Role of TH2B Serine 12 Phosphorylation in Mouse Spermatogenesis

---

A thesis submitted for partial fulfillment of degree of

MASTER OF SCIENCE (Biological Sciences)

as part of Integrated PhD program

by

Raktim Roy

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore-560064

March 2017

DECLARATION

I hereby declare that the work described in this thesis entitled '**Role of TH2B Serine 12 Phosphorylation in Mouse Spermatogenesis**' is the result of investigations carried out by myself under the guidance of **Prof. M.R.S Rao** at Chromatin Biology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

Raktim Roy

Jawaharlal Nehru Center for Advanced Scientific Research
Jakkur Campus, Jakkur Post
Bengaluru 560 064, INDIA

Office Tel: +91 (80) 2208 2754
Office Fax: +91 (80) 2208 2758
+91 (80) 2208 2468
Email:
mrsrao@jncasr.ac.in

Prof. MRS Rao
Honorary Professor and SERB Distinguished Fellow
Molecular Biology and Genetics Unit

CERTIFICATE

This is to certify that the work described in this thesis entitled **'Role of TH2B Serine 12 Phosphorylation in Mouse Spermatogenesis'** is the result of investigations carried out by Mr. Raktim Roy at Chromatin Biology laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

Prof. MRS Rao

ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my mentor and guide, Prof. M.R.S. Rao who has given me this wonderful opportunity to work in one of the emerging and crucial fields of biology. His constant guidance and insightful discussions inspired me to pursue this field of research and helped me to develop and sharpen my scientific ideas. He has always been supportive and has never pressurized me or anyone in the laboratory. He has always given us the freedom to work at our own pace and in the process made all of us independent. His inspiring talks on science and how it should be done motivated me to make science my ultimate goal in life. I am very much thankful to him for letting me work in this project and for guiding me so profoundly.

Secondly, I would like to thank my senior and colleague, Aditya Mahadevan, with whom I have worked in this project. His mentorship helped me to get well-versed with various techniques and ideas for tackling specific scientific problems. Whenever I faced a blockade in my work, he was always there to encourage and guide me to help me mitigate the problem(s). He never made me feel that he was a senior; instead I felt that we were both of the same age. He was the first and only person to whom I always approached for discussing both scientific and non-scientific problems. I am very much grateful to him for helping me all the way in this project and for being there always whenever I needed his help.

Two people who deserve mention are my colleagues and past roommates, Monoj Adhikari (TSU) and Arindam Ray (MBGU). They made my stay in JNCASR more memorable and enjoyable. They are more of a brother-like figure to me, who helped and guided me whenever I was in some kind of problem. Going out with them helped me to forget my sadness and made me smile. After a tiring day when I returned to room, they were always there to make me forget my problems and cheered me up. I am very much thankful to both of them for giving me memories I can never forget.

I would also like to thank all the faculties of MBGU who have been instrumental in shaping my scientific rigor and ideas. The coursework provided by them was truly unique and informative that helped me develop new ideas. I would specially like to thank Prof. Tapas K. Kundu for his brilliant coursework and guidance that helped me develop special interests towards Chromatin biology.

I am very much thankful to the Central facilities at MBGU, especially Confocal and FACS facility. Suma Ma'am (Confocal) was very kind and taught me a lot about the process of confocal microscopy. Her guidance and discussions were immensely helpful to me without which it would have been very difficult for me to analyse confocal images. Swaroopa Ma'am

(FACS) has been a constant source of patience because the protocol I was trying to standardise was very difficult and required a lot of expertise. She has always been there with me from the beginning and gave me various inputs on how to improve the protocol. I always went to her whenever I was facing some problems with the protocol and she was always there to sort it out. I would also like to take this opportunity to thank Dr. R.G. Prakash of the Animal Facility. Whenever I needed mice for my experiments he had always made it a priority and delivered them at the right time. Without his assistance it would have been very difficult to carry out the experiments.

I am thankful to my present and past lab members including Aditya, Utsa, Bhavana, Neha, Debosree, Brian, Shrinivas, Shubhangini, Dhanur, Arun, Subhendu, Shalini for being such helpful labmates and creating an environment where it was fun to work. During my rotation they made my stay in the lab comfortable and helped me get acquainted with it. I will always remember the enjoyable moments spent with them.

I am grateful to my classmates Akash, Aditi, Jigyasa, Neha, Neelakshi, Sarika and Zeenat for being such wonderful friends with whom I had spent some memorable moments during coursework. The outings and birthday celebrations with them were truly priceless and I thank all of them for being such helpful and delightful friends. I would specially like to thank two my departmental colleagues, Gaurav and Greg from the Autophagy laboratory. Both of them have been very helpful towards me and provided me with a smile whenever I felt lost or depressed. Without both of them it would have been very difficult for me to overcome the various hurdles.

Finally, I would like to thank my parents and my sister without whose constant support and encouragement it wouldn’t have been possible to arrive at this point. My parents’ support from the beginning was truly encouraging and their patience towards me was amazing. They had never tried to impose anything on me and instead gave me the freedom to choose my path of career. My sister, who is currently living in Bangalore, is always encouraging me and outing with her was both truly insightful and enjoyable. She always had faith in me and this faith of her made me truly love my work and I am very much grateful to her.

CONTENTS

Chapter 1: Introduction

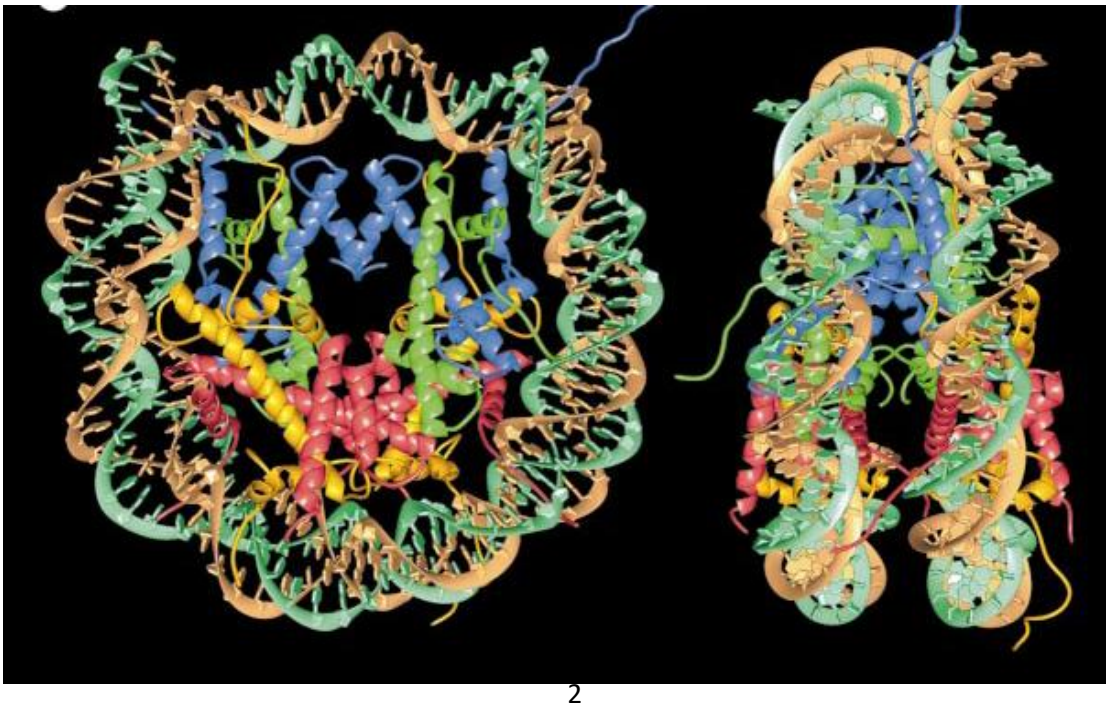
Introduction

1.1. Nucleosome structure

The nucleosome is the basic fundamental subunit of chromatin. It consists of 146bp of DNA, corresponding to 1.75 turns of DNA wrapped around an octamer of histone proteins (single tetramer of H3-H4 and two H2A-H2B dimers). The nucleosome of chromatin is thought of as a 'beads on a string' structure[1]. Each neighboring nucleosome is separated by a stretch of linker DNA containing histone H1. Nucleosomes are folded through a series of successively higher order structures eventually forming the chromosome and which ultimately regulates gene expression.

The primary level of chromatin compaction is the 11 nm fiber which is formed by multiple nucleosomes present in a row. The 11 nm fiber further folds upon itself to produce the 30 nm chromatin fiber forming a two helix structure which is the secondary level of compaction. There are two models to explain the structure of the 30 nm fiber at the molecular level- zig zag and the solenoid model. The zig zag model implies interactions between alternate nucleosomes. The solenoid model is characterized by interactions between consecutive nucleosomes. The tertiary level of compaction is formed by the further twisting of the 30 nm fiber to form the fiber which has loops and some of them which are attached to a protein scaffold. The quaternary level of chromatin structure is the three-dimensional arrangement of chromosomes inside the nucleus[2]. The structural force to bring about the kinks in the DNA is brought about by a large number of hydrogen bonds by masking the negative charge on the phosphate groups of DNA.

A)



B)

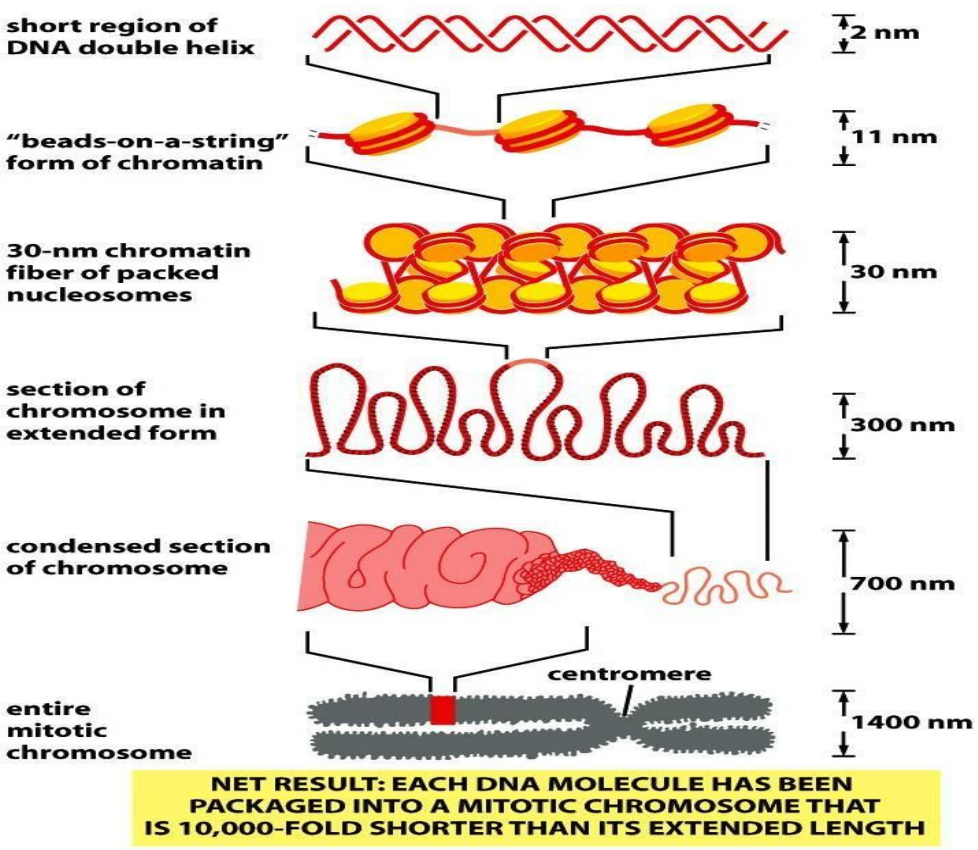


Fig 1: Basic nucleosome structure and higher order chromatin structure-A) Nucleosome core particle: ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B) **B)** Hierarchy of formation of the higher order chromatin structures **(Adapted from Luger. K. *et al*. *Nature* (1997), Alberts Molecular Biology of the Cell, 5th Edition)**

1.2. Histone variants in spermatogenesis

Histone variants are non-canonical (non-allelic) variants of the histones, representing one or a few amino acid differences and are usually expressed at very low levels compared with their conventional counterparts. They are deposited throughout the cell cycle. Till date, histone variants of all the core histones (except H4) and the linker histone H1 have been discovered[3]. The histone variants play various roles like regulating gene expression (H2AZ), in DNA repair and recombination (H2AX), kinetochore assembly (CENP-A), etc. For example, γH2AX which is a variant of the canonical histone H2A plays a role in DNA repair and recombination. For example, CENP-A which is a variant of H3 is present in the centromere region and serves as a platform for the assembly of kinetochore proteins. CENP-A is targeted to the centromere by its conserved histone fold domain and it differs greatly from other H3 variants in the N-terminal tails[4]. Such specialized functions carried out by the histone variants is due to the combination of differences in amino acid sequences and their specific chaperone proteins that deposit them at their unique locations.

