *et al.*,1997) and CENP-A in bull sperm (Palmer *et al.*,1990). Furthermore, many early studies indicated that the histones H4, H3, H2A and TH2B were located in human sperm (Gusse *et al.*,1986; Zalensky *et al.*,2002). However, the analysis of human sperm nuclear proteome by using LC-MS/MS revealed that the histones are the largest family of the mature sperm nuclear proteins and consists of 39 proteins (Table 1-1) (de Mateo *et al.*,2011). Several studies show that histones have a non-random distribution pattern within the nucleus of human (Li *et al.*,2008), and mouse sperm (Pittoggi *et al.*,1999). In addition, many studies have indicated that some of these histones carry acetyl or methyl modifications, while H3 is methylated (mono Me H3K4) in mouse (Govin *et al.*,2007), and (di,tri Me H3K4) in human (Hammoud, S. *et al.*,2009). H4 is acetylated (mono Ac H4K8, mono Ac H4K12) in mouse (van der Heijden *et al.*,2006).

1.5.2.2.1 Function of sperm histone

Many functions have been proposed for histones which are retained in the sperm nucleus. Firstly, previous studies suggested that these histones might play a role in embryo development. Recently, this hypothesis has been supported by two provisional studies. Using Chromatin immune precipitation-microarray analysis (ChIP-chip) and Chromatin immune precipitation-sequencing (ChIP-seq) Hammoud and his team (Hammoud, S. *et al.*,2009) have indicated that sequences for genes involved in embryonic development are packaged by histones. Furthermore using micrococcal nuclease digestion and ChIP-chip on human and mouse sperm another study indicated that endonuclease sensitive regions in nucleosomal chromatin are enriched with gene regulatory zones that include promoter and CCCTC binding factor (CTCF) sequences (Arpanahi *et al.*,2009). The results of both studies suggest that sperm histones may play epigenetic roles in embryo development. The second proposed function of paternal histones is that chromocentre histones in mouse sperm might play an important role in the re-establishment of heterochromatin/euchromatin partitioning after fertilization (van der Heijden *et al.*,2006).

A recent study in *Xenopus* oocyte extracts noted that the histone CENP-A (centromere protein) in the human sperm nucleus was redistributed to the peripheral parts after protamine removal (Mudrak *et al.*,2009). To some extent, the results of this study support the earlier findings, and from both studies we understand it is possible that the centromeric histone in the paternal nucleus plays a part in chromatin re-localization during pronucleus formation. The third function of sperm histone has been proposed by Sánchez-Vázquez and his group (Sanchez-Vazquez *et al.*,2008) in which the observation of *in vitro* nuclear decondensation indicated that H1 mediated chromatin condensation and play a key role of DNA condensation.

No	Histone name	No	Histone name
1	Histone H2A type 1	21	Histone H2B type 1-K
2	Histone H2A type 1-A	22	Histone H2B type 1-IL
3	Histone H2A type 1-B/E	23	Histone H2B type 1-M
4	Histone H2A type 1-C	24	Histone H2B type 1-N
5	Histone H2A type 1-D	25	Histone H2B type 1-O
6	Histone H2A.V	26	Histone H2B type F-S
7	Histone H2A.Z	27	Histone H2B type 2-E
8	Histone H2A.x	28	Histone H2B type 2-F
9	Core histone macro-H2A.1	29	Histone H2B type 3-B
10	Histone H2A-Bbd type 1	30	Histone H3
11	Histone H2A Bbd type 2/3	31	Histone H3.1t
12	Histone H2A type 2-A	32	Histone H3.1
13	Histone H2A type 2-B	33	Histone H3.2
14	Histone H2A type 2-C	34	Histone H3.3
15	Histone H2B type 1-A	35	H3-like centromeric protein A
16	Histone H2B type 1-B	36	Histone H4
17	Histone H2B type 1-C/E/F/G/I	37	Histone H4-like protein type G
18	Histone H2B 1-D	38	Testis-specific H1 histone
19	Histone H2B type 1-H	39	Histone H2A type 2-F
20	Histone H2B type 1-J		

Table 1-1 Human sperm histones.

1.5.3 Sperm chromatin remodelling after fertilization

After fertilization and as the maternal pronucleus commences DNA replication, sperm chromatin undergoes dramatic remodelling from the compacted, inactive form to the relatively relaxed, accessible and transcriptionally active form. Microscopic analysis of male pronucleus formation in mammals revealed that the sperm nucleus undergoes three morphological changes during this short period. In the first stage, during maternal anaphase II, the paternal chromatin expands about three-fold, then in the second stage during maternal telophase II the paternal chromatin condenses to about a half of the previous stage. In the third stage the chromatin is completely decondensed, and dispersed to about ten times its original size (Adenot *et al.*,1991; Mclay *et al.*,2003; Wright and Longo,1988). Similar behaviour of human sperm chromatin was noted in heterologous systems using a *Xenopus* oocyte extract (Mudrak *et al.*,2009).

Protamine replacement by histones correlates with the morphological changes in sperm chromatin after fertilization. Immunocytochemical analysis revealed that the protamine is completely removed from sperm pronucleus by the end of maternal anaphase II (Rodman *et al.*,1981). Many studies have also found that inactivation of the protamine removal pathway leads to a failure of DNA decondensation (Mclay *et al.*,2003; Perreault,1992). For example, inhibition of the reduction of inter-protamine sulphhydryl bonds by inactivation of sulphhydryl dependent enzymes or oocyte glutathione depletion resulted in failure to remove protamines and aberrant chromatin remodelling (Mclay *et al.*,2003; Perreault,1992).

Early studies indicated that sperm chromatin decondensation after fertilization is mainly controlled by the oocyte environment. Mclay and his group (Mclay *et al.*,2003) discussed these findings in their review and the main highlights are summarised here. Firstly, paternal chromatin remodelling depends on the oocyte stage at fertilization, as many studies found that sperm DNA remained highly condensed when the oocyte was fertilized at prophase I (pre mature stage) (Clarke,1986; Maeda *et al.*,1998). Secondly, when the maternal pronucleus was removed from the oocyte at prophase I , the sperm chromatin failed to de-condense (Balakier and Tarkowski,1980). Thirdly, when protein synthesis was inhibited during oocyte meiotic maturation, the sperm chromatin started the first remodelling stage but was unable to complete the other stages (McLay and Clarke.,1997).

There are many interesting differences between paternal and maternal genome during pronucleus formation. These differences raise the hypothesis that each genome is programmed in a different way. The most interesting difference is that the male pronucleus is transcriptionally more active than the maternal one (Aoki *et al.*,1997; Rastelli *et al.*,2001) indicating that perhaps some of the sperm genome is structurally ready for transcription, and the recent findings indicated that sperm promoters are enriched by active histone modification strongly support this idea (Brykczynska *et al.*,2010; Hammoud *et al.*,2009; Hammoud, S. *et al.*,2009). In addition, there is clear variation in genome methylation. Unlike the female pronucleus, the male genome is demethylated shortly after

fertilization in many mammalian species. Immunocytochemical staining of early mouse embryos showed that the DNA methylation signal of paternal chromatin disappeared less than 6hr post fertilization (Mayer,2002; Santos *et al.*,2002). A similar result was obtained using DNA sequencing and PCR amplification (Oswald *et al.*,2000). However, there was no change in DNA methylation of the sheep pronucleus (Young and Beaujean,2004). Moreover, male and female pronuclei differ in their histone acetylation. In contrast to maternal chromatin, the paternal genome is characterized by H4 hyperacetylation in the early stage of the first cell cycle, but this hyperacetylation then decreases, which might be because the acetylation is important for the accumulation of H3 and H4 within sperm chromatin (Adenot,1997; Verreault,2000).

1.6 Epigenetic roles of sperm in embryo development

1.6.1 DNA methylation

DNA methylation is the modification that occurs by the addition of a methyl group to DNA cytosine, and this reaction is regulated by a family of proteins known as DNA methyltransferase (DNMT) (Eden and Cedar,1994; Jenkins and Carrell,2011). While some DNMT members notably DNA methyltransferase A3a (DNMA3a) and DNA methyltransferase A3b (DNMA3b) are responsible for the catalytic reactions of DNA methylation, others like DNA methyltransferase A3I (DNMA3I) and DNA methyltransferase A1 (DNMA1) work to establish and maintain methylation marks on genes (Bestor,1992; Okano *et al.*,1999). DNA methylation at particular locations of specific genes leads to a decrease in the ability of the polymerase to bind to promoters, which results in blocking transcription and gene activity.

DNA methylation is a remarkable epigenetic feature in germ lines and embryo development, and plays critical roles in gametogenesis and normal embryo growth. One of the most important functions of DNA methylation is gene imprinting. Embryo developmental processes are delicate systems based on the activity of some genes and silencing of other genes at specific stages. DNA methylation helps in the regulation of gene activity to ensure appropriate embryo development (Ng and Bird,1999; Panning and Jaenisch,1996; Walsh *et al.*,1998). Many studies have focussed on the effects of abnormal methylation on gametogenesis and embryogenesis. The most critical factor in DNA

methylation is the DNMT protein family, as any disturbance in the mechanism of action of these proteins will lead to abnormal DNA methylation and subsequently abnormal gene expression and embryo development. There are many studies supporting this hypothesis. For example, knocking out DNMT gene in the mouse resulted in a decrease in the normal level of DNA methylation, which led to many significant molecular changes including chromosome inactivation and activation of imprinted genes. Morphologically, the embryos developed abnormally (Bestor,1992; Panning and Jaenisch,1996; Walsh *et al.*,1998). Similar results were observed by blocking DNMT3a, DNMT3b, and DNMT3L. The knocking out of these proteins remarkably resulted in a decrease in male germ cell production in the offspring (Kaneda *et al.*,2004; La Salle *et al.*,2007).

1.6.2 Sperm Nuclear matrix attachment region

Loop domains of sperm and somatic cell chromatin are characterized by the binding with nuclear matrix (Choudhary *et al.*,1995; Linnemann *et al.*,2009; Ward *et al.*,1989) and this attachment plays an important role in DNA replication and gene expression by changing the chromatin loop domains (compacted form) to functional loops (accessible form) (Dijkwel and Hamlin,1995; Ostermeier *et al.*,2003). Many studies suggest that the loop domain structures are located throughout sperm nucleus, and every protamine DNA toroid contains one chromatin loop domain (Sotolongo *et al.*,2005; Ward,1993; Ward *et al.*,1989). Interestingly, the sperm nuclear matrix is associated with many regulatory factors such as chromatin modifiers (topoisomerase), transcription factors and RNAs (Carrey *et al.*,2002; Har-Vardi *et al.*,2007; Johnson *et al.*,2011; Lalancette *et al.*,2008). Microarray analysis revealed that the sperm nuclear matrix is enriched in promoter and exon sequences (Johnson *et al.*,2011). Many additional studies have shown that the sperm nuclear matrix has a significant role in early embryo development. Ward and his group (Ward *et al.*,1999) treated mouse sperm nuclei with 'stale' dithiothreitol (DTT) and alkyltrimethylammonium bromide to remove the sperm perinuclear theca, acrosome and tail. Such 'stripped' sperm nuclei supported embryo development following injection into the egg. In contrast, nuclei washed with fresh reagents failed to support embryo development. In the first case the sperm nuclear matrix was weakened but not destroyed, while in the second case it was completely destroyed.

In another study, the sperm nuclei was extracted with DTT and high salt concentration to detach all nuclear proteins, and then treated with endonucleases and Deoxyribonuclease I (DNAase I) to remove the DNA not bound to nuclear matrix. The sperm nuclei were then injected into an oocyte. The researchers noted that these nuclei could form pronuclei and start DNA synthesis. In contrast, injection of extracted DNA on its own did not initiate paternal pronucleus formation and DNA synthesis (Shaman *et al.*,2007).

1.6.3 Role of histone variants and histone modifications in embryonic development

Histone modifications are important elements in the epigenetic regulation of embryo development, so any paternal histone delivered into the oocyte might be a component of epigenetic control of embryogenesis. Several recent studies have provided evidence that the retained histones in mature sperm might play a role in embryo development. The analysis of localization of the canonical histones, testis histone variants and histone modifications revealed that histones are located at particular regions in human sperm. In an excellent study, Hammoud and his team (Hammoud *et al.*,2009; Hammoud, S. *et al.*,2009) found that sperm nucleosomes are significantly enriched at developmental regulatory regions. Moreover , it was noted that nucleohistone regions are enriched for regulatory regions such as promoters and CTCF binding sites for embryologically significant genes in human (Arpanahi *et al.*,2009), and mouse sperm (Saida *et al.*,2011).

1.6.3.1 Histone variants

ChIP-chip analysis of testis variant H2B (TH2B), indicated that this variant is significantly localized to promoter regions of 0.3% of genes (Hammoud *et al.*,2009). Functional annotation analysis showed important enrichment of TH2B peaks at genes for spermatogenesis, ion channels, and fertilization (Hammoud *et al.*,2009). The second histone variant that is thought to play a role in embryo development is H2A.Z. In embryonic stem cells this variant is mainly located at the promoters and regulates cell differentiation (Creyghton *et al.*,2008). However, ChIP-seq analysis indicated that this variant is mainly located at pericentric heterochromatin in the human sperm genome, but without any importance in functional annotation analysis (Hammoud *et al.*,2009). This gave

rise to a suggestion that the H2A.Z might have a structural role in sperm chromatin.

1.6.3.2 Histone modifications

The combination of ChIP on chip and deep sequencing analysis, revealed that nucleosomal histone modifications are located at developmental regions. For example, Histone 3 di methyl lysine 4 (H3K4me2) is enriched at developmental promoters, while H3K27me3 marks inactive development promoters in early embryogenesis. In addition, Histone 3 tri methyl lysine 4 (H3K4me3) is located in Homeobox genes (Hox), non-coding RNAs, and imprinted regions (Hammoud *et al.*,2009). In a similar study, it was found that active and inactive histone methylation mark different promoters in mouse and human sperm (Brykczynska *et al.*,2010). The results of this study clearly showed that H3k27me3 marks developmental promoters, whereas H3k4me2 marks promoters of spermatogenesis and cellular homeostasis genes. Based on the above information, it is reasonable to assume that alterations in histone epigenetic information in the sperm may lead to abnormal embryo development. To test this hypothesis Hammoud and his team (Hammoud *et al.*,2011) analyzed the histone modifications in the sperm of infertile men, with unexplained poor embryogenesis during *in vitro* fertilization (IVF)treatments. Unlike in normal sperm, the results showed clearly that the nucleosomes are randomly distributed in the infertile men and there was also a dramatic reduction in the retention of H3K37me and H3K4me in developmental promoters. These results provide additional evidence that histone modifications in sperm are important factors in embryo development.

1.6.3.3 Sperm RNA

The mature sperm delivers many RNAs to the oocyte during fertilization, and the function of these RNAs is still not clear. However some recent studies discussed the role of sperm RNAs in the development process, and many possible epigenetic roles in embryo development were highlighted (Dadoune,2009; Johnson *et al.*,2011; Ostermeier *et al.*,2002; Rassoulzadegan *et al.*,2006). Traditionally, it was thought that these RNAs are residual from gene expression during spermatogenesis. However the use of microarray analysis profiling human sperm RNAs suggests that the sperm delivers particular paternal mRNAs into the oocyte, that are associated with

embryogenesis (Ostermeier *et al.*,2002; Ostermeier *et al.*,2004). Furthermore, micro array analysis of bovine sperm RNAs showed that 4.5% of RNAs are specific to mature sperm and are not found in spermatids and that more than half of spermatid RNAs are selectively degraded from the mature sperm (Gilbert *et al.*,2007). These results argue strongly against a passive residual aspect for sperm RNA retention.

The comparison between sperm, oocyte and embryo RNAs showed that some specific sperm RNAs are not found in the oocyte of human and hamster (Avendano *et al.*,2009; Johnson *et al.*,2011; Kocabas *et al.*,2006), but are detectable in embryos (Avendano *et al.*,2009; Johnson *et al.*,2011). A recent survey of small RNA in human sperm revealed that some miRNAs are not found in the ovary, and functional annotation analysis showed that the top 10 functions of these RNAs are embryo, tissue and organ development (Krawetz *et al.*,2011). Furthermore, the predicted target genes of these RNAs are NOTCH1 and DLL1 (Krawetz *et al.*,2011). These genes are known to have essential roles in somite formation and the segmentation of vertebrate embryos (Krebs *et al.*,2003; Zhang *et al.*,2002).

1.7 Thesis aims

There is growing evidence that the retained histones in the mature sperm of human are localised non randomly at developmental regulatory regions, this result suggest that these histone might play epigenetic function during embryogenesis. However, the knowledge about this topic still incomplete, and there are many questions need to be answer.

The main aim of this thesis is to investigate the possible epigenetic roles of mature sperm retained histones and modified histones on embryo development in the experimental animal models. The result of this study might help to use the experimental animal models in more advanced research in this topic. To achieve the main aim, the following subset of aims were investigated:

- Investigate the retention of histone and/or histone modifications in the mature sperm of *Drosophila melanogaster* (fruit fly), *Bos Taurus* (Bovine) and *Mus musculus* (mouse).

- Investigate the chromatin compartmentalization into nucleosomal and nucleoprotamine chromatin in the sperm of *Drosophila melanogaster* and bovine.
- investigate the positioning of retained nucleosomes and modified histones within the genome of *Drosophila melanogaster* sperm, and thereby assess its possible epigenetic role in embryo development.
- Investigate the association of histone retention in sperm of *Drosophila melanogaster* with the epigenome of embryo development.

Chapter 2: Materials and Methods

2.1 *Drosophila melanogaster* culture

Two transgenic *Drosophila melanogaster* flies (H2A.v tagged with Red Fluorescent Protein (H2A.v-RFP) and protamine tagged with Green Fluorescent Protein (protamine-GFP)) were received from Dr Helen White Cooper (School of Bioscience, Cardiff University). In addition, wild type flies were received from Professor Elwyn Isaac (Institute of Integrative and Comparative Biology, University of Leeds). The flies were grown and maintained on standard medium (Jazz-mix, Fisher Scientific), supplied by addition of yeast and at room temperature.

2.2 Sample slides preparation for Immunocytochemistry and microscopic analysis

2.2.1 Poly-l-lysine slides preparation

Poly-l-lysine coated slides were prepared to provide higher adhesion, and to reduce sperm loss during immunocytochemistry steps. The coated slides were prepared according to the protocol described by (Huang *et al.*, 1983) as follows. Firstly the slides were washed twice with Milli-Q water for 5 minutes, then transferred to washing solution (33g Sodium Hydroxide (NaOH) pellets, 200 ml 95% ethanol, 133 ml Milli-Q water) and shaken gently for two hours. The slides were then rinsed with Milli-Q water for 15 minutes.

In the second step, the slides were transferred into a plastic container, then immediately poly-l-lysine solution (60 ml poly-l-lysine, 35 ml 1X phosphate Buffer Solution (PBS), 250 ml Milli-Q water) was poured over them and the container was shaken gently for thirty minutes. Next, the slides were washed again with Milli-Q water, after which the slide racks were placed in a 60 °C oven until the slides were completely dry, when they were stored in a sealed plastic slide box.

2.2.2 *Drosophila melanogaster* Testis and Sperm slides preparation

Young males flies were used as the source of spermatogenic cells for H2A-RFP and protamine-GFP analysis and immunocytochemistry. The flies were dissected under the dissecting microscope, using fine forceps and tungsten needles (0.2 mm) in a drop of 1X PBS. The reproductive system was removed and cleaned, then transferred into another drop of PBS. After that, the

accessory glands were removed and the testis and seminal vesicles were transferred to another drop of PBS. In this drop the seminal vesicles were separated from the testes and both were transferred to separate poly-l-lysine coated slides. The seminal vesicle was split using forceps, then the sample was left one minute to allow sperm to swim out and obtain a good distribution on the slides. Testes were squashed with the coverslip and immersed in liquid nitrogen before the coverslip was removed with forceps. In the next step the slides were dried on the bench for 30 minutes, after which the samples were ready.

2.2.3 Mouse sperm

Sperm were collected from CD4 mouse (8-10 weeks). The vas deferens was cut open to allow sperm to swim out into 2 ml of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO®), and then the sample was centrifuged at 600 g for 10 minutes and the supernatant removed. Then 2 ml DMEM was added gently to the sample and it was incubated for 20 minutes at 37 °C to allow motile sperm to swim up. The supernatant was transferred to a new tube, centrifuged and the sperm suspended in 100 µl PBS.

2.2.4 Bovine

The sperm straw was retrieved from liquid nitrogen and opened in a 15 ml tube. The sample was then washed twice in 10 ml BSA by centrifugation at 1000 g for 5 minutes. A Percoll separation procedure was then used to collect normal sperm according to our lab protocol. Percoll (Sigma Aldrich) concentrations were 90% and 45% and the centrifugation was at 1000 g for 20 minutes.

2.2.5 Sperm decondensation

100 µl of decondensation solution (0.2% triton X-100, 10 mM Dithiothreitol (DTT), 400 U/ml heparin with 1 mM PhenylMethylSulfonyl Fluoride (PMSF)) was pipetted onto *Drosophila* sperm sample then it was incubated in a humid box for 30 minutes at 4 °C. After that the cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes and the slides were washed in 1 x PBS 3 times for 5 minutes. Next, the slides were dried and stored in a sealed box at 4 °C until use. The sperm de-condensation process in mouse and bovine are similar to the above steps for *Drosophila melanogaster*, the differences being that the de-condensation solution for mouse sperm consisted of 0.2% triton X-100, 2.5 mM DTT, 100 U/ml heparin, and for bovine 0.2% triton X-100, 10 mM DTT, 100 U/ml heparin (occasionally with 1 mM PMSF added), and the slides

were incubated in a humid box at 4 °C (10 minutes for mouse sperm and 30 minutes for bovine sperm), and human sperm was used as positive control (on a separate slide) for mouse and bovine.

2.3 Microscopic investigation of H2A.v-RFP and protamine1-GRF signal in the testis of *Drosophila melanogaster*

The slides prepared as described in section 2.2.2 were used to investigate the changes in H2A.v-RFP and Protamine1 signals in the germ cells during spermiogenesis, a Fluorescent microscope (Leica LEITZ DMRB) was used in this step.

2.4 Immunocytochemistry

Abcam[®] immunocytochemistry protocol (abcam,2012) was used with some modifications in this experiment. Firstly, using somatic cells of the seminal vesicle as an internal control, the sperm sample was hydrated with 1X PBS for 1 minute, then permeabilised with 0.25% Triton X-100 for 5 minutes, and then the sample was washed with 1X PBS, 3 times for 5 minutes, followed by the use of low fat milk (Marvel[®]) as a blocking agent at a concentration of 3% for 60 minutes in a humid box. Primary antibody (table 2.1) was diluted in 1% Bovine Serum Albumin (BSA) at a dilution of 1:50, then the samples were incubated with the antibody in a humid box overnight at 4 °C (2 slides were used as negative control). In the next step the sample was washed 4 times in BPS for 5 minutes in each wash, after that the slide was incubated in the secondary antibody conjugated with Fluorescein Isothiocyanate (FITC) or Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) (table 1) in 1% BSA (1: 1500) for an hour at room temperature in a dark box (secondary antibodies only were applied to one of the negative control samples). After one hour the slides were washed with 1% PBS 4 times for 5 minutes each in a dark box, then incubated with 30µl of 0.5µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes then rinsed with PBS. The coverslip was then mounted with a drop of glycerol and sealed by super glue and stored in the dark at 4 °C.

No	Antibody	type	Company
1	Anti core histone antibody (sheep)	Primary antibody	Abcamab7832-15
2	Anti histone H3 antibody (rabbit)	Primary antibody	Upstate # 06-755
3	Anti histone H4 antibody (rabbit)	Primary antibody	Cell Signaling #2595
4	Anti histone H2A antibody (rabbit)	Primary antibody	Cell Signaling #2572
5	Anti histone H2B antibody (rabbit)	Primary antibody	Cell Signaling #2722
6	Anti acetyl H3 antibody (rabbit)	Primary antibody	Upstate #06-599
7	Anti acetyl H4 antibody (rabbit)	Primary antibody	CelBiochem D0016404
8	Anti H3k27me3 antibody (rabbit)	Primary antibody	Millipore 07-449
9	Anti protamine 1 antibody (goat)	Primary antibody	Santa cruze Sc 23-107
10	Anti sheep IgG antibody (FITC)	Secondary antibody	Abcam ab6743-1
11	Anti goat IgG antibody (FITC)	Secondary antibody	Santa cruze Sc 20-24
12	Anti rabbit IgG antibody (FITC)	Secondary antibody	
12	Anti rabbit IgG antibody (TRITC)	Secondary antibody	

Table 2-1 List of primary and secondary antibodies used immunocytochemistry and western blot.

2.5 Histone protein extraction

2.5.1 Mouse and Bovine sperm

Approximately 10^7 cells (bovine or mouse sperm) were washed twice in PBS, then the pellet was washed with 200 μ l solution containing 0.02% Triton X, 1M Tris pH 8.0, 1M Magnesium Chloride ($MgCl_2$), then centrifuged at 10,000 rpm for 5 minutes at 4°C. The pellet was then washed again in PBS containing 1mM PMSF, then centrifuged again as above. After that, the pellet was suspended in 50 μ l solution containing 20 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 100mM Tris- hydrochloric acid (Tris-HCl) pH 8.0, and mixed by pipette, then 1 volume (50 μ l) of solution containing 6M guanidine hydrochloride (GuHCl), 575 mM DTT was added and mixed well by vortex. In the next step the reaction was stopped by adding 5 volumes cold (-20°C) absolute ethanol and incubated 10 minutes at -20°C. This incubation was followed by centrifugation at maximum speed for 15 minutes to remove the supernatant, after which 500 μ l 0.5 M hydrochloric acid (HCl) was added to the pellet which was then mixed well and incubated at 37°C for 5 minutes, then mixed again and incubated for another 5 minutes. After that, the sample was centrifuged at maximum speed for 10 minutes, and the supernatant (containing

the protein) was transferred to a new pre-cooled tube containing 125 µl 100% trichloroacetic acid (TCA) and incubated for 30 minutes at 4°C to precipitate the protein. Finally the sample was centrifuged to remove TCA and washed twice in 500 µl acetone containing 1% β-mercaptoethanol then the protein was dried at room temperature for 10 minutes and resuspended in 20 µl SDS sodium dodecyl sulfate (SDS) sample buffer.

2.5.2 *Drosophila melanogaster* sperm

Histone protein was extracted from *Drosophila melanogaster* sperm by incubating sperm cells (isolated from 100 males) in 10 mM DTT and 0.02% triton X for 10 minutes, then the sample was washed twice in PBS. After that the sample was boiled in SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA) for 15 minutes. The sample was then centrifuged at maximum speed for 15 minutes to pellet DNA (mucus-like liquid) and resistant cell parts, and the supernatant transferred to a new tube. After that, 10 µl antibody-coated beads (anti H3 and H4 anti bodies) were added to the supernatant, and it was incubated for 2 to 4 hours while rotating at 4°C, followed by incubation at 70°C for 20 minutes to separate histone protein from the beads and the antibodies, then the sample was processed for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

2.6 Sodium dodecyl sulphate (SDS PAGE) and Western Blot

2.6.1 SDS PAGE

Acrylamide gel was prepared as follows: firstly, the separation gel was prepared by mixing 1.5 ml deionised water, 1.25 Tris pH 8.8, 50 µl 10% SDS, 2 ml 30% Acrylamide/Bis, 75% µl ammonium persulphate and 10 µl temed, then the gel was left to solidify for 30 minutes. After that, the stacking gel was prepared above the separation gel by mixing 3 ml deionised water, 1.25 Tris-HCl pH 8.8, 50 µl 10% SDS, 0.7ml 30% Acrylamide/Bis, 75% µl ammonium persulphate and 6 µl Tetramethylethylenediamine (TEMED), then 2 ml was layered above the separating gel and left at room temperature for an hour. In the next step, the protein samples were loaded onto the gel and run at 200 V for 45 minutes in 1X SDS running buffer (15g/L of Tris base, 72g/L Glycine, 3g/L SDS). The gel was processed for western blotting.

2.6.2 Western blot

Nitrocellulose membrane and 8 filter papers were soaked in transfer buffer (24mM Tris base, 192 mM glycine, 20% methanol) for 10 minutes, then a transfer sandwich was prepared from cathode to anode direction as follows: 4 wet filter papers, the gel, Nitrocellulose membrane, 4 wet filter papers. After that the protein was transferred to transfer buffer at 30 V for 70 minutes. Then the gel was washed twice in PBS/0.1% Tween, followed by blot blocking in PBS/0.1% Tween, 5% milk powder for 2 hours at 4°C. The blot was then washed 3 times for 15 minutes each in PBS/0.1% Tween. After that the blot was incubated with PBS/0.1% Tween, 5% milk powder and 10 µl primary antibody (1:1000) (Table 2.1) overnight at 4°C, then washed 3 times for 15 minutes each in PBS/0.1% Tween, and incubated with PBS/0.1% Tween, 5% milk and 5 µl secondary antibody (anti rabbit Alkaline Phosphatase (AP) conjugated antibody) for an hour at room temperature. In the final step the blot was washed 3 times for 15 minutes with PBS/0.1% Tween and treated with alkaline phosphatase chromogenic (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT)) (abcam/ab7413) until the appropriate band appears, then the blot was imaged using a gel documentation system (Bio rad / Gel Doc™ XRT imaging system).

2.7 Gel silver staining

Gel silver staining was done as described in (Morrissey,1981) with some modifications. Firstly the gel was incubated in a solution containing 5% ethanol and 5% acetic acid for 2 hours and washed three times in distilled water for 10 minutes each time. The gel was then incubated in silver stain solution A (1ml 2N NaOH, 20 ml water, 1.3 ml ammonia) solution B (3 ml Silver nitrate (AgNO₃) 17ml water) for 60 minutes. For the next step, the gel was washed three times in water for 5 minutes each time, then incubated in developer solution (1 ml of 1% citric acid in 100 ml water, 0.1 ml formaldehyde) for few minutes until the bands were clearly seen, then the reaction was stopped by stop solution (2% acetic acid), and the gel was imaged using a gel documentation system (Bio rad/Gel Doc™ XRT imaging system).

2.8 Fluorescence in Situ Hybridization (FISH)

2.8.1 Digestion of sperm DNA by Micrococcal Nuclease (MNase)

2.8.1.1 Bovine sperm

Sperm chromatin digestion procedure was done according to our lab protocol (Arpanahi *et al.*,2009) with some modification. The sperm sample was thawed and washed twice in PBS then centrifuged at 900 g for 10 minutes and then the sample was fractionated on a Percoll density gradient into two sub groups. The Percoll density gradient was prepared by layering 2 ml 45% percoll solution on to 2 ml 90% percoll solution. 500 µl washed sperm sample was layered on the top of the Percoll gradient and it was centrifuged at 600 g for 20 minutes. The pellet containing normal sperm was re-suspended and washed twice in PBS. Afterwards, two tubes of about 5×10^6 cells were prepared and one of them was used as a negative control. In both tubes the cells were resuspended in 1ml 50 mM Tris-HCl, pH 8.0, 2mM PMSF, 10 mM DTT and 1% cetyltrimethylammonium bromide (CTAB) (Sigma), and incubated on ice for 30 minutes to remove the sperm tails. Then the nuclei were pelleted at 650 g for 5 minutes, and washed in PBS free of calcium (Ca^{2+}), magnesium (Mg^+), 2mM PMSF. In the next step the samples were centrifuged again at 650 g for 5 minutes and incubated in PBS 0.5% Triton X-100 for 10 minutes on ice, then centrifuged and washed in PBS (2mM PMSF). The nuclei were then pelleted at 650g for 5 minutes.

After that, the pellet of each tube was incubated in 1 ml PBS, 2mM PMSF, and 10 mM DTT, for 30 minutes at 37°C. The nuclei were digested by adding 0.6 mM Calcium Chloride (CaCl_2) and 6 units of micrococcal nuclease (Fermentas) (micrococcal nuclease was not added to the negative control) for 5 minutes at 37°C. The activity of micrococcal nuclease was stopped by adding 5 mM of EDTA, then the samples were agitated for 30 minutes at 37°C to allow soluble (digested) DNA to leach out from the nuclei. Following the centrifugation at 2,500g for 5 minutes, each sample was divided into two parts: the first being soluble DNA (the supernatant) and the second being insoluble DNA (the pellet). The insoluble DNA was re-suspended in stop buffer, then both soluble and insoluble DNA were incubated in 20 mg/ml proteinase K, 0.5% (w/v) SDS overnight at 55°C, followed by adding 10 mg/ml of Ribonuclease (RNAase) and

incubating for one hour at 37°C. In the final step the DNA was extracted by phenol chloroform (1:1), and precipitated by ethanol. The DNA was then dissolved in Tris-EDTA (TE) buffer and stored at -20°C.

2.8.1.2 *Drosophila melanogaster* sperm

Similar to the steps described in above section, but in *Drosophila*, the sperm sample did not undergo Percoll gradient separation, and the sperm number is approximately 10^6 in each of negative control and micrococcal nuclease treated sample.

2.8.2 Fluorescence probe preparation

FISH Tag™ DNA kit from Invitrogen was used in this step, and the procedure was run according to the manufacturer's protocol as follows:

2.8.2.1 Nick translation reaction

Firstly, DNase I working solution was prepared by adding 89 µl nuclease free water, 10 µl 10X nick translation buffer, and 1 µl DNaseI, then the mixture was gently agitated. After that the nick preparation reaction was prepared by adding 5 µl 10X nick translation buffer, 5 µl 0.1M DTT, 5 µl 10X DNA nucleotide mix, 1 µg DNA sample, 1.7 µl DNA polymerase I, 4 µl DNase I working solution, and water to a final volume of 50 µl. The mixture was mixed gently and incubated on the thermocycler at 15°C for 2 hours, then 50 µl nuclease free water was added to the reaction and mixed thoroughly by vortex to inactivate DNase I.

2.8.2.2 Purifying the amine-modified DNA

400 µl binding buffer was added to the reaction in the previous step, then the entire volume (500 µl) was added to a spin column, and the column was centrifuged at 13,000 g for 1 minute, then washed with 650 µl washing buffer with ethanol and centrifuged twice at maximum speed to remove any residual wash buffer. The spin column was placed in a new 1.7 ml collection tube, 55 µl nuclease free water was added to the centre of the column and the column was incubated at room temperature for 2 minutes, then centrifuged at maximum speed for 1 minute after which the purified amine-modified DNA was collected from the collection tube.

2.8.2.3 Ethanol precipitation of the amine modified DNA

In this step, 39 µl nuclease free water, 10 µl 3 M of sodium acetate, and 1 µl glycogen were added to the purified amine-modified DNA, followed by the addition of 250 µl 100% ethanol, and the sample was stored at -20°C for 30

minutes before centrifuging at maximum speed for 10 minutes. The supernatant was removed and the pellet was washed twice with 400 μ l 70% ethanol. In next step the sample was allowed to dry at room temperature, then 5 μ l nuclease free water was added and incubated at 37°C for 5 minutes and vortexed to ensure full re-suspension of the DNA sample. The concentration was determined and adjusted by addition of water to around 0.2 μ g/ μ l.

2.8.2.4 Labelling the amine-modified DNA with fluorescent dye

In this step, the DNA sample (5 μ g) was denatured by incubating it for 5 minutes at 96°C, then placing on ice for 3 minutes and centrifuging at 10,000 g for 3 minutes. 3 μ l of sodium bicarbonate solution was then added to the sample. Reactive dye was re-suspended in 2 μ l dimethylsulfoxide (DMSO), vortexed well and transferred to the DNA sample, then the mixture was mixed well and centrifuged briefly and incubated for 1 hour in dark at room temperature. After that 90 μ l water was added to the sample, before proceeding immediately to the next step.

2.8.2.5 Purifying the fluorescent dye labelled DNA

400 μ l binding buffer was added to the reaction, then the entire volume (500 μ l) was added to spin column and the column was centrifuged at maximum speed for 1 minute, then washed with 650 μ l of washing buffer with ethanol and centrifuged twice at maximum speed to remove any residual wash buffer. The spin column was placed in a new 1.7 ml collection tube and 55 μ l nuclease free water was added to the centre of the column which was then incubated at room temperature for 2 minutes and then centrifuged at maximum speed for 1 minute, and the purified amine-modified DNA was collected from the collection tube.

2.8.2.6 Ethanol precipitation of the amine modified DNA

39 μ l nuclease free water, 10 μ l of 3 M of sodium acetate, and 1 μ l of collagen were added to purified amine-modified DNA, followed by addition of 250 μ l 100% ethanol. The sample was stored at -20°C for 30 minutes and centrifuged at maximum speed for 10 minutes. The supernatant was removed and the pellet was washed twice with 400 μ l 70% ethanol. In the next step the sample was allowed to dry at room temperature, then 5 μ l nuclease free water was added to the sample and incubated at 37°C for 5 minutes and vortexed to ensure full resuspension of the DNA sample. The concentration was determined and the DNA sample was stored at -20 °C until hybridization.

2.8.3 Preparation of slides for hybridization

100 µl Percoll-fractionated sperm sample was spread on an ethanol cleaned slide and left to dry at room temperature. The sperm sample was decondensed using 10 mM Tris HCl (PH 7.8) and 10mM DTT in a humid box for 40 minutes at room temperature. The slides were washed in 1x PBS for 5 minutes and left to dry at room temperature. In the next step the sperm cells were denatured using 70% formamide/2x sodium chloride citrate (SSC) at 72 °C for 5 minutes. Then the slides were dehydrated in 70%, 90% and 100% ethanol for 2 minutes each and allowed to air dry at room temperature.

2.8.4 Hybridization procedure

The DNA probe was resuspended in TE buffer (final concentration 4ng/µl), then DNA probe–hybridization buffer (DPHB) was prepared by adding 1 µl 20 x SSC, and 6.5 µl formamide to 2.5 µl DNA probe. The DPHB was denatured by incubation for 5 minutes at 72°C and cooled on ice. In next step, 10 µl of DPHB were dropped on the slide (sample), and covered by a small piece of parafilm and the slides were incubated overnight at 37°C in a humid box. The slides were incubated in 0.4 x SCC/0.3% Tergitol type NP-40 (NP-40) at 37 °C for 2 minutes, followed by incubation 2 x SCC/0.1% NP-40 for 1 minute at room temperature, washed quickly in water, then dried in the dark. In the final step the samples were stained by DAPI, then one drop of antifade reagent was applied before covering the sample with a coverslip and proceeding to imaging using an epi-Fluorescent Zeiss Axiovert microscope (Carl Zeiss Ltd, Hertfordshire, UK).

2.9 Chromatin immunoprecipitation - Microarray (ChIP on chip)

This protocol was designed for samples with low number of sperm (10^5 - 10^6 cells) as described in (Dahl and Collas,2008) with some modification (figure 2.1).

