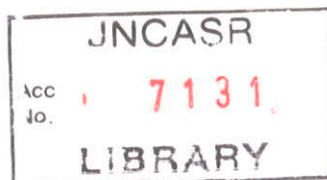


**Chromatin Remodeling During Spermiogenesis:  
Molecular Components Involved in Nuclear Import of  
Transition Protein 2 (TP2) and Role of Acetylation in  
Modulating TP2 Function**



A Thesis

Submitted for the Degree of

**Doctor of Philosophy**

By

**PRADEEPA MADAPURA MARULASIDDAPPA**



Molecular Biology and Genetics Unit  
Jawaharlal Nehru Center for Advanced Scientific Research,

Jakkur, Bangalore -560064, India

January 2009

## **Certificate**

I hereby declare that the work embodied in this thesis entitled **“Chromatin Remodeling During Spermiogenesis: Molecular Components Involved in Nuclear Import of Transition Protein 2 (TP2) and Role of Acetylation in Modulating TP2 Function”** has been carried out by Pradeepa Madapura Marulasiddappa under my supervision at Molecular Biology and Genetics Unit, Jawaharlal Nehru Center for Advanced Scientific Research, Bengaluru and that it has not been submitted for any degree or diploma to any other institution.

**Prof. M.R.S. Rao**

**Bengaluru**

**Date:**

## **Declaration**

I hereby declare that this thesis entitled “**Chromatin Remodeling During Spermiogenesis: Molecular Components Involved in Nuclear Import of Transition Protein 2 (TP2) and Role of Acetylation in Modulating TP2 Function**” is an authentic record of research work carried out by me under the guidance of Prof. M. R.S. Rao at the chromatin biology laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru.

In keeping with the norm of reporting scientific observations, due acknowledgement has been made whenever work described here has been based on the findings of other investigators. Any omission owing to oversight or misjudgment is regretted.

**Pradeepa M M**

**Bengaluru**

**Date**

***...To my Parents***

## Acknowledgements

*It is my pleasure to express my sincere gratitude to my research supervisor, Prof. M.R.S. Rao, for introducing me to scientific problems and guiding me during the various phases of my PhD student life with constant encouragement and stimulating discussions throughout. I am especially thankful to him for giving me an insight into several areas and sharing his excitements. I thank you Sir, for all your constant assistance, guidance and the tremendous support professionally and personally that you gave me in the lab and thank you for everything.*

*I thank the Chairmen and all the faculty members of MBGU for making MBGU the wonderful place it is. I thank Prof. Tapas K Kundu for critical discussions and suggestions at various stages of my work and also for useful reagents. My sincere thanks to Prof. Anuranjan Anand and Dr. Shipra Agarwal for various helpful discussions, their concerns and frequent queries on my progress. I acknowledge the CSIR, Government of India for granting me fellowship and DBT and JNCASR for funding my research.*

*Special Thanks to Kishore for his reagents and discussions through out my stay at JNC. I am thankful to Manju, Sathish and Barath for their help in generating reagents. I am thankful to Suma at JNCASR, who patiently and expertly assisted in capturing images. I also thank Anitha, JNCASR DNA sequencing facility for providing the results on time. I thank Roopa, for help in MALDI-TOF analysis and Prakash and Prof. Dipankar Chatargy for help in MALDI-TOF analysis. I am thankful to generous gift of clones from several Scientists*

*I am especially thankful to my seniors Dr. Ullas, Dr. Kumaran, Dr. Sneha, Dr. Nishanth, who interacted with me during initial period of my PhD. We have had great time in scientific and non scientific discussions. I would like to thank all my present and past members of the lab –Keerthi, Ramesh, Gayatri, Surbhi, Bharath, Mishra, Dr. Shiv Shankar, Manju, Sathish, Dr. Anil, Dr. Jayashree, Dr. Shritapa, Dr. Shilpa, Uma, Nikhil, Chethan, Manohari. Muniraju and Nagendra Prasad, Nishitha. My lab mates have been fantastic friends, too, and I cherish the many wonderful times spent in their company both within and out of the lab. I am also thankful to Mr. Muniraju and Nagendra Prasad for their help.*

*I acknowledge my batchmates: Krishanpal, Dr. Kiran, Rinki, Subhro, Venky and Vani and MBGU friends Rahul, Anand, Dr. Ashish, Dr. Ram, Dr. Prashanth, Dr. Kavitha, Dr. Arpitha, Dr. Debjani, Dr. Swaminathan, Dr. Chandrima, Vinay, Javaid, Thanuja, Shrikanth, Arif, Jayasha, Selvi, Dr. Ravindra, Dr. Lingu, Mamta, Nishtha Arunima, Mukthi, and Varun for all their supports. Non MBGU friends: Arun, Anamika, Dr. Kabra, Manohar, Pavan, Sathish KM, Gopal, Dhritiman, Saikrishna, Sai Ram, Madhu, Shreyas, Discussions with them on*