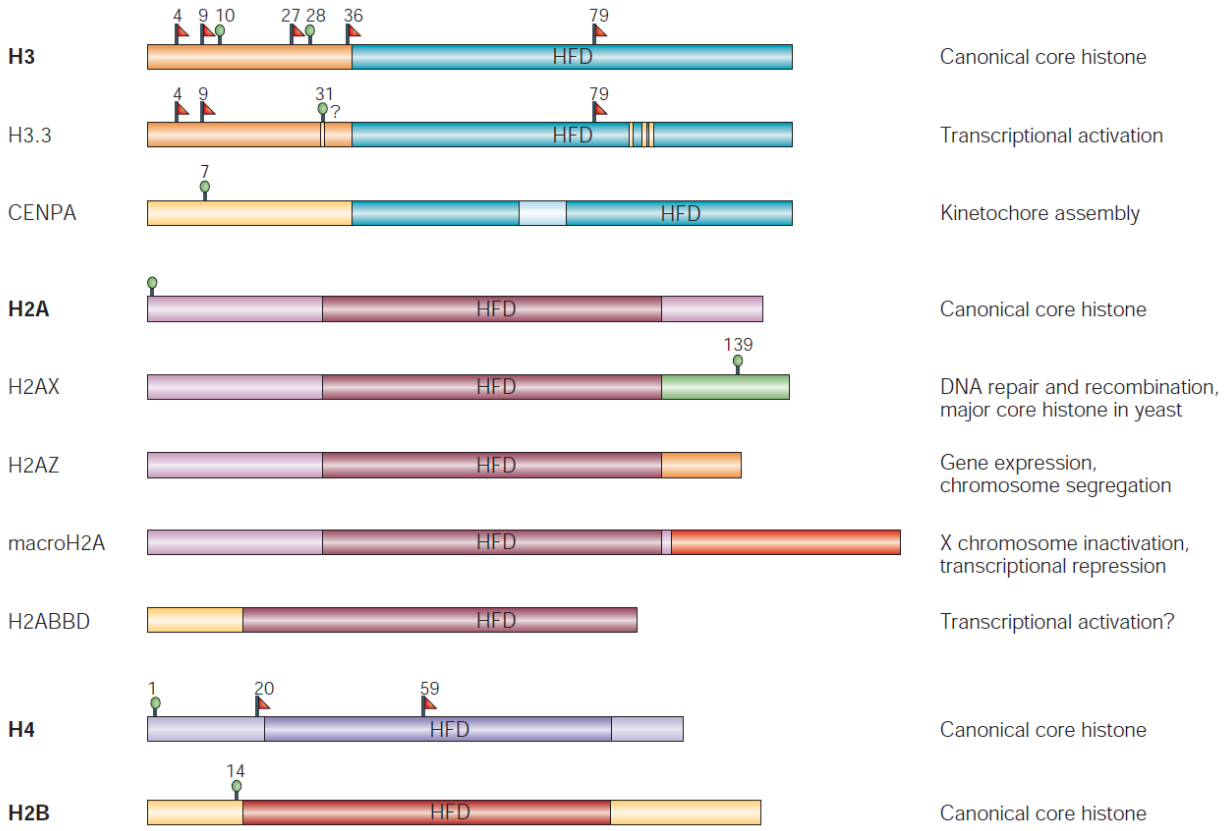


Fig 2: Various histone variants- The various histone variants and their roles. The major core histones contain a conserved histone-fold domain (HFD). In addition, they contain N- and C-terminal tails that harbour sites for various post-translational modifications. **(Adapted from Sarma and Reinberg, *Nature Reviews Molecular Cell Biology* (2005))**

Mammalian spermatogenesis is an excellent model for studying histone variants because it has been shown that the testis expresses both core and linker histone variants in a stage-specific manner. TH2B, the testis-specific variant of the canonical histone H2B, replaces almost 85% of H2B in spermatocytes[15]. In pachytene spermatocytes, about 55% of the linker histone H1 is replaced by the variant H1t which is present until elongating spermatids[5]. Also, another linker histone variant HILS1 which has been detected in mouse and human, is expressed in the later stages of spermatogenesis[5]. Variants of H3 and H2A are also present[5].

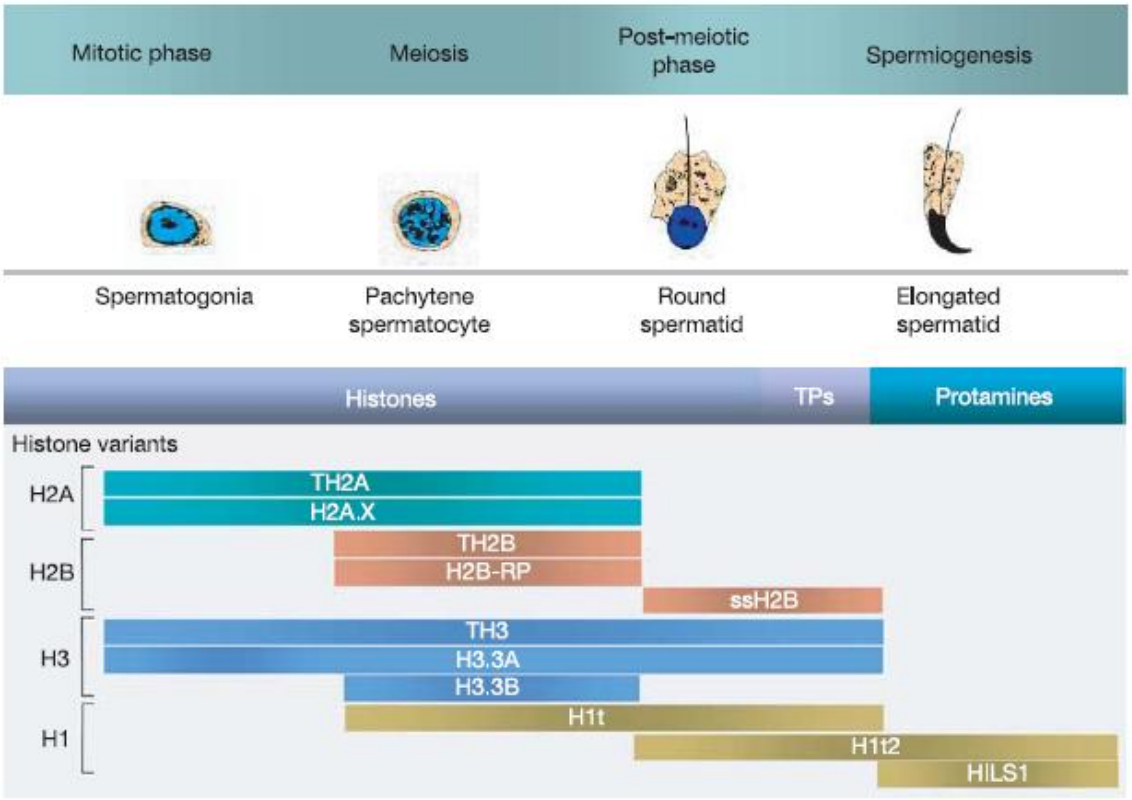


Fig 3: Histone variants in spermatogenesis- Unique chromatin remodelling during the development of male germ cells. Spermatogonia generate preleptotene spermatocytes, which enter meiosis. Next, round spermatids enter the spermiogenic phase. Germ cells contain many histone variants, which are expressed at different stages of male germ cell development. During the last phase of spermiogenesis, chromatin is highly compacted, a process that includes the replacement of most histones with sperm-specific transition proteins (TPs), which are subsequently replaced by protamines. The various histone variants that are expressed in the various stages are shown. **(Adapted from Sarah Kimminutes & Paolo Sassone-Corsi, *Nature* (2005))**

1.3. Spermatogenesis

Spermatogenesis is the generation of a transportable male genome placed in the nucleus of spermatozoa, which is capable of leaving and surviving the parent organism. This process is characterized by three main distinct stages: **premeiotic**, **meiotic** and **postmeiotic** stages. The premeiotic stage is characterized by the division of spermatogonial cells that undergoes DNA replication and extensive differentiation to give rise to tetraploid pre-leptotene spermatocytes. In the next stage i.e meiotic stage, the spermatocytes produced from spermatogonia undergo meiosis I to yield secondary spermatocytes, which rapidly undergoes meiosis II to generate round, haploid spermatids. The third and the final stage i.e. postmeiotic stage is where the maturation of the spermatids occurs. There occurs a global nuclear remodeling where most of the histones are first replaced by Transition Proteins (TPs), which are finally replaced by highly basic proteins known as protamines in the process known as spermiogenesis where extensive chromatin remodeling and the formation of mature spermatozoa take place[6]. Although the majority of the histones are replaced by

protamines in the mature spermatozoa, there still exists some histone-bound DNA which is required for activation of genes required for early development[7].

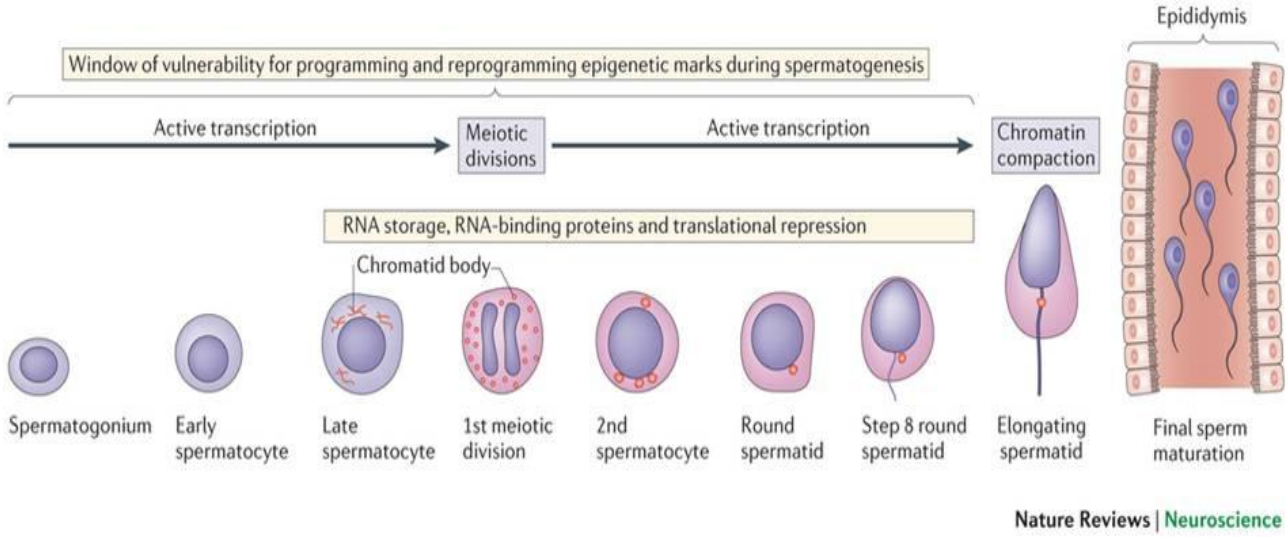


Fig 4: Various stages of spermatogenesis- The different stages of spermatogenesis and sperm maturation. Active transcription and storage of RNA is ongoing through the spermatogonium, spermatocyte and spermatid stages. In the final stages of sperm maturation transcription is halted and most of the histones are replaced by protamines. **(Adapted from Tracy L. Bale, *Nature Reviews Neuroscience* (2015))**

1.4. Meiotic recombination

Meiosis is the process of formation of haploid gametes which are required for sexual reproduction. It is characterized by various stages of which the most important is Prophase I. It is the longest process that occurs during the meiotic stage, leading to diversity of alleles in the offspring. It is characterized by the stages Prophase I, Metaphase I, Anaphase I and Telophase I. Prophase I is further divided into leptotene, zygotene, pachytene, diplotene, and diakinesis. Homologous pairing, meiotic recombination, and active transcription are the main processes that occur during this phase[8]. Proper chromosome segregation is also an essential process to give rise to viable gametes. Prophase I is a tightly regulated process involving various protein complexes.