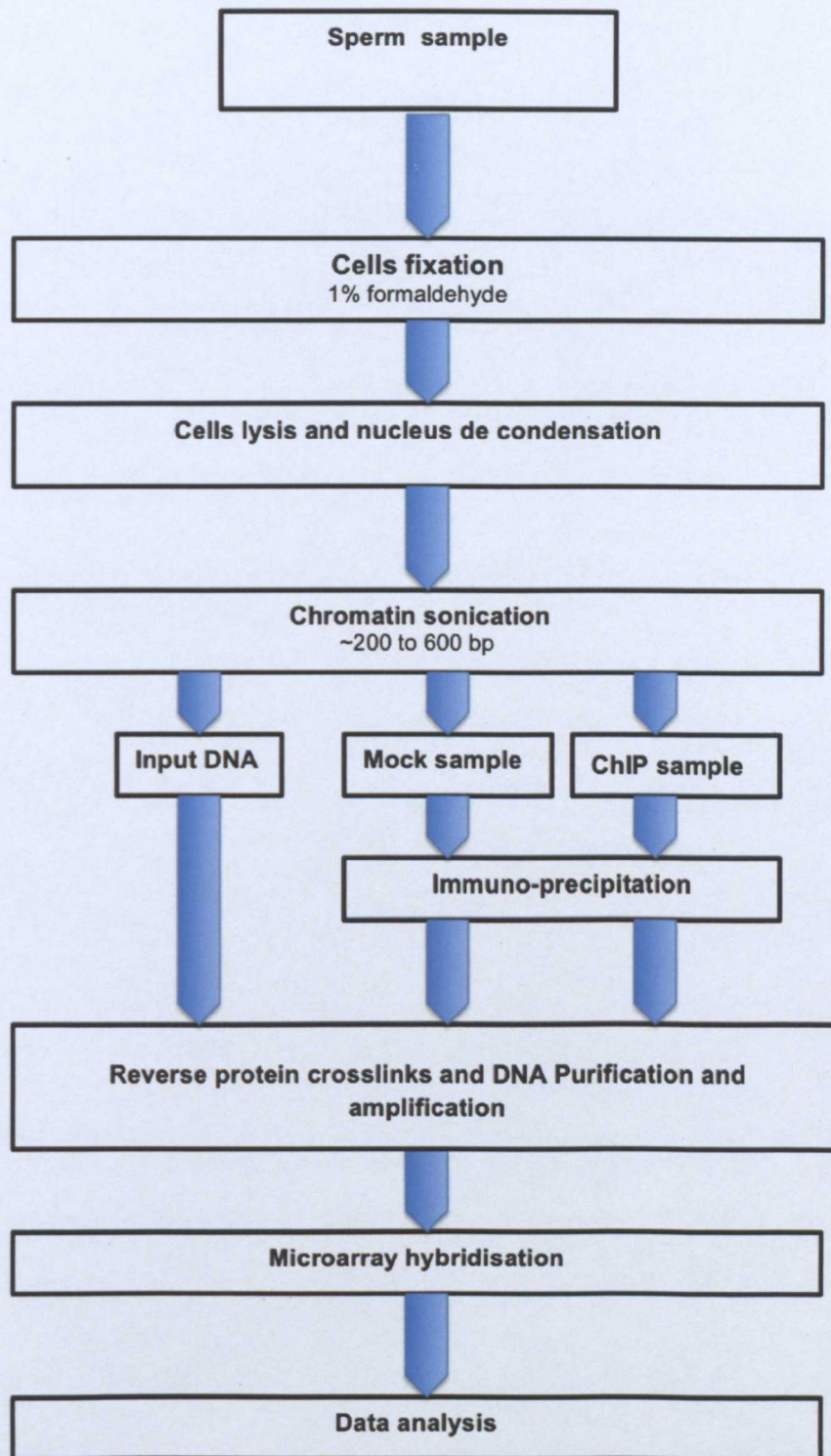


Figure 2-1 ChIP on chip procedure scheme.

2.9.1 *Drosophila melanogaster* Sperm isolation for ChIP

Sperm isolation is a critical step because of the small size of the seminal vesicles. To avoid any contamination with somatic cells, large size males (mid age) with full seminal vesicles were selected as the source of sperm. Generally, males were dissected on a clean slide with a drop of PBS under the

dissecting microscope by using fine forceps. All sperm collection steps were done in PBS.

Firstly, the male reproductive system was isolated from the body, transferred and washed in a new PBS drop, and the testes were separated from the accessory glands. Before sperm isolation the testes were transferred onto a new slide and the seminal vesicles were separated. One end of the seminal vesicle was anchored with forceps and the sperm were pushed out by pressing down with a second pair of forceps (figure 2.2). The sperm were then pulled out and collected as a filamentous ball. Finally, these sperm balls were collected in freezing medium and stored at -70°C after snap freezing on dry ice. To assess the quality of the sperm isolation procedure and to test the purity of the sperm samples from any contamination by somatic cells, random samples of sperm were stained by DAPI and examined using the fluorescence microscope (figure 2.2).

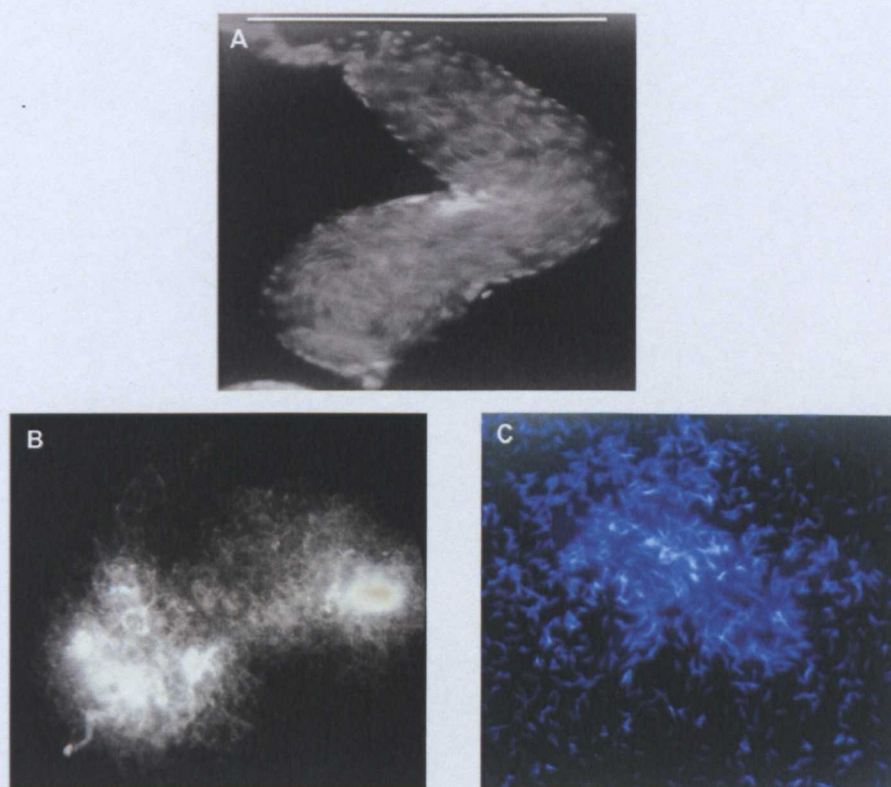


Figure 2-2 *Drosophila melanogaster* seminal vesicle and isolated sperm. A: full seminal vesicle, B: sperm out of seminal vesicle, C: isolated sperm stained with dapi.

2.9.2 Cell Fixation

For each ChIP replicate sperm (isolated from about 100 males) sample was thawed at room temperature, then centrifuged for 5 minutes at maximum speed at 4°C , and washed in $500\ \mu\text{l}$ cold PBS. The cells were suspended in $100\ \mu\text{l}$

PBS in a 1.7 ml Axygen "Maximum Recovery" low retention tube. The sperm were fixed by adding 6.25 μ l of 16% formaldehyde to give 1% formaldehyde for 10 minutes at room temperature. The reaction was stopped by adding 4 volumes cold PBS + 0.125 M glycine. In the next step, the cell were centrifuged for 5 minutes at maximum speed at 4°C, then washed twice in 500 μ l cold PBS and centrifuged at maximum speed for 5 minutes at 4°C.

2.9.3 Cell Lysis

Fixed cells were resuspended and incubated for 1 hour at room temperature in 500 μ l cold chromatin washing buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 10 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), and 0.25% Triton x-100), 10mM DTT, 0.5% (w/v) SDS, and fresh protease inhibitors (1/1000 dilution 10 mg/ml aprotinin, 1/1000 dilution of 5 mg/ml leupeptin, and 1/100 dilution of 50 mM PMSF). The DTT was then quenched by adding 5 mM N-ethylmaleimide and the cells were spun down for 10 minutes at maximum speed at 4°C. In the next step, the cells were suspended twice in 100 μ l cold chromatin washing buffer B (10 mM HEPES pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200mM Sodium Chloride (NaCl) and 0.01 % Triton x-100) and fresh protease inhibitors, then the cells were spun down for 10 minutes at maximum speed at 4°C.

2.9.4 Cell Sonication

In this step the cells were suspended in 100 μ l of sonication buffer (1% Triton X-100, 25 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, and 0.25% SDS) plus protease inhibitors. Then the sample was sonicated by a MEM Soniprep 150 sonicator at 6 micron amplitude in ice for 5 cycles of 20 seconds sonication followed by 40 seconds cooling. 5 cycles were sufficient to generate chromatin of ~300 to 800 bp (figure 2.3). The sonicated chromatin was diluted with two volumes of chromatin dilution buffer (1% Triton X-100, 25 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, and 7.5% glycerol) plus protease inhibitors. Then the samples were stored at -70 °C after snap freezing on dry ice.

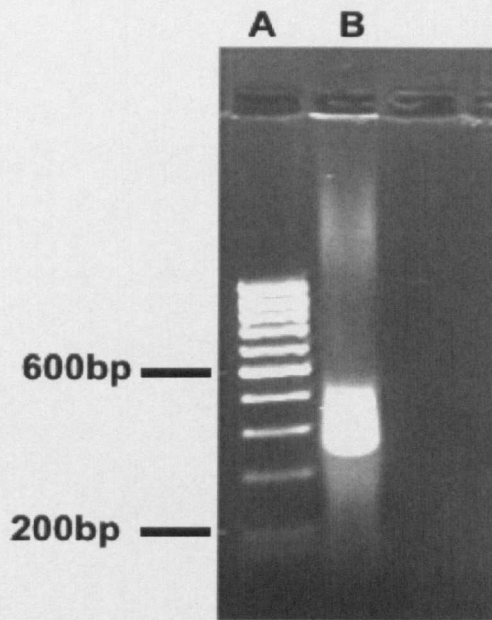


Figure 2-3 Sonicated chromatin.

2.9.5 Immunoprecipitation (IP)

2.9.5.1 Antibody and bead preparation

10 μ l Dynabeads-Protein G were used per IP reaction, the Dynabeads were transferred to a 1.5 ml tube, the buffer was removed by using magnet, then the beads were washed twice with 500 μ l of cold 100 mM sodium phosphate pH 8.0 and resuspended in 10 μ l 100 mM sodium phosphate pH 8.0 and 0.5%BSA. This was followed by addition of 5 μ g antibody (Table 2.2) per reaction and incubation for 2 hours while rotating at 4°C. The free antibodies were then removed using a magnet, and the beads were washed twice in 500 μ l cold 100 mM Sodium Phosphate pH 8.0 and resuspended in 10 μ l 100 mM sodium phosphate pH 8.0 and stored on ice until ready.

Histone H3 antibody, ChIP grade, 2650S	Cellsignal
Histone H4antibody, ChIP grade, Ab12079	Abcam
Histone H3K27me3 antibody, ChIP grade, Ab6002	Abcam
Histone H3K36me3 antibody, Chip grade, Ab9050	Abcam
Histone H3K9me3 antibody, Chip grade,Ab8898	Abcam
Anti rabbit IgG (secondary antibody), 31210	Thermo scientific

Table 2-2 list of the antibodies used in ChIP.

2.9.5.2 Chromatin preparation and precipitation

500 μ l aliquots of chromatin were thawed, mixed well and centrifuged for 5 minutes at maximum at 4°C, then transferred to new tube (leaving behind approximately 10 μ l to avoid contamination by the solid particles in the pellet). 10 μ l antibody-coated beads was then added and 25 μ g herring sperm DNA was added to each sample, before incubating for 2 to 4 hours while rotating at 4°C. The unbound chromatin was removed using the magnet, and the beads were suspended in 500 μ l chromatin washing buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl) plus fresh inhibitors, and incubated on ice for 10 minutes. The buffer was then removed and the beads were washed twice in chromatin washing buffer 2 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0, and 500 mM NaCl) plus fresh inhibitors for 10 minutes while rotating in the cold room.

In the third washing step, the beads were suspended in chromatin washing buffer 3 (0.25 M lithium chloride (LiCl), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0 and 1% NP40) plus fresh inhibitors for 10 minutes while rotating in the cold room. Finally, the pellet was washed twice with TE/NaCl pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA and 50 mM NaCl) while rotating for 10 minutes in the cold room during each wash. In the second wash the beads were transferred to a new 1.7 ml Axygen low retention tube.

2.9.5.3 Elution of the antibody-chromatin complex from the beads

Antibody-chromatin complex was eluted by re-suspending the beads in 50 μ l 1% SDS, 0.1 M NaHCO₃ pH 10.1, and incubating for 15 min while rotating at room temperature. The eluate was then transferred from the beads to a new 1.7 ml Axygen low retention tube, and the extraction was repeated in 50 μ l as above. The final eluate volume of both extractions was 100 μ l.

2.9.6 Reverse protein crosslinks and DNA Purification

For each 100 μ l of the eluate, 4 μ l 5 M NaCl and 5 μ l 0.2 M EDTA were added and incubated overnight at 55 °C. 4 μ l 2 M Tris pH 6.5 was then added to reduce the pH to 8.3, then 1 μ l 10 mg/ml RNase A was added and incubated for 30 minutes at 37 °C, followed by the addition of 1 μ l 10 mg/ml proteinase K and incubation for 4 hours at 55 °C.

2.9.7 Preparing input DNA sample

To prepare input DNA, a 200 μ l sample of sonicated chromatin was collected before freezing, and 10 μ l 3 M sodium acetate (CH_3COONa) pH 7.0 and 10 μ l 10 % SDS were added to each 100 μ l of input DNA, followed by incubation at 55 $^\circ\text{C}$ overnight. Following this, 1 μ l 10 mg/ml RNase A was added and the sample was incubated at 37 $^\circ\text{C}$ for 1 hour. Finally, 10 μ l 10 mg/ml Proteinase K was added per 100 μ l of chromatin and incubated at 55 $^\circ\text{C}$ for 4 hours.

2.9.8 DNA purification using Sigma Genelute™ PCR Clean-Up kit

DNA was recovered by Genelute™ PCR Clean-Up kit according to company instructions as follows. Firstly, the binding column was inserted into a collection tube, then 0.5 ml column preparation solution was added, followed by centrifugation at maximum speed for 1 minute, then the eluate was discarded. In the second step, 5 volumes of binding solution were added to the DNA sample before the solution was transferred onto the binding column, followed by centrifugation at maximum speed for 1 minute, then the eluate was discarded. In the next step, 0.5 ml of diluted wash solution was added to the column, then centrifuged at maximum speed for 1 minute, before the eluate was discarded and the column centrifuged again at maximum speed for 2 minutes. In the last step, the column was transferred into new collection tube, and 50 μ l of elution solution was added to the column before incubating at room temperature for 2 minutes. Finally, the column was centrifuged at maximum speed for 2 minutes and the DNA collected from the collection tube and stored at -70 $^\circ\text{C}$.

2.9.9 Measurement of DNA concentration using NanoDrop 3300 Fluorospectro-meter and PicoGreen® Assay

This step was done using Nanodrop fluorospectrometer (Nanodrop® ND-3300) and Quant-iT™ PicoGreen® dsDNA protocol (Invitrogen). Firstly, Picogreen dye stock was diluted 200-fold by 1 x TE buffer, then a series of diluted DNA samples were prepared at final concentration 2x as follows: 100ng/ml, 50 ng/ml, 5 ng/ml, 500 pg/ml, 50 pg/ml, 5 pg/ml, and blank. Finally, one volume of Picogreen working solution was mixed with one volume of each serial DNA sample concentration, the blank and the unknown samples, and all were incubated at room temperature for 5 minutes. In the next step, the standard curve was prepared in the instrument using the blank and the serial DNA

sample concentrations. In the last step the concentrations of the unknown samples were measured based on this standard curve.

2.9.10 DNA amplification

Precipitated DNA was amplified using two steps. In the first step, a whole genome amplification (WGA) kit (GenomePlex[®] Single Cell, WGA4), and in the second step the DNA was re-amplified by GenomePlex[®] WGA Ramplification Kit (WGA3) from sigma to amplify the small amount of the DNA. In all amplification steps a negative and a positive control were run with the sample in all amplification steps.

2.9.10.1 DNA amplification by GenomePlex[®] Single Cell (WGA4)

The amplification was started with library preparation by adding 2 μ l single cell library preparation buffer (1x) to the sample. Then 1 μ l library stabilisation solution was added, followed by mixing and placing in the thermocycler at 95 °C, then cooling on ice. 1 μ l library preparation enzyme was then added to the sample, followed by mixing and brief centrifugation. In the next step, the sample was placed in the thermocycler and incubated according to the following temperature program: first, 16 °C for 20 minutes, then 24 °C for 20 minutes, then 37 °C for 20 minutes, then 75 °C for 20 minutes. The sample was then held at 4 °C. Finally the sample was removed from the thermocycler and centrifuged briefly. Subsequently, the sample went through amplification cycles. Firstly, the following reagents were added to the sample (14 μ l): 48.5 μ l nuclease free water, 7.5 μ l 10x amplification master mix, and 5.0 μ l WGA DNA polymerase, then the sample was mixed and centrifuged briefly. After that, the sample was placed in the thermocycler, and the system was optimized as follows: initial denaturation at 95 °C for 3 minutes, then 25 cycles as follow: denaturation at 94 °C for 30 seconds, annealing at 65 °C for 5 minutes and in the last step the sample was held at 4 °C. Then the DNA was recovered by Genelute[™] PCR Clean-Up kit.

2.9.10.2 DNA re-amplification by GenomePlex[®] WAG Ramplification Kit (WAG3)

To 10 μ l (1 ng/ μ l) WAG4 amplified DNA, a mix of chemicals were added as follows: 49.5 μ l nuclease free water, 3.0 μ l 10 mM deoxyribonucleotide triphosphate (dNTP) mix, 7.5 μ l 10x amplification master mix, and 5.0 μ l WAG DNA polymerase. The sample was then placed in the thermocycler, and the

system was optimized as follows: initial denaturation at 95 °C for 3 minutes, followed by 14 cycles of denaturation at 94 °C for 15 seconds and 5 minutes annealing at 65 °C, then the sample was held at 4 °C. After this was completed, the re-amplified DNA was purified by Genelute™ PCR clean-Up kit and the sample was stored at -20 °C.

2.9.11 Microarray hybridisation

This analysis was done by Flychip group (Flychip,2012)www. flychip.org.uk) according to Nimblegen process.

2.9.11.1 Preparation of 10X low-C dNTP mix

Labelling reaction of 10X low cytosine (C) dNTPs mix (5 mM Adenine (A), guanine (G), thymine (T) and 3 mM C- dNTP) was prepared by mixing of 25 µl 100 mM dNTA, 25 µl 100 mM dNTT, 25 µl 100 mM dNTG, and 15 µl 100 mM dNTC.

2.9.11.2 Klenow labelling

In this step two reactions were prepared per sample and control in 200 µl tubes in PCR block. In the first, 1 µg DNA was diluted in 31.2 µl water (Sigma), then 30 µl 2.5x Random primer reaction buffer (Bioprime labelling system kit) were added to the DNA sample. This was then incubated for 5 minutes at 100 °C and cooled on ice. In the second step a master mix was prepared by mixing 7.5 µl 10X low-C dNTPs mix, 4.5 µl Cyanine 3 (Cy3) or Cyanine 5 (Cy5) dCTP and 1.8 µl 40 U/µl Klenow (Bioprime system kit). Following this, 13.8 of the master mix was added to each DNA sample, mixed by pipette then incubated at 37 °C for 2-3 hours. The reaction was stopped by adding 7.5 stop buffer (Bioprime labelling kit). In the last step the pairs of Cy3 and Cy5 were combined together.

2.9.11.3 Fluorescent labelled probe purification

This step was used to purify the sample from unincorporated nucleotides and dye. The same reactions were mixed together in a 1.5 ml tube, then 17.25 µl 5 M NaCl, and 165 µl isopropanol were added to the tube before mixing by vortex and incubating for 10 minutes at room temperature in the dark. After that, the sample was centrifuged at maximum speed for ten minutes, then the supernatant was removed and the DNA pellet was washed in 500 µl ice cold 80% ethanol, and centrifuged again for 2 minutes at maximum speed. In the final step the supernatant was discarded, and the pellet was dried at room temperature in the dark for 5 minutes.

2.9.11.4 Sample quantification

DNA pellet was diluted in 50 µl water, centrifuged briefly and incubated for five minutes in dark, then mixed and centrifuged again. After that, DNA concentration was measured by Nanodrop, then 34 µg of test sample and 34 µg reference sample were combined together in one tube, and dried by speed vacuum, then stored at -20°C until use.

2.9.11.5 Hybridisation to 2.1M arrays

The hybridisation was done by using Nimblegen hybridisation kit and the alignment oligo provided in the hybridisation kit was aliquoted to 1.2 µl and stored at -20°C. Firstly, the hybridisation system was set at 42°C. for three hours to stabilise the temperature, after which the sample pair was suspended in 12.3 µl water. Then the hybridisation master mix was prepared by mixing 1.2 µl alignment oligo, 11.8 µl hybridisation component A and 29.5 µl 2x hybridisation buffer. In the next step, 31.7 µl hybridisation master mix was added to each sample pair, the samples were vortexed and centrifuged briefly, then incubated for 5 minutes at 95 °C in the hot block, followed by 42 °C for five minutes. After that the sample was vortexed and spun before loading.

2.9.11.6 Mixers preparation, sample loading and hybridisation

Array format that used in this study is 2.1 *Drosophila melanogaster* (Roche 05542308001). In this step, the HM1 mixer was fixed on the slide by using precision mixer alignment tools (supplied with hybridisation kit), then the mixer-slide was placed in the slide position of the hybridisation system. After that 41 µl sample was loaded on the slide, and hybridised at 42 °C for 20 hours. After that, the slide was washed three times in warm wash buffers (225 ml water, 25 ml wash buffer I,II or III, and 25 µl 1M DTT), for 2 mins in wash I, 1 minute for wash II, and 15 seconds in wash III. The container was shaken 20 times every 10 seconds. Then the slide was transferred into a slide box containing tissue paper in the base and centrifuged for 5 minutes at 1000 rpm, before proceeding directly to the next step.

2.9.11.7 Two colour array scanning

The slide was scanned by GenePix Axon scanner (GenePix software) with the following setting (532 photo multiplier tube (PMT) Gain =650, 635 PMT Gain =750, power (%) =100%, pixel size (µm) = 5, lines to average =1, focus position (µm) = 0). After the scanning the image proceeded to NimbleScan data analysis.

2.9.11.8 Data analysis

Raw data for all ChIP runs (test and mock) were generated from scanned images using the NimbleScan software and normalised by NimbleScan software, to remove the variance between the replicates and nonspecific binding signals. Test replicates were then averaged and subtracted from the mock signal. The generated normalised probe data was used directly in the genome wide analysis. Following that, the enriched intervals were extracted from normalised \log_2 ratio data by Galaxy tools (Blankenberg *et al.*,2010) according to the algorithm, $\log_2 \geq 2x$ the replicate median interval value using 3 probes as the minimum number in each interval (this minimum number covers 150 bp of DNA or a single nucleosome. The maximum distance between the probes was 45bp, and provided significantly enriched intervals with $P < .0001$, (χ^2).

The Biomart web site (Haider *et al.*,2009) and UCSC Genome Browser website (Fujita *et al.*,2011) were used as sources of *Drosophila melanogaster* genomic data (*D. melanogaster* Apr. Berkeley Drosophila Genome project, release 5 (BDGP R5/dm3)). The enriched intervals were annotated around Flybase genes (Transcription start sites, transcription termination sites, exons, introns, and promoters (Negre *et al.*,2011)part of modENCODE project). Gene ontology enrichment analysis was carried out using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7(Huang da *et al.*,2009), and the generated enriched GO terms (with $p < 0.0009$) were visualized by REVIGO web site (Supek *et al.*,2011).

2.10 quantitative Polymerase Chain Reaction (qPCR)

This step was done using The Applied Biosystems 7900HT Fast Real Time PCR System. In the first place the primers were designed from five selected enriched intervals of each ChIP (H3&H4, Histone tri methyl lysine 36 (H3K36me3), Histone 3 tri methyl acetyl 72 (H3K27me3) and Histone 3 tri methyl lysine 9 (H3K9me3)) and three samples of DNA were used with each primer (IP DNA, Input DNA, and Mock DNA). The qPCR reaction was prepared in 3 replicates with total volume 25 μ l as follows: 12.5 μ l Sybr®Green PCR master mix (Applied Biosystem), 1.25 μ l primer, 10.25 μ l water and 1 μ l DNA (sample). Then the values of delta Ct were extracted and used in the enrichment calculation (see chapter 5 section 5.2.6).

Chapter 3: Investigation of Histone and Histone Modifications in the Mature Sperm of *Drosophila melanogaster*, *Bos taurus* and *Mus musculus*.

3.1 Introduction

Genetic material undergoes dramatic changes in later stages of spermatogenesis as a part of sperm maturation in metazoan species. While the condensation of DNA is an important step of sperm maturity (Braun,2001; Hennig,2003) in most species, in others the DNA is organised in somatic cell-like pattern such as in crab species (Felgenhauer and Abele,1991; Medina and Rodriguez,1992). Among the first group, the DNA is compacted by two actions occurring at the same time, these events are histone replacement and DNA reorganisation.

DNA condensation has been studied most extensively in mammalian spermatogenesis. In early spermatogenesis the chromatin is wrapped around histones (Meistrich,1989), but after meiosis, histone proteins are replaced by testis-specific histone variants, which are then replaced by transition proteins (TPs). In late spermatids, TPs are replaced by protamines. Like histones, mammalian protamines are highly basic proteins, and comprise the main components of sperm nuclear protein (Balhorn *et al.*,2000; Kistler *et al.*,1996; Meistrich *et al.*,2003; Zhao *et al.*,2004). As a result of histone-protamine replacement, chromatin nucleosomal conformation is shifted into basic units with a toroidal (doughnut) shape (McCarthy and Ward,1999) that can package up to 50kb of DNA (Balhorn *et al.*, 2000).

3.1.1 Histones in *Drosophila melanogaster* sperm

Several studies have indicated that many metazoan species still retain some histone proteins in their mature sperm. For example, the histone H3 variant CENP-A in bovine sperm (Palmer *et al.*,1990), and H1 like protein in mollusc (Jutglar *et al.*,1991). In insects, the largest class in the animal kingdom, the chromatin condensation and histone-protamine replacement during spermatogenesis has been reported in several studies, but the information is still insufficient. It seems that histone-protamine replacement steps during insect spermiogenesis are similar to those observed in mammals, but there are differences in histone structure and other basic nuclear proteins. For example in

the house cricket (*Acheta domesticus*), the somatic histones are replaced by histone like protein (TH1, TH2), then by stable protamine like protein (keratinous protamine) (McMaster-Kaye and Kaye,1976). However, the pattern of chromatin fibre formation differs from that in mammalian spermiogenesis. While the chromatin is arranged in doughnut shape in mammals, in the house cricket the chromatin is arranged into parallel fibres during late spermoigenesis, then these fibres bind together to form one condensed fibre, which is more condensed in the posterior part than the anterior part (Kierszenbaum and Tres,1978). Little is known about histones occupancy in the mature sperm of insects. In recent study, a clear signal from histone 3 modification (H3K9me3) has been detected by immunocytochemistry in the mature sperm of mealy bug (*Planococcus citri*) (Bongiorni *et al.*,2009). In addition, previous studies showed that mature sperm of honey bees (*Apis mellifera*) contains H1 variant (Falco and Mello,1999; Mattos *et al.*,2006).

In *Drosophila melanogaster*, previous studies indicated that the sperm contains protamine A, protamine B and Male-specific transcript 77F protein (Mst77f) as main condensing proteins in the chromatin (Jayaramaiah-Raja and Renkawitz-Pohl,2005; Rathke *et al.*,2007; Russell and Kaiser,1993). It has been reported that histone proteins are replaced first by transition protein Tpl94D, then replaced by protamines A and B during spermiogenesis (Rathke *et al.*,2007). By using transgenic flies (H2A tagged with Green Fluorescent Protein (GFP), it has been noted that H2A protein is degraded during the later stages of the spermatid and has disappeared completely in mature sperm (Jayaramaiah-Raja and Renkawitz-Pohl,2005). However, in other studies, histone protein and its variants have been reported in mature spermatozoa of *Drosophila melanogaster*. Signals for histones H3.3B, H2Av, H3.3A,B and H4r have been detected in previous proteomic analysis study on *Drosophila* sperm by using mass spectroscopy (Dorus *et al.*,2006) (supplementary data table 1)). Moreover, the sperm of transgenic flies containing an H2A-tagged with Red Fluorescent Protein can be observed to fluoresce indicating the persistence of H2A in sperm chromatin (reviewed in (Miller *et al.*,2009).

3.1.2 Histone and its modification in the nucleus of mouse and bovine sperm

In human sperm, about 85 -95 % of the chromatin is compacted by protamines, while around 5-15 % is still bound by histones (Gatewood *et al.*,1987; Hammoud *et al.*,2009). Using in situ hybridisation and immunolocalisation Li *et al.*,2008 indicated that histone proteins are localised in the posterior part of the human sperm nucleus, and are therefore not randomly distributed. In comparison with human, mouse sperm retains less than 2% of its chromatin in histone compacted form (Gatewood *et al.*,1990), and the pattern of histone localisation is similar to that observed in human sperm (posterior part of the nucleus) (Pittoggi *et al.*,1999). Unlike human sperm, it was thought that nuclear H3 and H4 in mouse sperm lack acetyl modification, which disappears during the condensing spermatid stage (Govin *et al.*,2007). In bovine sperm, however, little is known to date about the retention of histone protein in the nucleus. Initial investigation demonstrated that mature bull sperm contains non-nuclear core histones H2A, H2B, H3 and H4 (Tovich and Oko,2003). These histones are produced in the latter stage of spermatid development, and are located in the perinuclear theca (a cytoskeletal structure that covers the nucleus of mammalian spermatozoa) (Tovich *et al.*,2004). While there is no evidence for the presence of the histones H2A, H2B, and H4 in the bovine sperm nucleus, clear signals from histones CENP-A (H3 variant) (Palmer *et al.*,1990), and H1 (Sanchez-Vazquez *et al.*,2008) were detected in bull sperm. It has been proposed that mouse and bovine sperm might contain acetyl H3 and acetyl H4; however, as an appropriate chromatin de-condensation step is a critical requirement for the detection of histone protein in compacted sperm nuclei but is usually omitted prior to immunocytochemistry, it is possible that these histones remain undetected.

3.1.3 The aims of experiments

The aims of the experiments in this chapter are firstly, to confirm the preliminary observations of histone retention in *Drosophila melanogaster* sperm (mentioned above). The result of this experiment will lead to the use of *Drosophila* as an experimental model in more advanced research. Secondly, to re-investigate many controversial points about the presence and the localisation of histone protein and its modifications in the nucleus of mouse and bovine sperm, by

immunocytochemistry using dithiothreitol (DTT) and heparin as de-condensation chemicals.

3.2 Materials and methods

3.2.1 Sample collection and slide preparation

Described in detail in chapter 2 in section in section 2.2.

3.2.2 Investigation of H2A.v-RFP and protamine-GFP in *Drosophila melanogaster* spermatogenesis

Described in detail in chapter 2 in section 2.3.

3.2.3 Immunocytochemistry

Described in detail in chapter 2 in section 2.4.

3.2.4 Histone extraction

Described in detail in chapter 2 in section 2.5.

3.2.5 SDS-PAGE gel

Described in detail in chapter 2 in section 2.6.1.

3.2.6 Western blot

Described in detail in chapter 2 in section 2.6.2.

3.3 Result

3.3.1 *Drosophila melanogaster* sperm

3.3.1.1 H2A.v-RFP signal

The H2A.v-RFP transgenic fly was used to analyse the dynamics of histone protein during spermatogenesis in *Drosophila*. This histone variant has already been described in the mature sperm of *Drosophila melanogaster* by both proteomic analysis and direct visual observation of fluorescing sperm nuclei (Dorus *et al.*, 2006; Miller *et al.*, 2009). In addition to the availability of this transgenic fly. As result of the observations of this study, spermiogenesis was classified into eight stages based on three criteria, namely, spermatid morphology, H2A.v-RFP signal pattern and nuclear condensation (chromatin compacting as result of protamine accumulation (figure 3.1, 3.2 ; Table 3.1).

The Florescence signal of H2A.v was clearly detected up to the mid stage of spermiogenesis (middle spermatid) (figure 3.1 A-D, 3.2 I). In the next stage (late spermatid I) H2A.v-RFP was localised as spots at the edges of the

spermatid nuclear sheet but a very weak signal was noted in other parts of the nucleus (figure 3.2 F). During the later stages, H2A.v spots became smaller, and unclear in mature sperm and only a very faint signal was detected in the mature sperm (figure 3.2 I).

No	Spermatid stage	Spermatid morphology	H2A.v-REF signal	Chromatin Condensation (chromatin compacting)
1	Round spermatid	Round	Throughout the nucleus	No condensation signal
2	Early Elongating Spermatid I	Squamous shape	Throughout the nucleus	No condensation signal
3	Early Elongating Spermatid II	Elongated, regular sides	Throughout the nucleus	No condensation signal
4	Early Elongating Spermatid III	elongated, one expanded side	Throughout the nucleus	No condensation signal
5	Middle spermatid	Full elongation, canoe shape	Throughout the nucleus	No condensation signal
6	Late Elongating Spermatid I	Contraction in the anterior part	Spots in the edges of the nucleus	Condensation signal in the anterior part
7	Late Elongating Spermatid II	Spear shape	Faint spots in the anterior part and clear spots in the posterior part	Full condensation in the anterior part
8	Mature sperm	Needle shape	Faint spots	Full condensation

Table 3-1 Spermiogenesis stages in *Drosophila melanogaster* Classification based on cell morphology, H2A.v-RFP signal and nucleus condensation.

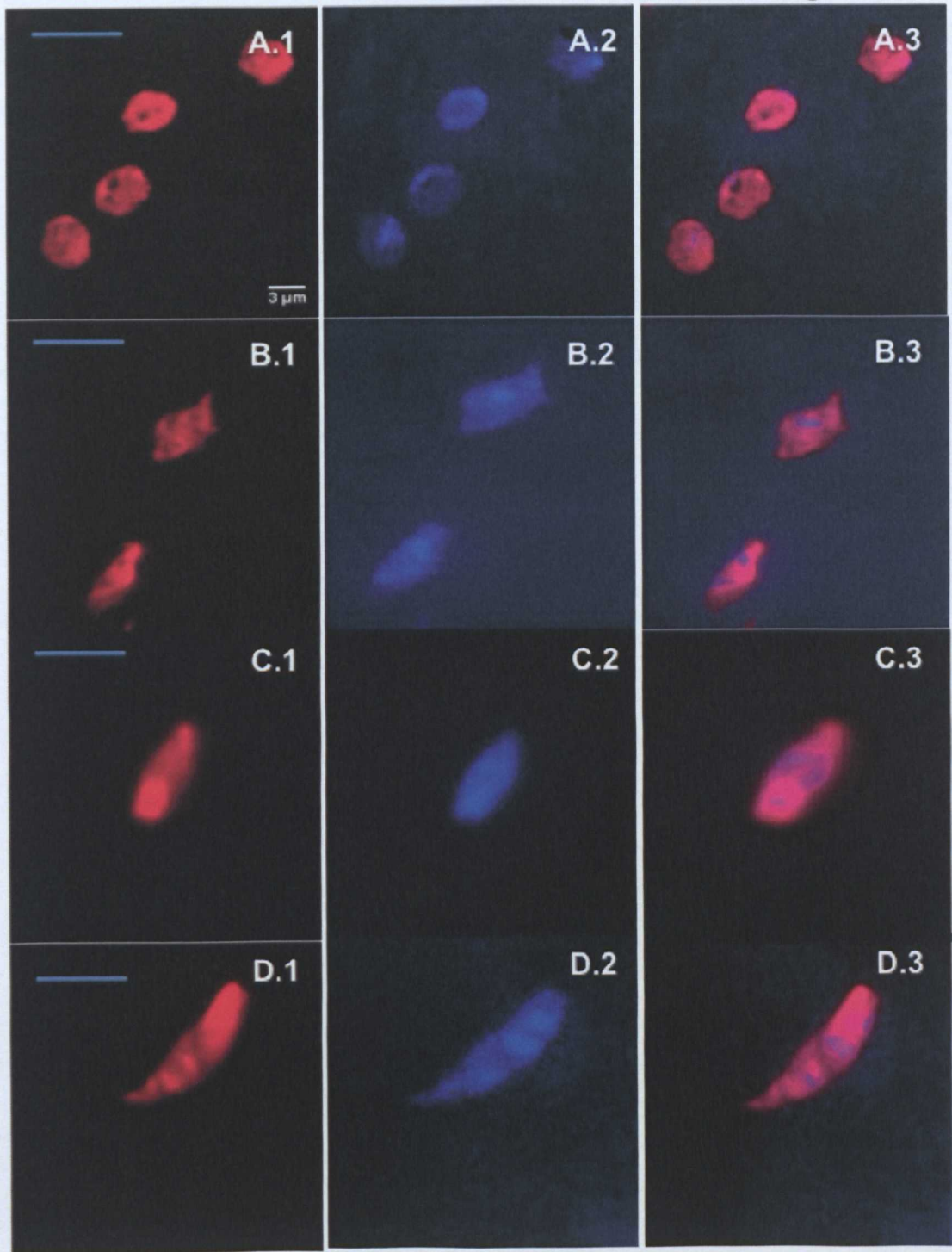


Figure 3-1 Spermiation stages of *Drosophila melanogaster* (part 1). A: Round spermatid, A.1; H2A.v-REF signal, A.2:dapi, A.3:merged. B: Early elongating spermatid I, B.1: H2A.v-REF signal, B.2: dapi, B.3: merged. C: Early elongating spermatid II C:1 H2A.v-REF signal, C.2: dapi, C.3: merged. D: Early elongating spermatid III, D.1: H2A.v-REF signal, D.2: dapi, D.3: merged, (scale bar 5 μ m).