***academic matters are cherished and the fun had . It is indeed a pleasure to be a part of this talented bunch of people. Special thanks to Dr. Kiran and Mrs. and Mr. Karmodia for many wonderful moments. I would also like to thank my department seniors from whom I have learnt many things.***

***Working in JNC has been great fun, thanks to all the wonderful JNCites. I thank all my friends on campus who have made my stay in the campus such a splendid one. I am thankful for various facilities at JNC; like Atomic force microscopy, comp-lab, library and animal house for their help. I thank JNC Administrative Office staffs, Administrative office Mr. Jayachandra and Academic Section, Academic, Complab, Library, Hostel, Mess, Canteen, Sports facility, Security, Transport, House keeping.***

***I am also thankful to all my GKVK friends with whom I have had the most beautiful times of my life in Bangalore. I am extremely lucky to have keerthi as my wife, we have been through so much together, and shared so many happy moments. I am very thankful to her for the constant and unconditional support and encouragement.***

***My parents, brother, sister, late grand parents and my mother in-law have always been very supportive and always gave me moral strength. I am extremely thankful to all of them. Whatever I have achieved in my life, is because of the blessings, love and encouragement of my entire family. I would like to thank all my teachers for bringing me up to aim high and believe in my abilities. Finally, I would like to thank the Almighty for providing me enough strength to carry on with my endeavors.***

***-Pradeepa MM***

# Contents

|           |   |    |
|-----------|---|----|
| i         | Declaration   |    |
| ii        | Certificate   |    |
| iii       | Acknowledgements  |    |
| iv        | Abbreviations   |    |
| <br>      |   |    |
| 1         | Introduction  | 1  |
| 1.1       | Spermatogenesis   | 1  |
| 1.1.1     | Nuclear protein transitions during spermatogenesis                                  | 2  |
| 1.1.2     | Premeiotic changes in chromatin   | 3  |
| 1.1.2.1   | Testis specific histone variants and chromatin structure of pachytene spermatocytes | 4  |
| 1.1.3     | Testis specific histones: TH2A, TH2B, TH3 and H1t                                   | 5  |
| 1.1.3     | Postmeiotic changes in chromatin  | 6  |
| 1.1.3.1   | Role of Transition proteins (TPs) during final stages of spermiogenesis.            | 8  |
| 1.1.3.2   | Transition proteins   | 8  |
| 1.1.3.2.1 | Transition protein 1  | 8  |
| 1.1.3.2.2 | Transition Protein 2 (TP2)  | 9  |
| 1.1.3.2.3 | Transition Protein 4 (TP4)  | 14 |
| 1.1.3.3   | Protamines  | 14 |
| 1.2       | Nucleocytoplasmic shuttling of proteins   | 16 |
| 1.2.1     | Nuclear import and export signals   | 17 |
| 1.2.2     | Molecular mechanism of the nuclear protein import cycle                             | 18 |
| 1.2.3     | Nuclear protein import pathways   | 19 |
| 1.2.4     | The classic nuclear protein import cycle  | 20 |
| 1.2.4.1   | Step 1 Cargo: Carrier import complex assembly                                       | 22 |
| 1.2.4.2   | Step 2 Translocation of importin cargo complex through nuclear pore complex         | 23 |
| 1.2.4.2.1 | Nucleoporins.   | 23 |

|           |  |           |
|-----------|--|-----------|
| 1.2.4.3   | Step 3 Import-complex disassembly                                    | 24        |
| 1.2.4.3.1 | Dissociation of the importin-:complex.                               | 25        |
| 1.2.4.3.2 | Cargo release.   | 25        |
| 1.2.4.4   | Step 4: Importin recycling   | 25        |
| 1.2.5     | Importins expression profiles during spermatogenesis                 | 25        |
| 1.2.6     | Nuclear import assay   | 26        |
| 1.3       | Chromatin structure and function                                     | 26        |
| 1.3.1     | Posttranslational modifications and their cellular function          | 27        |
| 1.3.1.1   | Enzymes involved in histone modification                             | 29        |
| 1.3.2     | Protein acetylation  | 31        |
| 1.3.2.1   | Histone Acetyltransferases (HATs)/ Lysine acetyl transferases (KATs) | 33        |
| 1.3.2.2   | Acetylation of non histone proteins                                  | 35        |
| 1.3.2.3   | Histone Deacetylases (HDACs) and Sirtuins                            | 38        |
| 1.3.2.4   | HDACs  | 38        |
| 1.3.2.5   | Sirtuins   | 39        |
| 1.3.3     | Protein Methylation  | 39        |
| 1.3.3.1   | Lysine Methylation.  | 40        |
| 1.3.3.2   | Arginine Methylation   | 41        |
| 1.3.4     | Association of Histone modification with chromatin function          | 42        |
| 1.3.4.1   | Transcription  | 42        |
| 1.3.4.2   | Replication  | 44        |
| 1.3.4.3   | Recombination  | 44        |
| 1.3.4.4   | DNA Repair   | 45        |
| 1.3.5     | ATP – dependent chromatin remodeling complexes                       | 45        |
| 1.3.6     | Nuclear chaperones   | 46        |
| 1.4       | AIMS and Scope of the present investigation                          | 48        |
| <b>2</b>  | <b>Materials and methods</b>   | <b>49</b> |
| 2.1       | Materials  | 49        |
| 2.2       | Antibodies   | 50        |
| 2.3       | <i>E. coli</i> Strains   | 50        |