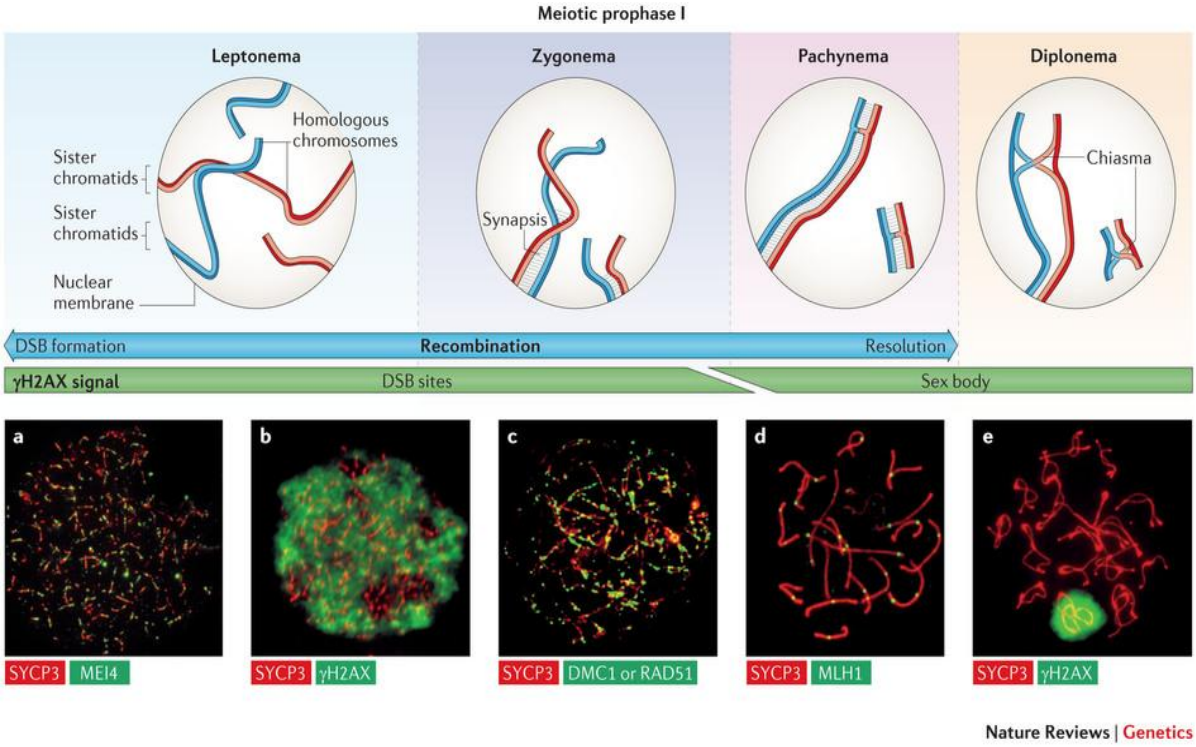


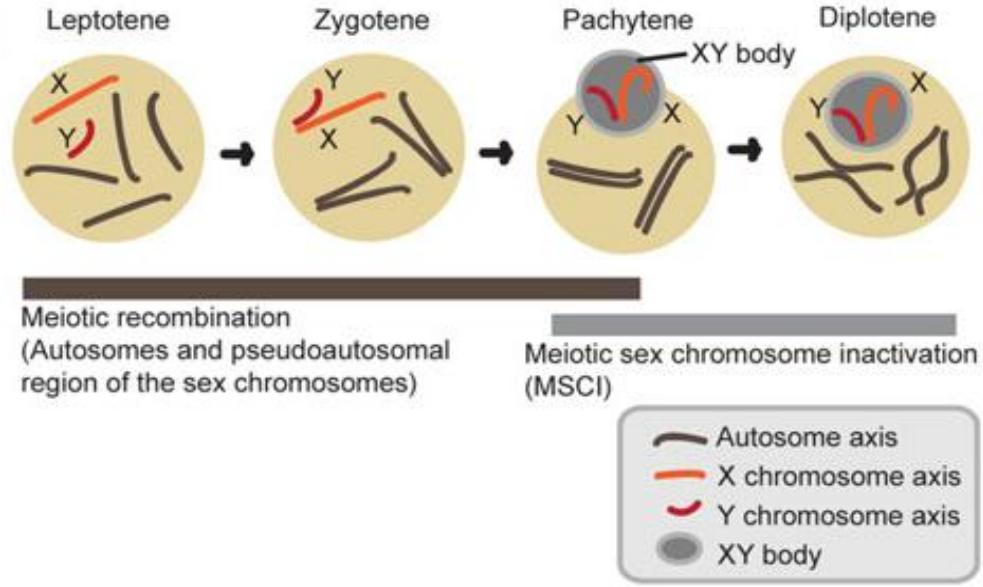
Fig 5: Various stages of meiotic recombination- Meiotic recombination starts with the formation of double-strand breaks (DSBs) at leptonema and is completed before the end of pachynema. Synapsis is initiated at zygonema (both chromosome ends are attached to the nuclear envelope at this stage). The bottom half of the figure shows immunofluorescence staining of synaptonemal complex protein 3 (SYCP3) and stage-specific signals on mouse spermatocyte spreads. **a** | Meiosis-specific protein MEI4-homologue (MEI4) localizes with SYCP3 at leptonema. **b** | H2AX is phosphorylated (γH2AX) by ataxia telangiectasia mutated (ATM) following DSB formation at leptonema. **c** | DNA recombinases DMC1 (meiotic recombination protein DMC1/LIM15 homologue) and RAD51 are localized at DSB repair sites at zygonema. **d** | MutL protein homologue 1 (MLH1) localizing at DSB sites are repaired by the interfering crossover pathway at pachynema. **e** | γH2AX is observed on sex body at diplonema. **(Adapted from Baudat, F., Imai, Y. & de Massy, B. *Nature Reviews Genetics* (2013))**

Meiotic recombination is initiated by the formation of programmed DNA DSBs catalyzed by Spo11 and other accessory proteins at the leptotene stage[9]. Upon the induction of DNA DSBs, H2AX becomes phosphorylated as serine 139 to become γH2AX, which signals the downstream processes for recombination. This phosphorylation is catalyzed by ATM kinase[10]. After this first level of phosphorylation of H2AX, recombination is further amplified in zygotene by the MRN (MRE11-Rad50-NBS1) complex. This complex further activates ATM kinase which in turn phosphorylates more H2AX, generating a positive feedback loop. There are multiple phosphatases which have been identified that negatively regulate this process[11]. Also, strand invasion takes place in zygotene which is mediated by Rad51 and Dmc1[9]. During pachytene, H2AX is phosphorylated at serine 139 by another PI3K kinase, ATR. This phosphorylation of H2AX by ATR kinase takes place in a specialized region known as the XY body. Due to less region of homology between X and Y chromosomes,

recombination is delayed and occurs only at specialized regions known as "Pseudoautosomal Region" or PAR[12]. The non-PAR is transcriptionally inactive. ATM-dependent phosphorylation of H2AX is a hallmark for autosomal recombination whereas ATR-dependent phosphorylation is unique to the XY body. In diplotene, the crossover and the non-crossover products get resolved[9].

H2AX, the histone variant of the canonical histone H2A, plays a crucial role in the initiation of meiotic recombination and in the formation of XY body. Spo11, which induces DSBs and initiates meiotic recombination, is also shown to be required for XY body formation as Spo11-null mice fail to form γH2AX foci in the XY body ultimately leading to failure of meiosis and male sterility.[13, 14].

A)



B)

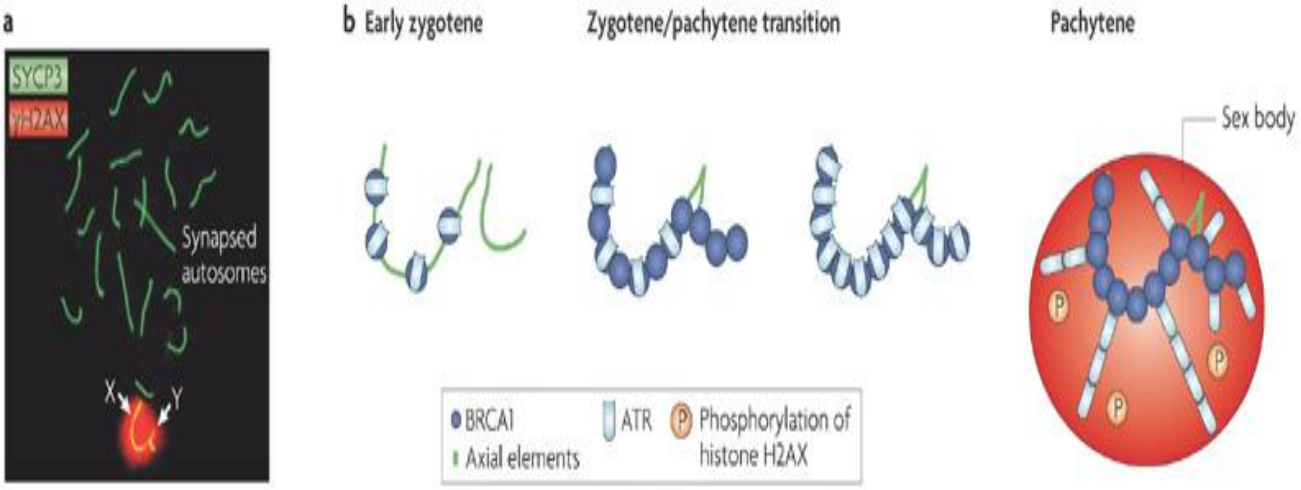


Fig 6: Formation of the XY body- A) Schematic representation of the XY body formation at various stages of meiotic Prophase I B) a | A pachytene spermatocyte stained for the chromosomeaxial element marker SYCP3 and for the phosphorylated form of the histone variant H2AX (γH2AX), which marks the transcriptionally silenced XY chromatin domain (the sex body). b | At the beginning of zygotene the DNA damage response protein BRcA1 and the checkpoint kinase ATR are already present at the sites of all meiotic double-stranded breaks (DSBs), including those on the unpaired X axis. At the zygotene/pachytene transition there is further DSB-independent recruitment of BRCA1 to the asynapsed X and Y axes, which in turn recruits additional ATR; ATR then spreads into the chromatin loops that are associated with the asynapsed X and Y axes and phosphorylates H2AX, triggering the chromatin changes that lead to transcriptional silencing**. (Adapted from Kato et al., *Hum Mol Genet.* (2015), Burgoyne et.al, *Nature Reviews Genetics* (2009))**

Recombination-induced DSBs do not occur randomly in the nucleus. They are localized to specialized chromatin regions called the recombination hotspots. About 94% of recombination hotspots are enriched in histone modification H3K4me3[15]. H3K4me3 associated transcription marks are distinct from the recombination related H3K4me3 marks[16].

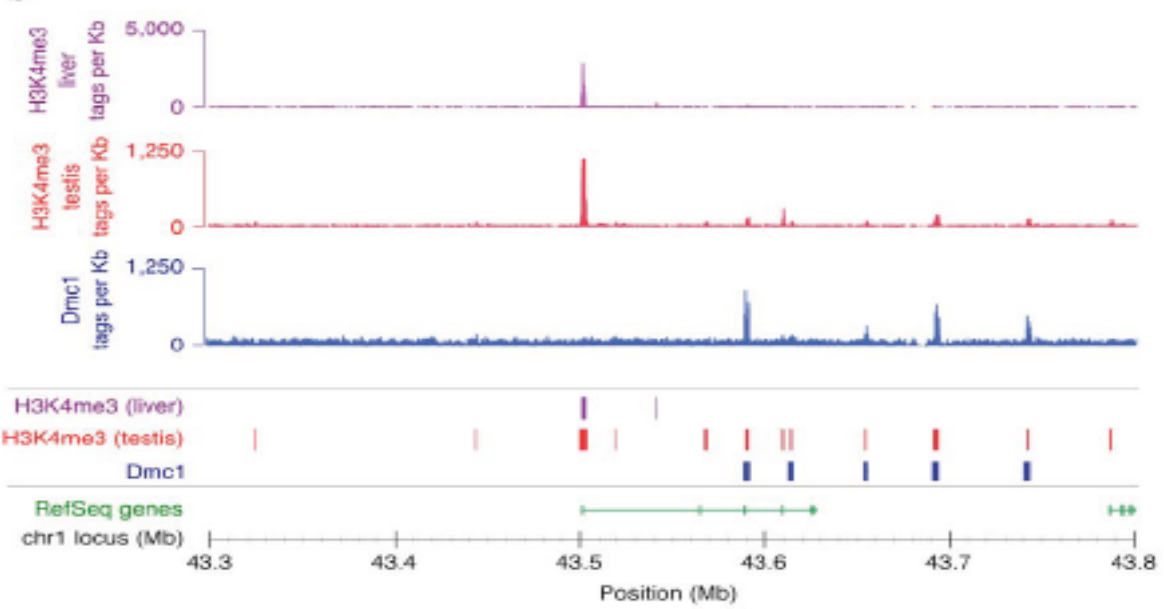


Fig 7: Specific H3K4me3 marks are associated with DSB hotspots- The vast majority of DSB hotspots overlap H3K4me3 marks, most of which are testis specific. DSB hotspots are associated with a set of H3K4me3 marks that are distinct from those at transcription start sites (TSSs) **(Adapted from Smagulova F. et al., *Nature* (2011))**

H3K4me3 formation is carried out by a specific methyltransferase, PRDM9, which mark the initiation sites of homologous recombination[15]. PRDM9 binds to recombination hotspots and reorganizes nucleosomes into a symmetrical pattern creating a nucleosome-depleted region[17]. Recently, reports have shown that along with H3K4me3, H3K36me3 also marks recombination hotspots, catalyzed by PRDM9[18]. This is the first report showing that a known epigenetic enzyme has both H3K4 and H3K36 trimethylation activity.