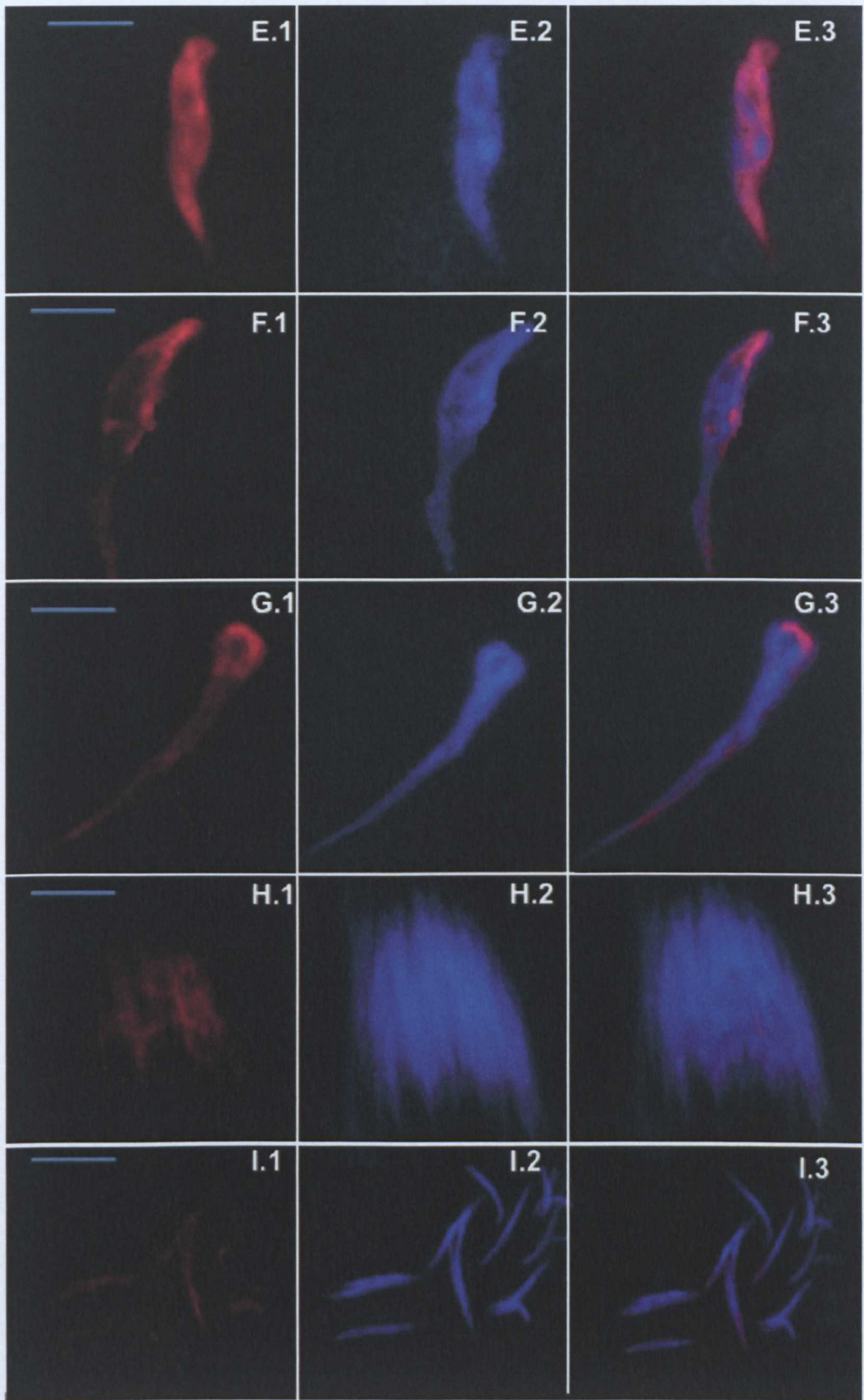


Figure 3-2 Spermiogenesis stages of *Drosophila melanogaster* (part 2). E : Middle spermatid, E.1: H2A.v-REF signal, E.2: dapi, E.3: merged. F: Late elongating spermatid I, F.1: H2A.v-REF signal, F.2: dapi, F.3:merged. G: Late elongating spermatid II, G.1: H2A.v-REF signal, G.2: dapi, G.3: merged. H: Bundle of mature sperm, H.1: H2A.v-REF signal, H.2: dapi, H.3: merged. I: Individual mature sperm, I.1: H2A.v-REF signal, I.2: dapi, I.3: merged (scale bar 5 μ m).

3.3.1.2 Protamine-GFP signal

The accumulation of protamine in the chromatin of *Drosophila melanogaster* during spermiogenesis was studied by using protamine-GFP flies. The protamine accumulation starts in middle spermatid stage, then the signal increases gradually to maximum level in last spermatid stage (figure 3.3). Meanwhile, the chromatin is compacted as a result of protamine binding. The interesting point in this process is that the protamine signal in the mature sperm does not have a uniform distribution pattern in the nucleus (figure 3.3,E). This means that many regions of the sperm genome are not fully packaged by protamine.

3.3.1.3 Histone Immunocytochemistry

An Immunocytochemical assay was used to detect the presence of histone proteins in the mature sperm of *Drosophila melanogaster*. Positive antigen signal was detected by using anti core histone antibody throughout the nucleus (figure 3.4). Although this signal was very weak, it was detectable using a 100X oil immersion objective and completely dark stage. A similar result was obtained by using anti individual histones antibodies (H2A, H2B, H3 and H4) (figure 3.6). However, no reaction was noted from anti protamine 1, anti-acetyl H3 and anti-acetyl H4 antibodies.

3.3.1.4 RFP immunocytochemistry

Immunocytochemistry for RFP bound to H2A.v was used to confirm the above histone immunocytochemical results. In mature sperm, unlike negative control (flies do not have RFP), the antibody against RFP gave a clear, detectable signal (Fig 3.5). It was noted here that the signal came from sperm inside the seminal vesicle, while sperm outside the vesicle did not give any signal.

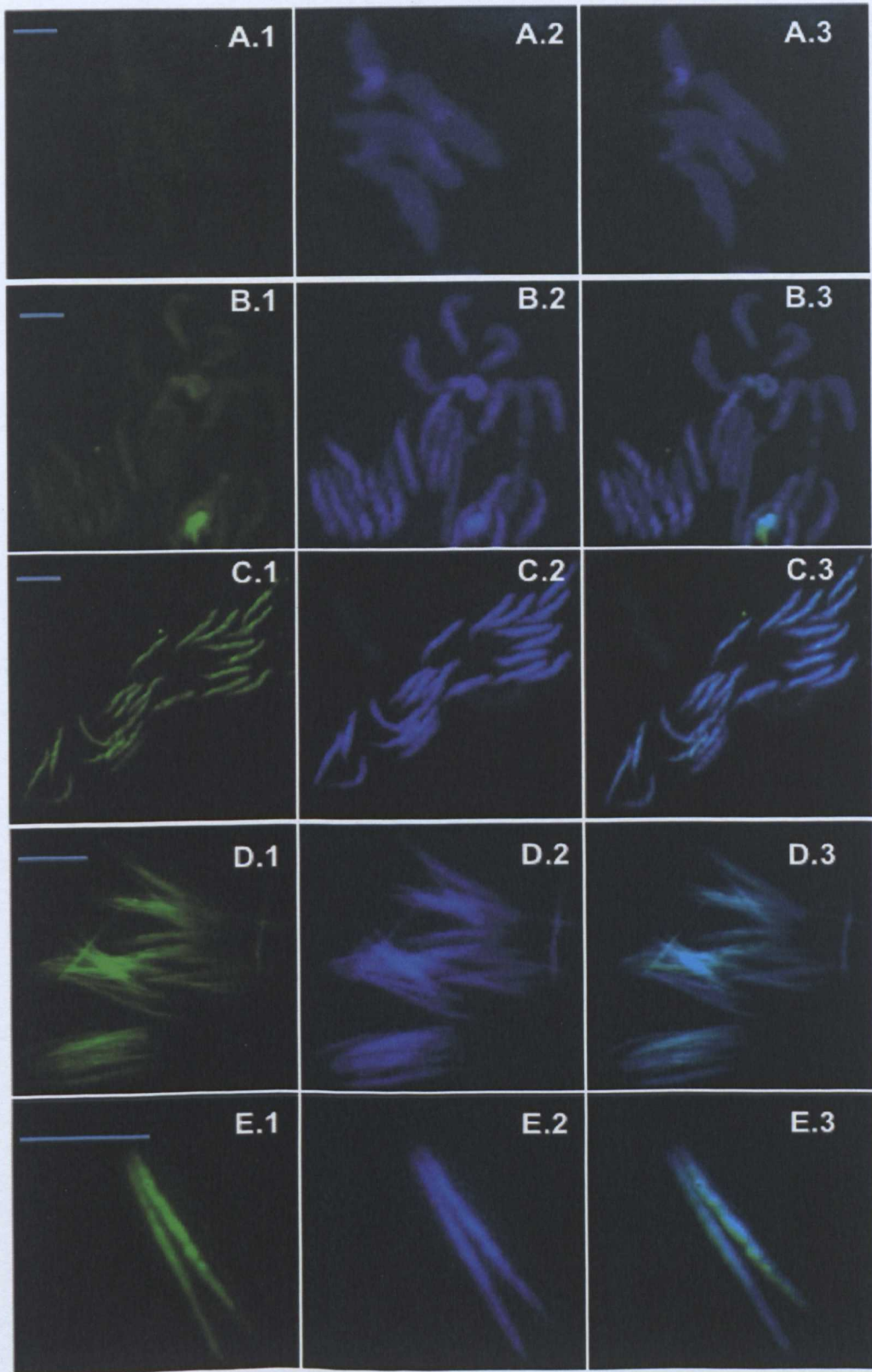


Figure 3-3 Protamine accumulation during spermiogenesis of *Drosophila melanogaster*. A: Middle spermatid stage, A.1: protamine-GFP signal, A.2: dapi, A.3: merged. B: Middle spermatid stage, B.1: protamine-GFP signal, B.2: dapi, B.3: merged. C: Late spermatid stage, C.1: protamine-GFP signal, C.2: dapi, C.3: merged. D: Bundle of mature sperm, D.1: protamine-GFP signal, D.2: dapi, D.3 merged. E: Individual mature sperm, E.1 protamine-GFP signal, E.2: dapi, E.3: merged. (scale bar 5 μ m).



Figure 3-4 Core histone signal in *Drosophila melanogaster* sperm, core histone: antibody signal (FITC), DNA: DNA stained by DAPI (blue), merge: merge of both histone and DNA signals. (scale bar 5 μ m).

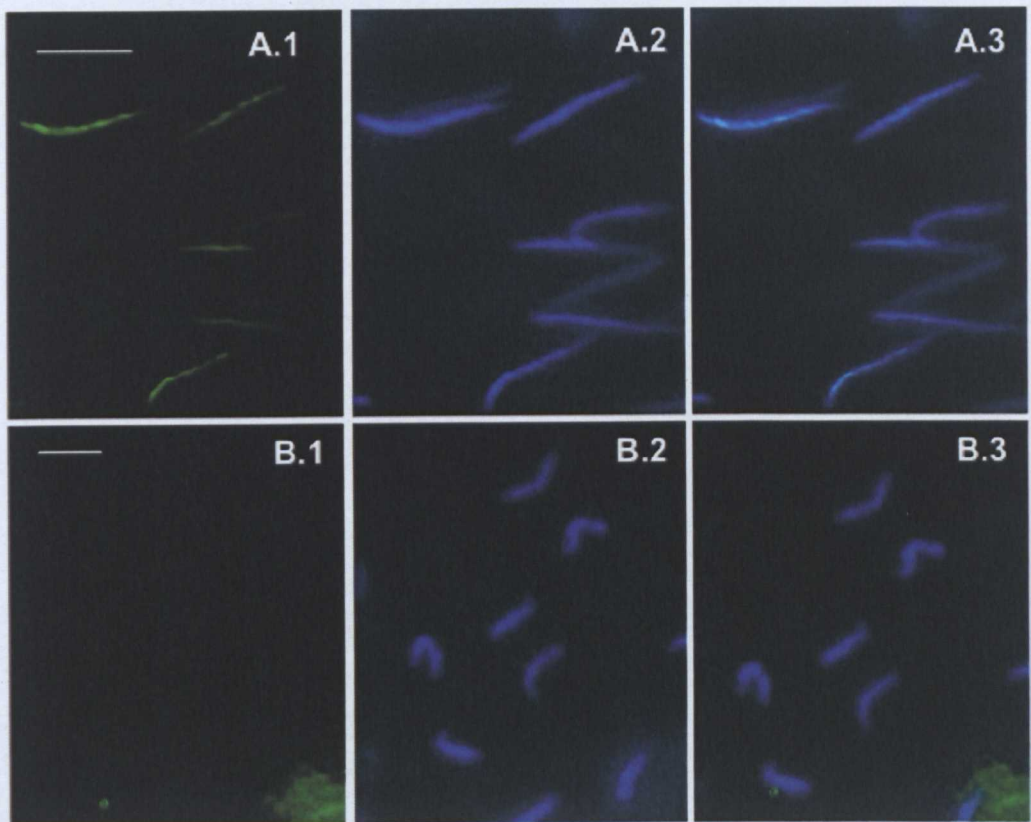


Figure 3-5 Immunocytochemistry of Red Fluorescent Protein (RFP) in mature sperm of *Drosophila melanogaster*. A: RFP, A.1: antibody signal, A.2: dapi, A.3: merged. B: Negative control, B.1: antibody signal, B.2: dapi, B.3: merged. (scale bar 5 μ m).

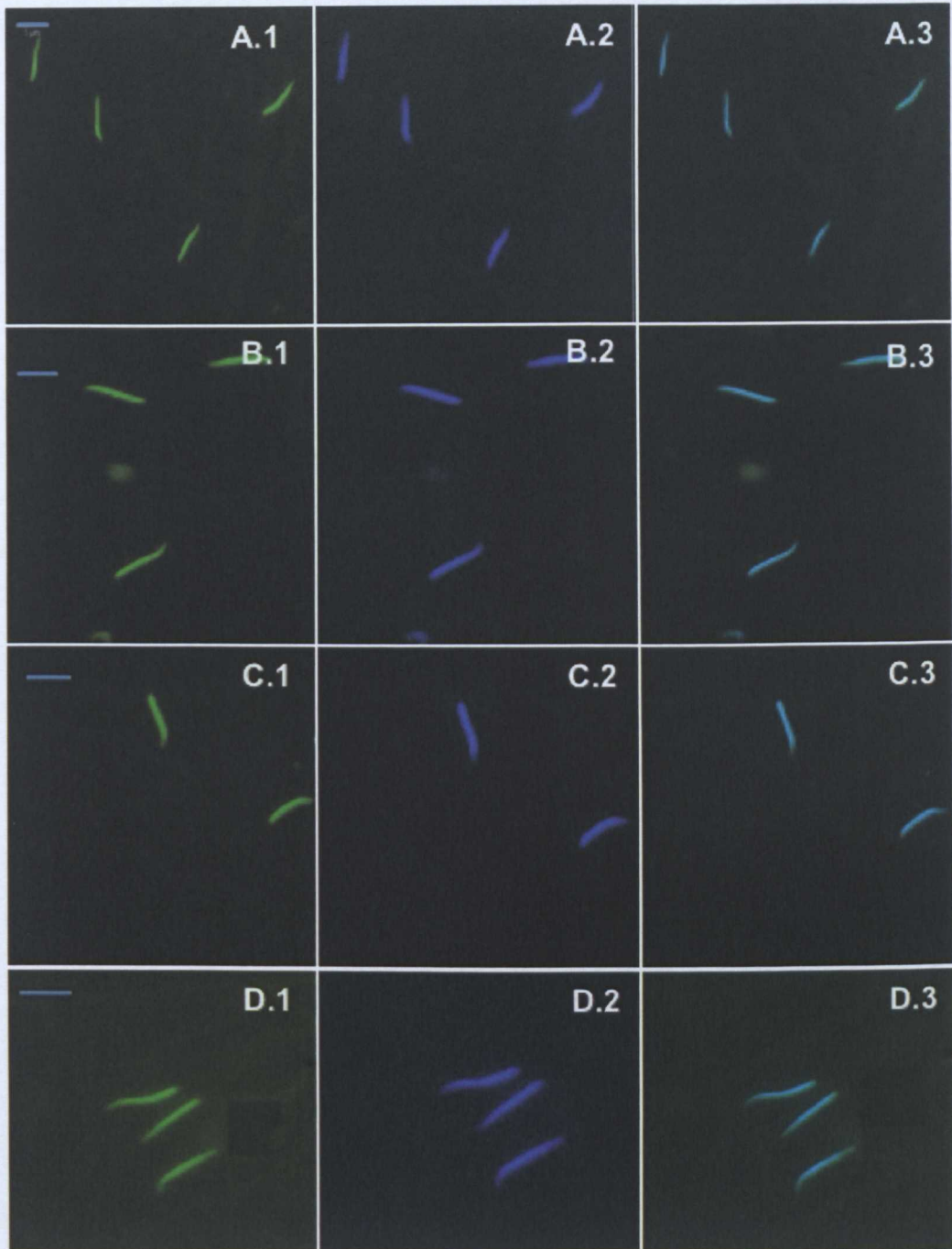


Figure 3-6 Histone signal in *Drosophila melanogaster* sperm. A: H2A, A.1: histone signal (FITC), A.2: dapi, A.3: merged. B: H2B, B.1: histone signal, B.2: dapi, B.3: merged. C: H3, C.1: histone signal, C.2: dapi, C.3: merged. D: H4, D.1: histone signal, D.2: dapi, D.3: merged. (scale bar 5 μ m).

3.3.1.5 SDS PAGE and silver staining

To further confirm the immunocytochemical results, histone protein was extracted by a delicate procedure from sperm collected from about 300 males, and the histone was precipitated by anti H3 and H4 antibodies. The resulting sample was run on 16% SDS gel. After silver staining (a highly sensitive method) many bands were stained within the histone position range (15-20

KDa) from *Drosophila* sperm (figure 3-7, lane B). This suggests that these bands came from protein precipitated by the antibodies. The same band range was obtained from bovine sperm histone (positive control) and whole *Drosophila* sperm protein extracted by boiling sperm in SDS buffer (figure 3-7, lanes C and D).

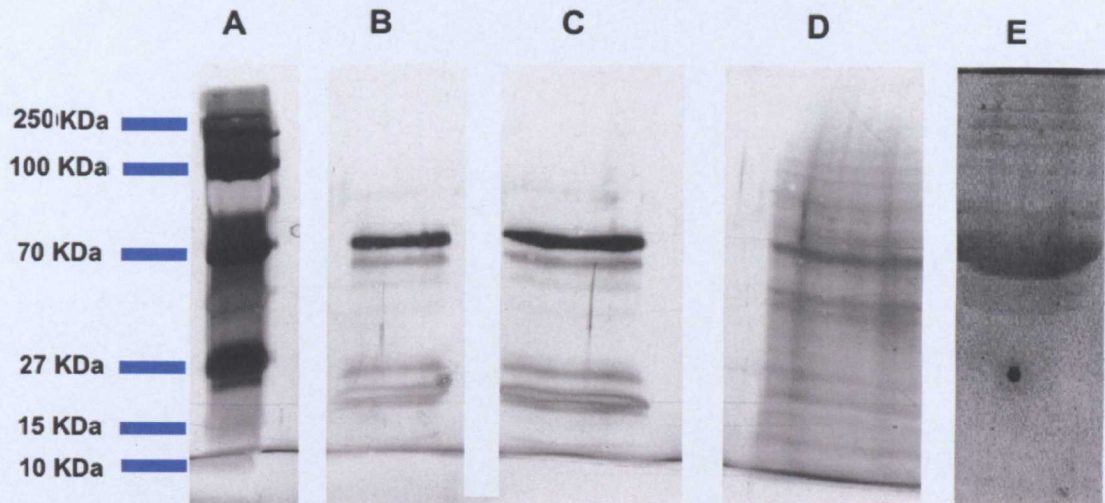


Figure 3-7 SDS-PAGE/silver stained gel. Lane A. protein marker. Lane B. *Drosophila melanogaster* sperm histone protein precipitated by anti H3 and H4 antibodies. Lane C. bovine sperm histone protein precipitated by anti H3 and H4 antibodies. Lane D. total *Drosophila melanogaster* sperm protein extracted by boiling in SDS buffer. Lane E. negative control (contain antibodies but no histone protein).

3.3.2 Mouse sperm

3.3.2.1 Immunocytochemistry

Immunofluorescent analysis was carried out by using motile epididymal sperm to detect the localisation of histone protein in the nucleus and to investigate whether the histones H3 and H4 carry the acetyl group or not. To achieve these goals the antibodies anti-core histone, anti-acetyl H3 and anti-acetyl H4 were used in this experiment. As can be seen from the images in Figure 3-8, the anti-core histone signal is distributed heterogeneously throughout the nucleus, and is mainly concentrated in the centre and posterior part of the nucleus. Interestingly, a clear positive reaction was obtained for anti-acetyl H4 and H3 antibodies, with many different patterns in the posterior central part of the nucleus (Figs 3.9 and 3.10 respectively).

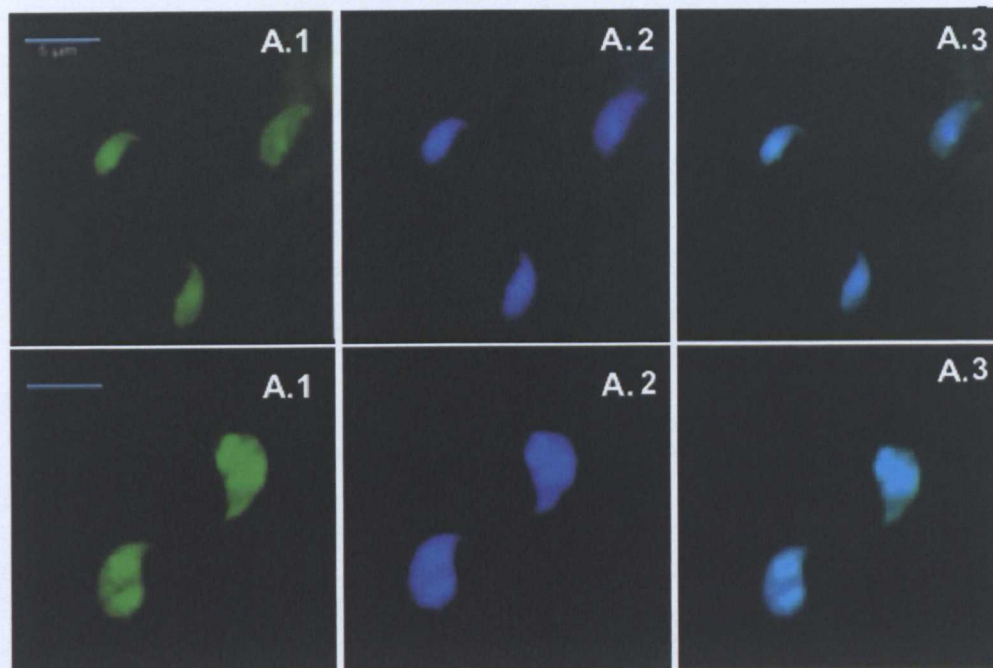


Figure 3-8 Immunofluorescent localisation of core histone in mouse sperm nucleus. A:1 histone signal (FITC). A.2: DNA stained by Dapi (blue). A.3: merged histone and DNA signals (scale bar 5 μ m).

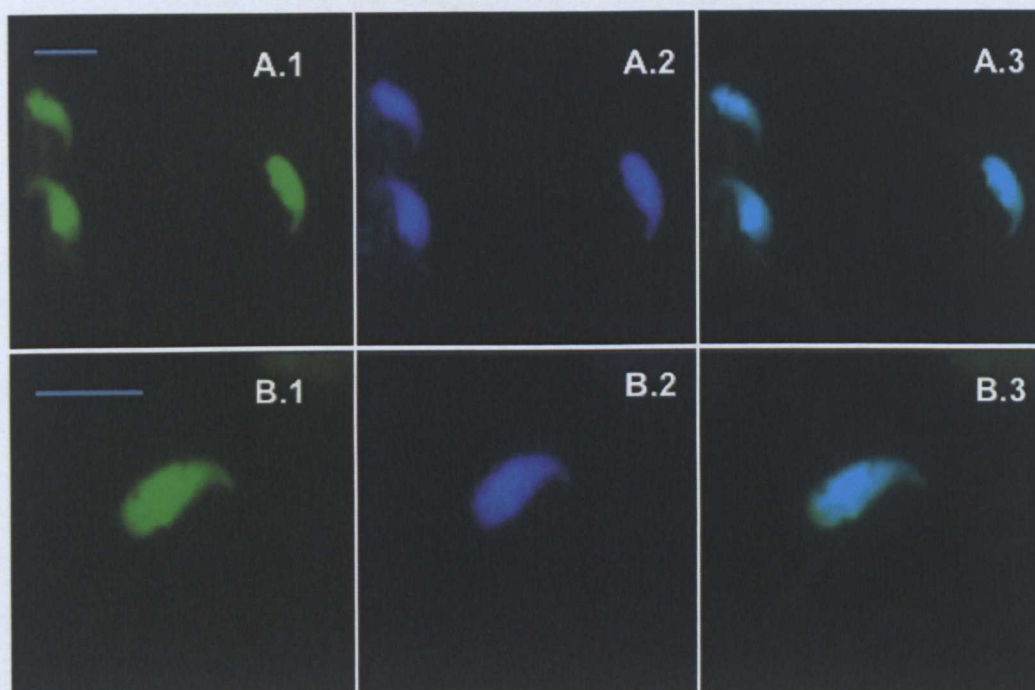


Figure 3-9 Immunofluorescent signal of acetyl H4 in mouse sperm nucleus. A.1 and B.1: histone signal (FITC), A.2 and B.2: DNA stained by Dapi (blue). A.3 and B.3 : merged histone and DNA signals (scale bar 5 μ m).

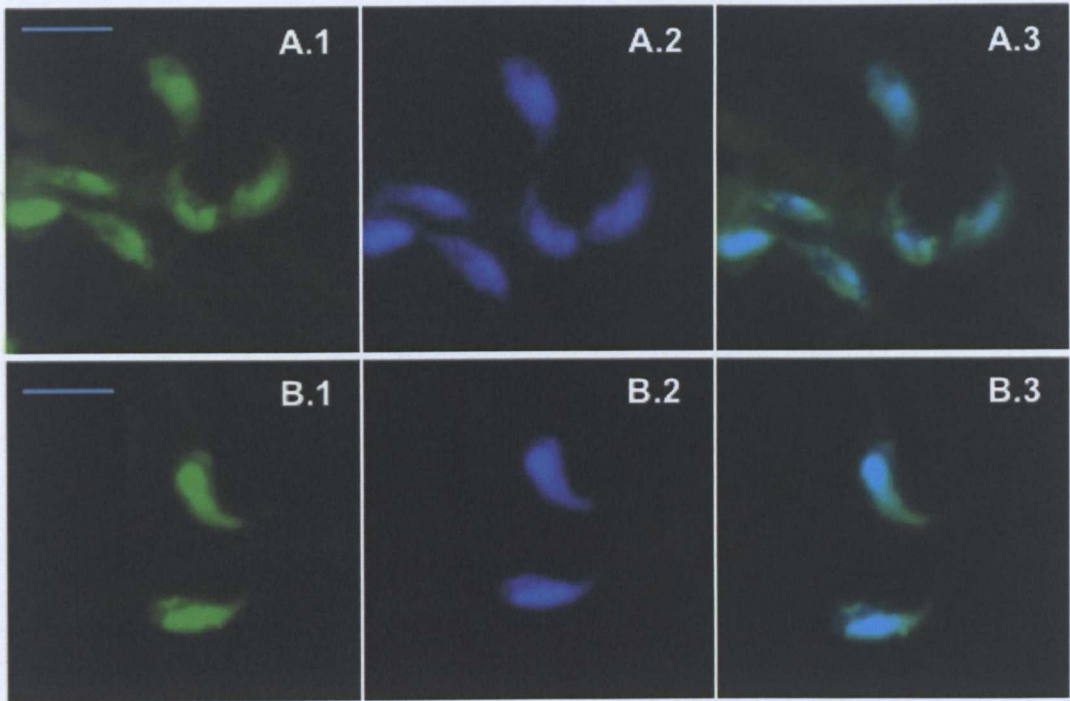


Figure 3-10 Immunofluorescence signal of acetyl H3 in mouse sperm nucleus. A.1 and B.1: histone signal (FITC), A.2 and B.2: DNA stained by Dapi (blue). A.3 and B.3 : merged histone and DNA signals (scale bar 5 μ m).

3.3.2.1 Western blot

Western blotting was used to confirm the presence of acetyl H3 in the mature sperm of mouse. The antibody reaction was captured on the nitrocellulose membrane within H3 range (14-20KDa) (figure 3-11).

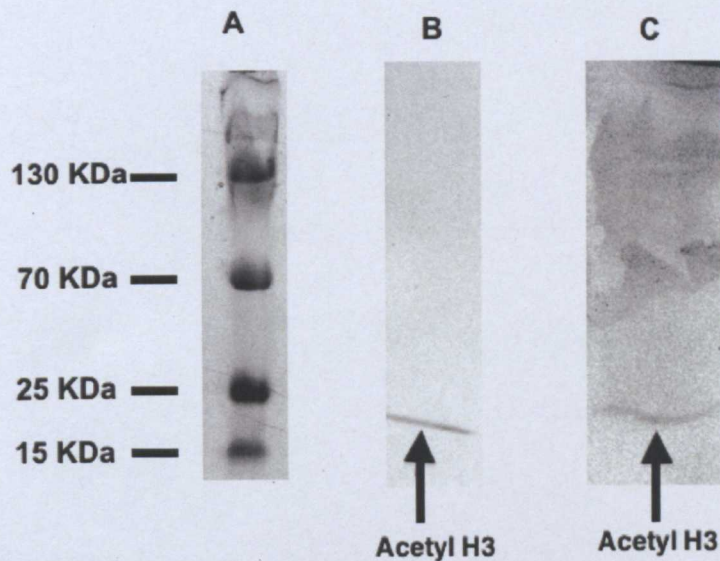


Figure 3-11 Western blot analysis of acetyl H3 in the mature sperm of mouse. Lane A. protein marker. Lane B. acetyl H3 signal from mouse sperm. Lane C. positive control :acetyl H3 signal from mouse testis cells.

3.3.3 Bovine sperm

3.3.3.1 Immunocytochemistry

It was a challenge to use immunocytochemical analysis to detect histone protein in the bovine sperm nucleus, because it is surrounded by a perinuclear theca, which is rich in histones H2A, H2B, H3 and H4 (Tovich and Oko, 2003). These histones might interfere with the nuclear histone signal. Therefore, this experiment was based on the use of antibodies for histones or histone modification that are usually localised in the nucleus, and have not been detected in perinuclear theca. Firstly, anti H2A, H2B, H3 and H4 antibodies were used to recognise the pattern of perinuclear theca histone signal, which was clear in histones H2B, H3 and H4 (figure 3-12). Antibodies for H1, acetyl H3, acetyl H4, H3K27me3 and core histone were then used to detect chromatin histones. Surprisingly, these antibodies gave a remarkable signal pattern different from those in the perinuclear theca (figure 3-13, 3-14). This difference was used to distinguish between the nuclear and the perinuclear histone signals. Although all histone signals are distributed throughout whole nucleus (figures figure 3-14), a greater intensity was noted in the annulus and posterior regions for core histone antibody (figure 3-13). Unlike acetyl H4, acetyl H3 and H3K27me3, the signal was weak which reflects the quantity present in the nucleus (figure 3-15).

3.3.3.2 western blot analysis

Western blotting was used to confirm the presence of acetyl H3 and H3K27me3 in mature bovine sperm. Antibody reaction was detected on the nitrocellulose membrane of both acetyl H3 and H3K27me3 within H3 range (14-20KDa) (figure 3-16).

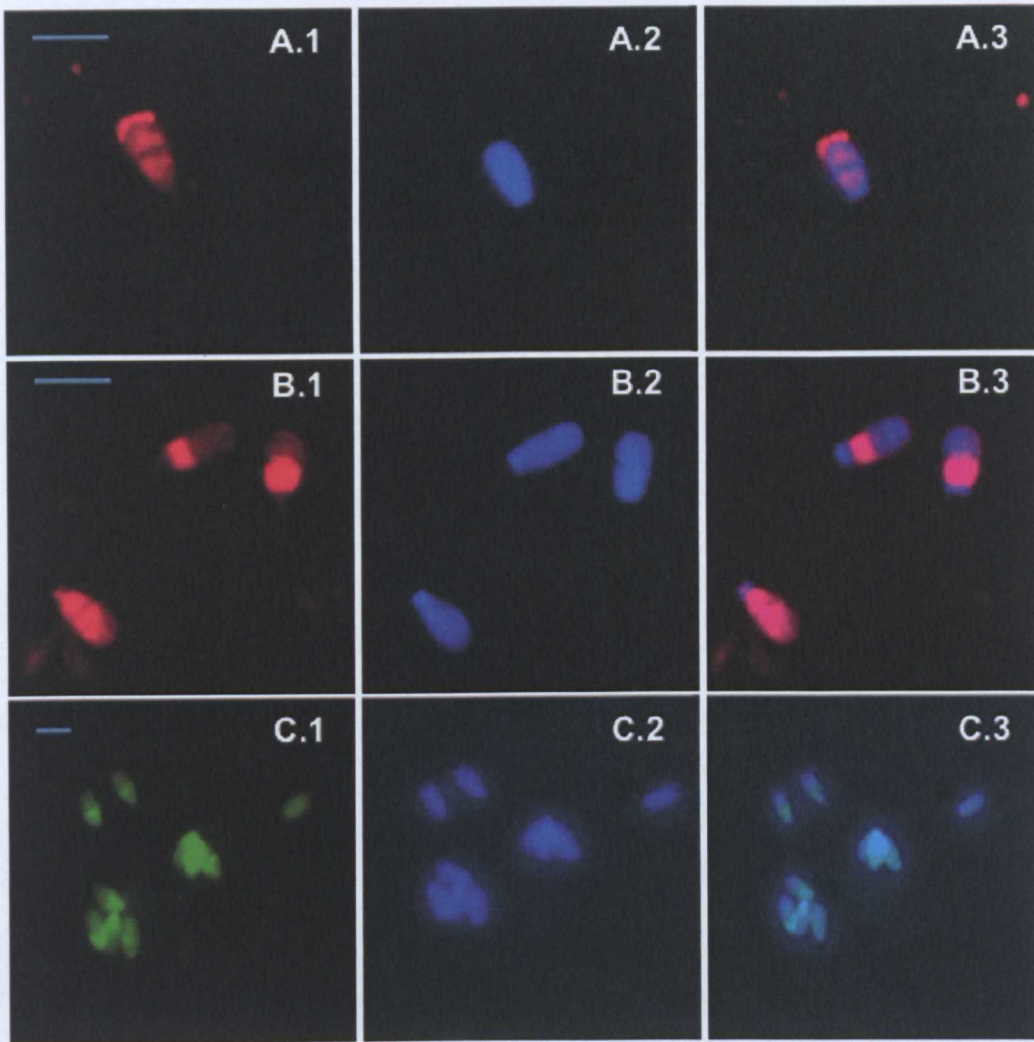


Figure 3-12 Immunofluorescence signal of H3, H4 and H2B in bovine sperm head (nucleus and perinuclear theca). A: H3 signal, A.1(TRITC) . A.2:DNA stained by Dapi (blue). A.3: merged histone and DNA signals. B: H4, B.1: H4 signal (TRITC), B.2: dapi, B.3: merged. C: H2B, C.1: H2B signal (FITC), C.2: dapi, C.3: merged. (scale bar 5 μ m).

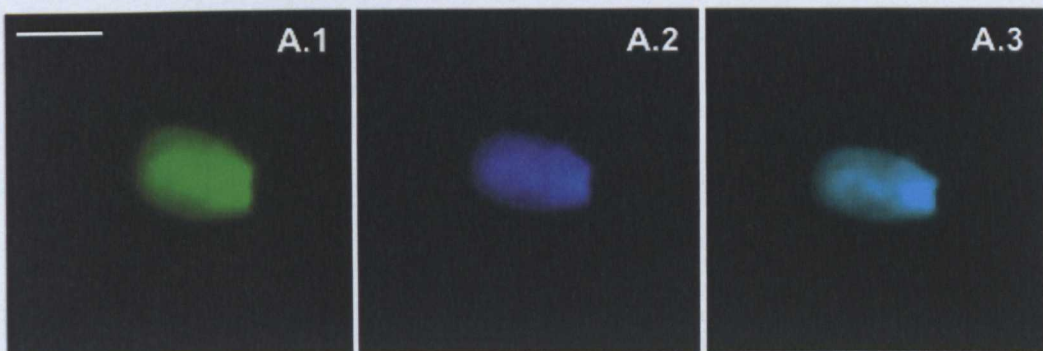


Figure 3-13 Immunofluorescence signal of core histone in bovine sperm nucleus. A.1: histone signal (FITC), A.2: DNA stained by Dapi (blue). A.3: merged histone and DNA signals (scale bar 5 μ m).

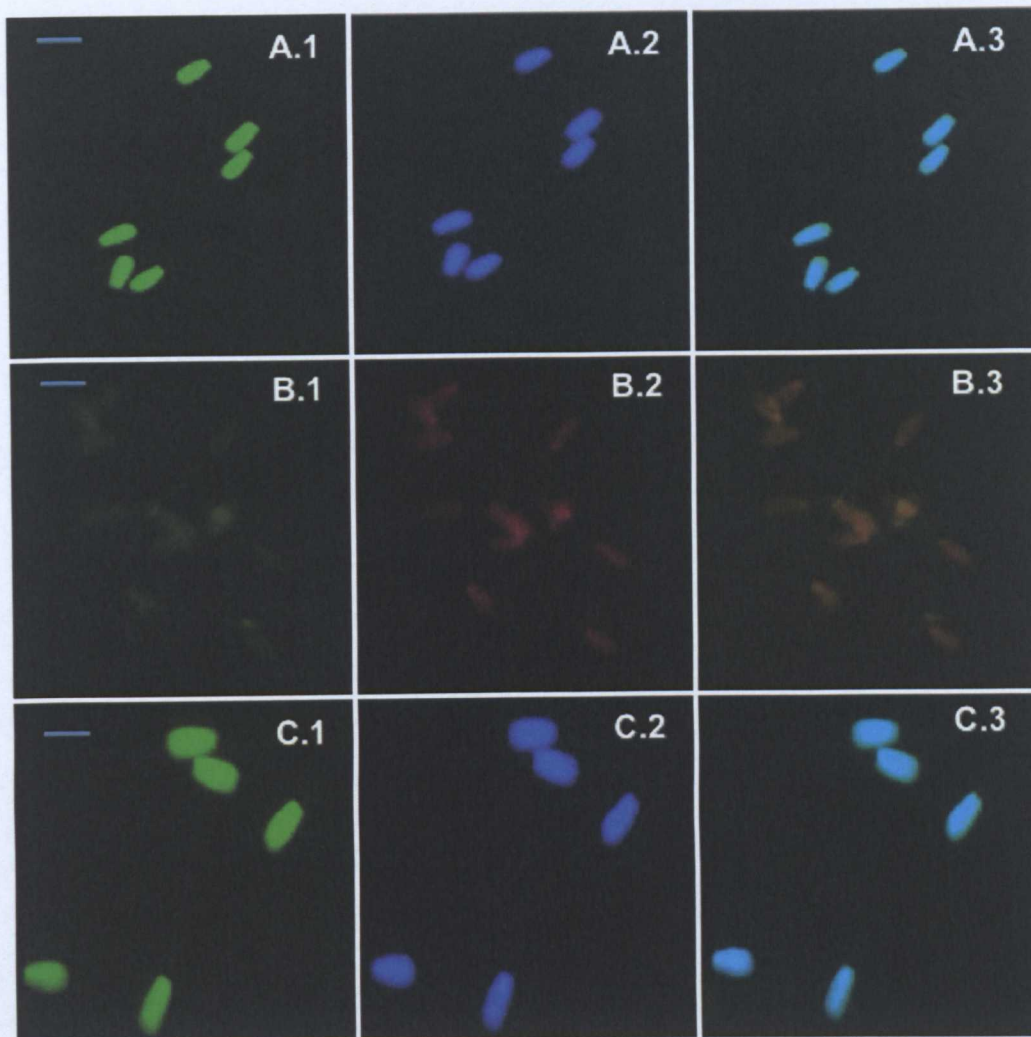


Figure 3-14 Immunofluorescence signal of H1, acetyl H3 and acetyl H4 in bovine sperm nucleus. A:H1, A.1: H1signal (FITC), A.2: DNA stained by Dapi (blue), A.3: merged histone and DNA signals. B: acetyl H3, B.1: acetyl H3 signal (FITC), B.2: DNA stained by propidium iodide (red), B.3: merged. C: acetyl H4 signal, C.1: acetyl 4 signal (FITC), C.2: DNA stained by Dapi. C.3: merged histone and DNA signals (scale bar 5 μ m).