|         |   |    |
|---------|---|----|
| 2.4     | Media for bacterial growth  | 50 |
| 2.5     | Methods   | 51 |
| 2.5.1   | Agarose gel electrophoresis of DNA                                      | 51 |
| 2.5.2   | Restriction enzyme digestion  | 51 |
| 2.5.3   | Polymerase chain reaction (PCR)   | 51 |
| 2.5.4   | Agarose gel purification of DNA fragments                               | 51 |
| 2.5.5   | Ligation reaction   | 51 |
| 2.5.6   | Preparation and transformation of competent cells                       | 52 |
| 2.5.7   | Plasmid isolation by alkaline lysis method                              | 52 |
| 2.5.8   | Cloning, protein expression and purification:                           | 53 |
| 2.5.8.1 | Purification of wild type TP2   | 53 |
| 2.5.8.2 | Isolation of Sonication Resistant Spermatid nuclei from rat testes      | 53 |
| 2.5.8.3 | Purification of endogenous TP2 from Rat testis                          | 54 |
| 2.5.8.4 | Cloning expression and Purification of GST NLS <sub>TP2</sub>           | 54 |
| 2.5.8.5 | Cloning expression and Purification of importins                        | 55 |
| 2.5.8.6 | Expression and purification of HATS                                     | 56 |
| 2.5.8.7 | Expression and purification of Histone chaperones NPM1,NPM3<br>and NAP1 | 56 |
| 2.5.8.8 | Cloning expression and Purification of Ran, VDR, PTB                    | 56 |
| 2.5.9   | Estimation of proteins  | 57 |
| 2.5.10  | SDS-polyacrylamide gel electrophoresis                                  | 57 |
| 2.5.11  | Acid urea polyacrylamide gel  | 58 |
| 2.5.12  | Western blot analysis   | 59 |
| 2.5.13  | Centrifugal elutriation of haploid spermatids                           | 60 |
| 2.5.14  | FACS analysis   | 60 |
| 2.5.15  | Preparation of cytosolic extracts                                       | 61 |
| 2.5.16  | Isolation of RNA  | 61 |
| 2.5.17  | Real time PCR analysis.   | 61 |
| 2.5.18  | In vitro import assay   | 63 |
| 2.5.19  | Immunofluorescence  | 64 |
| 2.5.20  | Immuno pull down assay  | 64 |

|          |   |           |
|----------|---|-----------|
| 2.5.21   | Glutathione S-Transferase (GST) pull down assays:   | 65        |
| 2.5.22   | Structural modeling of importin-4 and docking studies.  | 65        |
| 2.5.23   | <i>In vitro</i> acetylation reaction:   | 66        |
| 2.5.24   | Immunoprecipitation (IP)-acetylation assay:   | 67        |
| 2.5.25   | Immuno blotting and co localization studies:  | 67        |
| 2.5.26   | <i>MALDI-TOF analysis of TP2:</i>   | 68        |
| 2.5.27   | Circular Dichroism (CD) spectroscopy of protein-DNA complexes                                     | 69        |
| 2.5.28   | AFM analysis of nucleoprotein complexes   | 69        |
| 2.5.29   | Protein interaction studies   | 70        |
| 2.5.29.1 | Immuno precipitation with NPM3 antibody   | 70        |
| 2.5.29.2 | Immuno pull down experiments  | 70        |
| 2.5.29.3 | GST-NPM3 pull down assay  | 71        |
| 2.5.29.4 | Biotinylated TP2 peptide pulldown assay   | 71        |
| <b>3</b> | <b>Results</b>  | <b>72</b> |
| 3.1      | Characterization of Molecular Components Involved in Nuclear Import of Transition Protein 2 (TP2) | 72        |
| 3.1.1    | Expression profiling of importins at different stages of spermatogenesis.                         | 72        |
| 3.1.2    | Physical interaction of TP2 with importin-4   | 75        |
| 3.1.3    | Importin-4 mediates nuclear import of TP2   | 77        |
| 3.1.4    | Importin-4 present in the round spermatid cytosol facilitates TP2 import in to the nucleus.       | 79        |
| 3.1.5    | Nuclear Localization Signal of TP2 (residues 87-95) interacts with Importin-4                     | 81        |
| 3.1.6    | Importin-4 Mediates nuclear import of GST fused TP2 <sub>NLS</sub>                                | 83        |
| 3.1.7    | Modeling of importin-4 structure using importin $\beta$ 1 as template                             | 85        |
| 3.1.8    | Identification of TP2 <sub>NLS</sub> binding pocket in importin-4                                 | 86        |
| 3.1.9    | Mutational analysis to validate the identified TP2 NLS binding pocket                             | 88        |