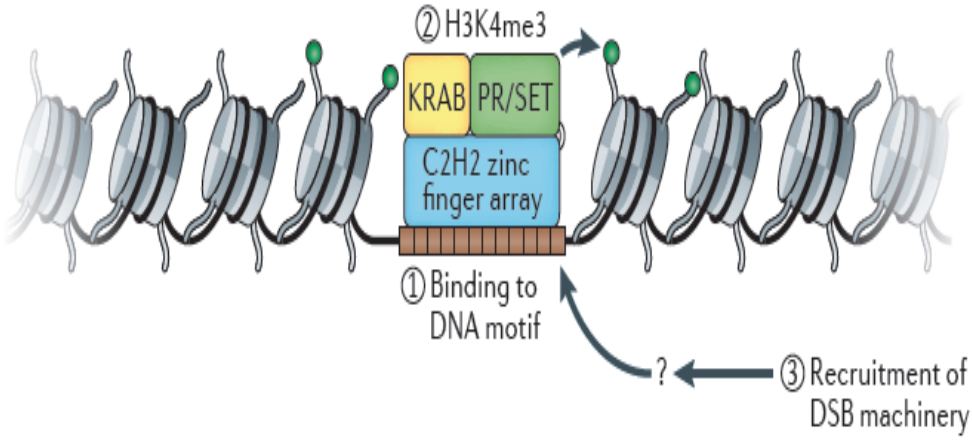


Fig 8: Model for the role of PRDM9 in meiotic DSB localisation- PRDM9 binds to a specific DNA motif (brown squares) through its C2H2 zinc finger array (blue oblong). Subsequently, the PR/SET domain (PRDI-BF1 and RIZ homologous region, a subclass of SET domains; green oblong) promotes histone H3 lysine 4 trimethylation (H3K4me3; green spheres) on adjacent nucleosomes. The Krüppel-associated box (KRAB) domain might carry out interactions with other recombination-related proteins. **(Adapted from Baudat, F., Imai, Y. & de Massy, B. *Nature Reviews Genetics* (2013))**

1.5. Posttranslational modifications of histones

Histone posttranslational modifications are one of the most crucial processes essential for providing dynamics to chromatin structure. Histones are usually modified at their flexible N-terminal tail which protrudes out from the octamer. A large number of histone posttranslational modifications have been identified which includes methylation, acetylation, phosphorylation, ubiquitinylation, etc[19]. which are carried out by respective enzymes. Histone posttranslational modifications bring about changes in the chromatin structure by disrupting DNA-histone interactions and by recruiting various reader proteins that carry out specialized functions. For example, histone acetylation for most of the cases is associated with transcriptionally active genes, whereas histone methylation is associated with both transcriptionally active genes (H3K36me3) and transcriptionally repressed genes (H3K27me3). Crosstalk between histone modifications i.e. the modification of one residue altering the ability of the second residue to be modified by its modification enzymes is said to occur[20]. Combinatorial patterns of histone modifications termed as 'Histone Code' has been suggested to specify distinct biological processes in terms of recruitment of specialized reader and effector molecules. The histone code is believed to be read by cellular machinery to regulate the biological functions and accessibility of chromatin DNA[21].

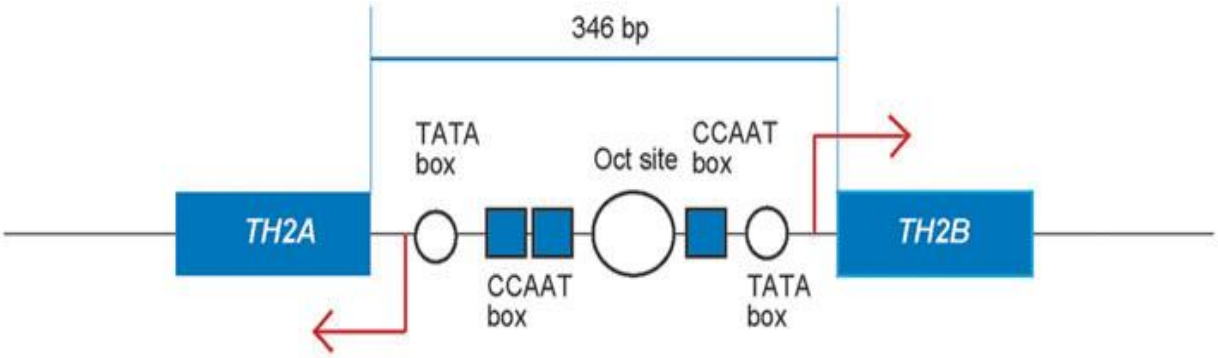
Table 1. Different Classes of Modifications Identified on Histones		
Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Fig 9: Histone posttranslational modifications and their roles- Overview of different classes of modification identified on histones. The functions that have been associated with each modification are shown. **(Adapted from Tony Kouzarides, *Cell* (2007))**

1.6. TESTIS-SPECIFIC H2B VARIANT TH2B

TH2B, the testicular histone variant of somatic H2B was first identified in 1975. It replaces 80-85 % of the somatic H2B in spermatocytes and in spermatids[22].The synthesis of TH2B occurs in a replication-independent manner. Both TH2B and TH2A genes share a common bidirectional promoter[23]. The amino acid sequence of TH2B is 85% similar to H2B, the variation lies only in the N-terminal residues of TH2B. It has also been previously shown from our laboratory that nucleosomes containing TH2B are less compact and exhibit lower stability than H2B containing nucleosomes. TH2B-containing nucleosomes are much more susceptible to Micrococcal nuclease digestion[24, 25].

A)



B)

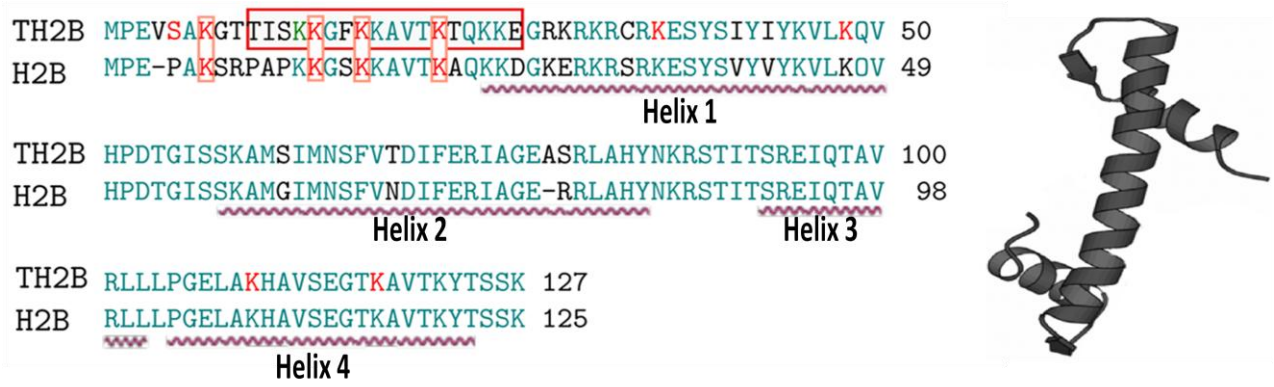


Fig 10: Testis-specific histone variant TH2B- **A)** Common bidirectional promoter of TH2A and TH2B genes **B)** Multiple sequence alignment of rat TH2B and H2B proteins, showing the different helix. The right figure shows the secondary structure of TH2B **(Adapted from Pentakota et.al, *Journal of Proteome Research* (2014))**

It has been experimentally shown that TH2B directs the histone to protamine transition in a stepwise manner[7]. The loss of TH2B cause increased expression of H2B and compensatory modifications on H3 and H4 to complement the function of TH2B and hence restoring normal sperm development. TH2B was found not be expressed exclusively in the testis. Shinagawa *et al* (26) showed that it is also enriched in the oocytes and it plays a role in reprogramming to induced pluripotent stem cells both in mouse[26] and humans[27].

We believed that histone PTMs could modulate the function of the testis-specific histone variants in germ cells. Various studies have focussed on understanding the post-translational modifications on testis-specific histone variants like TH2B, TP1[28], TP2[29], HILS1[30] etc. In this direction, Pentakota *et al* identified several PTMs on TH2B across the spermatocyte and spermatid stage using mass spectrometry. A computational 3D model of nucleosome containing TH2B was derived to study the spatial orientation of the PTMs and their effect on histone-histone and histone-NA contacts[31]. Also, the model revealed that the amino acid differences in the N-terminal tail and the posttranslational modifications acquired by some of the residues cause the destabilization of the nucleosome.

TH2B histone variant harbors various posttranslational modifications across the spermatocyte (meiotic) and the spermatid stages (post meiotic) of Rat testis. We hypothesized that the posttranslational modifications acquired by some of the residues in the amino terminal tail could contribute to the unique functions of TH2B compared to H2B in

spermatocytes. We chose Serine 12 phosphorylation of TH2B (will be referred to as TH2BS12P) for our study which was identified by Mass spectrometric analysis. The corresponding phosphorylation (Serine 14) on H2B has been shown to be involved in DNA repair in somatic cells. So we wanted to find out the biological role of Serine 12 phosphorylation. Previous studies from our lab have shown that in Rats TH2BS12P is enriched in XY body and it colocalizes with various proteins like Scp3, Spo11, Rad51 and γH2AX (partially) involved in recombination and which correspond to the unsynapsed axes of the XY body (Unpublished Data).

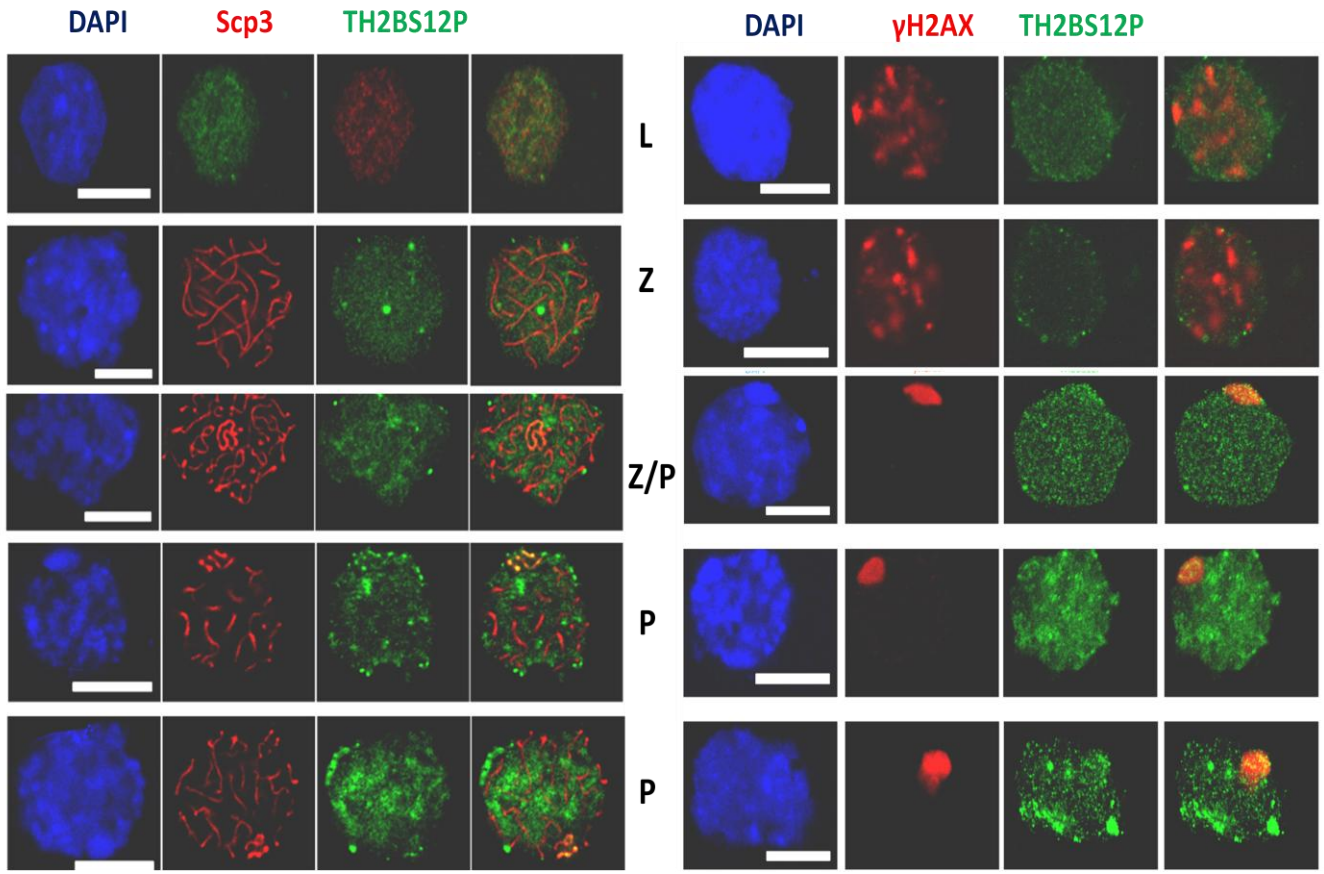


Fig 11: Earlier studies done on Rats- Immunofluorescence images of meiotic spreads showing the staining of TH2BS12P at various stages of meiotic prophase I (L-Leptotene, Z-Zygotene, P-Pachytene) with Scp3 and γH2AX, and the localisation of the modification to the XY body during Pachytene. Scale bars indicate 10 µm.