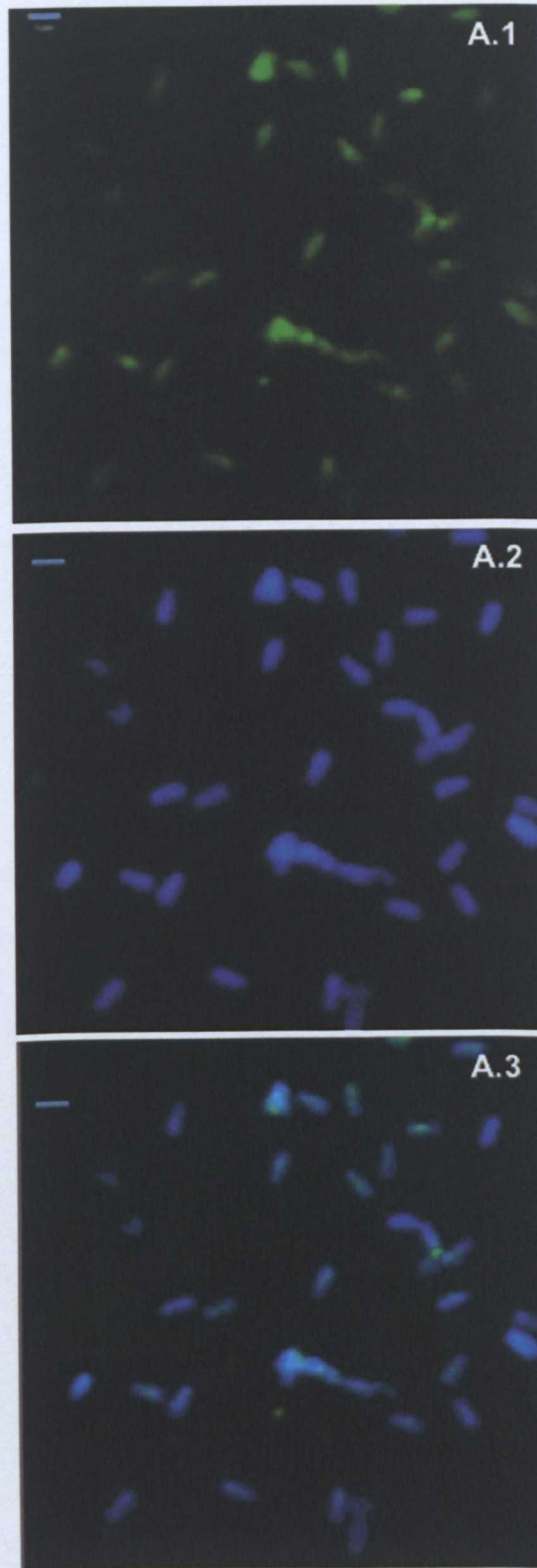


Figure 3-15 Immunofluorescence signal of H3K27me3 in bovine sperm nucleus. A.1: antibody signal (FITC), A.2: DNA stained by Dapi (blue), A.3: merged histone and DNA signals. (scale bar 5 μ m).

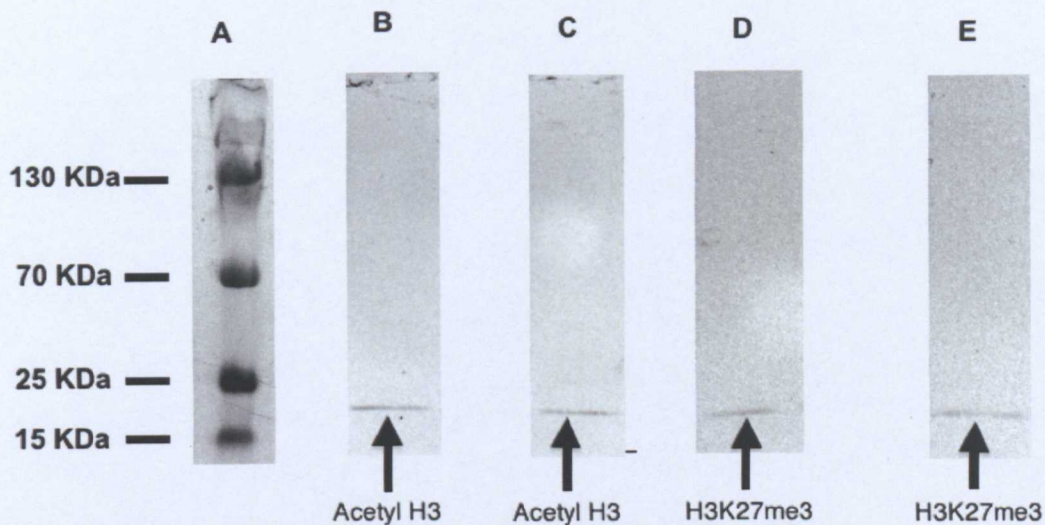


Figure 3-16 Western blot analysis of histone modifications (acetyl H3 and H3K27me3) in mature bovine sperm. Lane A: protein marker. Lane B: acetyl H3 signal from bovine sperm. Lane C: acetyl H3 signal from mouse testis cells. Lane D: H3K27me3 signal from bovine sperm. Lane E: H3K27me3 signal from mouse testis cells.

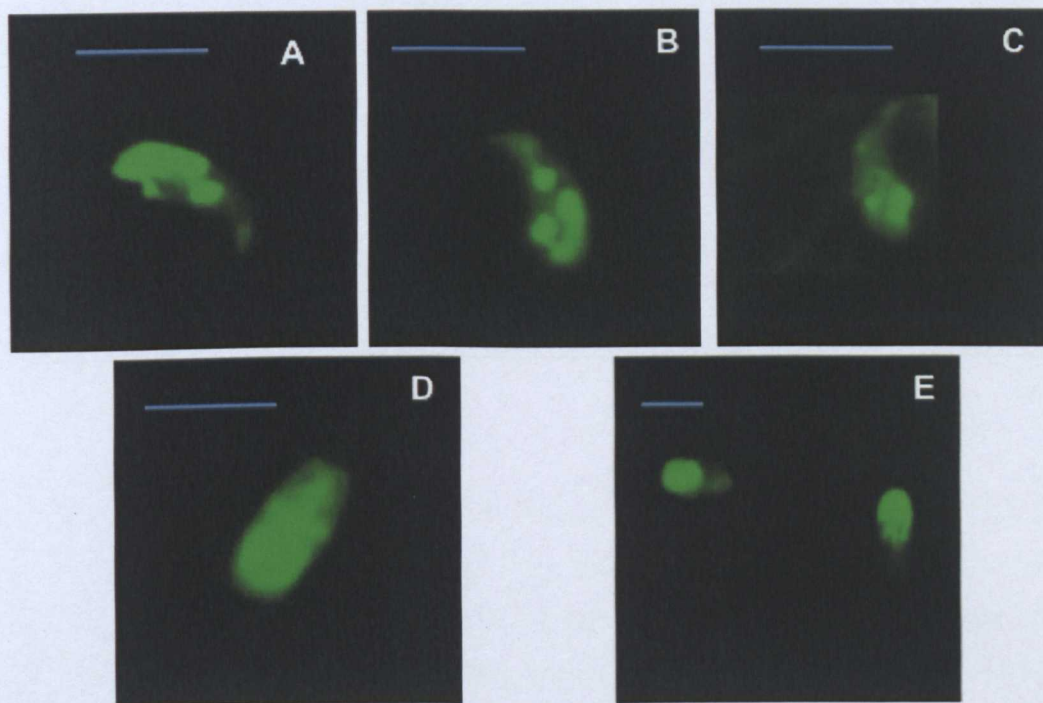


Figure 3-17 The effects of de-condensation chemicals (DTT and Heparin) on nucleoprotein signal. Upper row: A, B and C: show different signal patterns of acetyl H3 in mouse sperm. Lower row D and E: shows the signal of protamine 1 in bovine sperm, D: treated with 0.2% triton X-100, 2.5 mM DTT, 100 U/ml heparin for 10 minutes, E: treated with 0.2% triton X-100, 10 mM DTT, 100 U/ml heparin for 30 minutes. Note the disappearance of the protamine in the posterior part of the nucleus in E. (scale bar 5 μ m).

3.4 Discussion

3.4.1 *Drosophila melanogaster* sperm

In metazoan organisms, the forms of chromatin packaging in mature sperm are widely variable, and there are many models of histone replacement during spermiogenesis (Poccia,1986). Whereas some species have simple models (partial histone substitution) as in *Xenopus* (Dimitrov *et al.*,1994), in other species the model of histone replacement is more complex, where somatic histones are almost completely replaced by protamines (small and highly basic protein), such as in mammals (Meistrich *et al.*,2003).

In *Drosophila* the situation is controversial. Immunocytochemical and H2A-GFP observations indicated that the histones are completely removed from mature sperm (Akhmanova *et al.*,1997; Jayaramaiah-Raja and Renkawitz-Pohl,2005; Rathke *et al.*,2007). However, by using mass spectroscopy analysis, histones H3.3B, H2Av, H3.3A, B and H4r were detected in sperm proteome (Dorus *et al.*,2006) supplementary data table1). By comparison with immunocytochemical and Fluorescent Protein methods, mass spectroscopic results are more reliable because of their high sensitivity and specificity (Guerrera and Kleiner,2005). So this result removes any doubt about the presence of histone protein in mature sperm of *Drosophila melanogaster*. Moreover, supporting the results of Dorus *et al.* (2006), a fluorescing signal was reported in the individualised sperm of transgenic flies containing an H2A-tagged with Red Fluorescent Protein (reviewed in (Miller *et al.*,2009)).

Immunocytochemistry, H2A.v-RFP and SDS-PAGE/silver staining observations in this study have demonstrated that while most histones are removed during the late spermatid stages (late elongating spermatids I and II) (figure 3-2), some histones are still retained in the nucleus of the mature sperm (figures 3-2, 3-4, 3-5 and 3-6). These results are complementary to the previous observation by anti H2A.v-RFP antibody (reviewed in (Miller *et al.*,2009), and supports the mass spectroscopy result (Dorus *et al.*,2006) (supplementary data table 1). Hence, although the H2A.v-RFP signal was faint, it was detectable in mature sperm.

Signal weakness may be because the amount of H2A.v in the sperm nucleus is very low, and the signal is borderline with the brightness of the microscope field. However, immunofluorescence with anti-core histone antibody gave signals that could clearly be seen in mature sperm, after prolonged chromatin decondensation treatment with DTT and heparin. DTT-heparin treatment is absolutely essential to get a good reaction between histone and anti-histone antibodies. The concentrations of these chemicals and the period of treatment are critical points to achieve the right level of chromatin decondensation. From the observations of this study, it appears likely that previous studies failed to detect histone protein in insect or *Drosophila* sperm because the correct chromatin de-condensation was either not achieved or not attempted.

Initial investigations using an anti H3 antibody demonstrated that histone replacement starts in the posterior end of the elongated spermatid nucleus (Hauschteck-Jungen and Hartl,1982). However, from the images taken in this experiment (late spermatids I and II) (figure 3-2), it is readily apparent that histone replacement and chromatin condensation are initiated at the anterior part of the spermatid nucleus. This may be because H2A.v and H3 have different dynamics during histone-protamine replacement.

Two types of protamine were detected in *Drosophila* sperm, protamine A and protamine B. Although these protamines perform similar functions in insects and mammals, the chemical structure shows a considerable difference in amino acid sequences (Jayaramaiah-Raja and Renkawitz-Pohl,2005). Previous studies have suggested that the histones are removed completely from the chromatin of *Drosophila* spermatid as protamines accumulate (Rathke *et al.*,2007).

However, in this study, protamine A-GFP signal was captured in the middle spermatid stage (figure 3-3) before histone replacement (figure 3-2) and the signal was distributed through the whole nucleus. It was noted that the chromatin is completely condensed in the anterior part of the late spermatid II (figure 3-2) while the posterior part is still uncondensed, and the signal of H2A.v-RFP is clearly detectable. Therefore, these observations suggest that the protamines are accumulated *prior* to major histone replacement. Moreover, the

protamine signal in mature sperm was not uniformly distributed in the nucleus (Fig 3-3, E). This means that many regions of sperm genome may not be fully packaged by protamine, and that these regions might retain histones.

3.4.2 Bovine and mouse sperm

3.4.2.1 Decondensing chemicals effect

In vitro sperm de-condensation is a fundamental step for paternal nucleoprotein studies using immunocytochemistry. This is commonly achieved using DTT and heparin. While DTT is a reducing agent used to reduce disulfide bonds –S-S- in sperm protamine (Seligman *et al.*,1994), heparin destabilises the sperm membrane (Delgado *et al.*,1982). It was reported that these agents caused dramatic morphological changes in the sperm nucleus (Nakai *et al.*,2006). Furthermore, a recent study indicated that heparin released H1 from sperm chromatin, and its fluorescent signal decreased significantly with time (Sanchez-Vazquez *et al.*,2008). In this study, it was noted that many sperm lost their immunological signal (mostly or partially) as result of the de-condensation chemicals and the de-condensation process (figure 3-17). So this loss of target proteins from the sperm nucleus might possibly give incorrect or false-negative results.

3.4.2.2 Localisation of histones in the nucleus of mouse sperm

In the mouse sperm nucleus, retained histones comprise less than 2% of total nucleoproteins (Gatewood *et al.*,1990). Although the chromatin decondensation protocol was not used in these studies, initial investigation using immunocytochemistry demonstrated that H1 is distributed in the peripheral region of the nucleus (Pittoggi *et al.*,1999). However, another study showed that after chromatin decondensation by DTT and heparin, histones were located mainly in the centre of the nucleus. This result suggested that most nuclear histone is retained in the centric heterochromatin (van der Heijden *et al.*,2006). In this study a clear signal was noticed in the sperm nucleus in response to anti-core histone antibody (figure 3-8). Although this signal was distributed throughout the entire nucleus, there was a difference in the distribution of signal intensity. Whereas many parts showed a strong signal, other areas showed a weak signal. This might be the result of two possibilities. Firstly, it is possible that the heterogeneity of the signal pattern is a consequence of chromatin decondensation. Secondly, it is possible that a regional distribution of histones

variants in the nucleus results in this variation in histone intensity. For example, it was reported that the nucleosomes and acetyl H4 are concentrated in the chromocentre area in mouse sperm chromatin (van der Heijden *et al.*,2006) and a similar histone distribution was noted in this study (figures 3-9 and 3-10).

3.4.2.3 Histones in the bovine sperm nucleus

Several studies have indicated that the bovine sperm head contains histone proteins. These proteins are located in perinuclear theca histones (H2A, H2B, H3 and H4) ((2003,Tovich and Oko), and nuclear histones including CENP-A (Plammer *et al.*, 1990), and H1 (Sanchez-Vazquez *et al.*,2008). Nucleosomal histones have not been detected before which might be as a result of the genome compacting pattern, or the interference of perinuclear theca histones(Tovich and Oko,2003) In this study, to distinguish between these groups, we used antibodies to core (anti-H2A, H2B, H3, H4) and acetylated (anti acetyl H3, anti acetyl H4, anti H1, anti H3K27me3)histones which can help to distinguish between histones located within the nucleus and not in the preinuclear theca. Unexpectedly, the signal pattern of nuclear histones was distinguishable from those in the perinuclear theca (figures 3-12, 3-13 and 3-14). The core histone signal was distributed throughout the nucleus with more intensity in the sperm nuclear annulus (figure 3-13) and this localisation of the histones in the annulus is similar to the pattern seen in human sperm (Li *et al.*,2008). Although little is known about bovine nuclear histones, two histones (H1 and CENP-A) were detected previously, as mentioned above. It was reported that H1 is distributed throughout the nucleus and mediates chromatin condensation and decondensation (Sanchez-Vazquez *et al.*,2008), and CENP-A is localised in the centromeric region (Palmer *et al.*,1990).

3.4.2.4 Histone H4 and H3 modification

Previous investigations reported that histones (H3 and H4) are highly acetylated in human sperm. While this modification has proved controversial in mouse sperm, it has not been studied in bovine species. In this study anti acetyl H3 and H4 antibodies were used to investigate histone acetylation in the sperm of these experimental models. During mouse spermiogenesis, spermatid elongation (step 9-11) is marked with hyper acetylation in the core histones of H2A, H2B, H3 and H4. This acetylation disappears in condensing spermatids (Hazzouri *et al.*, 2000), suggesting that the histones of the mouse sperm do not carry acetyl modification. This result was supported by using anti acetyl H4

antibody, the signal for which disappeared completely in the condensing spermatid (Govin *et al.*,2007). In the condensing spermatids, protamines accumulated in the chromatin (Balhorn,2007), so in this situation the ability of the antibody to bind with the histone is decreased as result of chromatin condensation.

However, by using chromatin decondensation chemicals, a signal for acetyl H4 (H4K8ac and H4K12ac) was detected in the condensing spermatid and mature sperm of mouse, and it was suggested that acetyl H4 plays an epigenetic role in chromatin reorganisation after fertilisation (van der Heijden *et al.*,2006). A similar result for acetyl H4 has been detected in this research in both mouse and bovine sperm after chromatin de-condensation by DTT and heparin. Whereas acetyl H4 signal is distributed throughout the nucleus in bovine sperm, it is localised mainly in the centre of the mouse sperm nucleus. It is clear now that acetyl H4 was not detected in many studies because the protein was inaccessible to the antibodies using non-decondensed chromatin. These data concur with those derived using the (RFP) tagged H2A, where the fluorescent signal progressively disappears in elongating spermatids and in spermatozoa subjected to decondensation reagents. The former is due to both a genuine loss of H2A and a condensation-based change in the conformation of the RFP tag, reducing its sensitivity to fluorescent excitation and hence its brightness. The latter is due entirely to DTT and/or heparin based changes in RFP conformation, eliminating its excitation characteristics.

Unlike acetyl H4, little is known about acetyl H3 in metazoan mature sperm. Previous studies have shown that H3 is extensively acetylated in human sperm (Gatewood *et al.*,1990) but the details are not clear. Recently, another study on brachyuran crab sperm (with partially packaged chromatin) reported that the H3 is highly acetylated particularly on K9 and K18 (Kurtz *et al.*,2009). In this study, acetyl H3 has been noted in bovine and mouse sperm. The pattern of acetyl H3 signal is similar to acetyl H4 in mouse, so it is possible that acetylation of both H3 and H4 share a similar function in chromatin reorganisation.

In conclusion, firstly, *Drosophila melanogaster* is an excellent experimental model, and the observations using different techniques has indicated that the

chromatin of *Drosophila melanogaster* sperm contains histone protein. So using this result in advanced molecular analysis might help us to understand the possible roles of histones in fertilisation and embryo development. Secondly, taken together with recent data showing that histones are bound with DNA in particular sequences (Arpanahi *et al.*,2009; Hammoud, S. *et al.*,2009), the observations in bovine and mouse sperm suggest that histones, their variants and modifications, together with protamine are distributed throughout the sperm nucleus non randomly in a mosaic pattern and this pattern probably differs from species to species.

Chapter 4: Studying the location of histone bound DNA in *Drosophila melanogaster* sperm, and MNase soluble and insoluble DNA in Bovine sperm

4.1 Introduction

4.1.1 Chromatin organization in the mature sperm of mammals

The mature sperm of many species have a specialised nuclear structure in which the chromatin is super-compacted as a result of protamine binding. Sperm fertility may depend on the morphological and molecular structure of the chromatin and its organization within the nucleus. Hence, the normal localization of histone and protamine within the sperm genome is the most important factor in determining sperm quality. These points have been studied in human, mouse and few other animal experimental models. The position of sperm chromosomes has been analyzed by using FISH technique in many studies investigating the genome distribution within the human and mouse sperm nucleus (Haaf and Ward,1995; Hazzouri *et al.*,2000b; Mudrak *et al.*,2005; Zalenskaya and Zalensky,2004).

The results of these studies indicate that sperm chromosomes are not randomly distributed within the nucleus. For example, in human sperm the signals for chromosomes 1 and 6 were detected in the anterior part of the sperm, while chromosomes 2 and 5 were detected in the posterior end of the nucleus (Mudrak *et al.*,2005; Zalenskaya and Zalensky,2004). In addition, the main position of chromosomes X and Y is in the anterior half of the nucleus, in contrast to chromosome 18 which is mainly located in the posterior half (Hazzouri *et al.*,2000b; Luetjens *et al.*,1999; Zalenskaya and Zalensky,2004). On the other hand, some chromosomes (chromosome 13 for example) showed no particular localisation preference (Mudrak *et al.*,2005). These studies concluded that the non-random position of sperm chromosomes might play important roles in oocyte fertilization and early embryo development (Mudrak *et al.*,2005; Zalenskaya and Zalensky,2004).

The location of centromeres and telomeres is another approach that has been used to investigate chromatin organization within the sperm nucleus. FISH techniques have indicated that the telomeres are localized in the peripheral part

of the nucleus, while the centromeres are organized into a condensed form known as the chromo-centre in the centre of the nucleus (Haaf and Ward,1995; Hazzouri *et al.*,2000b; Mudrak *et al.*,2005; Zalenskaya and Zalensky,2000; 2004; Zalensky *et al.*,1995). Similarly to the chromosomal investigation, the centromeric / telomeric position tells us that the sperm genome is organized non-randomly.

4.1.2 Chromatin immunoprecipitation (ChIP)

The ChIP assay is a powerful and standard technique, widely used to identify DNA regions that bind to specific nuclear proteins. This technique was first developed in 1984 ((Gilmour and Lis,1984; 1985; 1986) to investigate the binding sites of RNA polymerase within the genome of *Drosophila melanogaster* and *Escherichia coli*. There are many types of ChIP assay, but the main types are N-ChIP and X-ChIP. The first uses native chromatin whereas the second uses fixed and fragmented DNA (Das *et al.*,2004; O'Neill and Turner,2003; Orlando,2000).

X-ChIP procedure (used in this study) consists of many steps. The main steps are crosslinking of protein bound DNA by using formaldehyde, DNA sonication (fragmentation) to small size (300-800 bp), and precipitation of chromatin bound protein by using specific antibody (Das *et al.*,2004). The significant point of using ChIP is that this technique can be combined with many techniques such as western blot (Wells and Farnham,2002), southern blot (Orlando *et al.*,1997), microarray (Weinmann *et al.*,2002) and PCR and real time PCR (Singal *et al.*,2002).

4.1.3 Micrococcal nuclease digestion

Micrococcal nuclease (MNase) digestion is a common and simple procedure to study the organisation of chromatin, particularly its accessibility to transcription factors and other DNA binding proteins (Henikoff *et al.*,2011). Generally, MNase was isolated first time from bacteria *Staphylococcus aureus*. Its molecular weight is about 18,6 kilo Dalton and it consists of 159 amino acids (Cuatrecasas,1967) (Cunningham,1959; Taniuchi,1967). The enzyme is active within the pH range of 7.0 – 10, and the optimum activity is at pH 9.2 (Cuatrecasas,1967).

MNase has been used widely to identify nucleosome positions within the genome, because of its ability to cut the linker DNA that connects between two neighbouring nucleosomes. In addition, it has weak activity against the DNA around the nucleosomes (Axel,1975; Clark,1971). Recently, MNase digestion has been coupled with microarray (Kharchenko *et al.*,2008; Lantermann *et al.*,2010), and deep sequencing strategies (Albert *et al.*,2007; Field *et al.*,2009). to provide information on chromatin organization and nucleosome position in relation to gene expression in the cells. Several studies have used MNase digestion successfully to investigate chromatin packaging in human and mouse sperm (Arpanahi *et al.*,2009; Saida *et al.*,2011; Zalenskaya and Zalensky,2000; Zalensky *et al.*,2002). The results revealed that the chromatin of human and mouse sperm is organized into two compartments. While the first one is soluble to MNase (to some extent relaxed nucleosomal chromatin), the second one is insoluble to MNase (less accessible and condensed nucleoprotamine chromatin) (Arpanahi *et al.*,2009; Saida *et al.*,2011).

4.1.4 The aim of the experiments

The aim of these experiments was to investigate histone localisation in the mature sperm nucleus, by using FISH technique based on ChIP of histone bound DNA and MNase digestion in *Drosophila melanogaster*, and MNase digestion of bovine sperm chromatin.

4.2 Material and methods

All materials and methods are described in detail in chapter 2 in section 2.8.

4.2.1 Samples

4.2.1.1 Sperms of *Drosophila melanogaster*

Described in detail in section 2.9.1.

4.2.1.2 Sperms of bovine

Described in detail in section 2.8.1.1

4.2.2 ChIP of histone bound DNA in the sperm of *Drosophila melanogaster*

Described in detail in section 2.9.

4.2.3 MNase of *Drosophila melanogaster* and bovine sperm

Described in detail in section 2.8.1.

4.2.4 Measurement of DNA concentration

Described in detail in section 2.9.9.

4.2.5 Whole genome amplification

Described in detail in section 2.9.10.

4.2.6 Gel electrophoresis

Described in detail in section 2.11.

4.2.7 Fluorescence in situ hybridization (FISH)

Described in detail in section 2.8.

4.3 Result

4.3.1 *Drosophila melanogaster* sperm

4.3.1.1 ChIP – FISH

Histone bound DNA precipitation results are described in detail in the next chapter. Fluorescence *in situ* hybridization was performed in order to assess the location of histone bound DNA in the nucleus of *Drosophila melanogaster* sperm. Histone bound DNA was precipitated by anti H3 and anti H4 antibodies, non specific anti body (secondary antibody) was added to negative control tube to evaluate the interference of non specific DNA which might bind to beads or antibodies. Labelled histone bound DNA showed clear specific labelling, the signal being distributed in patches throughout the nucleus (figure 4.1). However, the signal of labelled negative control sample is distributed throughout the nucleus, and showed no specific labelling (figure 4.1).

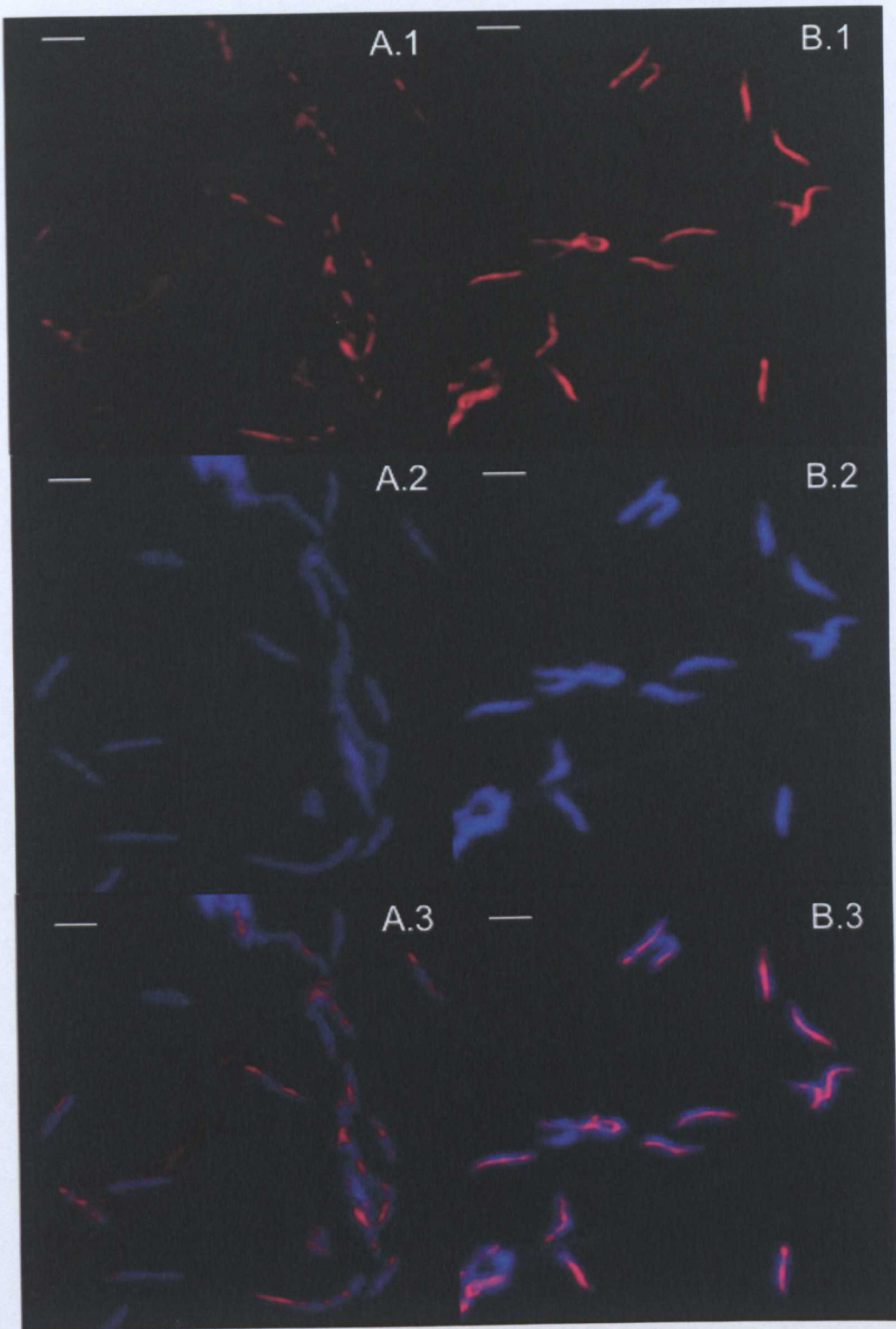


Figure 4-1 FISH Localization of ChIP-histone bound DNA in the sperm of *Drosophila melanogaster*. A.1: histone bound DNA signal, A.2: DNA signal (DAPI), A.3: merged histone bound DNA signal and DNA signal. B.1: Negative control signal (non specific antibody) B.2: DNA signal (DAPI), B.3: merged negative control signal and DNA signal.

4.3.1.2 Micrococcal nuclease digestion

Two samples of sperm were used in this experiment, each collected from about 100 males. The first was exposed to MNase and the second was used as a negative control. DNA produced from both samples was purified and quantified by nanodrop fluorospectrometry. The quantity of MNase-soluble DNA released by digestion of sperm nuclei was approximately 6.5 ng, with the remaining MNase-insoluble DNA accounting for 754 ng. Undigested (negative control DNA) was about 2 ng. After the amplification of all samples, 4 μ g of each sample was run on 1% agarose gel. Although DNA amplification leads to DNA fragments which stick together and run as a smear on the gel, the result showed variation in DNA behaviour on the gel indicating successful digestion. Firstly, MNase-soluble DNA ran as a smear but most of the DNA signal was located between 100 and 400bp, with evidence for a ladder-like arrangement and a most intense band at \sim 200bp, which could indicate mononucleosomes plus linker DNA. Secondly, MNase-insoluble DNA gave a signal mainly located in the upper half of the gel (more than 500bp). Thirdly, the negative control DNA ran from the top to the bottom with a stable signal (figure 4.2).

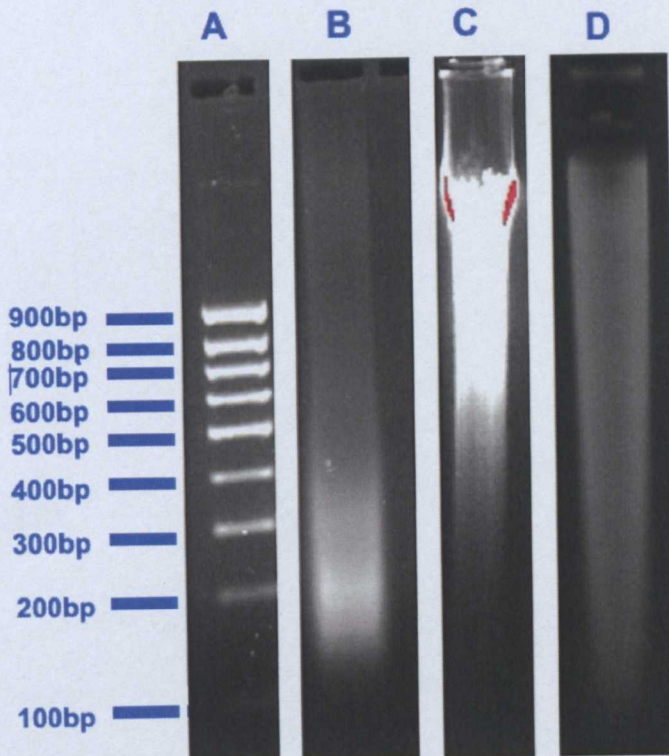


Figure 4-2 The difference between MNase-soluble DNA, MNase-insoluble DNA and negative control DNA of *Drosophila melanogaster* sperm in 1% agarose gel after amplification. A: DNA ladder, B: MNase-soluble DNA, C: MNase-insoluble DNA, D: supernatant DNA of negative control (no MNase was added).

4.3.1.3 MNase - FISH

To examine if the sperm of *Drosophila melanogaster* have regional variations in genome packaging, MNase soluble samples were labelled and hybridized with decondensed sperm. The observation indicated that there is no difference in signal distribution between soluble and insoluble MNase DNA (figure 4.3). Furthermore, the signal pattern in the negative control was similar to MNase treated DNA (figure 4.3).

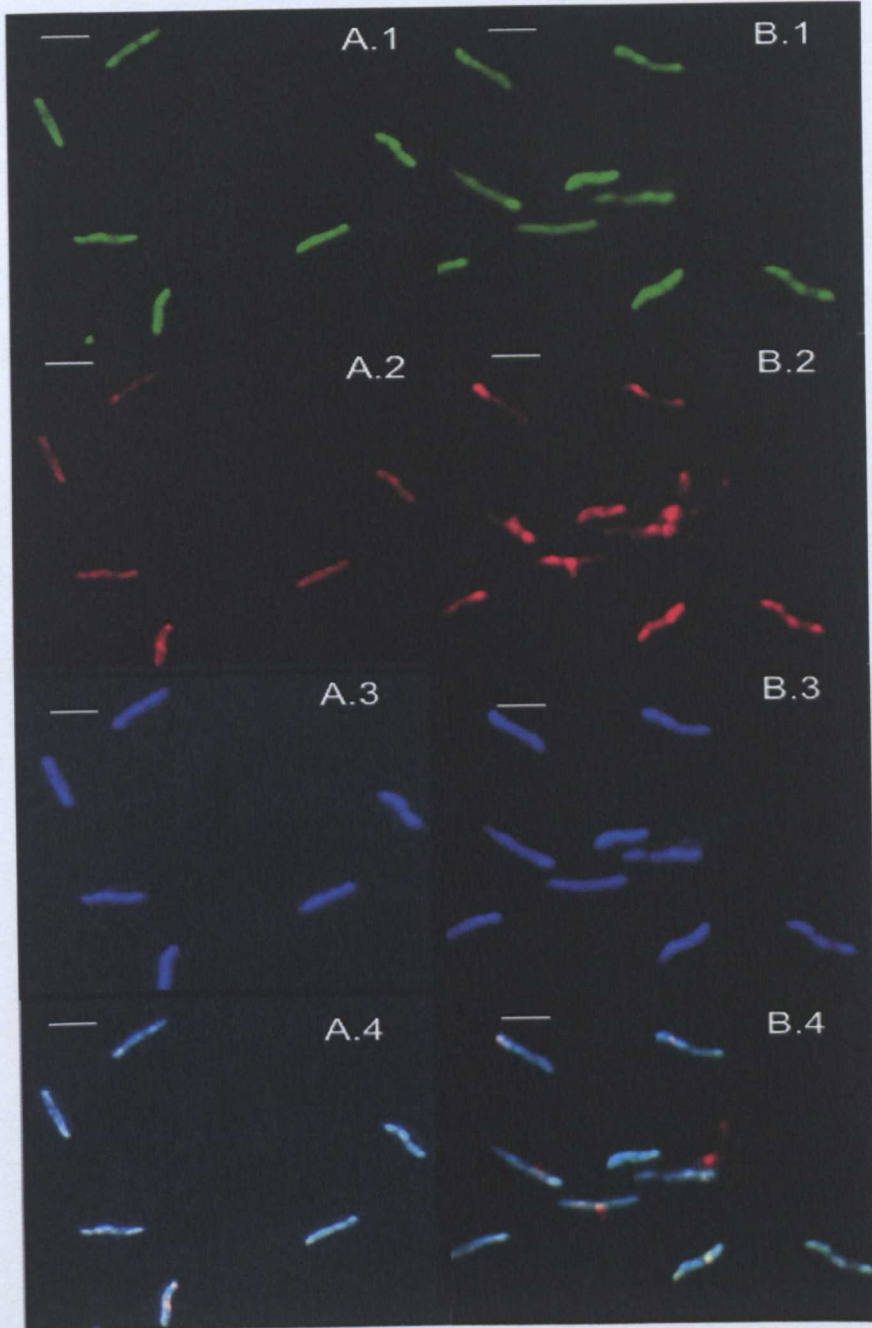


Figure 4-3 FISH localization of MNase-soluble and insoluble DNA in the sperm of *Drosophila melanogaster*. A.1: MNase-soluble DNA, A.2: MNase-insoluble DNA, A.3: DNA signal (dapi), A.4: merged. B.1: supernatant DNA of negative control (no MNase was added), B.2: pelleted DNA of negative control, B.3: DAN signal, B.4: merged.

4.3.2 Bovine sperm

4.3.2.1 Micrococcal nuclease digestion

After the purification of normal sperm by percoll gradient, three samples of about 2×10^7 sperm were separately digested by MNase. Three negative control samples were run in this experiment. DNA from all samples was purified and quantified by nanodrop spectrophotometry (for MNase digestion samples) and Nanodrop fluorospectrometry (for negative control samples). The average MNase-soluble DNA was 3.3 μg , and MNase-insoluble DNA was 205 μg . Negative control soluble DNA was about 20 ng. The P value of the difference between MNase-soluble DNA and the negative control was highly significant ($P < 0.001$).

To examine the quality of MNase digestion, all soluble, insoluble and negative control samples were run in 1% agarose (figure 4.4). The gel image clearly showed a clear difference between the soluble and insoluble DNA behaviour in the gel. While the soluble DNA ran to the bottom of the gel (between 150 - 400bp), the insoluble DNA ran slowly at the top of the gel (with size > 1000 bp). There was no signal for negative control in the gel.

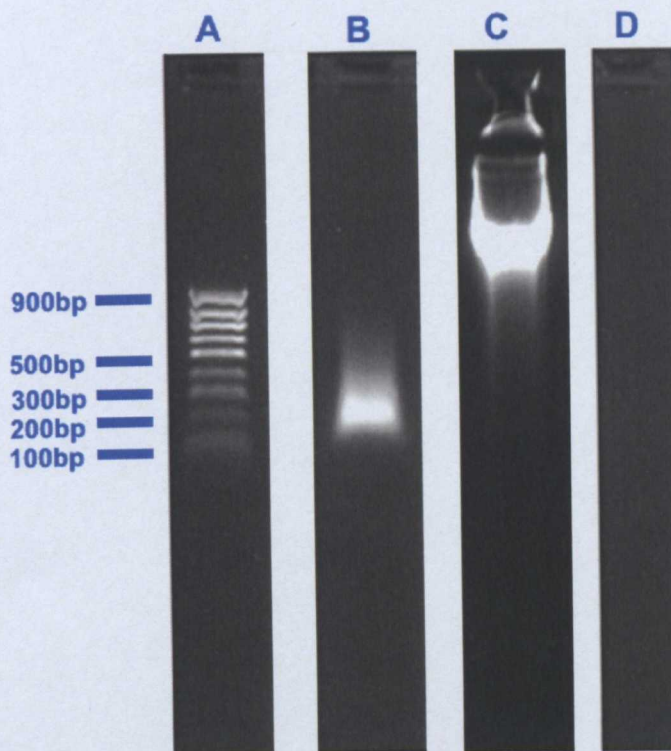


Figure 4-4 The difference between MNase-soluble and insoluble DNA of bovine sperm in 1% agarose gel. A: DNA ladder, B: MNase-soluble DNA, C: MNase-insoluble DNA, D: supernatant DNA of negative control (no MNase was added) no DNA signal .

4.3.2.2 MNase – FISH

To examine the location of MNase-soluble and insoluble DNA, the samples generated from MNase digestion were labelled and hybridized with decondensed sperm. The labelling procedure showed a clear difference in signal distribution between soluble and insoluble DNA (figure 4-5). While the soluble DNA signal was distributed through the entire nucleus with bright patches in the middle, the insoluble DNA signal was located in the middle of the nucleus. Moreover, both the soluble and insoluble DNA distribution patterns were different from the negative control signal (figure 4-5), suggesting that this DNA was specifically produced as result of MNase digestion.