1.7. Objectives

TH2BS12P has been shown to interact with proteins related to meiotic recombination and also coexist with histone marks in mononucleosomes. We wanted to understand the role of this modification in mouse, looking whether the function of TH2BS12P is conserved. Since it

has been seen in rats that TH2BS12P plays a role in meiotic recombination and coexists with various recombination associated histone marks, we wanted to see whether this interaction occurs at the meiotic recombination hotspots. We chose Mouse as the model to study the phenomenon, as the recombination hotspots sequences are well studied and characterized in mouse[32, 33, 34]. The objectives of the present study are:

1. Detection of TH2BS12P by Western Blotting and Immunofluorescence studies of this modification with various recombination-related proteins at various stages of Meiotic Prophase I.
2. Enrichment of TH2S12P at various recombination hotspots.
3. Purification of meiotic cells of various stages from adult mouse testes and study the dynamicity of TH2S12P at various stages of meiotic recombination.

Chapter 2: Materials and Methods

MATERIALS AND METHODS

2.1. Isolation of histones from adult mouse testis

Histones were isolated from adult C57BL/6J mice testis according to the protocol of Shechter *et.al* (35). Briefly, tissues were extracted from testis and were washed with 1X PBS. To the cell pellet Hypotonic buffer (10 mM Tris-Cl Ph 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1X mPIC) was added and was incubated for 30 minutes in cold room. Nuclei were pelleted at 10,000g for 10 minutes at 4^0C and were resuspended in 0.4 N H_2SO_4 overnight. After overnight incubation, centrifugation was done at 16,000g for 10 minutes at 4^0C. Histones were extracted from nuclei using Trichloroacetic acid (TCA) precipitation for 30 minutes on ice. The histones were pelleted at 16,000g for 10 minutes at 4^0C and were washed with ice-cold Acetone. After washing, the histone pellets were dissolved in 50 µl Autoclaved MilliQ water. The extracted histones were analysed on 15 % SDS-PAGE and the concentrations were determined with respect to BSA.

2.2. Western blotting

The proteins were separated by 15% SDS-PAGE gel electrophoresis and were transferred onto a nitrocellulose membrane using semi-dry transfer technique. The membrane was stained with Ponceau and its image was taken for reference. The membrane was blocked using 3 % BSA made in 1X TBS for 1 hour at room temperature. After blocking, the blots were incubated with the primary antibody of specified dilutions in 1 % BSA for overnight in cold room on a shaker. The blots were washed twice with 0.3% TBST for 5 minutes each. The blots were then incubated with HRP-conjugated secondary antibody, diluted in 1 % BSA made in 1X TBS, for 1hr at room temperature on a shaker. Blots were subjected to extensive washing with 0.3% TBST and they were developed using the ECL kit (Thermo Scientific).

2.3. Preparation of Meiotic spreads and Immunofluorescence

22-25 day C57BL/6J mice testes were decapsulated in 1X PBS. Tissue was pelleted at 1000 rpm for 7 minutes. Hypotonic buffer (17 mM Sodium citrate, 50 mM Sucrose, 30 mM Tris-cl pH 8.2) treatment was given for 30 minutes at room temperature in shaking conditions. The cell suspension was filtered using double layer cheese cloth and it was centrifuged at 1000 rpm for 7 minutes. The pellet obtained was dissolved in 100 mM Sucrose of appropriate volume and was incubated on ice for 5 minutes. The lysate was applied to Paraformaldehyde-coated slides containing 0.15 % Triton-X-100 and was kept in a humidified chamber for 2 hours.

The smears were rehydrated prior to blocking using 1X PBS for 10minutes. After rehydration, the slides were kept for blocking using 3% BSA 1 hour at room temperature in shaking conditions.

After blocking, the slides were incubated with TH2S12P (Rabbit) at a particular dilution prepared in 0.1% BSA. The slides were incubated in a humidified chamber overnight in cold room. After incubation with the first primary antibody, the slides were washed once with 1X PBS for 5 minutes. The slides were then incubated with second primary antibody (Mouse Scp3, Mouse γH2AX and Goat Spo11) for 1 hour at room temperature in a humidified chamber. 0.1 % PBST washes were given to the slides for 10 minutes. Depending on the host species of the primary antibody, the slides were incubated with Anti rabbit-568 Alexa Fluor secondary antibody for 1hr at room temperature. The slides were then washed with 1X PBS for 5 minutes and were incubated with the second secondary antibody Anti mouse-488 Alexa Fluor. Washes were given with 0.1 % PBST for 10 minutes. The slides were mounted with DAPI and the images were obtained by Zeiss confocal laser scanning microscope.

2.4. Isolation of Mononucleosomes from adult C57BL/6J mice testes

Mononucleosomes were isolated from mice testis according to the protocol by Montellier *et.al*. Briefly, 22-25 day old mice testes were decapsulated, homogenised in Lysis buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl pH 8.0, 0.03 % Triton-X-100, 340 mM Sucrose, 2 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1X mPIC) and were incubated on ice for 10 minutes. The nuclei which were obtained after centrifugation at 1500 rpm for 10 minutes were washed with the Wash buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl pH 8.0, 340 mM Sucrose, 1 mM DTT, 0.5 mM PMSF, 1X mPIC) and were centrifuged. Nuclei were resuspended in appropriate volume of MNase buffer (10 mM Tris-Cl pH 8.0, 10 mM KCl, 2 mM $CaCl_2$) and digestion was carried out at 37^0C with 5 units of MNase (Sigma). At various time points, aliquots were taken and digestion was stopped by immediate chilling and addition of 5mM EGTA. After digestion, centrifugation was performed at 2500 rpm for 10 minutes. The nuclei fraction isolated was resuspended in LSDB 250 buffer containing 20 % Glycerol, 50 mM HEPES, 3 mM $MgCl_2$, 250 mM KCl to which Proteinase K (20 mg/ml) was added and was kept at 55^0C overnight. After overnight incubation, samples were centrifuged at 20,000g 10minutes at 4^0C and supernatant was loaded on 1 % agarose gel to check the extent of MNase digestion.

2.5. Immunoprecipitation

For immunoprecipitation studies, 100µg of mononucleosomes was used for each IP reaction. 5% of the sample was used as input. The extracts were incubated with 4-5µg of the

following antibodies TH2BS12P (Rabbit), γH2AX (Mouse) overnight and this was the 'IP' sample. The corresponding pre-immune IgG was also added. This was taken as the negative control. Both the IP and the PIS samples were incubated at 4ºC overnight in shaking conditions. The beads were added on the next day. Protein G Dynabeads beads and Protein A Dynabeads beads were used for mouse and rabbit antibodies respectively. The lysate in both IP and PIS samples were added to the beads and were incubated at 4ºC for 2.5 hours in shaking conditions. Extensive washes with LSDB 250 buffer were given to the beads and DNA was extracted from them.

2.6. Isolation of DNA from immunoprecipitated mononucleosomes and Real-time PCR

Briefly, Elution buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1 % SDS) was added to the beads and was incubated at 65^0C for 30 minutes. The supernatant was collected and was centrifuged at 20,000g for 5 minutes. The samples were left for de-crosslinking overnight at 65^0C. The samples were treated with RNase A (40 µg/ml) for 1 hour at 37ºC and Proteinase K (100 µg/ml) for 2 hours at 55ºC, after which Phenol/Chloroform extraction was carried out. The hydrophilic portion was collected after centrifugation at 20,000g for 5 minutes. To the hydrophilic portion, 3 M Sodium acetate (pH 5.2), Glycogen and 100 % Ethanol were added and were incubated at -20ºC overnight. The samples were then centrifuged at 20,000g for 30 minutes at 4ºC and the supernatant was discarded. The pellet was washed with 80 % Ethanol and was centrifuged at 20,000g for 5 minutes at 4ºC. The supernatant was discarded and the pellets were left for drying. The pellets were then dissolved in an appropriate volume of MilliQ water and were kept at 65^0C for 10 minutes to resolve the pellet.

To check for enrichment of the modification TH2BS12P across the recombination hotspots, primers were designed against selected hotspots. The sequence for the hotspots was obtained by aligning the ChIP-Seq reads (obtained from ENCODE/LICR) of H3K4me3, H3K36me3 and Dmc1. 30 ng of DNA was used for each reaction.

2.7. Flow cytometry purification of meiotic cells from adult mice testes

FACS purification of meiotic cells was carried out by the protocol given in Getun *et.al*. (36) Briefly, decapsulated testes obtained from adult C57BL/6J mice were placed in GBSS (Gey's Balanced Salt Solution) containing 120 U/ml Collagenase Type I and 10 µl DNase I (1 mg/ml stock). The solution was shaken vigorously by hand until the testicular tubules start dissociating. It was then kept at 120 rpm for 15 minutes at 33ºC and the solution was decanted to remove the supernatant. After repeating this step gain, 50 µl of 50 mg/ml Trypsin (resuspended in 1 mM HCl) and 10 µl DNase I (1 mg/ml) was added to the cells

contained in GBSS and was shaken vigorously. Incubation was done at 120 rpm for 15 minutes at 33ºC, followed by gentle pipetting with Pasteur pipette. 40 µl Hoechst 33342 (10 mg/ml, resuspended in DMSO) was added to the cells along with 30 µl Trypsin and 10 µl DNase I (1 mg/ml) and was incubated at 120 rpm for 15 minutes at 33ºC. Foetal Bovine Serum (FBS) was added after this to inactivate Trypsin and final staining was performed with 50 µl Hoechst 33342 along with 10 µl DNase I (1 mg/ml). Final incubation was performed at 120 rpm for 15 minutes at 33ºC, followed by which the samples are passed through two 40 µm GBSS pre-wetted filters and Propidium iodide (PI) was added to the solution. Flow cytometry analysis was performed immediately after this in BD FACS Aria III.

Four samples were processed for the analysis, namely, “Stained”, “Unstained”, “Hoechst stained” and “PI stained”. The sample in the “Stained” tube was processed as given above. To the samples containing in “Hoechst stained” and “PI stained” either of the two dyes was added. The samples in the “Unstained” tube were devoid of both the dyes. Just prior to adding PI to the tube labelled “PI stained” a small aliquot of the sample was taken and was boiled at 65ºC for 10 minutes to kill the cells. After killing the cells, they were dispensed back into the tube.

- THE LISTS OF COMMERCIAL ANTIBODIES USED ARE GIVEN IN THE FOLLOWING TABLE:

Antibody	**Manufacturer**	**Catalogue number**
Anti-Scp3	Abcam	ab97672
Anti-γH2AX	Millipore	05-636
Anti-Spo11	Santa Cruz Biotechnology	sc-22476
Anti-TH2BS12P	Imgenex	-

- THE LISTS OF PRIMERS USED FOR REAL-TIME PCR ARE GIVEN IN THE FOLLOWING TABLE:

Region	**Forward Primer**	**Reverse Primer**
PAR Region 1	5’ GCCCAGACATCTAGAGTTTTC 3’	5’ GCCACGATCTTCTGGAGTG 3’
H3K36me3 PAR	5’ CAGGGAAATGCAAATCAGAAC 3’	5’ GCTTCTTGACGGGATGCATTG 3’
Pbx1 Peak	5’ ATACAGCTGGCTTGCTTGGT 3’	5’ CCCCCTTCCCCATAATACTG 3’
Hlx1 Peak	5’ GGTCGGTGTGAGTATTAGACG 3’	5’GGCTACTATACCTTATGCTCTG 3’
Chr1 Peak	5’ CCCACAGTTTCCTTCCGTCTT 3’	5’ CCTCTGTATTTCCTAGAACATG 3’

Chapter 3: Results and Discussions

RESULTS and DISCUSSIONS

3.1 TH2BS12P is present in the adult mice testes

TH2BS12P was found to be present in mice testes as observed from Western blotting done with histones isolated from mice testes.