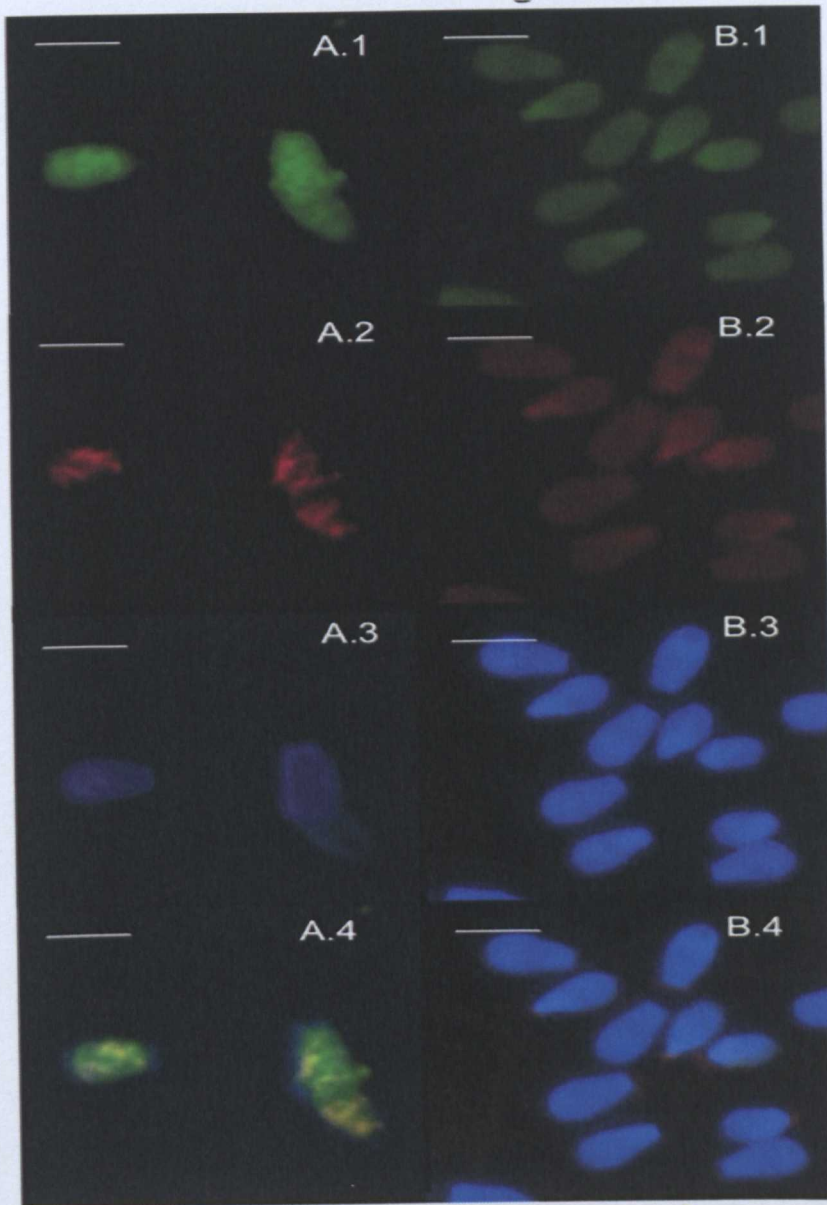


Figure 4-5 FISH localization of MNase-soluble and insoluble DNA in bovine sperm. A.1: MNase-soluble DNA, A.2: MNase-insoluble DNA, A.3: DNA signal (dapi), A.4: merged. B.1: soluble DNA of negative control (no MNase was added), B.2: insoluble DNA of negative control DNA (no MNase was added), B.3: DNA signal, B.4: merged.

4.4 Discussion

4.4.1 MNase - FISH of bovine and *Drosophila melanogaster* sperm

Previous studies indicated that MNase digests somatic cell chromatin to small fragments in periodical manner (soluble fragment sizes vary by multiples of approximately 200bp) (Felsenfeld,1978). Electron microscopy analysis revealed that these fragments have a “beads on a string” shape (a common nucleosome pattern) (Bonne-Andrea *et al.*,1984). The organization of human sperm DNA to histone or protamine bound chromatin suggests that the histone-bound DNA is in a more relaxed (open) conformation than the protamine-bound DNA. MNase was used successfully in many studies to solubilize the chromatin in the sperm from various animals including mouse (Pittoggi *et al.*,2001; Pittoggi *et al.*,1999; Saida *et al.*,2011), human (Nazarov *et al.*,2008) and zebra fish (Wu *et al.*,2011). Unlike in somatic cells, a large portion of mammalian sperm chromatin is resistant to MNase digestion (Nazarov *et al.*,2008; Saida *et al.*,2011), and the DNA released by MNase from human sperm is visualized as a nucleosomal ladder in agarose gels similar to somatic cells (Arpanahi *et al.*,2009; Nazarov *et al.*,2008).

The amount of MNase-soluble DNA in the sperm differs from species to species. While the digested DNA in human sperm was about 12% of total sperm DNA, it was very low (about 0.6%) in the mouse sperm (Arpanahi *et al.*,2009; Saida *et al.*,2011). This might be as a result of the variation of chromatin packaging between species. In this study, MNase digestion of bovine sperm nuclei produced about 1.6% soluble DNA. Unlike human sperm, this DNA gave one band with range around 200 bp in 1% agarose gel, without periodicity (figure 4-4). The size of the fragments suggested that this DNA may be generated from nucleosomes. Similar to bovine sperm, the digestion of *Drosophila melanogaster* sperm by MNase produced soluble DNA, but the amount that was produced from *Drosophila* sperm was very low (0.85%) because the original number of cells was small. This may be because the *Drosophila* sperm nucleus is less accessible to MNase than the bovine sperm nucleus. But similarly to bovine sperm the generated DNA mostly ran between 100 and 400 bp on the gel (Figures 4-2 and 4-4).

Many studies indicated that histone protein is distributed non-randomly in the sperm nucleus. Immunocytochemistry and FISH analysis of histone/ protamine distribution within the human sperm nucleus revealed that the histone protein is located in the posterior part of the nucleus, while the protamine is located throughout the whole nucleus (Li *et al.*,2008). In addition, in human sperm, the DNA precipitated by anti H4K12ac in ChIP-chip analysis showed a strong regional correlation with MNase-soluble DNA. Both regions gave similar gene ontology (Arpanahi *et al.*,2009). This result supports the idea that more relaxed sperm chromatin is enriched in nucleosomes. However, does the location of histone protein restricted to soluble chromatin? A preliminary answer was obtained in the previous chapter. Our immunocytochemistry results using anti core histone antibody clearly showed the histone signal distributed through the whole nucleus in the mouse and bovine sperm, with regional differences in the signal intensity in the middle of the nucleus. These differences might reflect a different packaging pattern of sperm chromatin (accessible and compacted regions).

In this experiment, the findings of FISH investigations in bovine sperm showed that there is a distinctive distribution between MNase soluble and insoluble DNA (figure 4-5). While the soluble DNA is located through the whole nucleus, the insoluble chromatin is distributed within strong patches in the middle of the nucleus. The location of insoluble chromatin is similar to the findings in mouse sperm (Saida *et al.*,2011) using the same techniques. However, there is a difference in the distribution of MNase-soluble DNA between bovine and mouse sperm. Whereas the probe signal is distributed through the whole nucleus in bovine sperm, it is localized to the nuclear periphery in mouse sperm (Saida *et al.*,2011). This difference might be the result of many factors. Firstly, it is possible that these sperm have a different packaging pattern. Secondly, as result of the variation in sperm structure, under the microscope we examine both sperm from different sides, with bovine sperm having an upper view, but mouse sperm having a lateral view. Thirdly, this difference may be the result of a variation in nuclear decondensation procedure or MNase protocol. Unlike bovine sperm, FISH analysis of DNA produced by MNase digestion from *Drosophila* sperm did not show any significant localization of soluble/insoluble DNA (Fig 4.3). Perhaps the fly sperm nucleus is not accessible to the MNase

enzyme, or as result of non specific DNA (DNA that did not generated by MNase) signal interferes.

A previous study showed that the chromatin sensitive to endonuclease digestion is highly enriched in binding sites for many transcription factors, such as Oct-1, Oct4, Ets-1, TBP, and C/EBP (Pittoggi *et al.*,2001). Supporting this result, recent microarray analysis revealed that MNase-soluble DNA is enriched in promoters and CTCF binding site sequences in both human (Arpanahi *et al.*,2009), and mouse sperm (Saida *et al.*,2011). Gene ontology analysis showed that soluble promoters and CTCF sequences have strong embryological significance (Arpanahi *et al.*,2009). These studies concluded that the MNase-soluble DNA in mouse and human sperm is enriched by nucleosomes and binding sites of many transcription factors, which might play roles in embryo development.

4.4.2 CHIP-FISH of histone bound DNA in *Drosophila melanogaster* sperm

In eukaryotic cells, subtle differences in the packaging of genomic DNA plays significant roles in the control of nuclear function, such as mRNA transcription, DNA replication and repair (Groth *et al.*,2007; Workman,2006). Nucleosomes are the main DNA packaging components in somatic cell chromatin organization (van Holde,1988) and are distributed through the nucleus in a regular pattern, wide genome analysis revealed that this periodical distribution (between 10-50 bp) differs from one cell type to another, or from species to species (Jiang and Pugh,2009; Rando and Chang,2009; Travers *et al.*,2010).

Unlike all other eukaryotic cells, the paternal germ cells from many species are characterized by the incomplete (in many species) elimination of nucleosome core particles from the genome during spermatozoal differentiation and maturation. The incomplete removal of nucleosomal histone differs from one species to another. For example, human sperm retain about ~10% of their chromatin in a nucleosomal conformation, while in mouse sperm, less than 2% of the chromatin remains in Hammoud, 2009; (Gatewood *et al.*,1990).

Previous investigations showed that the retained histone in the mature sperm has a regional distribution. For instance, immunolocalization analysis revealed that histone protein is greater in the posterior half of the human sperm nucleus (Li *et al.*,2008). In the same study the FISH investigation of chromosome 16

telomeric region which is enriched by H2B (Zalensky *et al.*,2002) is located within the immunofluorescent core histone signal region in the posterior half of the nucleus. The position of both signals in the same area supports the idea that histone protein is located in this region. Moreover, In mouse sperm the immunofluorescent signal of H1 is distributed peripherally around the nucleus (Pittoggi *et al.*,1999), while the core histone signal is distributed throughout the entire nucleus with strong patches in the middle of the sperm nucleus (Saida *et al.*,2011).

In previous chapter, the physical distribution of nucleohistone and nucleoprotamine compartments in the nucleus of *Drosophila melanogaster* sperm was studied by the investigation of the localization of H2Av-RFP and protamine-GRP signals through the nucleus, in addition to the immunolocalization of core histone and RFP (discussed in previous chapter). In this part of the study, the localization of histone bound DNA was investigated by the FISH technique and the results clearly showed that the nucleohistone area was located in patches throughout the nucleus (figure 4.1) which is in complete agreement with the previous result obtained by fluorescence protein and immunocytochemistry signals. All results suggest that histone bound DNA is organized into territories in the nucleus of *Drosophila melanogaster* sperm.

Chapter 5: Molecular landscape of histone protein and its modifications in the mature sperm of *Drosophila melanogaster*

5.1 Introduction

5.1.1 Histone protein occupancy in the genome

5.1.1.1 In eukaryotic cells

In eukaryotic cell, the chromatin is composed of deoxyribonucleic acid and nuclear proteins which have an influence on cell function and process such as gene activation/inactivation, DNA replication and cell differentiation (Kouzarides,2007). The nuclear protein might be classified as histone or non-histone proteins, with the histones (including their various post-translational modifications) arranged into nucleosomes. Although the nucleosomes are distributed throughout the genome, many studies have shown considerable differences in their geographical distribution and occupancy (Mavrigh *et al.*,2008; Tilgner *et al.*,2009). For example, genome wide analysis of nucleosome positioning suggests that nucleosomes mainly occupy exonic sequences rather than introns or pseudo-exons in the human and worm (*Caenorhabditis elegans*) (Tilgner *et al.*,2009). Similar to this, in the fly (*Drosophila melanogaster*) Nucleosomes containing H2A.Z are not incorporated upstream of the transcription start site, but enriched in downstream regions (Mavrigh *et al.*,2008). Moreover, modified histone analysis showed that while H3K36me3 nucleosomes are generally located in the 'body' of expressed genes (Kolasinska-Zwierz *et al.*,2009; Tilgner *et al.*,2009), H3k27me3 nucleosomes are located on silent genes (Boyer *et al.*,2006).

Nucleosomes might occupy regulatory regions in eukaryotic genomes such as: around Transcription Start Sites (TSS) (Bai *et al.*,2010; Bai and Morozov,2010; Svaren and Horz,1997), promoters (Imbalzano *et al.*,1994) and enhancers (Bonn *et al.*,2012). By this positioning, nucleosomes control gene activation/inactivation process; for example, the TATA box region in inducible promoters is frequently located immediately upstream of the TSS. The nucleosomes which normally occupy these regions during gene inactivation are removed during gene expression, exposing the TATA box to its cognate binding proteins (Bai and Morozov,2010; Imbalzano *et al.*,1994).

5.1.1.2 In sperm cell

Unlike the somatic cell, the majority of nucleosomes are removed from the sperm cell in most species and replaced with protamines (Meistrich,1989; Meistrich *et al.*,1978). Recently, micro-array analysis revealed that the majority of retained nucleosomes in human sperm are preferentially located on exons rather than introns (Nahkuri *et al.*,2009). Furthermore, immunocytochemistry and ChIP analysis showed that sperm nucleosomes are marked by many histone modifications such as H3K27me3, H3K4me3, H3K4me2 (Brykczynska *et al.*,2010; Hammoud *et al.*,2009), H3K9me (van der Heijden *et al.*,2006), H4K8ac and H4K12ac (Govin *et al.*,2007). In addition, computational analysis showed that the nucleosomes tend to favour positioning at GC-rich regions in both coding and non coding DNA (Vavouri and Lehner,2011).

Nuclease sensitive regions and microarray analysis showed that the histone protein is significantly enriched at regulatory regions with developmental function (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009). To elaborate, Hammoud *et al.*, (2009) found that endonuclease-sensitive regions in human sperm contain nucleosomes that package many gene sequences important for embryogenesis including developmental transcription factors. Moreover, they showed that histones are significantly retained at the promoters of miRNAs. Hammoud also showed that the histone variant TH2B binds to approximately 0.3% of sperm promoters, but without any significant enrichment in genes for developmental processes. Furthermore, the same study showed important localization of the modified histones H3K4me2, H3K4me3 and H3K27me3 at different developmental promoters (Hammoud *et al.*,2009).

Brykczynska *et al.*, (2010) looked at the genome wide distribution of human and mouse sperm nucleosomes, and concluded that while they are retained at regulatory regions, the retention is relatively minor. In addition, the modified histone H3K27me3 showed a strong association with TSS, but interestingly, H3K27me3 bound genes are not expressed during gametogenesis or early embryo stages. Unlike humans and mouse, zebra fish sperm DNA is organized on nucleosomes into differentially packaged domains that may be equivalent to the nucleosomal-protamine toroid of mammalian sperm (Wu *et al.*,2011). Using

this model, ChIP-chip analysis of histone bound DNA indicated that the modified histone H3K36me3 has different positions in sperm and somatic cells. While it is enriched at 3' coding region of active genes in somatic cells, it is significantly localized at the promoters of developmental genes in the sperm (Wu *et al.*,2011). This study suggested that the modified histones therefore perform different functions in the somatic and sperm cell genomes. Secondly, the result showed that the modified histones in the sperm are associated with specific gene expression at particular stages during embryo development. For example, while the H3K14ac and H3K4me2 modifications in fish sperm are located on genes expressed at early stages in embryogenesis (before mid blastula transition), the H3K27me3/ H3K4me3 are associated with genes activated after mid blastula transition (Wu *et al.*,2011). All above studies suggested that the histones retained in the mature sperm might play an epigenetic role during embryo development (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009; Wu *et al.*,2011).

5.1.1.3 The aim

The aim of this chapter is to investigate the positioning of retained nucleosomes and modified histones within the chromatin of *Drosophila melanogaster* sperm, and thereby assess its possible epigenetic role in embryo development.

5.2 Material and methods

5.2.1 Sperm sample isolation

See chapter 2 section 2.9.1.

5.2.2 Chromatin immuno-precipitation (Chip)

See chapter 2 section 2.9.

5.2.3 DNA amplification

See chapter 2 section 2.9.10.

5.2.4 Microarray hybridisation

See chapter 2 section 2.9.11.

5.2.5 Data analysis

Raw data for all ChIP runs (test and mock) were generated from scanned images using the Nimblescan software and normalised by Nimblescan software, to remove the variance between the replicates and nonspecific binding signals. Test replicates were then averaged and subtracted from the mock signal (see data analysis scheme figure 5.1). The generated normalised probe data was used directly in the genome wide analysis. Following that, the enriched intervals were extracted from normalised \log_2 ratio data by Galaxy tools (Blankenberg *et al.*,2010) according to the algorithm, $\log_2 \geq 2x$ the replicate median interval value using 3 probes as the minimum number in each interval (this minimum number covers 150 bp of DNA or a single nucleosome. The maximum distance between the probes was 45bp, and provided significantly enriched intervals with $P < .0001$, (χ^2).

The Biomart web site (Haider *et al.*,2009) and UCSC Genome Browser website (Fujita *et al.*,2011) were used as sources of *Drosophila melanogaster* genomic data (*D. melanogaster* Apr. Berkeley Drosophila Genome project, release 5 (BDGP R5/dm3)). The enriched intervals were annotated around Flybase genes (Transcription start sites, transcription termination sites, exons, introns, and promoters (Negre *et al.*,2011) part of modENCODE project). Gene ontology enrichment analysis was carried out using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7(Huang da *et al.*,2009), and the generated enriched GO terms (with $p < 0.0009$) were visualized by REVIGO web site (Supek *et al.*,2011)

5.2.6 Specificity of ChIP result

To validate the ChIP result, five enriched intervals of each ChIP precipitate were assessed by qPCR (Haring *et al.*,2007) (for qPCR procedure details see chapter 2), then the fold enrichment of each interval was calculated as follow:

$$\text{Fold enrichment above background} = 2^{(-\Delta\Delta Ct)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ IP} - \Delta Ct \text{ mock}$$

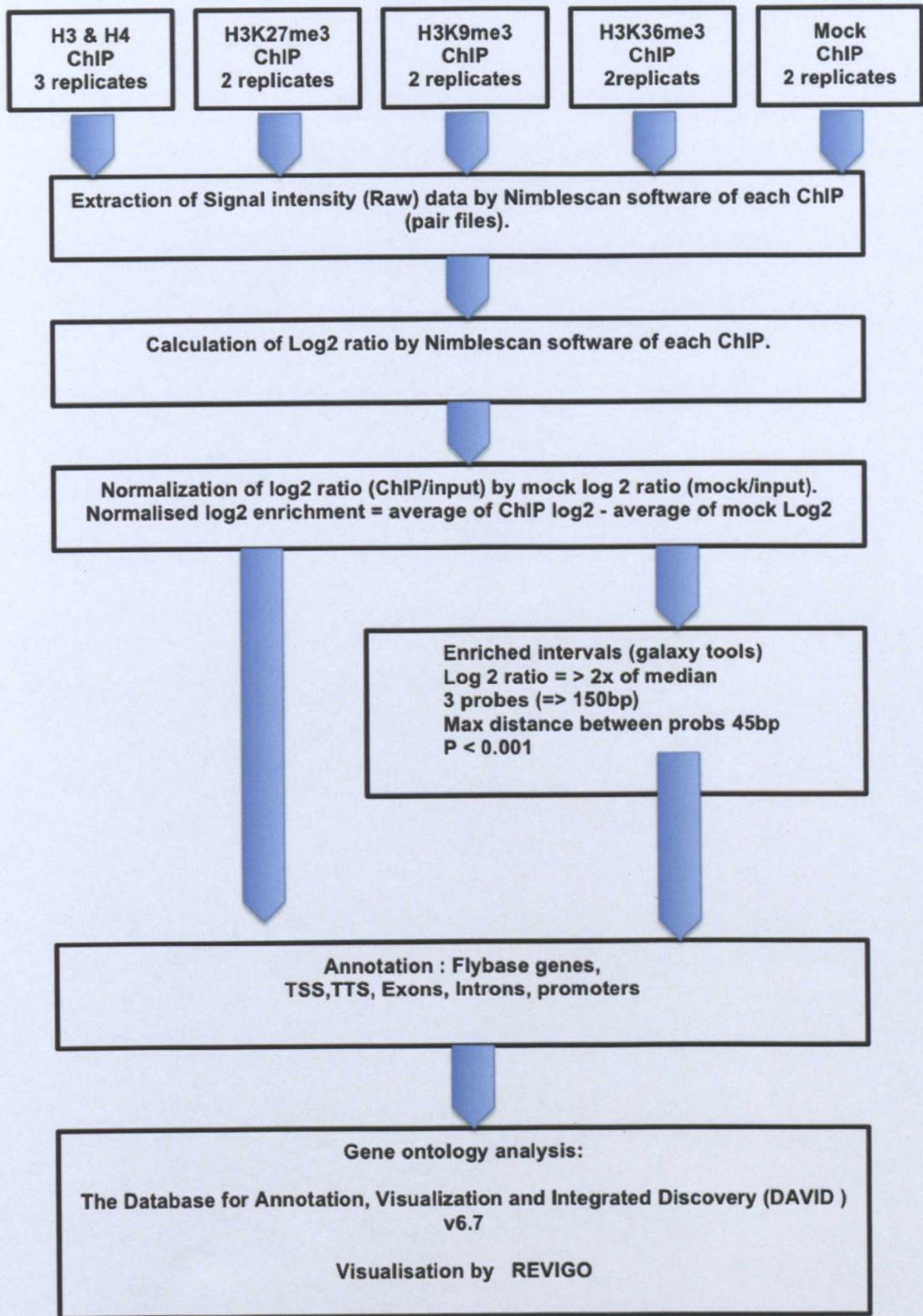


Figure 5-1 ChIP on chip data analysis scheme.

5.3 Result

5.3.1 Quality assessment

5.3.1.1 Chromatin immunoprecipitation

The precipitated DNA was quantified by Nanodrop fluorospectrometry using the Quant-iT™ PicoGreen® dsDNA protocol. See Table 5-1 for details of sample composition:

Description	Replicates	Antibody	Original DNA concentration	Chip-DNA concentration
H3&H4 ChIP	1	Anti H3 antibody	934 ng	89 pg
	2	Anti H4 antibody	905 ng	55 pg
	3	Anti H3& H4 antibodies	1002 ng	72 pg
H3K36me3 ChIP	1	Anti H3K36me3antibodies	870ng	67pg
	2	Anti H3K36me3antibodies	900ng	65pg
H3K27me3 ChIP	1	Anti H3K27me3antibodies	892ng	54pg
	2	Anti H3K27me3antibodies	970ng	86pg
H3K9me3 ChIP	1	Anti H3K9me3antibodies	954ng	49pg
	2	Anti H3K9me3antibodies	907ng	52pg
Mock ChIP	1	Secondary antibody (IgG)	750 ng	12 pg
	2	Secondary antibody (IgG)	800 ng	18 pg

Table 5-1 Precipitated DNA in ChIP replicates.

5.3.1.1 Microarray data

The quality of microarray data was assessed by generating a spearman correlation and scatter plot of NimbleScan generated log₂ ratio data (not normalised by mock) of the replicates in each ChIP (table 5.2) (for scatter plot see appendix 9.1). The visualisation of normalised averaged log₂ values in the UCSC Genome Browser showed that the replicates of H3 &H4 replicates have similar overlap (figure 5.2), and were overlapped with modified histone peaks (figure 5.3). Five enriched intervals of each ChIP precipitate were assessed by qPCR, and the calculation of fold enrichment of these intervals showed important enrichment of these regions in comparison with mock DNA, and the range of fold enrichment was between 2.8 and 7.8. (appendix 9.2).

ChIP	Replicates	Spearman correlation
H3 & H4	H3 vs H4	0.93
H3K27me3	Replicate 1 vs replicate 2	0.73
H3K36me3	Replicate 1 vs replicate 2	0.65
H3K9me3	Replicate 1 vs replicate 2	0.82

Table 5-2 Spearman correlation between ChIP replicates.

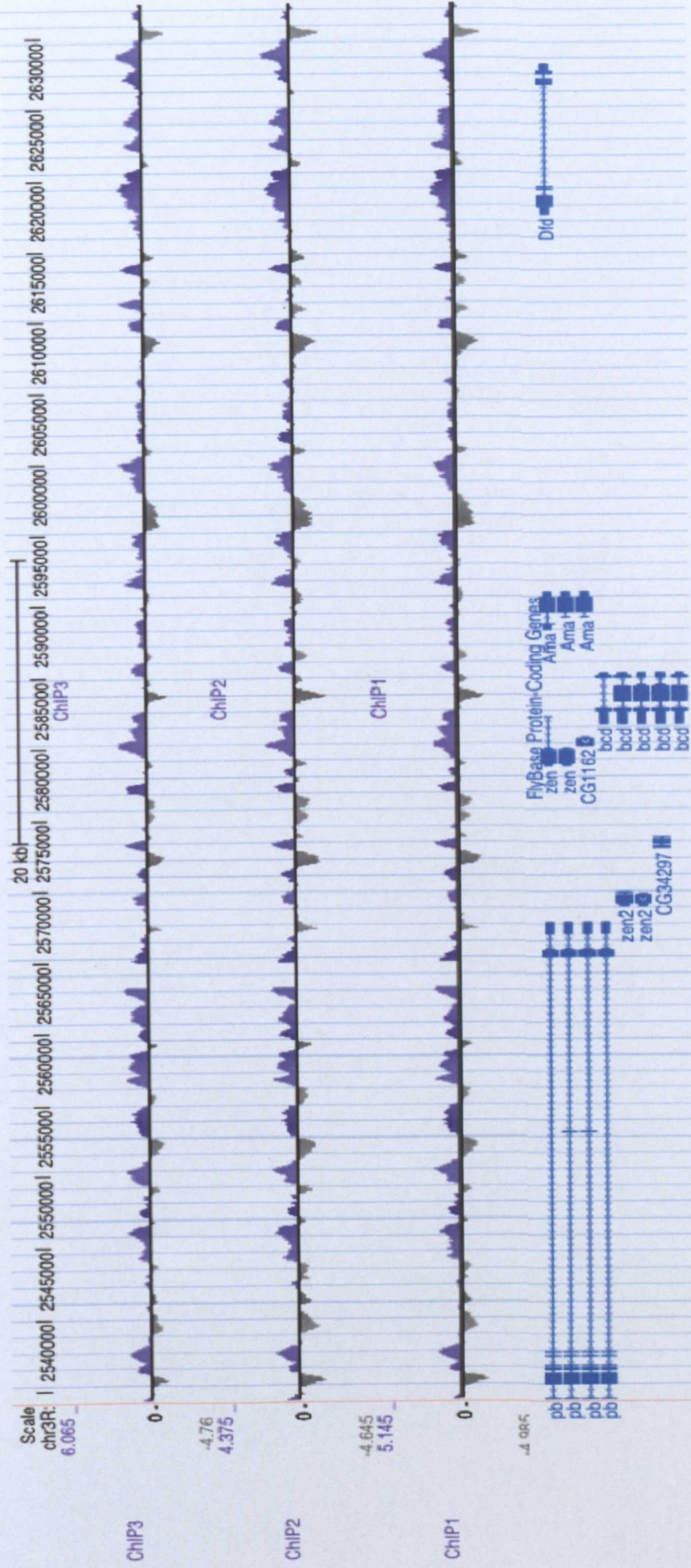


Figure 5-2 \log_2 ratio of H3&H4 replicates visualisation in UCSC Browser (red boxes show the overlap between replicates).

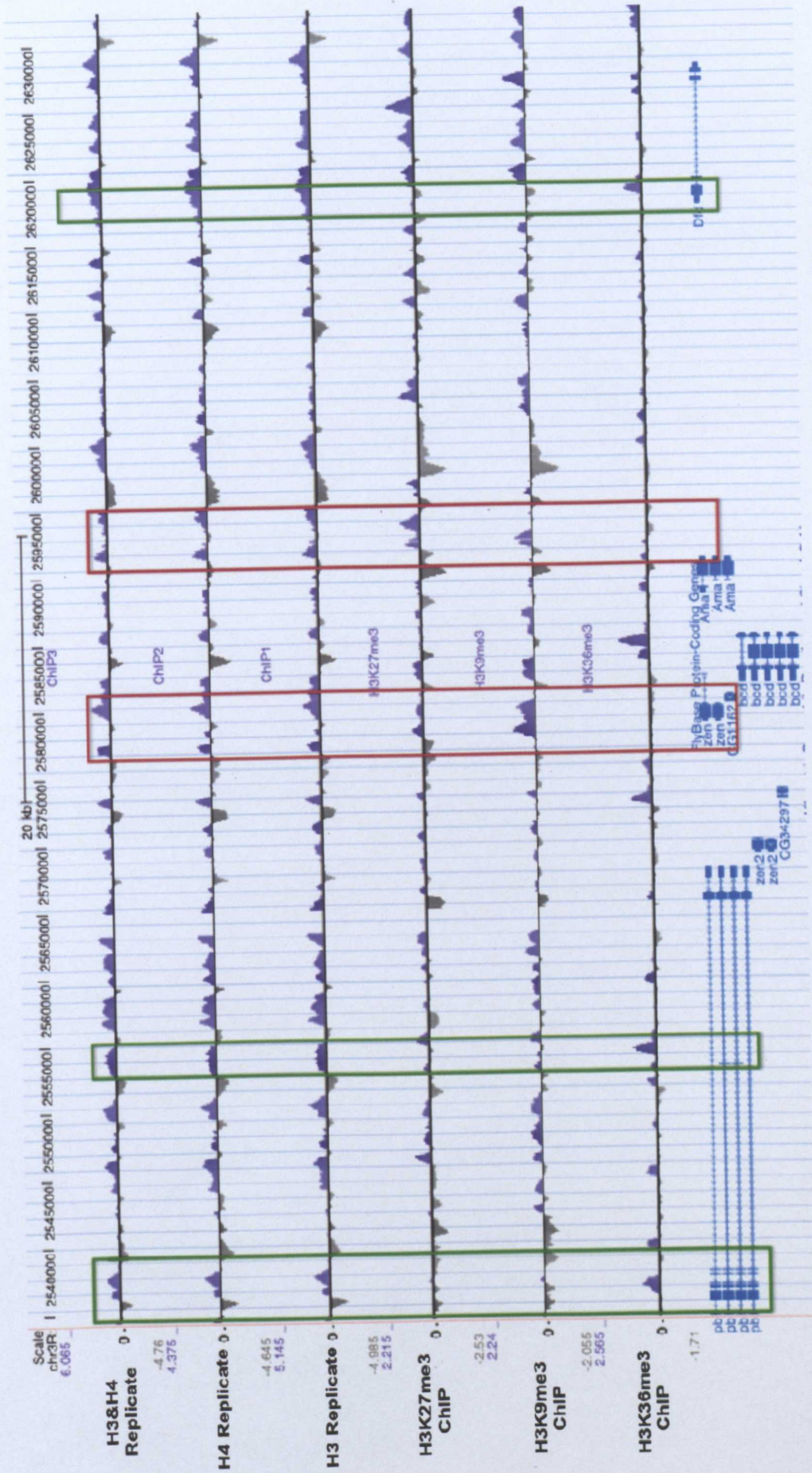


Figure 5-3 The overlapping of H3&H4 ChIP replicates with modified histones ChIP. (red boxes show the overlap between H3&H4 ChIP with H3K27me3 and H3K9me3, green boxes show the overlap between H3&H4 ChIP with H3K36me3).

5.3.2 Chromosomes packaging by nucleosomes in the sperm

Nucleosome retention in fly sperm was analysed using the averaged H3 and H4 ChIP data, and uploading the normalised \log_2 ratio file to the USCS browser. Statistical analysis showed that (as expected) the majority of nucleosomes are removed from the genome of *Drosophila melanogaster* sperm (the average \log_2 ratio <0 ; Figures 5.4 and 5.5), and the histone signal showed that nucleosomes are scattered throughout the genome with little enrichment in particular regions (figure 5.6). However, the analysis revealed that the individual sperm chromosomes have different nucleosomes retention patterns, and each single chromosome is not uniformly packaged, and the X chromosome is exceptionally histone poor chromosome (figures 5.5, 5.6)

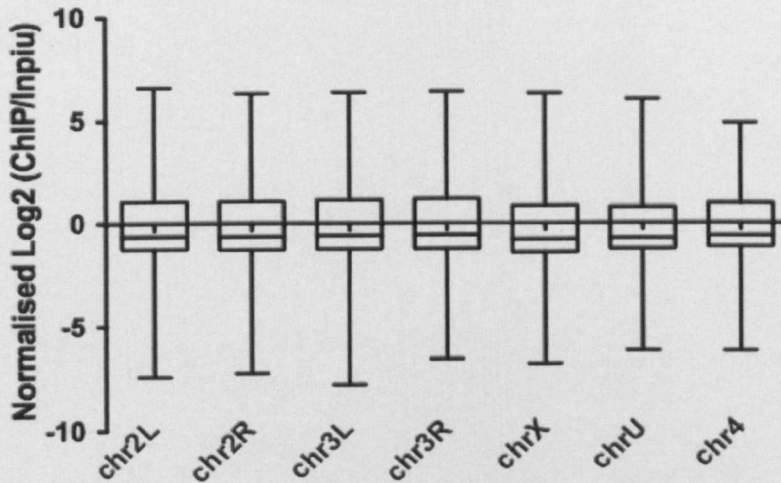


Figure 5-4 Plot of H3-H4 abundance in fly sperm chromosomes.

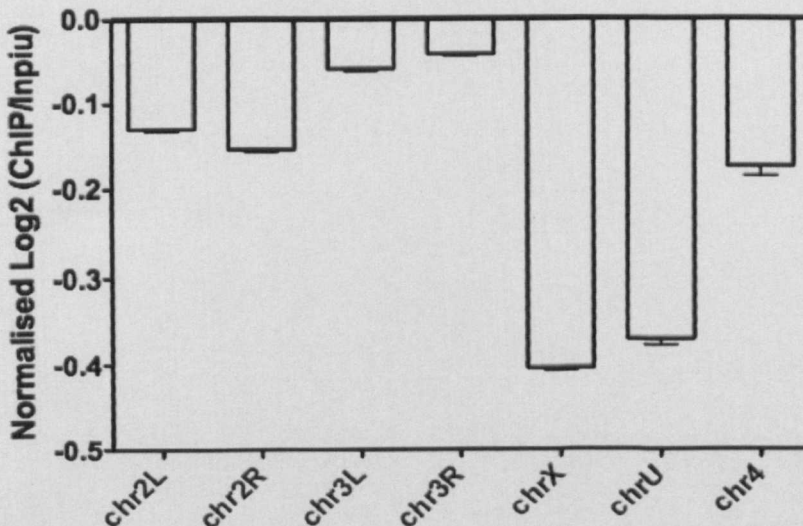


Figure 5-5 Averaged levels of H3-H4 abundance in fly sperm chromosomes

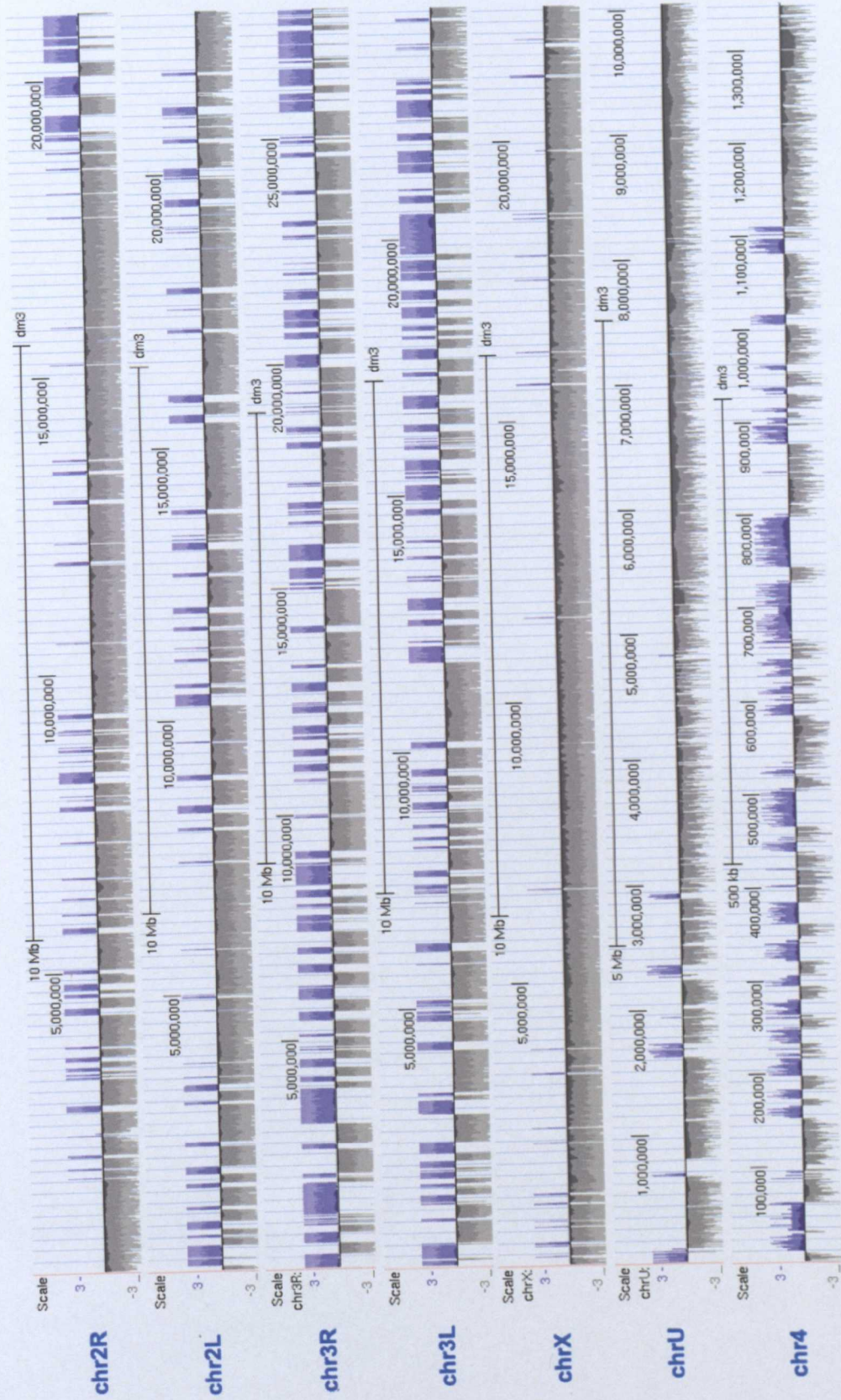


Figure 5-6 Normalised log₂ ratio data in UCSC browser shows regions enriched in nucleosomes in the fly sperm chromosomes (purple bars = the average of log₂ ratio above zero (histone enrichment), grey bars = the average of log₂ ratio below zero (histone depletion)).

5.3.3 Location of nucleosomes on exon and intron

The result of this study showed that average nucleosome signals (H3 and H4) located on the exons of *Drosophila melanogaster* sperm DNA are sharply and statistically significantly ($p < 0.0001$) higher than on introns (figures 5.7, 5.8, 5.9, 5.10, 5.11). On other hand, single chromosome analysis indicated that the X chromosome is significantly depleted of nucleosomes even in exons ($P < 0.0001$) in comparison to other chromosomes (figure 5.10). The modified histones showed different distribution patterns on exons and introns. While H3K9me3 has a similar distribution to H3 and H4, H3K36me3 and H3K27me3 are generally depleted in both regions (figure 5.7). Gene ontology analysis of H3&H4 enriched intervals on exons showed that the top significant enrichment terms are for ATP and DNA binding, but that genes for developmental processes are also enriched (table 5.3 and figure 5.12) (please see appendix 9.4 for full gene ontology list). Moreover, the enriched exon intervals containing modified histones (H3K36me3 and H3K27me3) showed similarity in many developmental GO terms, but also many distinctive developmental functions (tables 5.4, 5.5 and figure 5.13).