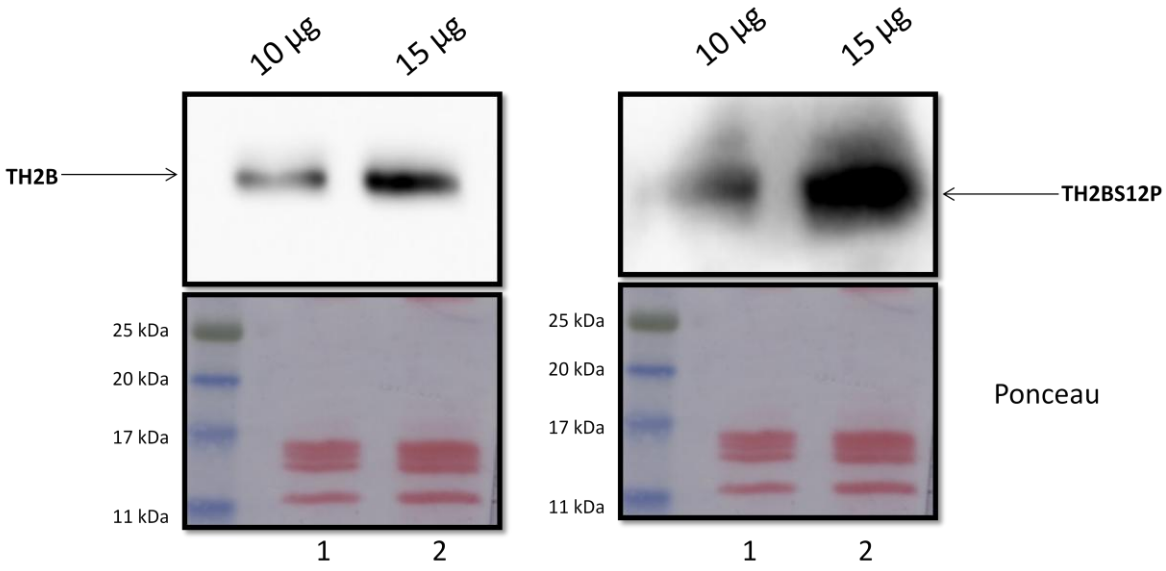


Fig 1: TH2BS12P is present in the mouse testis- Western blot images showing presence of TH2B and TH2BS12P in adult mice histones isolated from the testis. Lanes 1 and 2 indicate the two different histone concentrations.

Since the expression of TH2BS12P was confirmed in mice, we went forward to check for the localization of this modification at the various stages of Meiotic Prophase I with respect to various recombination-related proteins.

3.2. TH2BS12P shows the same localization pattern as in rats at various stages of meiotic recombination

Immunofluorescence studies of TH2BS12P were carried out to check whether the phenomenon that was seen in rats, was also conserved in the mice. Scp3 staining was used to differentiate the various stages of Meiotic Prophase I. Scp3 is a component of the axial/lateral element of the Synaptonemal Complex. It was interesting to observe that similar in rats, TH2BS12P was enriched in the axes of the XY body of mouse spermatocytes during Pachytene as colocalisation with Scp3 could be observed. This modification was also observed in the Leptotene and the Zygotene stages.

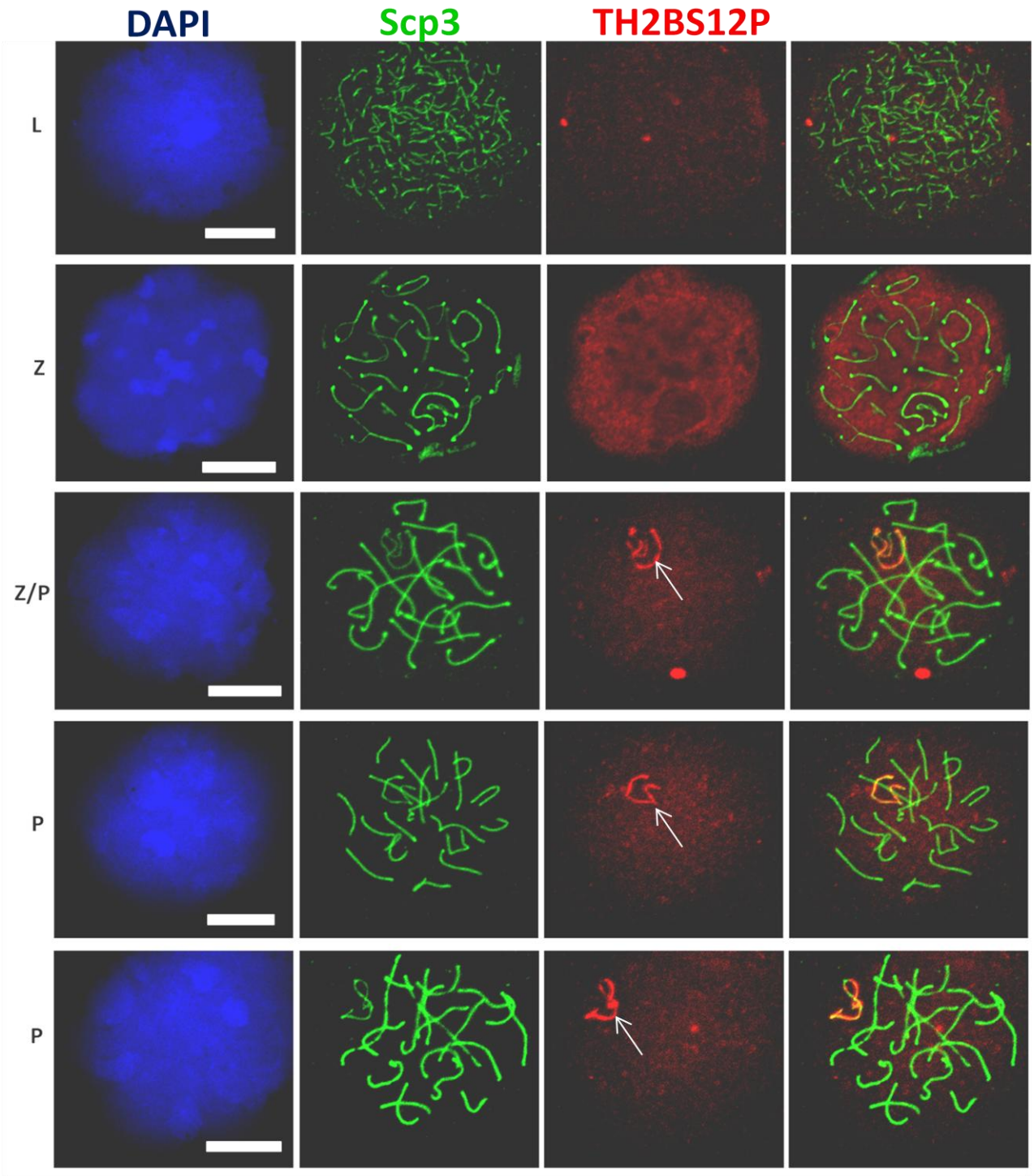
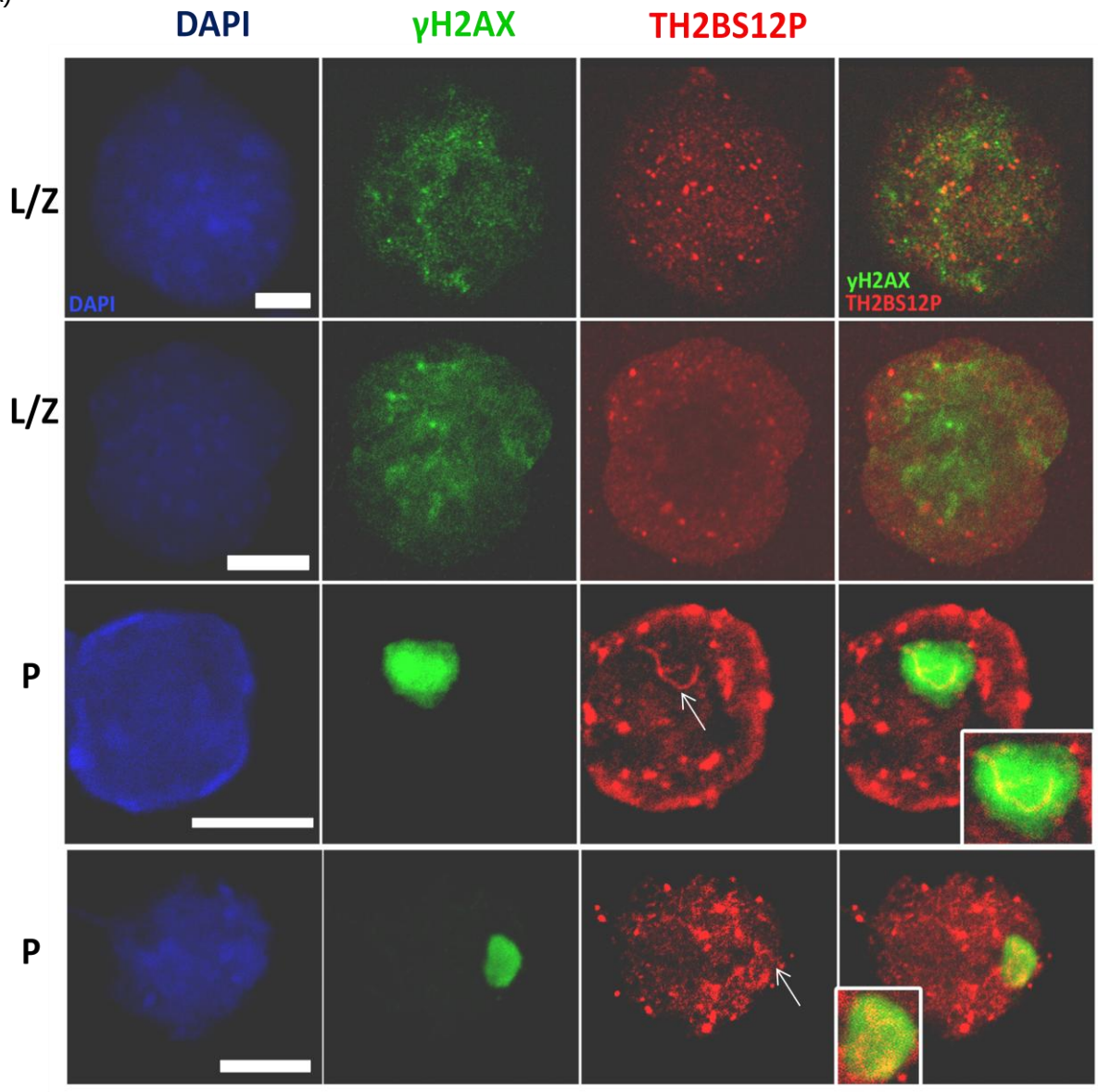


Fig 2: Immunofluorescence images of mice meiotic spreads Colocalisation studies showing TH2BS12P staining across various stages of Meiotic Prophase I with Scp3; L-Leptotene, Z-Zygotene, P-Pachytene. Arrows indicate the XY body. Scale bars indicate 10 µm.

As mentioned in the introduction, various recombination-related proteins such as γH2AX (marker for DSB) and Spo11 (forms DSB) play a role in the formation of XY body and becomes localized there during the pachytene stage. Spo11 is responsible for the formation of γH2AX which leads to the formation of XY body, loss of which leads to sterility[13, 14]. So we went ahead and carried out immunofluorescence studies with TH2BS12P, γH2AX and Spo11. We found out that TH2BS12P showed colocalisation with both γH2AX and Spo11 corresponding to the axes of the XY body during the pachytene stage.

A)



B)

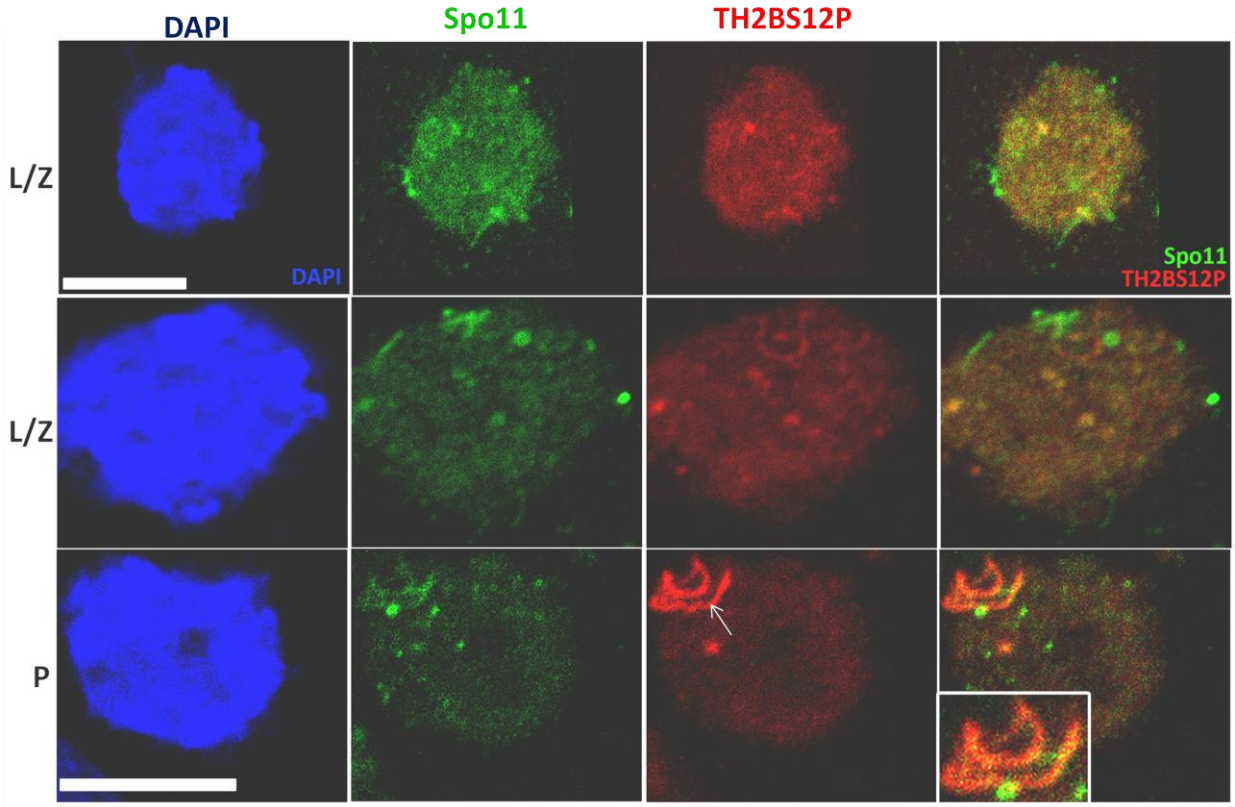


Fig 3: Immunofluorescence images of mice meiotic spreads Colocalisation studies showing TH2BS12P staining across various stages of Meiotic Prophase I **A)** With γH2AX, and **B)** With Spo11; L-Leptotene, Z-Zygotene, P-Pachytene. Arrows indicate the XY body. Scale bars indicate 10 µm.

Immunofluorescence studies led us to the conclusion that the role of TH2BS12P is conserved in the axes of the XY body as shown from the colocalisation with major players in meiotic recombination. As we can observe that the phenomenon is conserved in both rats and mice, we went ahead to see what is the status of TH2BS12P at the genomic level i.e. at the recombination hotspots.

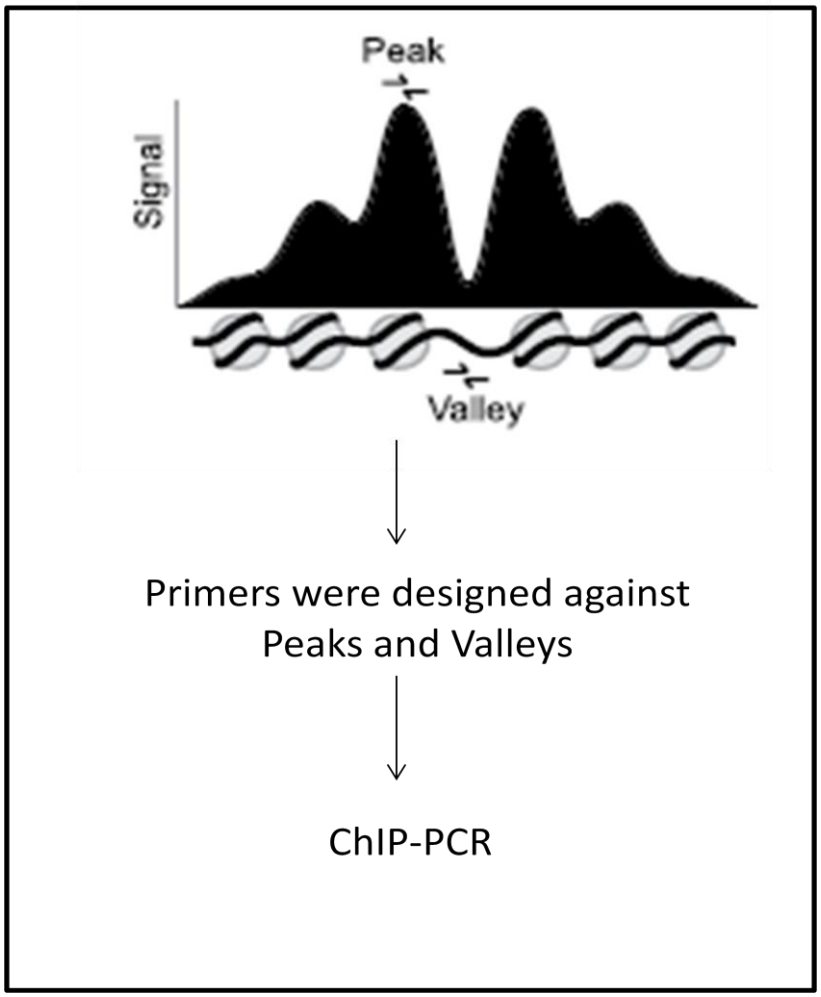
3.3. Enrichment of TH2BS12P across various meiotic recombination hotspots

As mentioned earlier in the introduction, recombination happens in certain regions of the genome known as “recombination hotspots”. PRDM9, a SET domain-containing methyltransferase, defines these hotspots by trimethylating H3 at Lysine 4 and Lysine 36 respectively. Also, PRDM9 binding creates Nucleosome-depleted Region (NDR) known as “Valleys” and creating a symmetrical pattern of nucleosomes on either side of valleys known as “Peaks”[18]. For our present study, primers were designed against the peak regions. Numerous studies on recombination hotspots have been done with mouse as the model,

since the recombination sequences are very well known and characterized in the mouse[32, 33, 34]. So we were interested in knowing the localization of TH2BS12P at various recombination hotspots, both on the autosomes and on the Pseudoautosomal Region (PAR), after confirming that indeed this modification is conserved in both rats and mice.

We chose three autosomal hotspots (Pbx1, Hlx1, and Chr1) and a hotspot in the Pseudoautosomal region which is the small region of homology between X and Y chromosomes. We wanted to check the enrichment level of TH2BS12P at these chosen hotspots by ChIP-PCR. As mentioned earlier, hotspot regions were chosen by aligning ChIP-seq reads of H3K4me3, H3K36me3 and Dmc1.

A)



B)

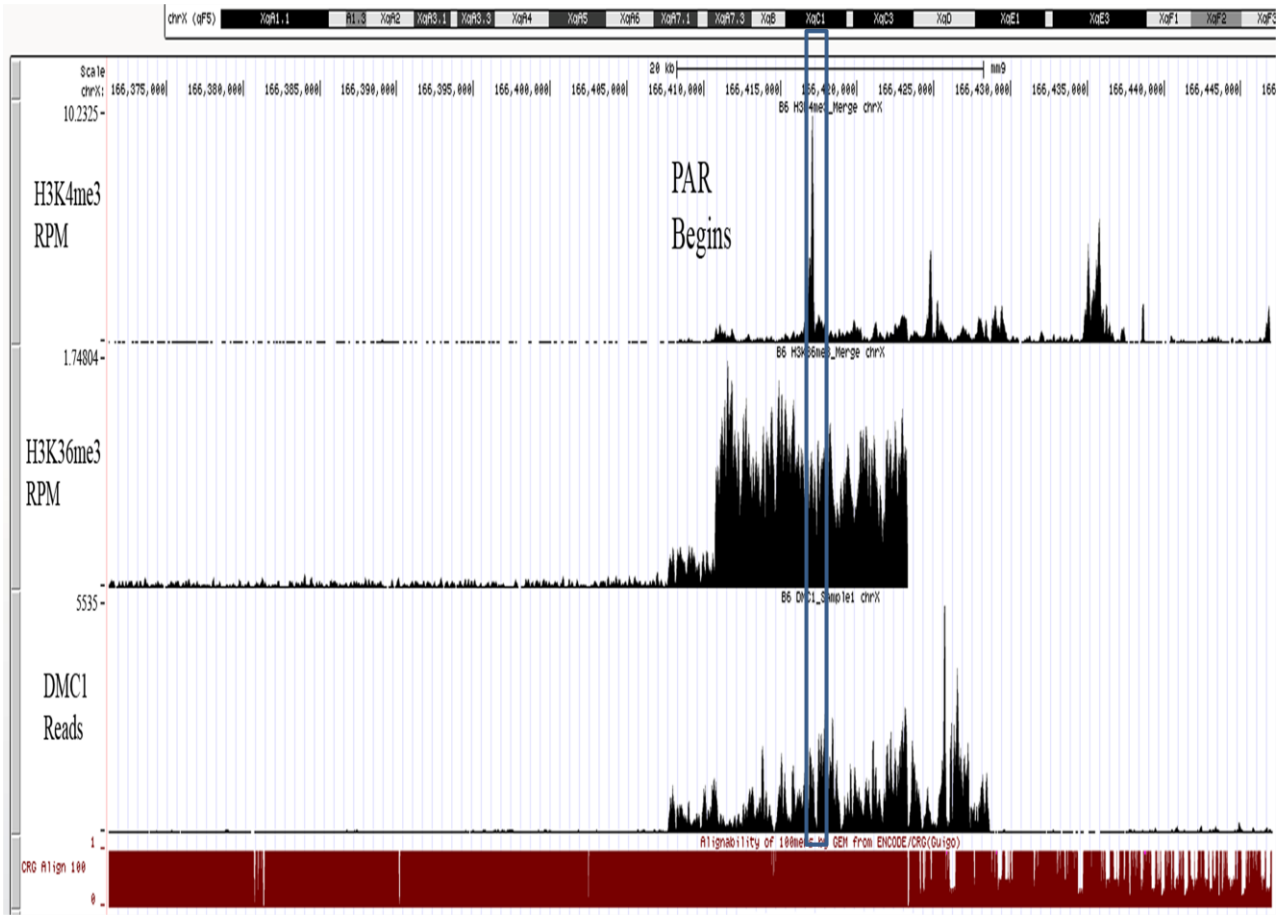


Fig 4: Meiotic Recombination Hotspots studies- A) Strategy of primer designing **B)** Selection of recombination hotspot sequences by aligning ChIP-seq reads of H3K4me3, H3K36me3 and Dmc1. **(Adapted from Baker, C. L., Walker, M., Kajita, S., Petkov, P. M. & Paigen, K. *Genome Res.*(2014), https://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=580163559_xTef7U0bWuP331T2o5gdlQZB6y4r&c=chr3&g=wgEncodeLicrHistone)**

TH2BS12P containing nucleosomes were isolated by Native ChIP to isolate the DNA bound by TH2BS12P histone marks. PCR was carried out to check for the presence of this modification at the PAR and autosomal recombination hotspots. Primers specific for the hotspots were used and the fold enrichment over Input sample was calculated.

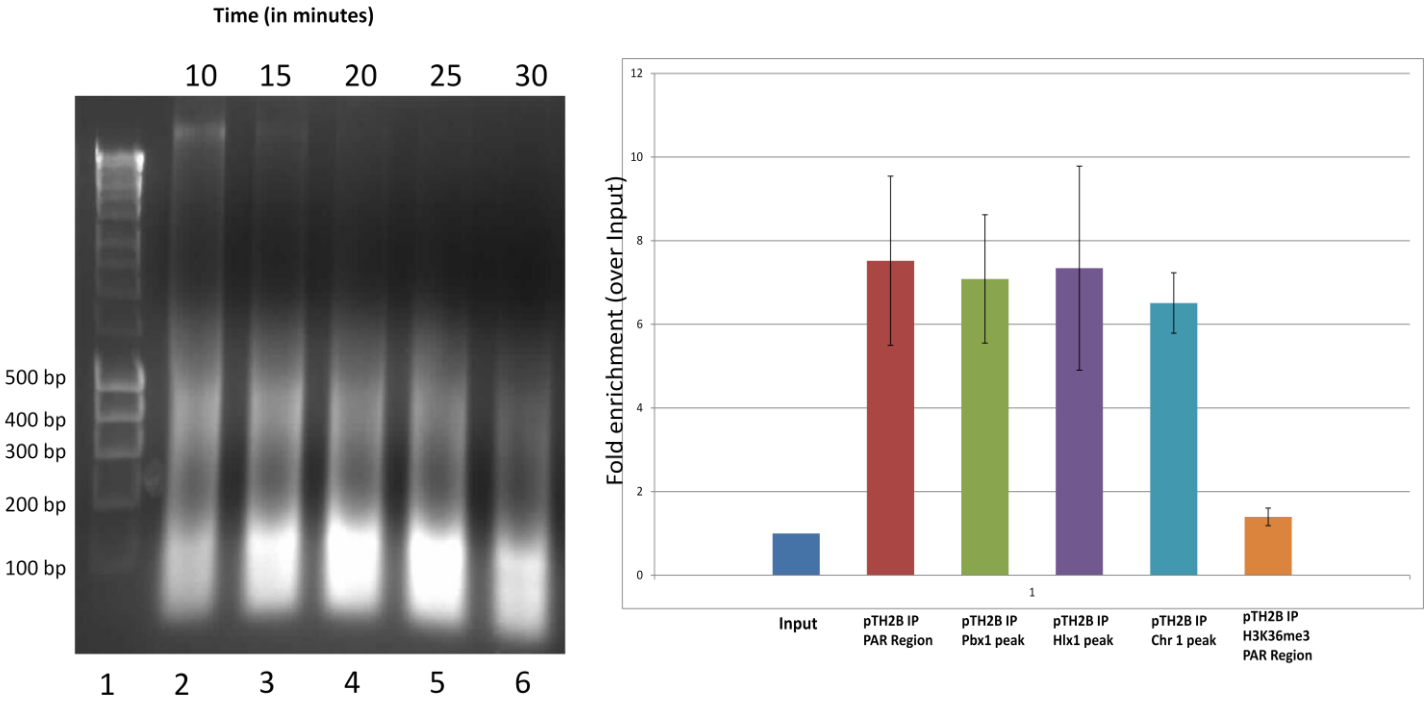


Fig 5: ChIP-PCR analysis of TH2BS12P in meiotic recombination hotspots- (Left) Mononucleosome digestion pattern. Lanes 1-6 indicate the various time points of digestion; (Right) Real-time PCR profile of TH2S12P enrichment at various recombination hotspots. Fold enrichment was calculated over Input. (n=2)

From our ChIP-PCR analysis, it is evident that TH2BS12P is highly enriched in the PAR i.e. the XY body. Also, we found enrichment of this modification at autosomal hotspots, confirming the Immunofluorescence data which ultimately suggests that this modification is involved with both recombination-related proteins and hotspot DNA. Since autosomal DSBs occur mostly at the leptotene stage we need pure populations of cells from the leptotene, zygotene, and pachytene stages to study PAR recombination or XY body formation.