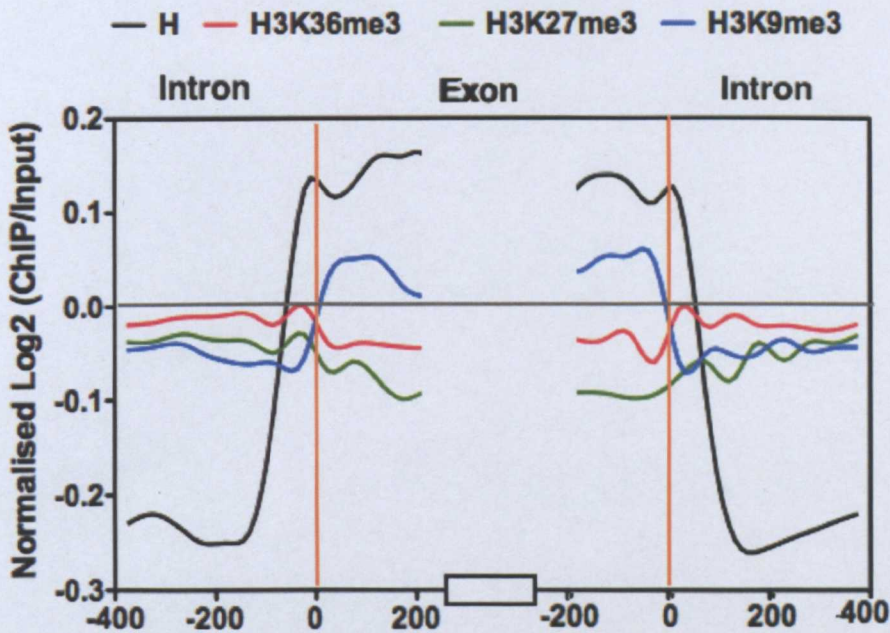


Figure 5-7 Distribution of nucleosomes and modified histones around intron /exon boundaries.

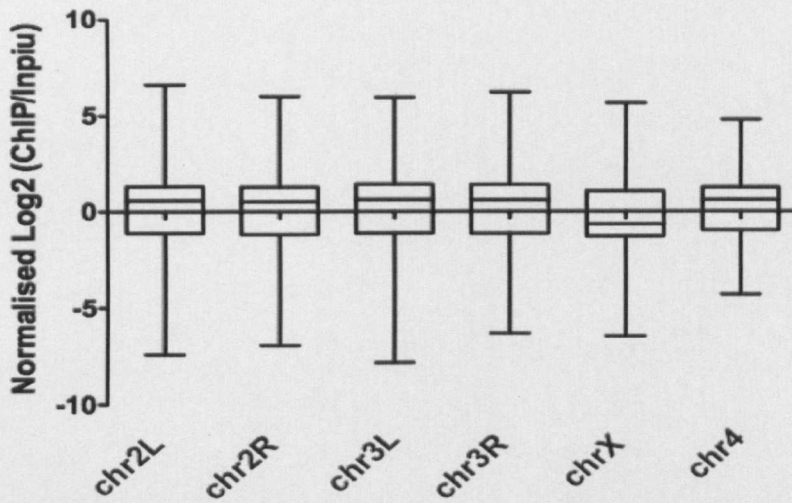


Figure 5-8 Nucleosome packaging in the exons of fly sperm chromosomes. The median (solid black bar), upper and lower quartile values are indicated.

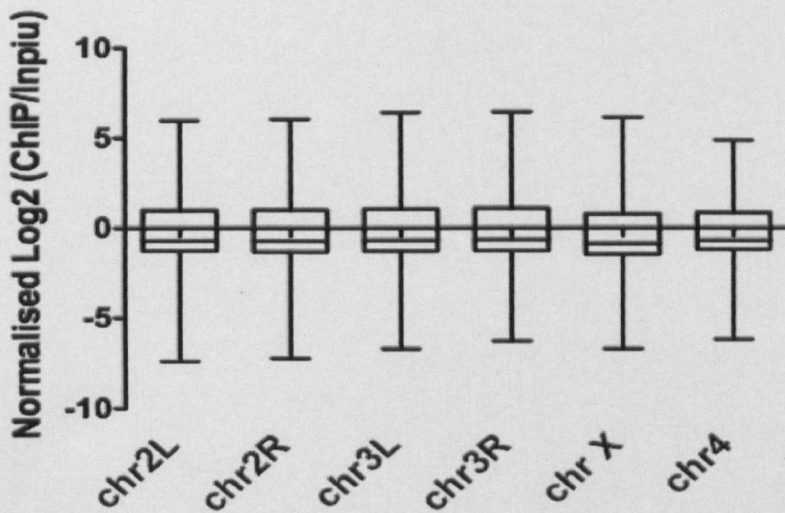


Figure 5-9 Nucleosome packaging in the introns of fly sperm chromosomes. The median (solid black bar), upper and lower quartile values are indicated.

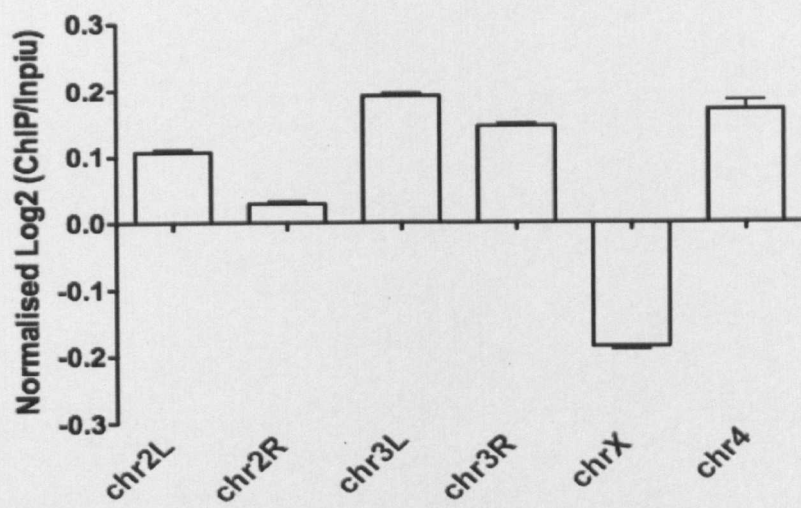


Figure 5-10 Averaged nucleosome occupancy in the exons of fly sperm chromosomes.

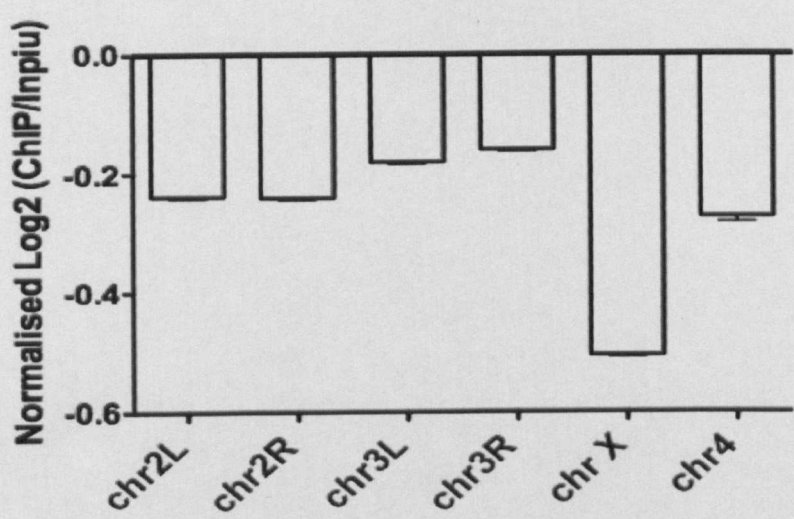


Figure 5-11 Averaged nucleosome occupancy in the introns of fly sperm chromosomes.

Top enriched term

Category	Term	Count	%	PValue	Fold Enrichment	Benjamini	FDR
GOTERM_MF_FAT	GO:0005524~ATP binding	462	7.51	2.12E-16	1.30	3.64E-13	3.77E-13
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	462	7.51	4.21E-16	1.29	3.64E-13	7.44E-13
GOTERM_MF_FAT	GO:0001882~nucleoside binding	499	8.11	2.03E-15	1.27	1.09E-12	3.35E-12
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	492	8.00	2.98E-15	1.27	1.23E-12	5.03E-12
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	493	8.01	7.77E-15	1.27	2.55E-12	1.31E-11
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	533	8.66	2.78E-11	1.21	7.59E-09	4.66E-08
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	533	8.66	2.78E-11	1.21	7.59E-09	4.66E-08
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	565	9.18	7.58E-11	1.20	1.78E-08	1.27E-07
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	722	11.7	2.32E-09	1.14	1.51E-06	3.46E-06
Developmental enriched terms							
GOTERM_BP_FAT	GO:0000902~cell morphogenesis	244	3.96	7.05E-09	1.31	1.20E-05	1.29E-05
GOTERM_BP_FAT	GO:0048666~neuron development	188	3.05	2.47E-06	1.29	0.001	0.004
GOTERM_BP_FAT	GO:0007411~axon guidance	83	1.35	9.35E-06	1.45	0.002	0.017
GOTERM_BP_FAT	GO:0006928~cell motion	165	2.68	1.22E-05	1.29	0.003	0.022
GOTERM_BP_FAT	GO:0000904~cell morphogenesis involved in differentiation	164	2.66	1.25E-05	1.29	0.003	0.022
GOTERM_BP_FAT	GO:0035215~genital disc development	24	0.39	1.46E-05	2.04	0.003	0.026
GOTERM_BP_FAT	GO:0030182~neuron differentiation	213	3.46	1.80E-05	1.24	0.003	0.032
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	136	2.21	2.51E-05	1.31	0.003	0.045

Table 5-3 The top enriched terms generated from enriched exon-containing nucleosome intervals following gene ontology analysis.

Category	Term	Count	%	PValue	Fold	Benjamini	FDR
GOTERM_BP_FAT	GO:0000902~cell morphogenesis	72	72	1.84E-07	1.8	4.04E-04	3.20E-04
GOTERM_BP_FAT	GO:0000904~cell morphogenesis and differentiation	53	53	1.18E-06	1.1	0.001	0.002
GOTERM_BP_FAT	GO:0030182~neuron differentiation	65	65	1.97E-06	1.9	0.001	0.003
GOTERM_BP_FAT	GO:0048858~cell projection morphogenesis	54	54	2.86E-06	2.8	0.001	0.004
GOTERM_BP_FAT	GO:0048667~neuron differentiation	50	50	3.15E-06	3.1	0.001	0.005
GOTERM_BP_FAT	GO:0048666~neuron development	57	57	3.40E-06	3.3	0.001	0.005
GOTERM_MF_FAT	GO:0005524~ATP binding	104	104	3.66E-06	3.6	0.002	0.005
GOTERM_BP_FAT	GO:0030030~cell projection organization	59	59	3.74E-06	3.7	0.001	0.006
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	104	104	4.01E-06	4.0	0.001	0.006
GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	76	76	4.96E-06	4.9	0.001	0.008
GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	76	6.5	4.96E-06	1.6	0.001	0.008
GOTERM_BP_FAT	GO:0048812~neuron projection morphogenesis	49	4.2	5.87E-06	1.9	0.001	0.010
GOTERM_BP_FAT	GO:0031175~neuron projection development	49	4.2	6.42E-06	1.9	0.001	0.011
GOTERM_MF_FAT	GO:0001882~nucleoside binding	111	9.5	7.07E-06	1.4	0.001	0.010
GOTERM_BP_FAT	GO:0032990~cell part morphogenesis	54	4.6	7.95E-06	1.8	0.001	0.013
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	109	9.4	1.02E-05	1.4	0.002	0.015
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	109	9.4	1.30E-05	1.4	0.002	0.019
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	43	3.7	1.62E-05	1.9	0.002	0.028
GOTERM_BP_FAT	GO:0048569~post-embryonic organ development	49	4.2	1.67E-05	1.8	0.002	0.029
GOTERM_BP_FAT	GO:0002009~morphogenesis of an epithelium	40	3.4	2.07E-05	2.0	0.003	0.035
GOTERM_BP_FAT	GO:0007444~imaginal disc development	62	5.3	2.07E-05	1.7	0.003	0.035

Table 5-4 The enriched gene ontology terms of H3K36me3 exon intervals.

Category	Term	Count	%	PValue	Fold	Benjamini	FDR
GOTERM_MF_FAT	GO:0005524~ATP binding	97	10.6	2.42E-08	2.4	1.75E-05	3.67E-05
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	97	10.6	2.80E-08	2.8	1.01E-05	4.25E-05
GOTERM_MF_FAT	GO:0001882~nucleoside binding	102	11.2	1.08E-07	1.0	2.60E-05	1.64E-04
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	101	11.0	1.30E-07	1.3	2.36E-05	1.97E-04
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	100	10.9	1.91E-07	1.9	2.75E-05	2.89E-04
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	109	11.9	3.97E-07	3.9	4.78E-05	6.01E-04
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	109	11.9	3.97E-07	3.9	4.78E-05	6.01E-04
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	112	12.3	2.21E-06	2.2	2.28E-04	0.003
GOTERM_MF_FAT	GO:0030695~GTPase regulator activity	28	3.07	4.15E-06	4.1	3.75E-04	0.006
GOTERM_MF_FAT	GO:0060589~nucleoside-triphosphatase	28	3.07	6.26E-06	6.2	5.03E-04	0.009
GOTERM_BP_FAT	GO:0030030~cell projection organization	48	5.27	1.06E-05	1.0	0.020	0.018
GOTERM_BP_FAT	GO:0030182~neuron differentiation	52	5.71	1.11E-05	1.1	0.010	0.018
GOTERM_BP_FAT	GO:0048858~cell projection morphogenesis	43	4.72	2.01E-05	2.0	0.012	0.034
GOTERM_BP_FAT	GO:0032990~cell part morphogenesis	44	4.83	2.06E-05	2.0	0.009	0.035
GOTERM_BP_FAT	GO:0007409~axonogenesis	31	3.40	2.13E-05	2.1	0.008	0.036
GOTERM_MF_FAT	GO:0000166~nucleotide binding	126	13.8	2.62E-05	2.6	0.001	0.039
GOTERM_BP_FAT	GO:0048666~neuron development	45	4.94	3.02E-05	3.0	0.009	0.05

Table 5-5 The enriched gene ontology terms of H3K27me3 exon intervals.

Category	Term	Count	%	PValue	Fold	Benjamini	FDR
GOTERM_MF_FAT	GO:0042624~ATPase activity, uncoupled	42	4.57	5.00E-10	4.9	3.55E-07	7.55E-07
GOTERM_MF_FAT	GO:0001882~nucleoside binding	107	11.6	6.65E-09	6.6	2.36E-06	1.01E-05
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	106	11.5	8.04E-09	8.0	1.91E-06	1.21E-05
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	105	11.4	1.22E-08	1.2	2.17E-06	1.84E-05
GOTERM_MF_FAT	GO:0005524~ATP binding	98	10.6	2.18E-08	2.1	3.11E-06	3.30E-05
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	98	10.6	2.54E-08	2.5	3.01E-06	3.84E-05
GOTERM_MF_FAT	GO:0016887~ATPase activity	55	5.98	2.51E-07	2.5	2.55E-05	3.79E-04
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	115	12.5	7.27E-07	7.2	6.46E-05	6.458
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	108	11.7	1.32E-06	1.3	1.04E-04	0.000
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	108	11.7	1.32E-06	1.3	1.04E-04	0.000
GOTERM_MF_FAT	GO:0042623~ATPase activity, coupled	47	5.11	3.62E-06	3.6	2.58E-04	0.000
GOTERM_MF_FAT	GO:0000166~nucleotide binding	129	14.0	1.15E-05	1.1	7.40E-04	0.000
GOTERM_MF_FAT	GO:0008092~cytoskeletal protein binding	36	3.91	1.20E-05	1.2	7.12E-04	0.000
GOTERM_CC_FAT	GO:0044430~cytoskeletal part	44	4.78	1.36E-05	1.3	0.005	0.005
GOTERM_MF_FAT	GO:0043167~ion binding	175	19.0	2.42E-05	2.4	0.001	0.001
GOTERM_MF_FAT	GO:0043169~cation binding	174	18.9	2.77E-05	2.7	0.001	0.001

Table 5-6 The enriched gene ontology terms of H3K9me3 exon intervals.



Figure 5-12 Relationship showing the similarity (correlation) of top biological process terms ($P < 0.0009$) of nucleosomes from enriched exons

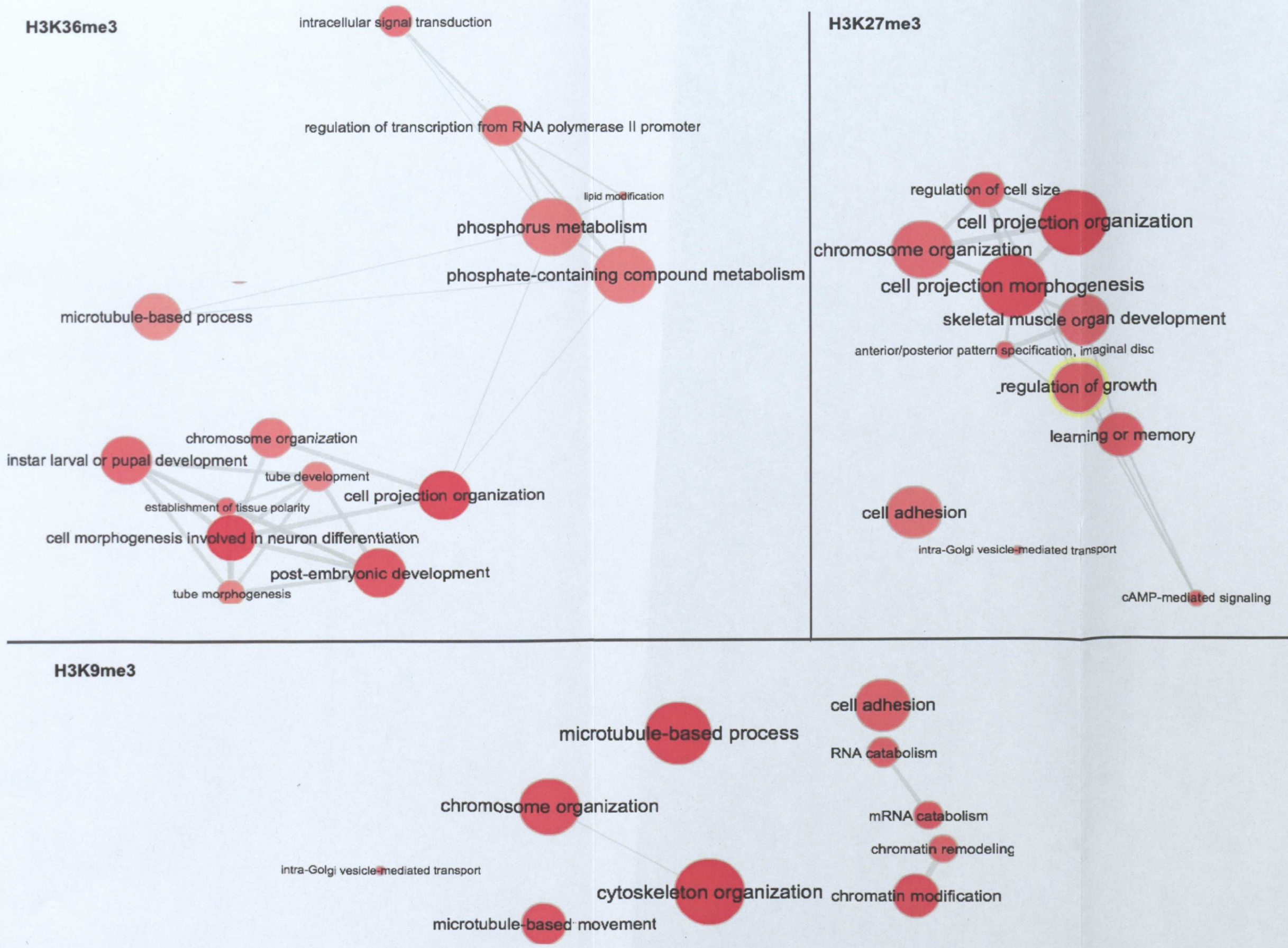


Figure 5-13 Relationship showing the similarity (correlation) of top biological process terms ($P < 0.0009$) of H3K36me3, H3K27me3 and H3K9me3 enriched exons.

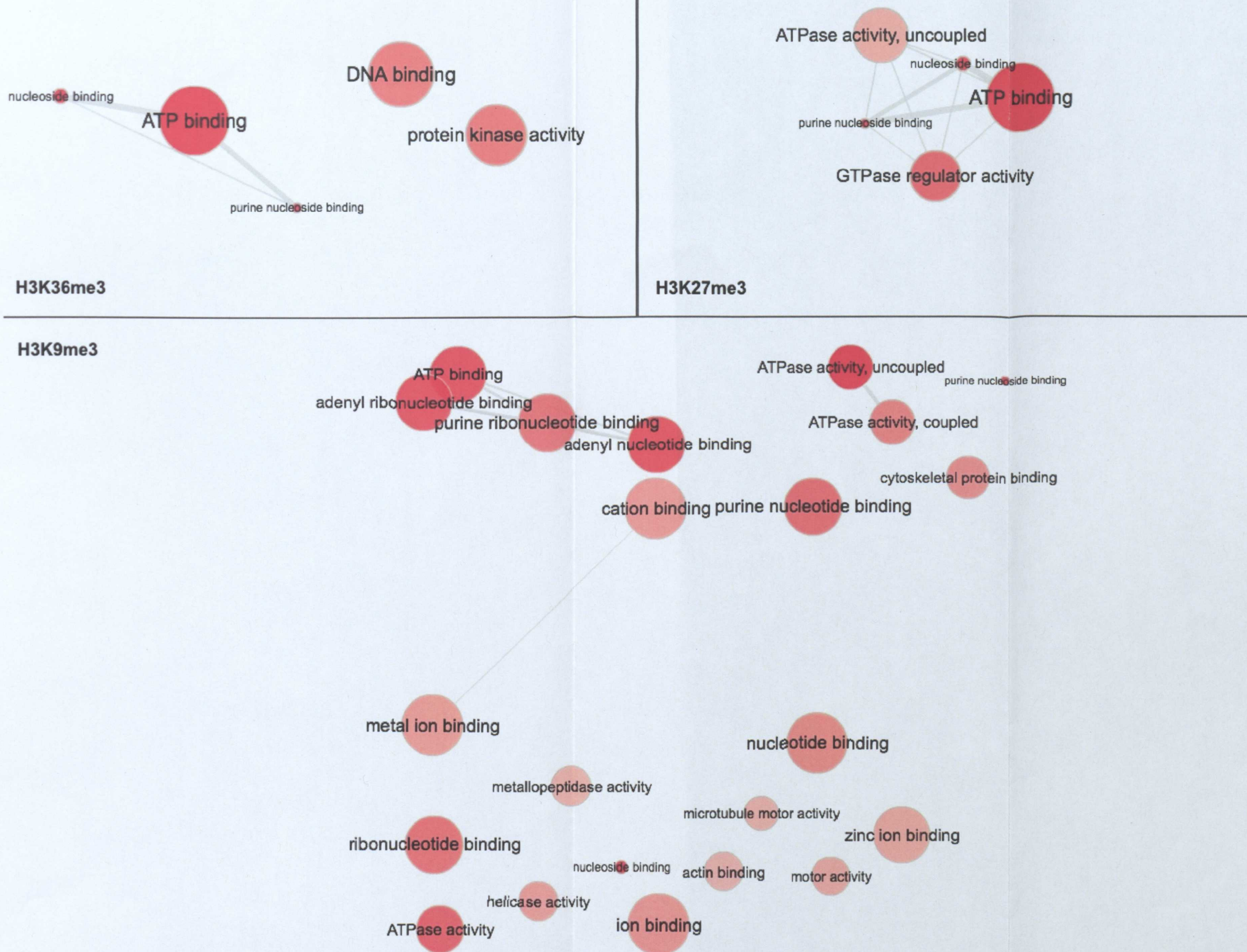


Figure 5-14 Relationship showing the similarity (correlation) of top molecular function terms ($P < 0.0009$) of H3K36me3, H3K27me3 and H3K9me3 enriched exons

5.3.4 Nucleosome occupancy in intron-exon boundaries

Although the nucleosome signals (H3 and H4) are depleted in intronic sequences, they are increased dramatically ($p < 0.0001$) (t test) at both 5' and 3' ends of the intron (100bp distant from the exon start), where signals are clearly distinguishable from the exon signal (figure 5. 7) and the positioning of peak centre is 0bp from both sides. In other hand, the modified histone H3K9me3 which is enriched on exon did not show the same figure around 0bp of intron-exon boundaries (figure 5. 7).

5.3.5 Nucleosomes occupancy in Transcription start sites (TSS) and Transcription termination sites (TTS)

In general, the plotting of \log_2 ratio of normalised probes showed significant depletion of nucleosomes in both TSS and TTS regions (figures 5.15). However, the enriched interval analysis around TSS (± 200 bp) revealed that some TSS regions are occupied by nucleosomes and modified histones (figure 5.16). Gene ontology analysis of these enriched regions did not show any significant GO terms.

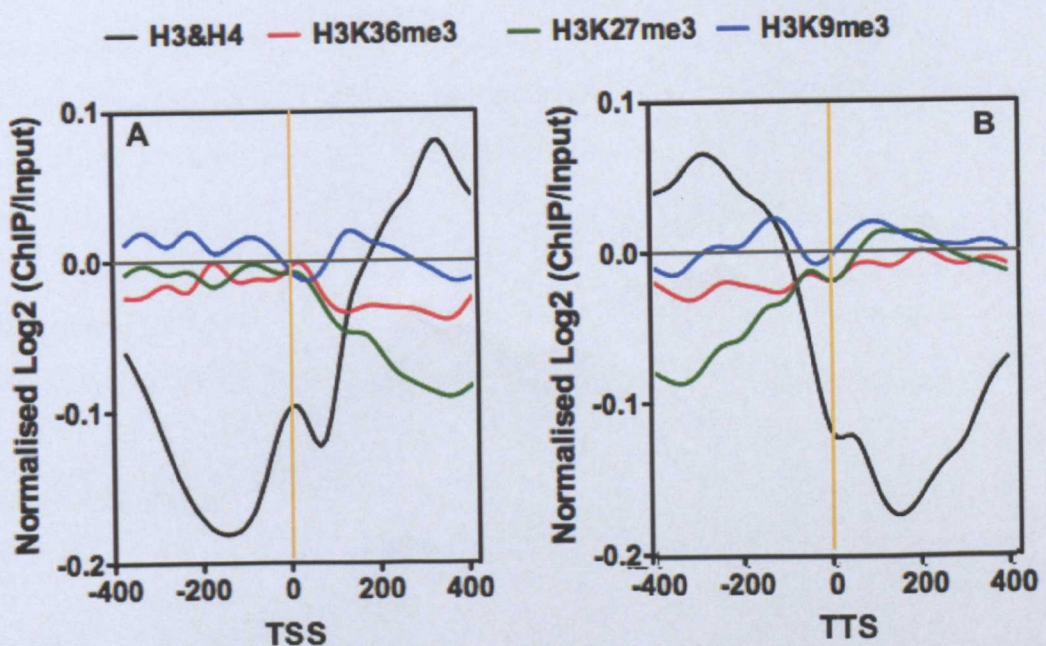


Figure 5-15 Distribution of nucleosomes and modified histones around the TSS and TTS.

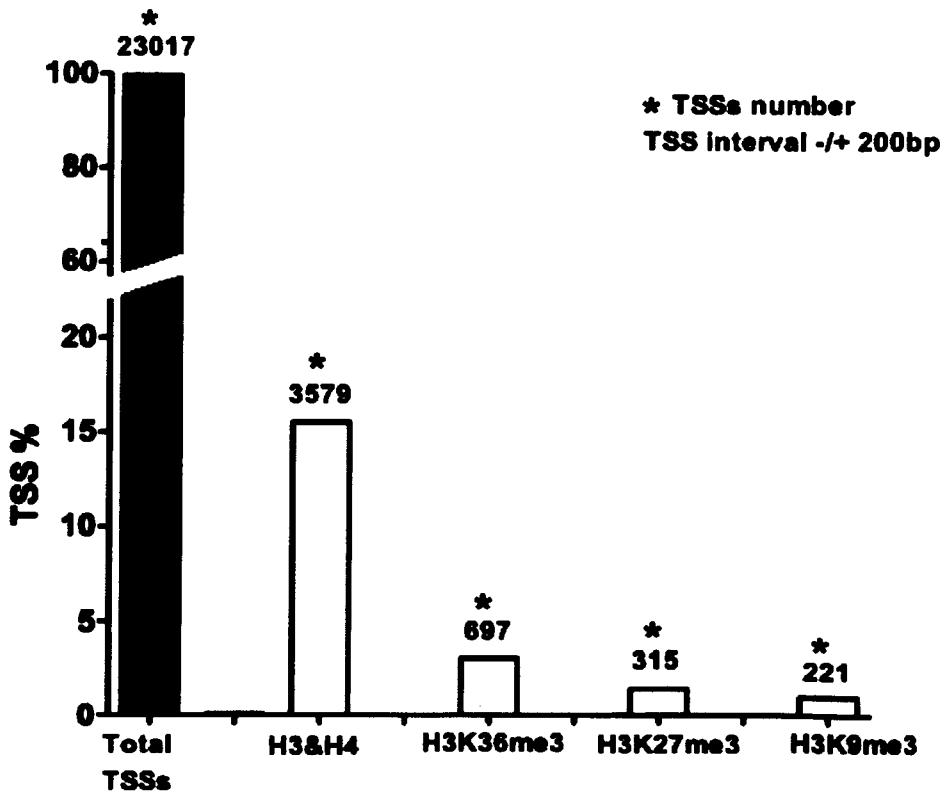


Figure 5-16 Nucleosome occupancy and histone modifications around TSSs (-/ + 200bp)

5.3.6 Nucleosomes occupancy in promoters

Based on recent analysis by modENCODE project group, *Drosophila melanogaster* genome has approximately 23017 TSS; however, only 8420 of the corresponding promoters for these TSSs have been identified and validated by RNA sequencing analysis. Moreover, the analysis revealed that a high proportion (approximately 27%) of these promoters in fly sperm chromatin are occupied by nucleosomes (figure 5.17). Gene ontology (GO) analysis revealed that these promoters have significantly enriched GO functions such as ATP binding and chromosomes organisation (table 5.7; figure 5.18; appendix 9.5). On other hand, the modified histones (H3K36me3, H3K27me3 and H3K9me3) showed only minor association with promoters (6.1%, 2.5%, 1.9% respectively) (figure 5.17) and without significant GO enrichment.

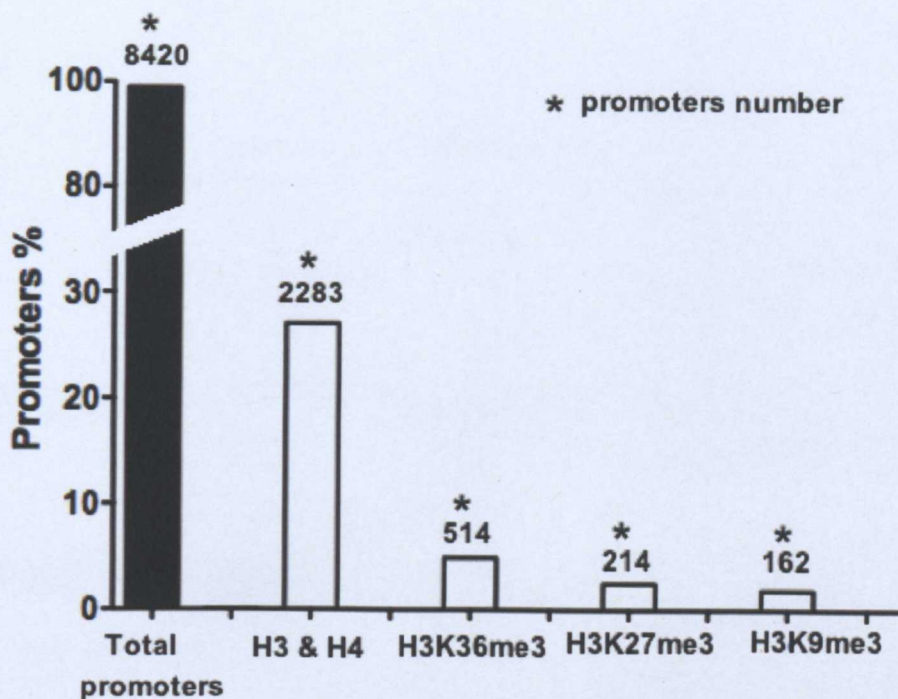


Figure 5-17 Promoters occupancy with nucleosomes and its modification.

Term	%	PValue	FDR
GO:0000166~nucleotide binding	13.7	3.38E-12	5.44E-09
GO:0000278~mitotic cell cycle	4.88	9.93E-12	1.77E-08
GO:0032555~purine ribonucleotide binding	10.9	1.89E-11	3.04E-08
GO:0032553~ribonucleotide binding	10.9	1.89E-11	3.04E-08
GO:0043933~macromolecular complex subunit organization	3.98	1.05E-10	1.87E-07
GO:0051276~chromosome organization	3.98	1.31E-10	2.32E-07
GO:0017076~purine nucleotide binding	11.4	1.41E-10	2.27E-07
GO:0005524~ATP binding	9.00	3.87E-10	6.22E-07
GO:0008104~protein localization	5.01	4.87E-10	8.66E-07
GO:0032559~adenyl ribonucleotide binding	9.00	5.01E-10	8.05E-07
GO:0007051~spindle organization	3.30	9.50E-10	1.69E-06
GO:0043228~non-membrane-bounded organelle	10.5	1.65E-09	2.38E-06
GO:0043232~intracellular non-membrane-bounded organelle	10.5	1.65E-09	2.38E-06
GO:0000226~microtubule cytoskeleton organization	3.98	3.28E-09	5.85E-06
GO:0007052~mitotic spindle organization	2.91	3.41E-09	6.07E-06

Table 5-7 Top gene ontology terms of nucleosome enriched promoters.

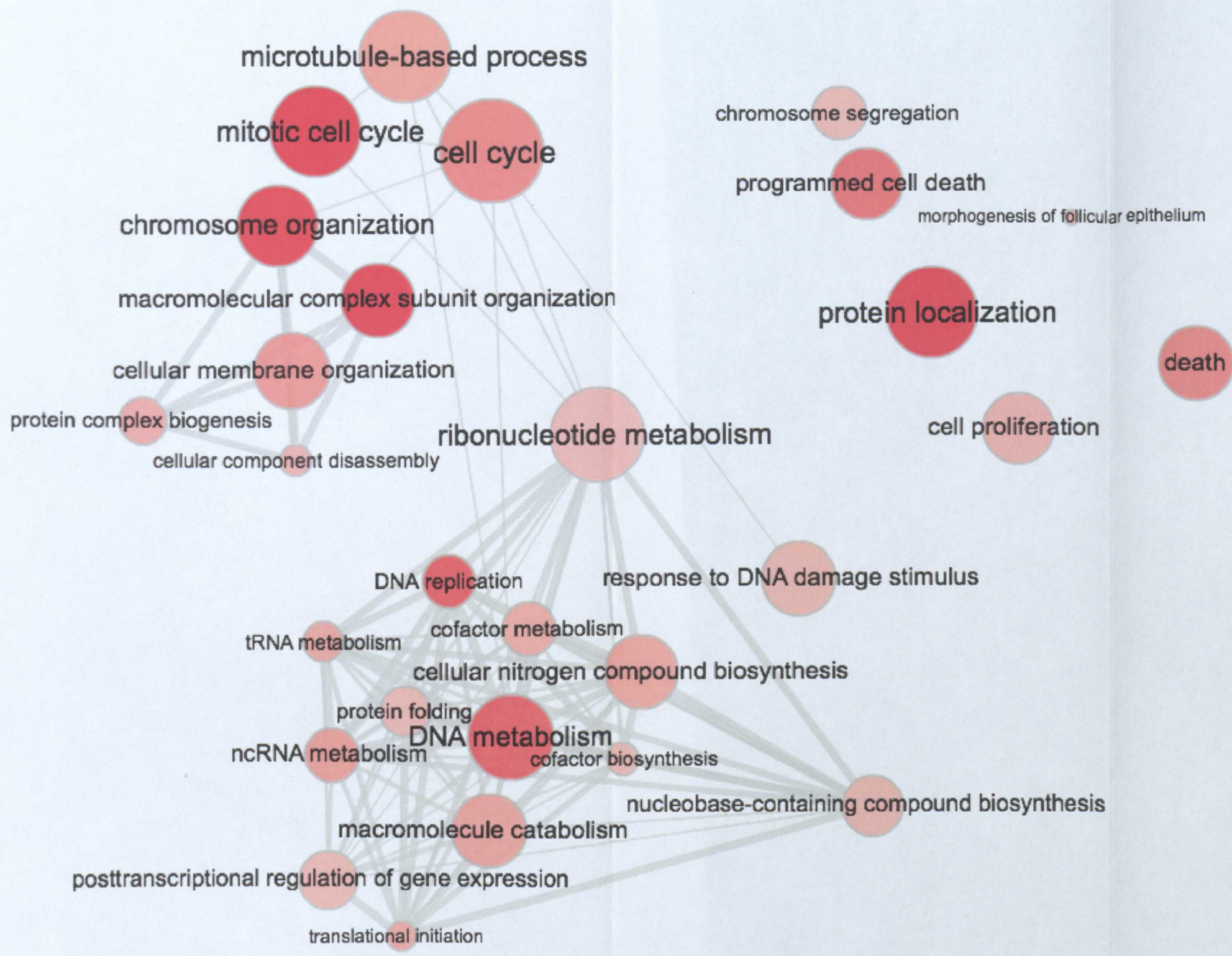


Figure 5-18 Relationship showing the similarity (correlation) of top biological process terms ($P < 0.0009$) of nucleosomes enriched promoters.

5.4 Discussion

5.4.1 Chromosomes packaging

The genome of the mature spermatozoa of the majority of mammals differs from that of their somatic cells in that almost all of the paternal DNA is packaged by protamines, with some residual nucleosomes remaining (estimated at 1- 10%, depending on species). The ChIP on chip results of this study showed that *Drosophila melanogaster* is another species whose sperm retain a nucleosomal compartment (figures 5.4, 5.5, 5.6). Previous studies showed that the positions of retained nucleosomes are scattered throughout but are not randomly distributed among the chromosomes (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009). Similarly, this study recorded that nucleosome signals are distributed throughout the genome but with little enrichment in many regions (figure 5.6). However, the statistical analysis revealed that the chromosomes are not equally packaged by histone, with some chromosomes being more enriched by nucleosomes than others. This might have structural (as result of chromosome position in the nucleus) or functional significance (epigenetic function as result of nucleosome position on the gene).

5.4.2 Positioning of nucleosomes on exons and introns

The precise position of the nucleosomes in the genome plays important roles in gene activity and recent studies indicate that they are enriched on exons rather than introns of somatic cell chromatin (Nahkuri *et al.*,2009; Tilgner *et al.*,2009), Similarly, the modified histone H3K36me3 is enriched in the exons of active genes (Kolasinska-Zwierz *et al.*,2009). However, unlike exons, the location of nucleosomes in introns has been thought to be nonspecific (Jiang and Pugh,2009). In human sperm, like the somatic cell, landscape analysis of histone positioning indicated that sperm nucleosomes are retained preferentially on exons rather than introns (Nahkuri *et al.*,2009). Like human sperm, the analysis on fly sperm chromatin showed that nucleosomes were more significantly located on exons rather than introns (figures 5.7, 5.8, 5.9, 5.10, 5.11). Furthermore, it was shown that nucleosome positioning on human sperm exons is independent of their modification, gene expression, and GC content (Nahkuri *et al.*,2009). In this study, the analysis of modified histone positions revealed that while H3K36me3 and H3K27me3 are significantly depleted from

exons and enriched in introns (figure 5.7), H3K9me3 has a similar distribution to H3 and H4. This variation in modified histone positioning on the intron and exons may have epigenetic consequences for embryo development. The observations from human sperm suggest that sperm exonic nucleosomes might be inherited from one generation to the next and might contribute to the occupancy of nucleosomes on zygotic exons that may assist in exon recognition and splicing selection during gene transcription (Nahkuri *et al.*,2009).

5.4.3 Intron/exon boundaries

Many studies have shown that nucleosomes peaks are located at the intron/exon boundaries, of many species including mammals, chicken, fish, worm, fly and yeast (Andersson *et al.*,2009; Chodavarapu *et al.*,2010; Choi *et al.*,2009; Tolstorukov *et al.*,2011). In this study, nucleosomal peaks were also clearly observed in intron/exon boundaries in the whole genome view of *Drosophila melanogaster* sperm (figure 5.7). The distances between nucleosome centres and intron/exon splice sites vary among these species. While centres are located on the exon side of some species, it is located on the intron side of other species (Andersson *et al.*,2009; Liu and Sun,2010). In the fly, the nucleosomal centre is located on the splice site (0bp) at both ends of the exon (figure 5.7). However, unlike our observation, (Liu and Sun,2010) reported that the nucleosomal peaks in the fly are located on the exon side. This differences most likely arises because of the use of different cell types with different types of genome organization and transcriptional activity.

It has been suggested that the positioning of nucleosomes at intron/exon boundaries create barriers against transcription elongation that may play role in the splicing process (Liu and Sun,2010). Moreover, it was shown that the nucleosomal peaks are depleted at both the start and the end sites of coding regions of highly expressed genes (Choi *et al.*,2009), supporting the previous suggestion that the nucleosomal peaks in these positions play epigenetic functions in transcriptional regulation (Schones *et al.*,2008). In agreement with the above suggestion, these results suggest that the retention of the nucleosomes in intron/exon boundaries of *Drosophila melanogaster* sperm might have epigenetic roles in the regulation of transcription elongation and splicing process during subsequent embryo development.

5.4.4 Nucleosomes occupancy around TSS

The mapping of nucleosome positioning in the human genome has shown that their occupancy is greater immediately upstream of the TSS and then decreases gradually (Boyle *et al.*,2008; Oszolak *et al.*,2007). Many studies showed that the location of the nucleosomes in the TSS play an important role in gene activity. For example, the sliding of the first nucleosome around 30 bp of the transcription start site can affect RNA II polymerase binding (Schones *et al.*,2008; Taft *et al.*,2009). In human (and fly) sperm, although transcriptionally inactive, there is evidence for a strong retention of nucleosomes at TSS particularly at GC rich regions (Vavouri and Lehner,2011). This study, shows that a large number of TSS (about 15%) (figures 5.15, 5.16) in *Drosophila melanogaster* sperm are also occupied by nucleosomes. The positioning of the nucleosomes around TSS of developmental genes in human sperm suggested that they might play epigenetic roles in embryo development (Vavouri and Lehner,2011). Although there appears to be no clear GO enrichment of the TSSs occupied by nucleosomes in *Drosophila*, these nucleosomes may still play a hidden epigenetic function during early embryo proliferation and development.

5.4.5 Promoters

Many studies have reported that promoter regions are occupied by nucleosomes in both somatic cells (Bai and Morozov,2010; Imbalzano *et al.*,1994) and mature sperm (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009; Wu *et al.*,2011). Moreover, the active and/or repressive modified histones are located on promoters of different gene functions and developmental pathways (Brykczynska *et al.*,2010; Hammoud *et al.*,2009; Wu *et al.*,2011). In this study, the analysis of genome packaging by histone in *Drosophila melanogaster* showed that the promoters in the mature sperm are also marked by nucleosomes and modified histones (figure 5.17), which is similar to the previous observation in human sperm (Arpanahi *et al.*,2009; Hammoud *et al.*,2009). GO analysis showed that these genes have complex function (figure 5.18), the most enriched terms were for chromosomes organisation and DNA metabolism. These result highlight also the possibility of epigenetic roles of the nucleosomes in the development stage.

Chapter 6: Evidence for histone retention in the sperm of *Drosophila melanogaster* being related to the epigenome of embryo development

6.1 Introduction

In mammals, the epigenetic memory of the mature gametes play important roles in embryonic development, this memory mainly consists of DNA methylation, histone modification and RNA molecules (Bonasio *et al.*,2010). The epigenetically encoded information undergoes re-programing twice during the life cycle of mammals, while the first resetting occurs in primordial germ cell of the testis and ovaries, the second occurs after fertilization (Migicovsky and Kovalchuk,2011). In this short introduction, the possible epigenetic role of modified histones in embryo development will be considered.

6.1.1 Histone epigenetic asymmetry in the zygote

There are many considerable epigenetic differences in the histones of maternal and paternal gametes (Morgan *et al.*,2005), suggesting that modified histones in each gamete have specific functions that differ from the other. To begin with, oocyte and somatic cell chromatin are organized in a form completely different from sperm; while the former have relaxed open chromatin wrapped around nucleosomes, sperm cell chromatin is organized in compact form as a result of protamine accumulation, and removal of the majority of histone protein (Dadoune *et al.*,2004). Moreover, the sperm nucleus delivers testis specific histone variants(Hammoud *et al.*,2009), suggesting that sperm histones might carry specific epigenetic message to the embryo.

Furthermore, both maternal and paternal germ cells chromatin are marked by active and repressor histone modification (Brykczynska *et al.*,2010; Gu *et al.*,2010; Hammoud *et al.*,2009), but these modifications are located in different genomic regions. Microarray analysis showed that human and mouse sperm are enriched by H3K4me2, H3K4me1 and H3K27me3 at the regulatory regions of developmental genes (Brykczynska *et al.*,2010; Hammoud *et al.*,2009). In contrast to sperm, there is no data available to show the particular positions of histone modifications in the oocyte genome, but immunocytochemical staining revealed that mature mammalian oocytes (metaphase II) are enriched by large

number of histones modification such as acetylation and methylation of H3 and H4, (reviewed in (Gu *et al.*,2010).

In addition, after fertilization both paternal and maternal genomes display different histone modification behaviour; while the histones of the male pronucleus undergo a more global acetylation process (Adenot,1997; Reik *et al.*,2001), the signal for histone modifications remains stable in female pronucleus (reviewed in(Morgan *et al.*,2005).

6.1.2 Epigenetic roles of histone during embryo development

In the course of embryo development, many histone modifications are recorded as important players in the early fate of embryonic cells, the differentiation and patterning of the tissues and the organs in animals. These include H3K27me3, H3K4me3, H3K14ac, H3K9me, H3K4me2, H2A ubiquitination, and H2B ubiquitination (Aday *et al.*,2011; Gibson *et al.*,2012; Joo *et al.*,2011; Lindeman *et al.*,2010). Moreover, these modified histones interact with other regulatory factors giving rise to the delicate and complex processes of morphogenesis(Joo *et al.*,2011). The epigenetics of modified histones was studied in different experimental embryonic models such as human embryonic stem cells (Lee *et al.*,2006), mouse embryonic stem cells (Boyer *et al.*,2006; van Arensbergen *et al.*,2010), marine annelid (Gibson *et al.*,2012), zebrafish (Aday *et al.*,2011; Lindeman *et al.*,2010), *Drosophila* (Bonn *et al.*,2012) and *Xenopus*, in different embryonic stages from zygote to organogenesis of many organs such as heart (Delgado-Olguin *et al.*,2006), nervous system (Song and Ghosh,2004) and pancreas (van Arensbergen *et al.*,2010).

6.1.2.1 Zygote genome activation (ZGA)

Immediately after fertilization, embryo development is dependent on maternal RNAs which decrease gradually, until the embryo start producing its own RNA by the process known as ZGA, (reviewed in (Bogdanovic *et al.*,2012; Tadros and Lipshitz,2009). The timing of ZGA differs among species; for instance, in mice, embryonic transcription starts the 2 cell stage; in zebrafish, it starts at the 512 cell stage and in *Xenopus* it takes place at the mid blastula stage (Bogdanovic *et al.*,2012).

Many studies examined the epigenetic roles of histone modification during ZGA. For example, in the zygote stage, chromatin preparation for gene activation is an important requirement for early embryonic stages and cell fate during organogenesis (Lindeman *et al.*,2011; Xu *et al.*,2011). ChIP- microarray analysis of histone modifications in the embryos of *Xenopus* (Akkers *et al.*,2009) and zebrafish (Aday *et al.*,2011; Lindeman *et al.*,2010) revealed that H3K4me3 and H3K27me3 are remarkably enriched at the period of ZGA. The possible developmental function of these histones at this time was highlighted by these studies. More recently, in the zebrafish, ChIP data on different cell division stages before ZGA showed that the active modified histone H3K4me3 (with or without H3K27me3 and H3K9me3) is located on the promoters of genes involved in developmental regulation and homeostasis (Lindeman *et al.*,2011). This study suggested that the modified histones have an epigenetic pre-patterning effect on the developmental gene activity programme.

6.1.2.2 Embryonic cells differentiation

Many investigations indicated that histone modifications serve a function in pluripotent stem cell differentiation to more specialized cells during embryogenesis (van Arensbergen *et al.*,2010). Among modified histones, the inactive mark H3K27me3 and the active mark H3K4me3 are the most common modifications uncovered during developmental process. In pluripotent embryonic cells these modified histones are located as bivalents (Bernstein *et al.*,2006; Mikkelsen *et al.*,2007). The ratio of this organisation (H3K27me3 : H3K4me3) is associated with embryonic cell specific differentiation, while loss of H3K27me3 leads to the activation of differentiation regulatory genes, loss of H3K4me3 result in suppression of gene expression (Bernstein *et al.*,2006; Mikkelsen *et al.*,2007).

Large scale molecular analysis indicated that H3K27me3 was localised specifically at developmental regulatory genes, those repressed by polycomb group (PcG) proteins in the embryonic stem cells of human and mouse (Boyer *et al.*,2006; Lee *et al.*,2006). Similarly, another study showed that PcG proteins and H3K27me3 control the differentiation programme of pancreatic beta cells of the mouse embryo (van Arensbergen *et al.*,2010).

Moreover, many studies have shown that the embryonic cells have different histone modifications, which might be associated with their fate. For example, μ Chip-microarray data of histone modifications in mouse pre-implantation embryo tissues indicated that inner cell mass and trophoectoderm have the epigenetic marks H3K27me3 and H3K4me3, but these modification are located in different promoters (Dahl *et al.*,2010), suggesting that this epigenetic variation may play developmental roles in embryonic cell differentiation.

6.1.2.3 In *Drosophila melanogaster* embryo

Like vertebrates, histone modifications in *Drosophila melanogaster* undergo dramatic changes during embryogenesis (Roy *et al.*,2010), reflecting the changes in genes activity during embryo developmental guiding. The focus on the dynamics of histone modifications during embryo development of *Drosophila* started early. Giancotti *et al.*,1984 studied the differences in histone modification level between 0-2hr and 18-hr stage embryos. The first stage is characterized by fast cell division and DNA replication but low level of transcription. In contrast, the second stage has slow mitotic and high rates of RNA synthesis. Many observations were recorded in this study; for example, the study showed that H3 and H4 are highly acetylated in early embryonic stages and that the signals are located outside the nucleus. The authors concluded that this histone modification is incorporated into newly synthesised DNA and plays a role in chromatin assembly but not in transcription. Secondly, while no modification marks H2B in both stages tested, H2A is clearly modified in both embryo stages and the study suggested that H2A modification might play important roles in mitosis and DNA replication but that (modified) H2B does not have any significance in these processes.

In last few years, protein chip- based microarray and ChIP-sequencing techniques have provided large scale molecular views, and at a high resolution of histone distribution in the chromatin of the *Drosophila melanogaster* embryo, in addition to uncovering much detail of modified histones and their likely roles in the development process. Many studies discussed the relationship between modified histones and genes regulatory region such as transcription start sites, promoters, enhancers, chromatin remodelers, and DNA polymerase binding sites during embryo development (Roy *et al.*,2010).

Such molecular landscape analysis of H3K9me, for example, revealed that this modified histone carries a specific epigenetic signature in *Drosophila* embryo chromatin (Yasuhara and Wakimoto,2008). In contrast to euchromatic regions, the study showed that the H3K9me2 is significantly enriched in expressed regions of heterochromatic genes and euchromatin - heterochromatin transitional region. The authors argued that this modified histone might work as mark of gene activation, not for gene silencing.

Embryo development is a very sensitive process that requires a fast and smooth transition of the chromatin state from one regulatory condition to another. Recent studies indicated that histone modifications play important roles in tissue specific activity (timing) during embryogenesis (Bonn *et al.*,2012). In this study using Batch isolate, a specific chromatin immune-precipitation approach (BiTS-ChIP) method (isolation of specific labelled cells from tissue) revealed that the modified histones H3K79me3 and H3K27ac activate developmental enhancers of mesodermal cells during the 6-8hr stage of *Drosophila* embryo development (Bonn *et al.*,2012). These modifications were not detectable in the stages 4-6hr and 8-10hr stages.

6.1.3 Histone in sperm of *Drosophila melanogaster*

The observation of this study (previous chapters) indicated that the mature sperm of fruit fly like human and mouse sperm still retain traces of histones and modified histones. In addition, these result are similar to previous findings by using H2A-RFP (Miller *et al.*,2009)and mass spectroscopy (Dorus *et al.*,2006). The analysis of this study in previous chapter suggested that these histones might play epigenetic role in embryo development.

6.1.4 The aim

The aim of this chapter is to analyse the possible association of ChIP- chip data of histone retention in the sperm of *Drosophila melanogaster* and the epigenetics of fruit fly gametes and embryos.

6.2 Materials and methods

This chapter is based on a deep bioinformatics (*in silico*) analysis. ChIP- chip data of histone retention in the sperm were compared with transcriptomic data of *Drosophila melanogaster* testis and sperm (Fischer *et al.*,2012),

maternal (oocyte) (Pilot *et al.*,2006), zygote (De Renzis *et al.*,2007), and chromatin remodeler binding sites (Moshkin *et al.*,2012).

6.3 Result

6.3.1 The association and enrichment of testis and sperm transcripts within nucleosome containing loci in sperm chromatin

The normalised \log_2 (ChIP/Input) data was compared with the abundance of testis and sperm transcripts within sperm chromatin. Based on the average of \log_2 ratios, these transcripts were associated with and enriched by nucleosomes (H3 & H4) and the modified histone H3K9me3 (figure 6.1). However, the analysis also showed that these transcripts were not associated with loci containing H3K36me3 and H3K27me3 (figure 6.1). For further confirmation, these transcripts were compared with the enriched interval lists of H3K36me3 and H3K27me3, which indicated that the male germ cell transcripts were absent from these lists (figure 6.2, 6.3)

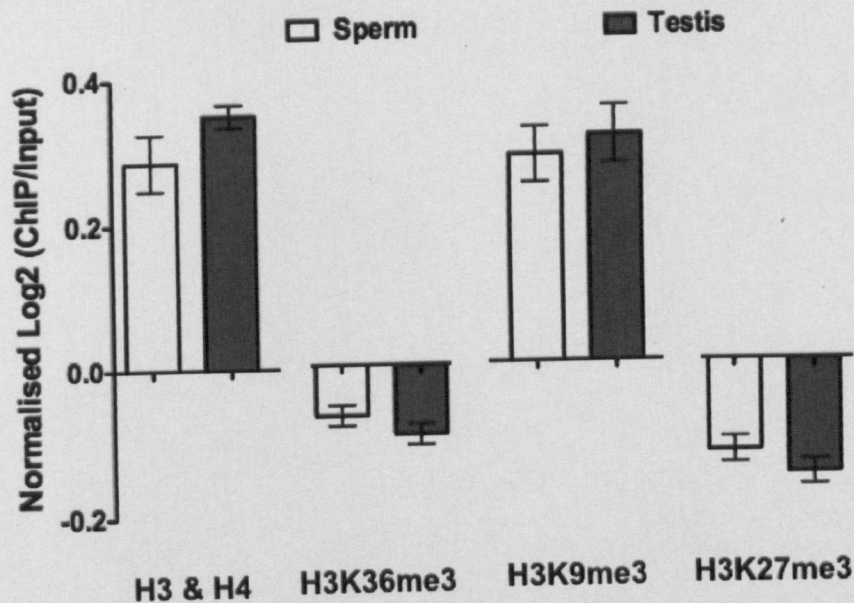


Figure 6-1 The general association of testis and sperm transcripts with gene loci containing nucleosomes and modified histones within the sperm genome.

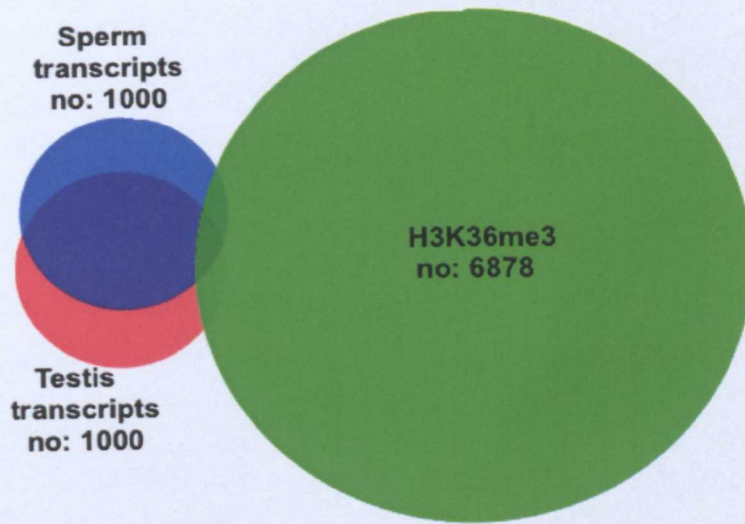


Figure 6-2 The weak overlap between H3K36me3 enriched intervals and the transcripts of testis and sperm suggesting that nucleosomes are not relics of prior gene expression during spermatogenesis.

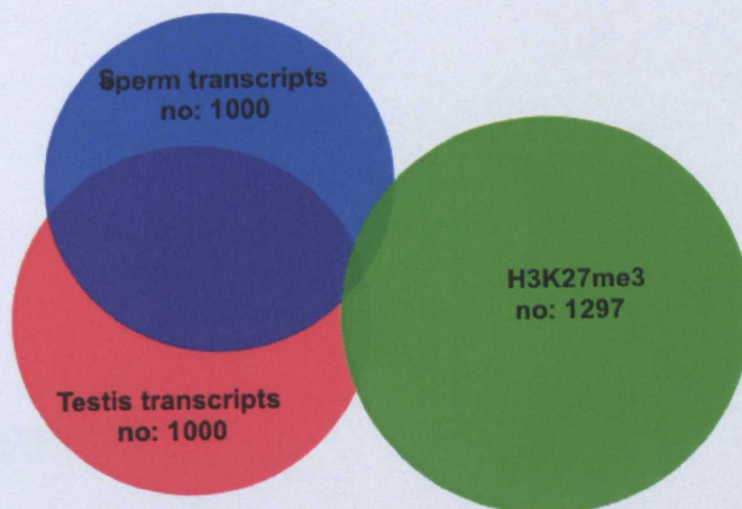


Figure 6-3 The overlap between H3K27me3 enriched intervals and the transcripts of testis and sperm.

6.3.2 The association of maternal and zygote transcripts with loci containing nucleosomes within sperm chromatin

The normalised \log_2 (ChIP/Input) data was used to analysis the association of maternal and zygotic transcripts with loci containing nuceosomes within the sperm chromatin. Based on the averaged \log_2 ratio, while maternal transcripts were associated generally with nucleosomes (H3 & H4), there was little association with the modified histones H3K36me3, H3K9me3 and H3K27me3 (figure 6.4). Moreover, the result showed that the early zygotic transcripts were more closely associated with H3K36me3 than the other modified histones (figure 6.4). Unlike early zygotic transcripts, the results indicated that transcripts expressed in late zygote were enriched by H3K9me3 and depleted from H3K36me3 (figure 6.4).

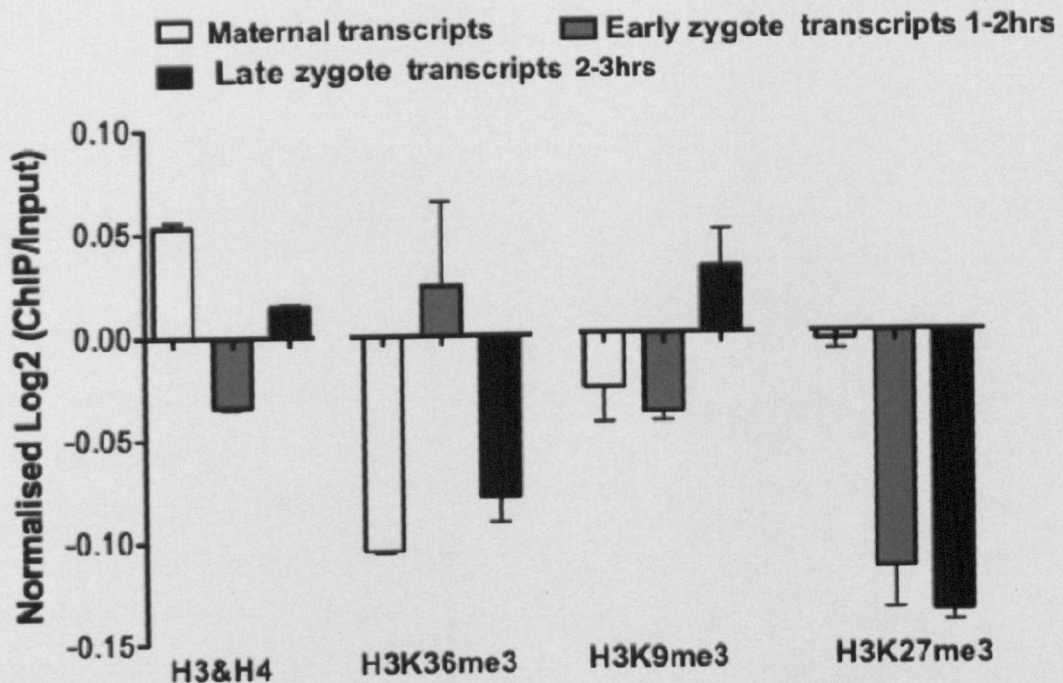


Figure 6-4 The association between maternal, early zygote and all pure zygote transcripts with gene loci containing nucleosomes and modified histones within the sperm genome.

6.3.3 The association of chromatin remodeler binding sites with loci containing nucleosomes within sperm genome

The normalised \log_2 (ChIP/Input) data was used to analysis the association of chromatin remodelers binding sites with nucleosomes loci within sperm chromatin, Based on the average of \log_2 ratio, only binding sites for the

chromatin remodeler NURD were associated with nucleosomes containing H3K27me3 (figure 6.5).

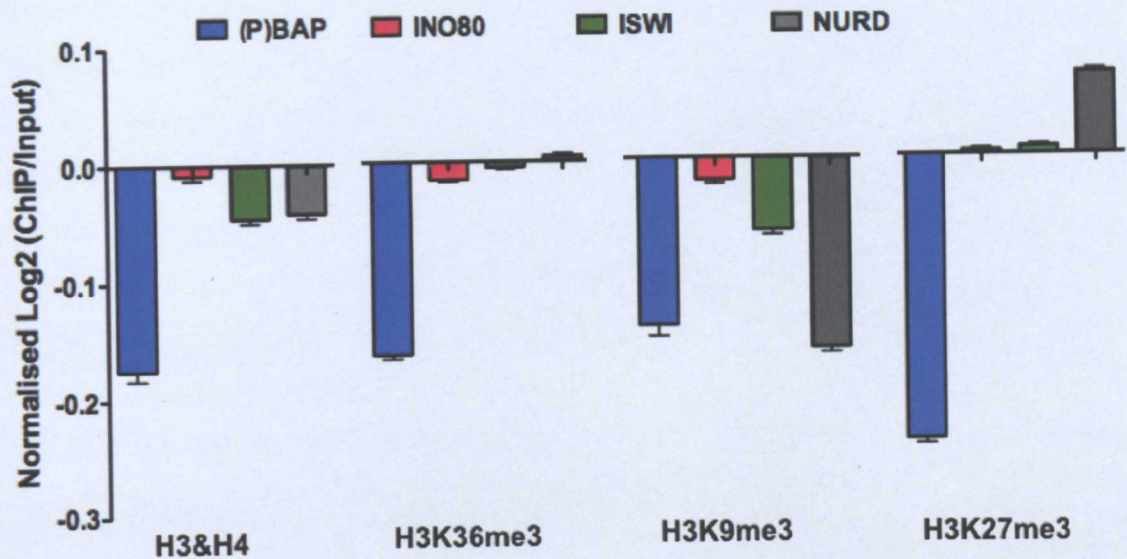


Figure 6-5 The association of chromatin remodeler ((P)BAP, INO80, ISWI and NURD) binding sites with nucleosomes and modified histones within the sperm genome.

6.4 Discussion

6.4.1 Gene loci containing H3K36me3 and H3K27me3 are not associated with male germ line transcripts

The first suggestion that was highlighted by this study was that the detection of histones in the chromatin of mature sperm might be related to the prior history of gene expression during spermatogenesis. In this scenario, certain nucleosome-packaged DNA escapes the general removal of histones during the nuclear compacting stage when nucleosomes are replaced by protamine-like proteins. In this regard, the comparison of gene sequences containing nucleosomes from fly sperm with the fly testis and sperm transcriptomes showed that the latter's gene sequences tend to be occupied by nucleosomes containing H3K9me3 (figure 6.1). In general, most fly testis transcripts tend to be restricted to expression in the male germ line (Barreau *et al.*,2008) (Fischer *et al.*,2012), so might their expression be switched off by the modified histone H3K9me3, which is a marker of inactive genes(Karimi *et al.*,2011). Furthermore, this epigenetic signal may be maintained during embryonic

development and delivered to the germ cells of next generation, as was shown recently in *Caenorhabditis elegans* (Furuhashi *et al.*,2010).

However, unlike the repressive H3K9me3, the analysis indicated that there was little association between either H3K36me3 or H3K27me3 and testis and sperm transcripts (figure 6.1), as these transcripts were not represented in the list of enriched regions containing these modification (figure 6.2, 6.3). This finding raised new question: if these modified histones do not represent previous history of gene expression during spermatogenesis then what is their function? The gene ontology analysis outlined in the previous chapter suggested that these modified histones might play distinctive epigenetic roles in embryo development and the results here also propose the same suggestion. Moreover, the results suggest that similar to mouse and human sperm, *Drosophila melanogaster* sperm carry a specific epigenetic message for embryo development to the egg (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009).

6.4.2 Sperm nucleosomes carry specific epigenetic signature related to genes expressed in the zygote

In *Drosophila melanogaster*, the genomes of the oocyte and early embryo are not transcriptionally active (Tadros and Lipshitz,2005), and the biological process of the first two hours of embryo development are mostly programmed by maternal mRNAs (Tadros *et al.*,2007b), with about 50% to 75% of total mRNAs represented in this pool (De Renzis *et al.*,2007; Lecuyer *et al.*,2007; Semotok *et al.*,2008; Tadros *et al.*,2007a). The comparison between genes enriched with nucleosomes in sperm with fly maternal transcripts shows that oocyte transcripts tend to be associated with genes that are occupied by bulk nucleosomes in sperm (figure 6.4). However, the same analysis failed to show this relationship with nucleosomes containing modified sperm histones (H3K36me3, H3K9me3 and H3K27me3). These finding suggest that sperm carry a specific epigenetic message to the egg. Many studies in many experimental models such as *Xenopus* (Akkers *et al.*,2009), and zebrafish (Aday *et al.*,2011; Lindeman *et al.*,2010) have indicated that modified histones might play epigenetic roles during ZGA. These studies suggest that many modified histones including H3K4me3 and H3K27me3 mark the zygote chromatin in the period of genome activation. For further analysis, the possible

epigenetic involvement of these modified spermatozoal histones in the zygote stage was investigated *in silico*. Interestingly, the results indicated that late zygote transcribed genes (De Renzis *et al.*,2007) are enriched in H3K9me3 (figure 6.4); however, a small set of zygote transcribed genes which are early zygote transcribed genes (1-2hr)(De Renzis *et al.*,2007) were depleted in H3K9me3 and enriched in H3K36me3 instead. The zygote transcribed genes can be divided into two groups; the first group, enriched with H3K36me3 (active transcription signal) are transcribed in the early zygote stage. The second group, enriched by H3K9me3 (inactive transcription signal) are transcribed in late zygote stage. Thus, the localisation of modified sperm histones and the presence of equivalent and overlapping modifications in zygotic genes suggest that the sperm epigenetic signature might play a role in zygotic genome activation.

6.4.3 H3K27me3 marks NURD binding sites

In this study, the possible interaction between sperm nucleosomes and chromatin remodelers was assessed. Chromatin remodelers are ATP-dependent chromatin remodeling complexes that mediate nucleosomes sliding, assembly, ejection and restructuring (Chaban *et al.*,2008; Clapier and Cairns,2009; Dechassa *et al.*,2010; Floer *et al.*,2010; Moshkin *et al.*,2012). The *in silico* analysis revealed that the binding sites of chromatin remodeler, NURD are enriched in H3K27me3 (figure 6.5). Previous studies indicated that chromatin remodelers bind to specific modified histones (Clapier and Cairns,2009; Ho and Crabtree,2010; Moshkin *et al.*,2012), hence the enrichment uncovered here suggests the possibility that H3K27me3 blocks the DNA binding or mediates the activity of NURD. In addition, it has been found that chromatin remodelers such as NURD (and possibly others) play important roles in gene expression during embryo developmental processes by controlling nucleosomes repositioning (Ho and Crabtree,2010; Kaji *et al.*,2006; Moshkin *et al.*,2012). Based on the above information, these observations suggest that modified sperm histone H3K27me3 might play an epigenetic role during embryo development by interacting (positively or negatively) with the chromatin remodeler NURD.

Chapter 7: General discussion

The general aim of this study was to investigate the possible epigenetic role of retained nucleosomes in the mature sperm during embryogenesis. To achieve this aim the study objectives were organised as follows. The many controversial points regarding the retention of histone protein and its modification in the mature sperm were considered in Chapter Three and included investigations of *Drosophila melanogaster* (fly), *Bos taurus* (bull) and *Mus moluscus* (mouse) sperm using microscopic, immunocytochemical and biochemical tools. In Chapter, Four, fly and bovine sperm were selected as experimental models for more detailed investigations of chromatin partitioning into the nucleosomal and protamine compartments. MNase-FISH and CHIP-FISH techniques were used for this purpose. In Chapter Five, fly sperm were used for molecular landscape analysis of histone binding sites within the sperm genome using CHIP on chip technique for the first time. Finally, in Chapter, Six, the relationship between the CHIP on chip data generated in this study with available data on the fly zygote and embryo was investigated.

7.1 Retention of nucleosomes in the mature sperm and chromatin compartmentalisation

Genetic material undergoes dramatic changes in the later stages of spermatogenesis. The most remarkable event is the replacement of nucleosomal histones by protamine or protamine-like proteins as a part of sperm maturation (Braun,2001; Hennig,2003). Although a previous study indicated that the histones are removed completely from the mature sperm of *Drosophila melanogaster* (Jayaramaiah-Raja and Renkawitz-Pohl,2005), the microscopic and biochemical investigations of this study (Figures 3.1, 3.2, 3.4, 3.5 and 3.6) revealed that they are detectable in the nucleus of fly sperm (similar to mouse and bovine sperm) that retain histone proteins. This observation is consistent with the previous findings using mass spectroscopy and flies expressing H2A-RFP (Dorus *et al.*,2006; Miller *et al.*,2009). In agreement with this finding, many studies indicated that although the majority of nucleosomes are removed during sperm maturation, some histones are retained in the mature sperm of many species including marsupials and mice (Pittoggi *et al.*,1999; Soon *et al.*,1997), the bull (Palmer *et al.*,1990), human (Gusse *et al.*,1986; Zalensky *et al.*,2002) and mealy bug, *Planococcus citri* (Bongiorni *et al.*,2009). Previous study has indicated that about 15% of somatic

cell nucleosomes are maintained in the mature sperm of human (Gatewood *et al.*,1987; Hammoud *et al.*,2009). In this study, to estimate the percentage of nucleosomes those might retained in the mature sperm of fruit fly, the enriched ChIP on chip intervals of histones were overlapped (in silico, overlap with > 50 bp) with nucleosomes positions in embryonic cell (bearing in mind the dynamic of the nucleosomes), which mapped by MNase digestion (Mavrigh *et al.*,2008), the result showed (similar to human) about 12 % of somatic cells nucleosomes are still retained in the mature sperm.

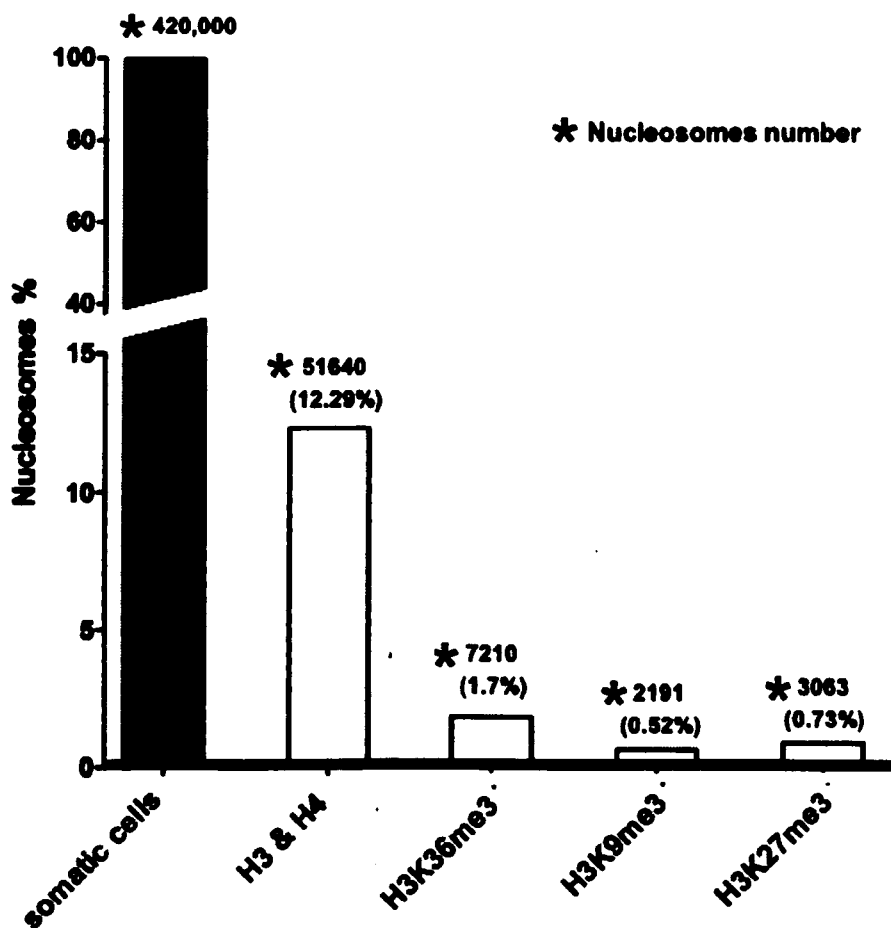


Figure 7-1 The overlap between nucleosomes enriched region in *Drosophila melanogaster* sperm with nucleosomes position in somatic cell.

Proteomic analysis of the human sperm nucleus using LC-MS/MS indicated that histones comprise the largest family of nuclear proteins in the mature sperm and consist of 39 members (de Mateo *et al.*,2011). It has been suggested that these histones and their higher order nucleosomes might play important roles in embryo development (Arpanahi *et al.*,2009; Hammoud *et al.*,2009). This high diversity of histones in the sperm genome reflects their complexity and their

possible epigenetic function during embryogenesis. In addition to *Drosophila melanogaster* sperm, this study provided further evidence that nucleosomes are retained in the genome of bovine sperm. Previous studies showed that H1 and CENP-A mark bovine sperm chromatin (Palmer *et al.*,1990). The immunocytochemical investigations reported in this study reaffirmed the presence of histones H2A, H2B, H3 and H4 in the mature bovine sperm nucleus (Figures 3.11, 3.12, 3.13), similar to the pattern found in human sperm (Li *et al.*,2008) where the highest intensity of histone signal in bovine sperm was located in the annulus part of the nucleus (Figure 3.12).

In the fifth chapter, the result showed that the chromosomes are not equally packaged by histone, with some chromosomes being more enriched by nucleosomes than others (figure 5.6). It was concluded that this might have structural (as result of chromosome position in the nucleus) or functional significance (epigenetic function as result of nucleosome position on the gene). Among fly sperm chromosomes, the analysis showed that chromosome X is mostly depleted from histones than other chromosomes, previous study was indicated that chromosome X (like human) in *Drosophila melanogaster* is inactivated early during spermatogenesis before the other chromosomes (Hense *et al.*,2007), the authors suggested that the X chromosome inactivation might occur as result of a major structural change, rather than by transcription repressors binding (Hense *et al.*,2007). The observation in this study propose that possibly the removal of the nucleosomes from X chromosomes start earlier than others as part of chromosome inactivation process.

In this thesis, the investigation of epigenetic marks in the experimental animal models was a basic step. Modified histones (active/inactive epigenetic marks) have been detected in the sperm of many species including human (H3K4me2, H3K4me3 and H3K27me3) (Hammoud *et al.*,2009), mouse (H3K9, H4K9 and H4K12) (Govin *et al.*,2007; van der Heijden *et al.*,2006), and insects (mealy bug (H3K9me3; (Bongiorni *et al.*,2009). These modifications add a potentially new coding layer to the sperm epigenetic information delivered to the zygote. Gene ontology analysis showed that the modified histones in human have a different function (Hammoud *et al.*,2009). In this study, the results showed that the chromatin of bovine and mouse sperm retain modified histones such as

acetylated H3, acetylated H4 and H3K27me3 (Figures 3.8, 3.9, 3.13 and 3.14). These modifications vary in distribution between the two species. The immunocytochemical signals showed that the pattern of acetyl H3 is similar to acetyl H4 in mouse (in the centre of the cell), so it is possible that the acetylation of both H3 and H4 share a similar function in chromatin reorganisation in mouse and bull spermatogenesis. However, in bovine sperm, H3 and H4 acetylation signal localisations differ from those in mouse (in the whole cell) (Figures 3.8, 3.9, 3.13 and 3.14). This suggests that H3 acetylation might have a different function in bovine sperm, or it is possible that the centromeric chromatin in bovine is arranged in a different pattern from that observed in mouse sperm. Unlike H3 and H4 acetylation, H3K27me3 signals tend to be located in the centre of the nucleus rather than the peripheral regions (Figure 3.14). This specific variation of modified histone positioning within the sperm genome supports the previous suggestion that the nucleosomes are non-randomly distributed in the sperm nucleus.

In this study, unlike bulk canonical histones, which were clearly visible, immunocytochemistry failed to detect modified histones in *Drosophila melanogaster* sperm, perhaps due to uncontrollably high background interference. However, significant precipitation of DNA bound by the modified histones H3K27me3, H3K36me3 and H3K9me3 (figure 5.1) was achieved. This finding and the previous H2A-RFP, histone immunocytochemical and biochemical observations of this research encouraged the use of *Drosophila melanogaster* sperm as an experimental model for further analysis.