3.4. FACS purification of meiotic cells from adult mice testes

To study the dynamics of TH2BS12P at the recombination hotspots at various stages of Meiotic Prophase I, it is necessary to get pure populations of meiotic cells, from spermatogonia to diplotene stage. Elutriation can achieve up to 70-75 % purity of meiotic cells which is not suitable to study the dynamics at the recombination hotspots. Also, it is very difficult to isolate preleptotene, leptotene, zygotene cell populations from elutriation. FACS methodology[32, 36] allows one to isolate meiotic fractions from adult mice to more than

95 % purity in a single experiment. This technique separates meiotic cells based on the variations of size, chromatin structure and DNA content of the various meiotic cells. Two dyes, Hoechst 33342 and Propidium Iodide (PI) are used in combination to distinguish between dead and live cells respectively. Hoechst 33342 uptake depends on the chromatin structure and DNA content of the cells and gives a clear picture of the different meiotic cell populations[37]. So we resorted to this technique to further explain the results obtained from ChIP-PCR.

A) B)

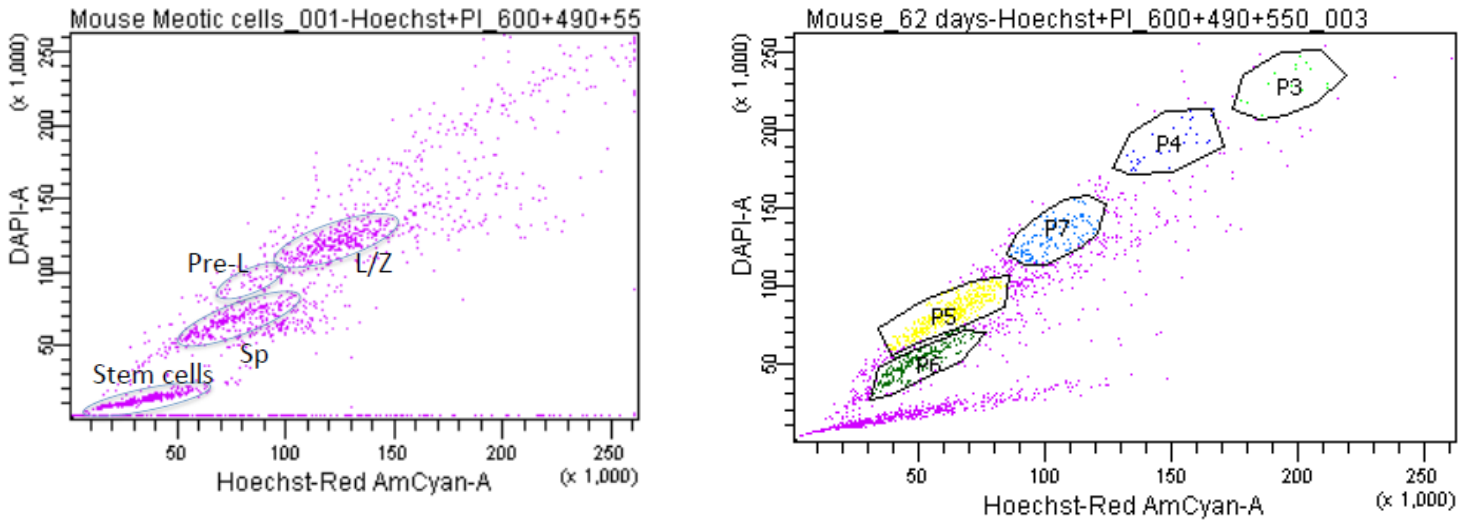


Fig 6: Flow cytometry analysis of mouse meiotic cells- Flow cytometry profile obtained from **A)** 30 days old mouse testis; Sp-Spermatogonia, Pre-L-Preleptotene, L/Z-Leptotene-Zygotene **B)** 60 days old mouse testis; P6-Spermatogonia, P5-Preleptotene, P7- Leptotene-Zygotene, P4-Pachytene, P3-Diplotene

FACS technique was found to work where we could visualize the various meiotic cell populations. The less enrichment of pachytene and diplotene cells is due to the lower age of the mouse whereby all the populations of meiotic cells are not developed yet. We plan to carry out this methodology with older day mice (at least 10 weeks old) in order to see the various meiotic cell populations at high enrichment.

To summarize, we have shown that TH2BS12P is conserved in both rat and mouse and from our recombination studies we have shown that this modification is indeed present in recombination hotspots where maximum enrichment was found to be in the axes of the XY body. Since we had also found enrichment of TH2BS12P in some autosomal hotspots, we used FACS to isolate pure populations of meiotic cells and we plan to perform ChIP-seq studies from the purified cell populations, as different processes occur at different stages of

meiotic recombination. Also, pure cell populations obtained from FACS will help us better understand TH2BS12P and its biological role in a homogenous population of cells.

3.5. FUTURE OBJECTIVES

1. Dynamics of TH2BS12P at various recombination hotspots using FACS-sorted meiotic cell populations.
2. Purify TH2BS12P-containing nucleosomes and address the histone PTMs that occur in those nucleosomes.
3. Carrying out ChIP-seq to better understand the ChIP-PCR results that we had obtained.

Chapter 4: References

REFERENCES

1. Luger. K. *et al*. Crystal structure of nucleosome core particle at 2.8 Å resolution. *Nature*. **389,** 251-260 (1997).
2. Woodcock, C. L. & Ghosh, R. P. Chromatin Higher-order Structure and Dynamics. 1–26 (2017).
3. Sarma and Reinberg, Histone variants meet their match. *Nature Reviews Molecular Cell Biology*, **Vol 6** (2005).
4. Bernstein, E. & Hake, S. B. The nucleosome: a little variation goes a long way. *Biochem. Cell Biol.* **84,** 505–507 (2006).
5. Govin *et al*. The role of histones in chromatin remodelling during mammalian spermiogenesis. *Eur. J. Biochem*. **271**, 3459-3469 (2004).
6. Gaucher, J. *et al.* Bromodomain-dependent stage-specific male genome programming by Brdt. *EMBO J.* **31,** 3809–20 (2012).
7. Montellier *et al*. Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes & Development*. **27**, 1680-1692 (2013).
8. Handel and Schimenti. Genetics of mammalian meiosis: regulation, dynamics and impact on fertility. *Nature Reviews Genetics*. **Vol 11** (2010).
9. Székvölgyi, L., Ohta, K. & Nicolas, A. Initiation of meiotic homologous recombination: Flexibility, impact of histone modifications, and chromatin remodeling. *Cold Spring Harb. Perspect. Biol.* **7,** 1–18 (2015).
10. Bernard de Massy. *et al*. Initiation of Meiotic Recombination: How and Where? Conservation and Specificities among eukaryotes. *Annual Reviews of Genetics.* **47,** 563-599 (2013).
11. Keogh, M.C. *et al*. A phosphatase complex that dephosphorylates gamma H2AX regulates DNA damage checkpoint recovery. *Nature* **26,** 497-501 (2006).
12. Burgoyne, P. S., Mahadevaiah, S. K. & Turner, J. M. a. The consequences of asynapsis for mammalian meiosis. *Nat. Rev. Genet.* **10,** 207–216 (2009).
13. Carofiglio, F. *et al.* SPO11-Independent DNA Repair Foci and Their Role in Meiotic Silencing. *PLoS Genet.* **9,** (2013).
14. Bellani, M. A., Romanienko, P. J., Cairatti, D. A. & Camerini-Otero, R. D. SPO11 is required for sex-body formation, and Spo11 heterozygosity rescues the prophase arrest of Atm-/- spermatocytes. *J Cell Sci* **118,** 3233–3245 (2005).
15. Baudat, F., Imai, Y. & de Massy, B. Meiotic recombination in mammals: localization and regulation. *Nat. Rev. Genet.* **14,** 794–806 (2013).
16. Smagulova, F. *et al.* Genome-wide analysis reveals novel molecular features of mouse recombination hotspots. *Nature* **472,** 375–378 (2011).

17. Baker, C. L., Walker, M., Kajita, S., Petkov, P. M. & Paigen, K. PRDM9 binding organizes hotspot nucleosomes and limits Holliday junction migration. *Genome Res.* **24,** 724–732 (2014).
18. Powers, N. R. *et al.* The Meiotic Recombination Activator PRDM9 Trimethylates Both H3K36 and H3K4 at Recombination Hotspots In Vivo. *PLoS Genet.* **12,** 1–24 (2016).
19. Kouzarides. Chromatin modifications and their function. *Cell*. **128**, 693-705 (2007).
20. Jung-Shin Lee. The Language of Histone Crosstalk. *Cell*. **142** (2010).
21. Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403,** 41–45 (2000).
22. Meistrich, M. L., Bucci, L. R., Trostle-Weige, P. K. & Brock, W. A. Histone variants in rat spermatogonia and primary spermatocytes. *Dev. Biol.* **112,** 230–240 (1985).
23. Huh, N. eung, Hwang, I., Lim, K., You, K. hee & Chae, C. bom. Presence of a bi-directional S phase-specific transcription regulatory element in the promoter shared by testis-specific TH2A and TH2B histone genes. *Nucleic Acids Res.* **19,** 93–98 (1991).
24. Rao, B. J., Brahmachari, S. K. & Rao, M. R. S. Structural organization of the meiotic prophase chromatin in the rat testis. *J. Biol. Chem.* **258,** 13478–13485 (1983).
25. Rao, B. J. & Rao, M. R. S. DNase I site mapping and micrococcal nuclease digestion of pachytene chromatin reveal novel structural features. *J. Biol. Chem.* **262,** 4472–4476 (1987).
26. Shinagawa, T. *et al.* Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. *Cell Stem Cell* **14,** 217–227 (2014).
27. Huynh, L. M., Shinagawa, T. & Ishii, S. Two Histone Variants TH2A and TH2B Enhance Human Induced Pluripotent Stem Cell Generation. *Stem Cells Dev.* **25,** 251–8 (2016).
28. Pradeepa, M. M. & Rao, M. R. S. Chromatin remodeling during mammalian spermatogenesis: role of testis specific histone variants and transition proteins. *Soc. Reprod. Fertil. Suppl.* **63,** 1–10 (2007).
29. Baskaran R. & Rao M. R. S. Interaction of Spermatid-specific Protein TP2 with Nucleic Acids, In Vitro. *J. Biol. Chem.* **265,** 21039–21047 (1990).
30. Mishra, L. N., Gupta, N. & Rao, S. M. R. Mapping of post-translational modifications of spermatid-specific linker histone H1-like protein, HILS1. *J. Proteomics* **128,** 218–230 (2015).
31. Pentakota, S. K., Sandhya, S., P. Sikarwar, A., Chandra, N. & Satyanarayana Rao, M. R. Mapping post-translational modifications of mammalian testicular specific histone variant TH2B in tetraploid and haploid germ cells and their implications on the dynamics of nucleosome structure. *J. Proteome Res.* **13,** 5603–5617 (2014).

32. Getun, I. V, Wu, Z. K., Khalil, A. M. & Bois, P. R. J. Nucleosome occupancy landscape and dynamics at mouse recombination hotspots. *EMBO Rep.* **11,** 555–560 (2010).
33. Lange, J. *et al.* The Landscape of Mouse Meiotic Double-Strand Break Formation, Processing, and Repair. *Cell* **167,** 695–708.e16 (2016).
34. Baudat *et al.* PRDM9 is a Major Determinant of Meiotic Recombination Hotspots in Humans and Mice. *Science.* **327,** 836-840 (2010).
35. Shechter, D., Dormann, H. L., Allis, C. D. & Hake, S. B. Extraction, purification and analysis of histones. *Nat Protoc* **2,** 1445–1457 (2007).
36. Getun, I. V, Torres, B. & Bois, P. R. J. Flow cytometry purification of mouse meiotic cells. *J. Vis. Exp.* e2602 (2011).
37. Bastos, H. *et al.* Flow cytometric characterization of viable meiotic and postmeiotic cells by Hoechst 33342 in mouse spermatogenesis. *Cytom. Part A* **65,** 40–49 (2005).