In this study, the microscopic analysis of H2A.v-RFP and protamine1-GFP in *Drosophila melanogaster* sperm showed that these proteins are localised in clusters (patches) (Figures 3.1 and 3.2). These findings support the argument that these proteins package the sperm chromatin into nucleosomal and protamine DNA compartments, respectively. For further confirmation, the painting of sperm chromatin with histone bound DNA using CHIP-FISH techniques (Figure 4.1) and the visualisation of normalised log₂ ratio (CHIP/input) in UCSC Browser (Figure 5.6) showed agreement with the microscopic analysis. These observations suggest that nucleosomes organise sperm DNA into territories in *Drosophila melanogaster* sperm. The organisation

of sperm chromosomes into territories was described previously in human sperm (Hazzouri *et al.*,2000b; Zalenskaya and Zalensky,2004). Indeed, the organisation of sperm chromatin into histone and protamine rich domains was discussed in many studies using different techniques (Arpanahi *et al.*,2009; Li *et al.*,2008; Nazarov *et al.*,2008; Saida *et al.*,2011). Immunocytochemistry and FISH analysis of histone/ protamine distribution within the human sperm nucleus revealed that the histone proteins are located in the posterior part of the nucleus, while the protamine is located over the whole nucleus (Li *et al.*,2008). Moreover, similar findings of FISH investigation in mouse sperm showed that MNase soluble and insoluble DNA arise from distinctive compartments. While the soluble DNA is located in the peripheral parts of the nucleus, the insoluble chromatin is located more deeply towards the interior of nucleus (Saida *et al.*,2011). In addition to the fly, the MNase-FISH analysis in this study showed that the chromatin of bovine sperm is organised in a similar pattern (histone bound and protamine bound DNA regions) (Figure 4.5) to that of human and mouse chromatin. All data (including this research and that of previous studies) suggested that histone bound DNA plays an important role in the non-random organisation (compartmentalisation) of the sperm chromatin.

7.2 Nucleosome positioning in sperm chromatin

Genome wide analysis provides a comprehensive map of chromatin structure in the somatic cells of *Drosophila melanogaster* (Kharchenko *et al.*,2011; Roy *et al.*,2010). Previous studies have indicated that the modified histones play important roles in chromatin organisation and gene expression activity (Felsenfeld and Groudine,2003; Mendenhall and Bernstein,2008), recent study has indicated that the modified histones and non histones proteins play important roles in organising somatic cell chromatin into combinatorial chromatin (state) at different levels from single regulatory unit to chromosomal level in *Drosophila melanogaster* (Kharchenko *et al.*,2011), and the modified histones work together to get particular chromatin state (Kharchenko *et al.*,2011).

In this study, ChIP-microarray showed that sperm nucleosomes are retained at gene regulatory regions such as promoters and TSS. In addition to exons and intron/exon boundaries in *Drosophila melanogaster* (Figures 5.7, 5.8, 5.9, 5.15, 5.16 and 5.17), this positioning is similar to the observation of histone binding in

the mouse and human sperm genome (Arpanahi *et al.*,2009; Brykczynska *et al.*,2010; Hammoud *et al.*,2009; Nahkuri *et al.*,2009). Moreover, the analysis showed that while many genes are highly enriched by histones (figure 7.2), other genes are completely depleted from nucleosomes.

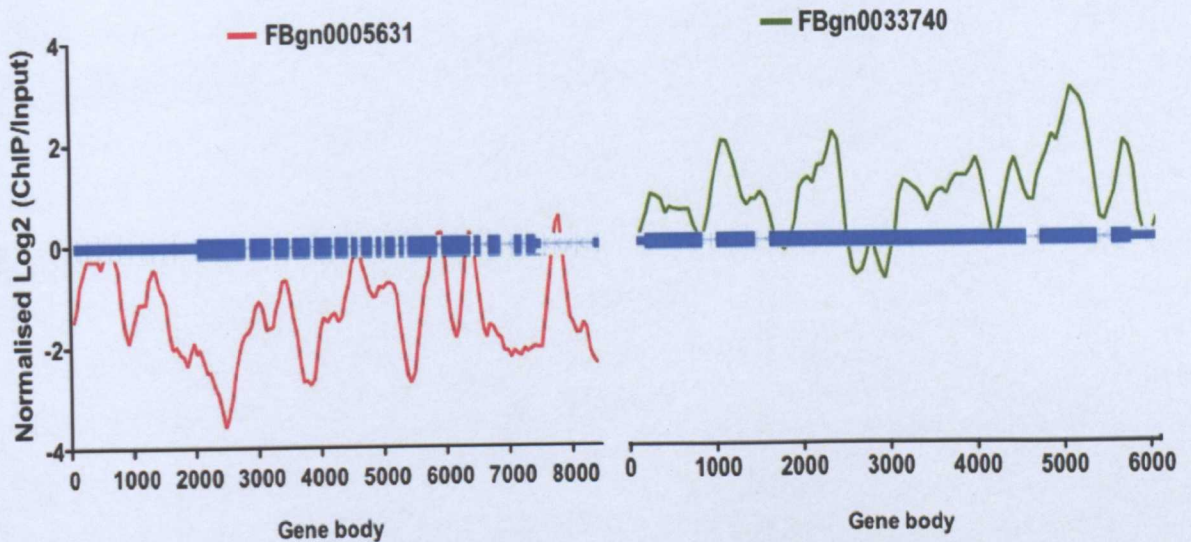


Figure 7-2 comparison between \log_2 ratio of nucleosomes enriched and depleted genes . Red line: nucleosomes depleted gene, green line: nucleosomes enriched gene.

The prediction of molecular function (gene activation or inactivation) of these nucleosomes and their modified histones is very difficult, because there is the possibility that each modified histone might perform both functions. For example, the modified histone H3K36me3, which is more prevalent at the 3' ends of genes is known as an epigenetic mark for actively transcribed genes but has also been shown to negatively affect gene transcription (Furuhashi *et al.*,2010; Kouzarides,2007), and (Carrozza *et al.*,2005; Furuhashi *et al.*,2010). Moreover, it has been shown that H3K36me3 binds with histone H3K36 methyltransferase maternal effect sterile (MES)-4 and inhibits the activity of germ cell development genes during embryogenesis in *Caenorhabditis elegans* (Furuhashi *et al.*,2010). Interestingly H3K36me3 in human sperm is enriched at the same 3' ends (Nahkuri *et al.*,2009) suggesting that this epigenetic mark might carry a negative epigenetic mark to the zygote. It seems that the position of the modified histone and the binding with other proteins plays a role in determining the molecular function (gene suppressor or repressor) of the modified histone.

The microarray analysis in this project revealed that the nucleosomes are preferentially retained on exons rather than introns in the sperm of *Drosophila melanogaster*. This result is similar to previous findings on human sperm (Brykczynska *et al.*,2010; Nahkuri *et al.*,2009) and somatic cells (Nahkuri *et al.*,2009; Tilgner *et al.*,2009). Recently, many studies revealed that the positioning of the nucleosomes and the specific histone modification on the exon play important roles in exon expression in somatic cells (Andersson *et al.*,2009; Kolasinska-Zwierz *et al.*,2009; Tilgner *et al.*,2009), and specific modified histones mediate in splice site selection in many genes (Luco *et al.*,2010). The exact mechanism of nucleosome action on exon expression is still not clear, but one previous study suggested that nucleosomes in exons have a role in chromatin structural changes during the heterogeneous nuclear RNA (hnRNA) splicing process (Tilgner *et al.*,2009). The same authors indicated that these nucleosomes control exon expression by changing their position from internal to an upstream region. The enrichment of nucleosomes on the exon, coupled with the depletion upstream of the acceptor site would lead to active expression, while depletion would have repressive effects (Tilgner *et al.*,2009). However, another study indicated that the positioning of nucleosomes on exons is independent of the gene expression level (Nahkuri *et al.*, 2009).

Unlike in the somatic cell, it has been known that the sperm cell is transcriptionally inert and expresses no signal of gene expression; so what could be the function of nucleosome occupancy on sperm exons? It was suggested (Nahkuri *et al.*, 2009;) that the positioning of nucleosomes on exons might be a general phenomenon in expressed DNA regions in somatic cells, but a different explanation might be required to explain such positioning in sperm cell DNA. The observation in this research raised many questions: Why, for example, are some exons still enriched in nucleosomes and others not? For example, this study showed that nucleosomes are strongly depleted from the exons of chromosome X (Figure 5.10). Why are particular modified histones more enriched on exons than others in some specific genes? The answer is incomplete, but GO analysis of nucleosome enriched exons revealed cell morphogenesis and differentiation suggesting that these nucleosomes might

have epigenetic roles during embryo development by controlling the expression of these exons. Moreover, a previous study suggested that such epigenetic information might be transferred to the next generation (Nahkuri *et al.*, 2009), and guide the status (expression) of zygotic exons during differentiation and development (Nahkuri *et al.*, 2009).

7.3 The hidden fate of sperm nucleosomes during embryogenesis

After fertilisation, sperm histones are eclipsed by the huge numbers of maternal and embryonic nucleosomes that come to fore, and the single cell epigenome will be distributed within a large number of embryo cells. The questions are; do sperm histones exert an epigenetic action within the embryonic genome and where and when do they do so? Until now this has not been studied experimentally, perhaps because of the experimental difficulties in following the fate of a few sperm histones in embryonic tissues. However, many paternal histones have been detected after fertilisation and up to zygote S phase starts. These include H4K8ac and H4K12ac in the human (van der Heijden *et al.*, 2006) and testis-specific variants H2AL1 and H2AL2 in the mouse (Wu *et al.*, 2008). This study tried to address the question using gene ontology analysis of histone and modified histone enriched DNA intervals. The common feature of the gene ontology results is that most nucleosomal enriched genes are involved in the later stages of embryogenesis (cell morphogenesis and differentiation) (Figures 5.12 and 5.13). The same analysis showed that although the modified histones share many gene ontology terms, they have distinctive developmental functions. For example, H3K36me3 is more specific in neuron differentiation and post embryonic development, whereas H3K27me3 is more involved in growth regulation and skeletal muscle organ development. The expression of H3K27me3 enriched genes in late embryogenesis rather than early stages (pre-implantation) was also noted for the modified histones from mammalian sperm (Brykczynska *et al.*, 2010).

In this study, the result showed similarity of gene ontology terms among modified histones. This means that these genes may be occupied by more than one histone modification (bivalent), or (less likely) it may result from sperm polymorphisms (different sperm cells have a different modified histone occupancy pattern). A previous study showed that bivalent modified histones mark the mouse sperm genome (Brykczynska *et al.*, 2010), and it is likely that

this also happens in human sperm (Johnson *et al.*,2011). This bivalent histone is commonly observed in embryo cells of many experimental models during embryogenesis (Vastenhouw and Schier,2012).

The promoters of many genes expressed during spermatogenesis are occupied by nucleosomes in mouse sperm (Brykczynska *et al.*,2010) and it was suggested that these nucleosomes did not have any role in embryo development but are retained from the historic activity of these genes during spermatogenesis (Johnson *et al.*,2011). However, testis and maternal RNA transcripts tend to be associated with genes that are occupied by bulk nucleosomes (containing H3 and H4) in sperm (figures 6.1, 6.2, 6.3 6.4); however, unlike H3 and H4, the analysis indicated that there was little association between either H3K36me3 or H3K27me3 and these transcripts. These findings appear to suggest that sperm may carry a specific epigenetic message to the egg that does not depend on these histone modifications. However, in this study, the *in silico* analysis indicated that H3K36me3 is enriched in genes expressed by the early (1-2hr) zygote; figure 6.4), while genes transcribed in the late (>2hr) stage zygote (De Renzis *et al.*,2007) are depleted in H3K36me3 and enriched in H3K9me3 instead. Thus, the localisation of modified sperm histones and the presence of equivalent and overlapping modifications in zygotic genes suggest that the sperm epigenome might be important in zygotic genome activation. There is another possible fate of these nucleosomes. A recent study indicated that the maternal modified histone H3K36me3 epigenetic mark in primordial germ cells of *Caenorhabditis elegans* is maintained as an inactive mark during embryonic stages and is transferred to primordial germ cells of the new generation (Furuhashi *et al.*,2010). This study suggests the same mechanism may exist for sperm nucleosomes particularly those marking genes involved in spermatogenesis.

7.4 Conclusion

This analysis of histone/nucleosome retention in the mature sperm chromatin of *Drosophila melanogaster* supports the previous suggestion of the possible epigenetic role of histone protein and its chemical modification in embryonic development. Firstly, the enrichment of the nucleosomes on particular exons might carry a hidden epigenetic program from sperm to embryo. Secondly, the retention of the nucleosomes at promoter and intron/exon boundaries might

control gene expression after fertilization or during embryogenesis. Thirdly, the localisation of modified sperm histones and the presence of equivalent and overlapping modifications in zygotic genes suggest that the sperm epigenetic signature might play a role in zygotic genome activation. Fourthly, the enrichment of embryo development terms in gene ontology analysis indicates that the nucleosomes may carry epigenetic messages for embryogenesis.

7.5 Further studies

Although this study highlighted some possible epigenetic functions of the retained nucleosomes in the sperm of *Drosophila melanogaster*, there are many points that need to be studied to support and to fill the gaps of the results of this study:

- The results showed that the enrichment of H3 & H4 is far greater than the modified histones. These nucleosomes (H3&H4) might contain other histone modifications. So the study of other histone modifications such as acetylation might bring new results.
- The investigation of paternal histone signal in the timeline of the male pronucleus is an important step in the investigation of the epigenetic role of paternal histones in embryogenesis. There are many basic techniques that may help in this experiment, such as mating the male H2A.v-RFP fly with the wild type female; or using a culture system by injecting sperm with broken tails into the oocyte or into oocyte extracts.
- Knocking out of protamine genes in *Drosophila melanogaster* decreases the fertility and increases abnormal embryogenesis (Rathke; personal communication). Identifying the protamine binding sites in the sperm of *Drosophila melanogaster* should be possible by comparison with the data generated during this study and will provide greater insight into the epigenetic role of histone retention in embryo development.

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Appendixes

Appendix 1: scatter plot of micro array data

Spearman correlation:

H3 vs H4	0.93
H3 vs (H3&H4)	0.89
H4 vs (H3&H4)	0.92

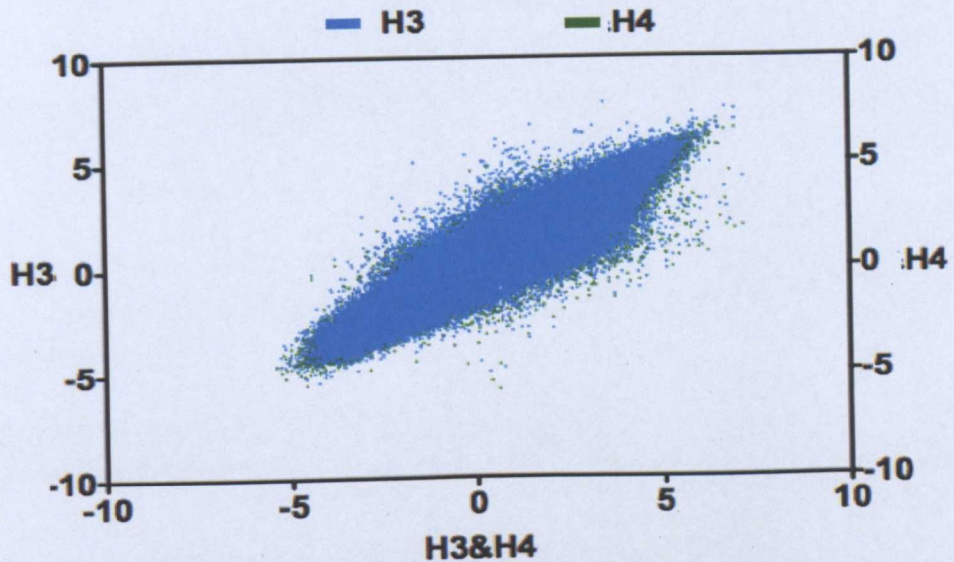


Figure 9-1 Scatter plot of \log_2 ratio of H3 & H4 replicates

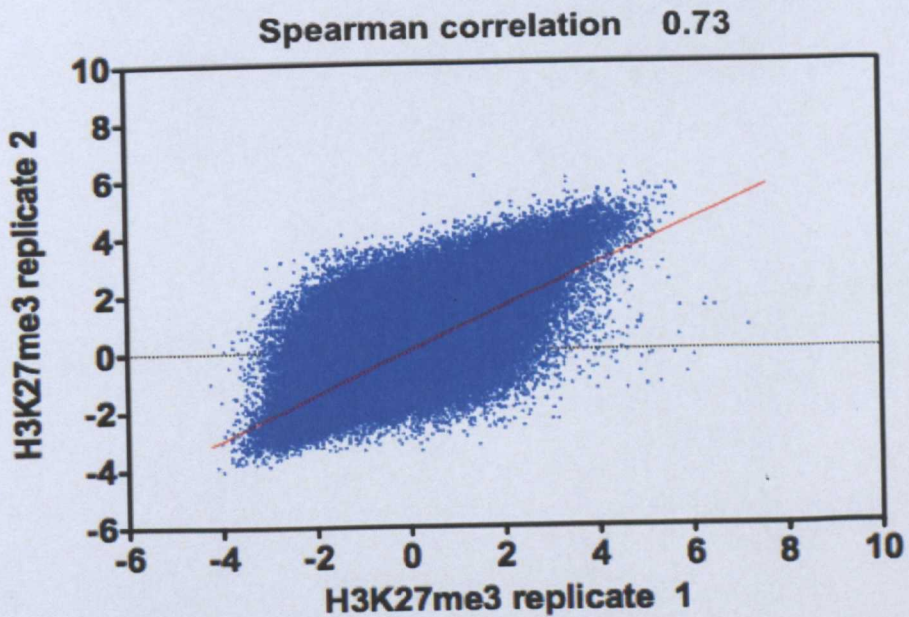


Figure 9-2 Scatter plot of \log_2 ratio of H3K27me3 replicates.

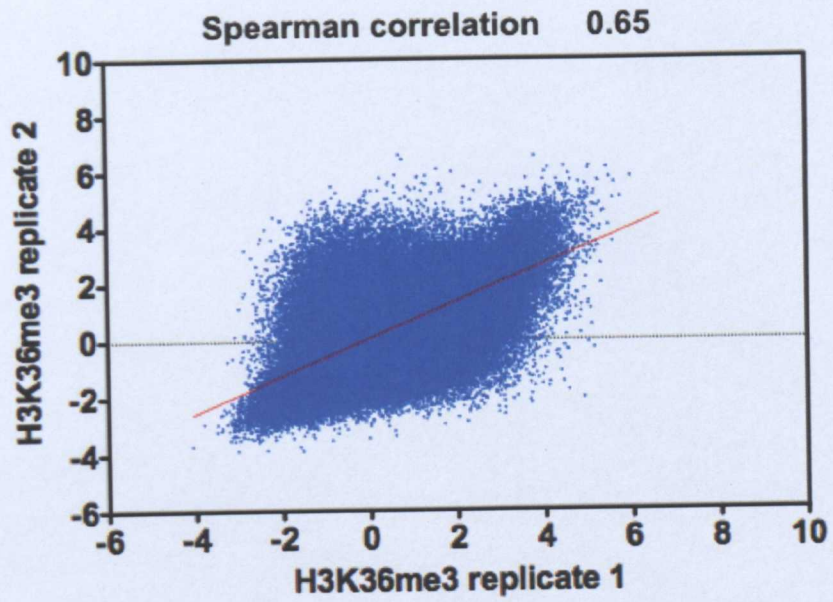


Figure 9-3 Scatter plot of \log_2 ratio of H3K36me3 replicates

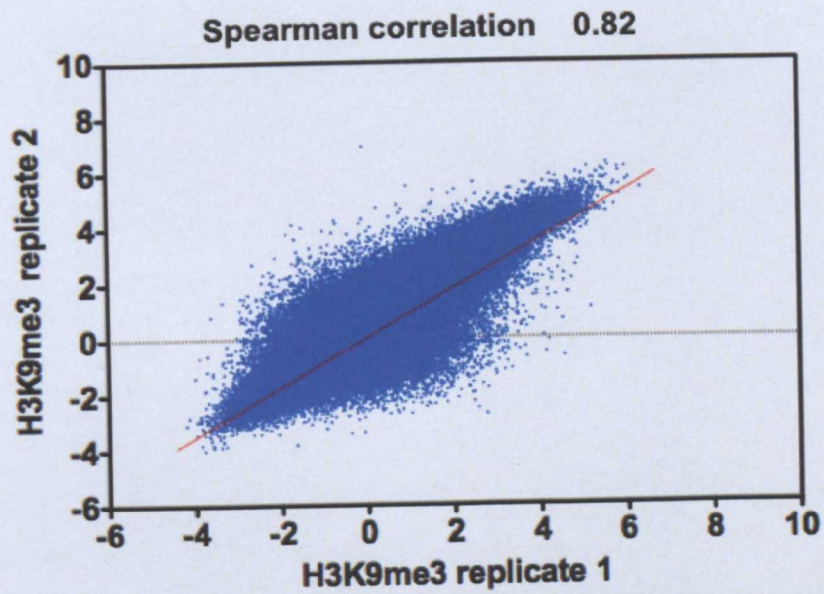


Figure 9-4 Scatter plot of \log_2 ratio of H3K9me3 replicates

Appendix 2: qPCR fold enrichment of selected enriched ChIP intervals

H3 & H4 ChIP				
Interval position	Input Ct	Mock Ct	ChIP Ct	Fold enrichment
chr2R_9553239_9553461	23.942	25.973	24.416	2.8
chr2L_17981878_17982138	24.08	26.3	24.4	3.73
chr3L_336988_337257	24.36	26.5	24.15	5.09
chr3R_7238392_7238661	23.6	26.6	24.3	4.92
chrX_17471588_17471974	23.84	26.64	24.48	4.46
H3K36me3 ChIP				
Interval position	Input Ct	Mock Ct	ChIP Ct	Fold enrichment
chr2L_11263675_11264075	25.73	28.94	27.09	3.6
chr2L_14879048_14881652	25.61	29.19	27.16	4.08
chr3L_12182624_12183064	26.44	30.09	27.12	7.83
chr3R_7579176_7579411	25.62	28.79	27.13	3.16
chrX_16345901_16346136	25.79	28.79	27.06	3.31
H3K27me3 ChIP				
Interval position	Input Ct	Mock Ct	ChIP Ct	Fold enrichment
chrX_20808375_20809055	25.97	29.82	27.1	6.58
chr2R_9278952_9279617	25.29	29.06	27.01	4.14
chr2R_16079263_16079923	25.61	28.99	27.01	3.94
chr3L_20105417_20106042	25.6	29.72	27.07	6.27
chr3L_23230629_23231249	25.77	29.37	27.22	4.43
H3K9me3 ChIP				
Interval position	Input Ct	Mock Ct	ChIP Ct	Fold enrichment
chr3R_5092623_5093403	24.18	26.64	24.33	4.95
chr3L_20015001_20015666	23.88	26.41	24.41	4.00
chrX_3802545_3803210	24.13	26.68	24.49	4.56
chr3L_13147755_13148380	24.12	26.57	24.56	4.02

chr3L_12426705_12427380	23.64	26.72	24.39	5.02
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Appendix 3: Primers used in qPCR

H3 & H4 ChIP		
Peak position	Primer	
chr2R_9553239_9553461	Left	AAACTCTTAGGAAATGCATA
	Right	TTCTTTGCAGTCTTCGCATC
chr2L_17981878_17982138	Left	ATGCCCAAATGCGATTAAGA
	Right	ACACTAGAAGGGGGGCATGGT
chr3L_336988_337257	Left	GCGTCACAAGCAATTAGCAG
	Right	CGGGGAGACTGTCACCTCTA
chr3R_7238392_7238661	Left	CTGCAATTCCAAAACGACTG
	Right	GCCATAGCCTGCAAATGAAT
chrX_17471588_17471974	Left	ACAGGTAATCGCCATCGTTC
	Right	CCATGACTAACAGCCCATGA
H3K36me3 ChIP		
Peak position	Primer	
chr2L_11263675_11264075	Left	ATTGACTGCCAAGTCCTGCT
	Right	GTGAGAGCCACCCTAATGGA
chr2L_14879048_14881652	Left	AAGCCAATGAAGTCCTGTGC
	Right	CCGCGGTTTCAAGTTACAGT
chr3L_12182624_12183064	Left	GAGGACGAGCCTTTGAACTG
	Right	ATAATGGCGTCTGGAGCATC
chr3R_7579176_7579411	Left	GGCGATACTTGCGATAGTCC
	Right	ATGAGCACAGCAAGCAACAG
chrX_16345901_16346136	Left	TTCATATCCGCAGACGACAA
	Right	CTGCAGCTCCTTCAGCTTCT
H3K27me3 ChIP		
Peak position	Primer	
chrX_20808375_20809055	Left	AAAACCTTCGCCATGTATGC
	Right	GAACAAGGTCTCCAGCAAGC
chr2R_9278952_9279617	Left	GCTGATTCGCTGGCTGTAAT
	Right	TTCAAAGGGATAGCGTTGC
chr2R_16079263_16079923	Left	CCGCATTTCTTACGCAGATT
	Right	CTGGAGGAAAGGAGCAACTG
chr3L_20105417_20106042	Left	CTGGCCGAATTCTGAATGTT
	Right	CAGCGATTCCAATTCCAAC

chr3L_23230629_23231249	Left	TGGCATTGGCTAGGAGTACC
	Right	AAACAATGCAACTCCGACCT
H3K9me3 CHIP		
Peak position	Primer	
chr3R_5092623_5093403	Left	CTCTGGACCTGGACTTGGAG
	Right	AGCCCATGGCAATAAGTTTG
chr3L_20015001_20015666	Left	GATCCGTCGAATGTTCTGGT
	Right	AGTTGTTCCGGTCCGTGGTAG
chrX_3802545_3803210	Left	TCCCGCCGATTAATCATAAC
	Right	GTCAGGTGAAGGGATTCCAA
chr3L_13147755_13148380	Left	CCTCGAAGCATCCTGACACT
	Right	TTCAATGCAATCAACGGTGT
chr3L_12426705_12427380	Left	ACAGAAATCAGGAGCCGTGT
	Right	TCTCTGTCACACCCACTGGA

Appendix 3: Gene ontology analysis of H3 and H4 enriched intervals

Category	Term	Count	%	PValue	Fold Enrichment	Benjamini	FDR
GOTERM_MF_FAT	GO:0005524~ATP binding	462	7.51	2.12E-16	1.30	3.64E-13	3.77E-13
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	462	7.51	4.21E-16	1.29	3.64E-13	7.44E-13
GOTERM_MF_FAT	GO:0001882~nucleoside binding	499	8.11	2.03E-15	1.27	1.09E-12	3.35E-12
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	492	8.00	2.98E-15	1.27	1.23E-12	5.03E-12
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	493	8.01	7.77E-15	1.27	2.55E-12	1.31E-11
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	533	8.66	2.78E-11	1.21	7.59E-09	4.66E-08
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	533	8.66	2.78E-11	1.21	7.59E-09	4.66E-08
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	565	9.18	7.58E-11	1.20	1.78E-08	1.27E-07
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	722	11.7	2.32E-09	1.14	1.51E-06	3.46E-06
GOTERM_CC_FAT	GO:0016021~integral to membrane	711	11.5	2.87E-09	1.14	9.33E-07	4.29E-06
GOTERM_MF_FAT	GO:0043167~ion binding	950	15.4	3.28E-09	1.12	6.72E-07	5.51E-06
GOTERM_MF_FAT	GO:0043169~cation binding	946	15.3	3.36E-09	1.12	6.12E-07	5.64E-06
GOTERM_BP_FAT	GO:0007166~cell surface receptor linked signal transduction	333	5.41	4.21E-09	1.26	1.43E-05	7.69E-06
GOTERM_BP_FAT	GO:0000902~cell morphogenesis	244	3.96	7.05E-09	1.31	1.20E-05	1.29E-05
GOTERM_MF_FAT	GO:0000166~nucleotide binding	662	10.7	1.75E-08	1.15	2.87E-06	2.94E-05
GOTERM_MF_FAT	GO:0046872~metal ion binding	917	14.9	2.42E-08	1.12	3.62E-06	4.07E-05
GOTERM_CC_FAT	GO:0005886~plasma membrane	424	6.89	7.24E-08	1.19	1.57E-05	1.08E-04
GOTERM_MF_FAT	GO:0046914~transition metal ion binding	728	11.8	2.74E-07	1.13	3.75E-05	4.61E-04
GOTERM_MF_FAT	GO:0008270~zinc ion binding	563	9.15	3.04E-07	1.15	3.84E-05	5.11E-04

GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	272	4.42	4.23E-07	1.25	4.79E-04	7.73E-04
GOTERM_BP_FAT	GO:0007155~cell adhesion	106	1.72	5.10E-07	1.45	4.33E-04	9.31E-04
GOTERM_BP_FAT	GO:0016568~chromatin modification	73	1.18	1.18E-06	1.55	7.99E-04	0.002
GOTERM_MF_FAT	GO:0042623~ATPase activity, coupled	187	3.04	1.71E-06	1.28	2.00E-04	0.002
GOTERM_BP_FAT	GO:0016192~vesicle-mediated transport	209	3.39	2.25E-06	1.27	0.001	0.004
GOTERM_MF_FAT	GO:0016887~ATPase activity	211	3.43	2.34E-06	1.25	2.56E-04	0.003
GOTERM_BP_FAT	GO:0048666~neuron development	188	3.05	2.47E-06	1.29	0.001	0.004
GOTERM_MF_FAT	GO:0042624~ATPase activity, uncoupled	123	2.00	4.16E-06	1.34	4.27E-04	0.006
GOTERM_BP_FAT	GO:0030030~cell projection organization	195	3.17	5.35E-06	1.27	0.002	0.009
GOTERM_BP_FAT	GO:0006897~endocytosis	144	2.34	5.37E-06	1.33	0.002	0.009
GOTERM_BP_FAT	GO:0010324~membrane invagination	144	2.34	5.37E-06	1.33	0.002	0.009
GOTERM_BP_FAT	GO:0006468~protein amino acid phosphorylation	144	2.34	7.10E-06	1.32	0.002	0.012
GOTERM_BP_FAT	GO:0007411~axon guidance	83	1.35	9.35E-06	1.45	0.002	0.017
GOTERM_BP_FAT	GO:0006928~cell motion	165	2.68	1.22E-05	1.29	0.003	0.022
GOTERM_BP_FAT	GO:0000904~cell morphogenesis involved in differentiation	164	2.66	1.25E-05	1.29	0.003	0.022
GOTERM_BP_FAT	GO:0051056~regulation of small GTPase mediated signal transduction	61	0.99	1.26E-05	1.55	0.003	0.023
GOTERM_BP_FAT	GO:0035215~genital disc development	24	0.39	1.46E-05	2.04	0.003	0.026
GOTERM_BP_FAT	GO:0006281~DNA repair	65	1.05	1.48E-05	1.52	0.003	0.027
GOTERM_BP_FAT	GO:0022610~biological adhesion	108	1.75	1.78E-05	1.37	0.003	0.032
GOTERM_BP_FAT	GO:0030182~neuron differentiation	213	3.46	1.80E-05	1.24	0.003	0.032

GOTERM_BP_FAT	GO:0006259~DNA metabolic process	124	2.01	1.86E-05	1.34	0.003	0.033
GOTERM_BP_FAT	GO:0048858~cell projection morphogenesis	171	2.78	2.12E-05	1.27	0.003	0.038
GOTERM_BP_FAT	GO:0051276~chromosome organization	153	2.48	2.14E-05	1.29	0.003	0.039
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	136	2.21	2.51E-05	1.31	0.003	0.045
GOTERM_BP_FAT	GO:0016044~membrane organization	170	2.76	2.75E-05	1.27	0.004	0.050

Appendix 4: Gene ontology analysis of the promoters enriched with H3 and H4.

Category	Term	Count	%	PValue	Fold	Benjamini	FDR
GOTERM_MF_FAT	GO:0000166~nucleotide binding	321	13.7	3.38E-12	1.3	3.88E-09	5.44E-09
GOTERM_BP_FAT	GO:0000278~mitotic cell cycle	114	4.88	9.93E-12	1.8	2.68E-08	1.77E-08
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	255	10.9	1.89E-11	1.4	1.08E-08	3.04E-08
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	255	10.9	1.89E-11	1.4	1.08E-08	3.04E-08
GOTERM_BP_FAT	GO:0043933~macromolecular complex subunit organization	93	3.98	1.05E-10	1.9	1.42E-07	1.87E-07
GOTERM_BP_FAT	GO:0051276~chromosome organization	93	3.98	1.31E-10	1.9	1.18E-07	2.32E-07
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	266	11.4	1.41E-10	1.4	5.39E-08	2.27E-07
GOTERM_MF_FAT	GO:0005524~ATP binding	210	9.00	3.87E-10	1.4	1.11E-07	6.22E-07
GOTERM_BP_FAT	GO:0008104~protein localization	117	5.01	4.87E-10	1.7	3.29E-07	8.66E-07
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	210	9.00	5.01E-10	1.4	1.15E-07	8.05E-07
GOTERM_BP_FAT	GO:0007051~spindle organization	77	3.30	9.50E-10	1.9	5.13E-07	1.69E-06
GOTERM_CC_FAT	GO:0043228~non-membrane-bounded organelle	247	10.5	1.65E-09	1.3	8.48E-07	2.38E-06
GOTERM_CC_FAT	GO:0043232~intracellular non-membrane-bounded organelle	247	10.5	1.65E-09	1.3	8.48E-07	2.38E-06
GOTERM_BP_FAT	GO:0002226~microtubule cytoskeleton organization	93	3.98	3.28E-09	1.8	1.48E-06	5.85E-06
GOTERM_BP_FAT	GO:0007052~mitotic spindle organization	68	2.91	3.41E-09	2.0	1.32E-06	6.07E-06
GOTERM_BP_FAT	GO:0034621~cellular macromolecular complex subunit organization	70	3.00	4.26E-09	1.9	1.44E-06	7.57E-06
GOTERM_MF_FAT	GO:0030554~adenyl nucleotide binding	219	9.39	7.28E-09	1.4	1.39E-06	1.17E-05

GOTERM_CC_FAT	GO:0005739~mitochondrion	162	6.94	9.21E-09	1.4	2.37E-06	1.33E-05
GOTERM_MF_FAT	GO:0001883~purine nucleoside binding	219	9.39	1.26E-08	1.4	2.06E-06	2.02E-05
GOTERM_BP_FAT	GO:0006260~DNA replication	41	1.75	1.46E-08	2.4	4.38E-06	2.60E-05
GOTERM_MF_FAT	GO:0001882~nucleoside binding	220	9.43	1.65E-08	1.3	2.36E-06	2.65E-05
GOTERM_BP_FAT	GO:0007010~cytoskeleton organization	128	5.48	1.78E-08	1.5	4.81E-06	3.17E-05
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	72	3.08	3.64E-08	1.8	8.93E-06	6.48E-05
GOTERM_BP_FAT	GO:0012501~programmed cell death	54	2.31	1.34E-07	2.0	3.03E-05	2.39E-04
GOTERM_CC_FAT	GO:0005811~lipid particle	82	3.51	1.35E-07	1.7	2.32E-05	1.95E-04
GOTERM_CC_FAT	GO:0030529~ribonucleoprotein complex	113	4.84	1.75E-07	1.5	2.26E-05	2.53E-04
GOTERM_CC_FAT	GO:0033279~ribosomal subunit	59	2.53	4.08E-07	1.8	4.20E-05	5.90E-04
GOTERM_CC_FAT	GO:0044429~mitochondrial part	119	5.10	4.46E-07	1.5	3.83E-05	6.45E-04
GOTERM_CC_FAT	GO:0044445~cytosolic part	49	2.10	4.50E-07	2.0	3.31E-05	6.51E-04
GOTERM_BP_FAT	GO:0065003~macromolecular complex assembly	74	3.17	8.00E-07	1.7	1.66E-04	0.001
GOTERM_BP_FAT	GO:0007049~cell cycle	153	6.56	8.36E-07	1.4	1.61E-04	0.001
GOTERM_BP_FAT	GO:0015031~protein transport	81	3.47	9.45E-07	1.6	1.70E-04	0.001
GOTERM_BP_FAT	GO:0008219~cell death	54	2.31	1.06E-06	1.9	1.78E-04	0.001
GOTERM_BP_FAT	GO:0016265~death	54	2.31	1.31E-06	1.9	2.08E-04	0.002
GOTERM_BP_FAT	GO:0045184~establishment of protein localization	82	3.51	1.40E-06	1.6	2.10E-04	0.002
GOTERM_CC_FAT	GO:0005840~ribosome	63	2.70	1.71E-06	1.7	1.10E-04	0.002
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	61	2.61	2.17E-06	1.7	2.76E-04	0.003
GOTERM_CC_FAT	GO:0031974~membrane-enclosed lumen	152	6.51	2.42E-06	1.3	1.38E-04	0.003
GOTERM_CC_FAT	GO:0070013~intracellular organelle lumen	148	6.34	3.48E-06	1.3	1.79E-04	0.005
GOTERM_CC_FAT	GO:0043233~organelle lumen	148	6.34	3.48E-06	1.3	1.79E-04	0.005
GOTERM_BP_FAT	GO:0016044~membrane organization	88	3.77	3.71E-06	1.5	5.27E-04	0.006

GOTERM_CC_FAT	GO:0005700~polytene chromosome	37	1.58	4.97E-06	2.1	2.33E-04	0.007
GOTERM_BP_FAT	GO:0006396~RNA processing	86	3.68	5.03E-06	1.5	6.78E-04	0.008
GOTERM_BP_FAT	GO:0006325~chromatin organization	54	2.31	5.39E-06	1.8	6.93E-04	0.009
GOTERM_BP_FAT	GO:0022402~cell cycle process	135	5.78	5.94E-06	1.4	7.29E-04	0.010
GOTERM_MF_FAT	GO:0008270~zinc ion binding	250	10.7	6.33E-06	1.2	7.26E-04	0.010
GOTERM_BP_FAT	GO:0000279~M phase	120	5.14	9.14E-06	1.4	0.001072	0.016
GOTERM_BP_FAT	GO:0007017~microtubule-based process	105	4.50	9.23E-06	1.4	0.001	0.016
GOTERM_BP_FAT	GO:0022403~cell cycle phase	123	5.27	1.28E-05	1.4	0.001	0.022
GOTERM_BP_FAT	GO:0016568~chromatin modification	39	1.67	1.37E-05	2.0	0.001	0.024
GOTERM_BP_FAT	GO:0034660~ncRNA metabolic process	44	1.88	1.51E-05	1.9	0.001	0.026
GOTERM_BP_FAT	GO:0034984~cellular response to DNA damage	32	1.37	1.67E-05	2.1	0.001	0.029
stimulus							
GOTERM_MF_FAT	GO:0016887~ATPase activity	100	4.28	1.89E-05	1.4	0.001	0.030
GOTERM_BP_FAT	GO:0070727~cellular macromolecule localization	69	2.95	2.01E-05	1.6	0.001	0.035
GOTERM_BP_FAT	GO:0006897~endocytosis	72	3.08	2.10E-05	1.6	0.001	0.037
GOTERM_BP_FAT	GO:0010324~membrane invagination	72	3.08	2.10E-05	1.6	0.001	0.037
GOTERM_CC_FAT	GO:0031967~organelle envelope	97	4.15	2.15E-05	1.4	0.01	0.031
GOTERM_BP_FAT	GO:0030163~protein catabolic process	59	2.53	2.31E-05	1.7	0.002	0.041
GOTERM_CC_FAT	GO:0031975~envelope	97	4.15	2.45E-05	1.4	9.70E-04	0.035
GOTERM_BP_FAT	GO:0016192~vesicle-mediated transport	100	4.28	2.55E-05	1.4	0.00214907	0.045
GOTERM_CC_FAT	GO:0044427~chromosomal part	73	3.13	2.96E-05	1.5	0.00108651	0.042
GOTERM_CC_FAT	GO:0005694~chromosome	87	3.73	3.44E-05	1.5	0.00118060	0.049