|         |  |            |
|---------|--|------------|
| 3.1.10  | Nuclear import of TP 1 is a passive process:   | 90         |
| 3.2     | Identification of the nuclear chaperone interacting with TP2.  | 91         |
| 3.2.1   | Nucleoplasmin 3 is up regulated in meiotic and postmeiotic cells<br>and co localizes with TP2 in elongating spermatids | 92         |
| 3.2.2   | Nucleoplasmin 3 (NPM3) interacts with TP2 <i>in vivo</i> .   | 93         |
| 3.2.3   | NPM3 interacts <i>in vitro</i> with TP2 specifically   | 94         |
| 3.2.4   | NPM3 interacts with TP2 in the C-terminal domain of TP2  | 95         |
| 3.3     | Role of Acetylation in Modulating TP2 Function.  | 96         |
| 3.3.1   | TP2 is acetylated <i>in vivo</i>   | 96         |
| 3.3.2   | Co localization of TP2 with acetylated lysine  | 98         |
| 3.3.3   | p300/CBP and PCAF acetylate TP2 <i>in vitro</i>  | 99         |
| 3.3.4   | p300 protein is present in post meiotic haploid cells  | 101        |
| 3.3.5   | <i>In vivo</i> p300 complex present in rat haploid nuclear extracts<br>acetylates TP2                                  | 102        |
| 3.3.6   | TP2 gets acetylated in the C-terminal DNA condensing domain.   | 103        |
| 3.3.7   |  | 105        |
|         | Effect of Acetylation on TP2 mediated DNA condensation   |            |
| 3.3.7.1 | Circular Dichroism Studies on TP2 mediated DNA compaction  | 105        |
| 3.3.7.2 | Atomic force microscopy (AFM) visualization of TP2 and Ac-TP2<br>DNA complexes.  | 106        |
| 3.3.8   | The effect of acetylation on TP2 on NPM3 interaction   | 107        |
| 4       | <b>Discussion</b>  | <b>109</b> |
| 4.1     | Importin-4 is up-regulated in post meiotic haploid cells   | 109        |
| 4.2     | Importin-4 is involved in nuclear import of TP2 in haploid cells   | 110        |
| 4.3     | The structural studies of Importin $\beta$ family proteins   | 111        |
| 4.4     | Structural modelling of importin-4 and docking of TP2 <sub>NLS</sub> to<br>importin-4                                  | 113        |
| 4.5     | Nuclear Import of TP1 is a passive process   | 115        |
| 4.6     | TP2 Interact with putative histone chaperone NPM3 <i>in vivo</i>   | 115        |
| 4.7     | TP2 is acetylated <i>in vivo</i>   | 116        |

|      |   |     |
|------|---|-----|
| 4.8  | p300 and PCAF acetylates TP2 <i>in vitro</i>  | 117 |
| 4.9  | Acetylation of TP2 reduces TP2 mediated DNA condensation.   | 118 |
| 4.10 | The interaction is blocked by acetylation on TP2  | 118 |
| 4.11 | Working model explaining sequence of events including nuclear import of TP2, involvement of p300 mediated acetylation in modulating TP2 function. | 119 |
| 5    | Summary   | 121 |
| 6    | References  | 124 |
| 7    | List of publications  | 145 |

## CHAPTER 5

# SUMMARY

---

Mammalian spermiogenesis is rather a unique differentiation process wherein nuclear morphology and chromatin structure changes dramatically. During this process many testis specific basic proteins appear on chromatin and the nucleoprotein transition occurs in two steps. In the first step highly basic proteins called the transition proteins that are synthesized postmeiotically replace somatic and testis specific histones before ultimately themselves getting replaced by protamines. Transition proteins have specifically evolved in mammals and appear during the stages 12-15.

The most prominent of these transition proteins are transition protein 1 (TP1) and transition protein 2 (TP2). TP2 is a 13 kDa (114 amino acids) highly basic zinc-metalloprotein. TP1 is a very small, highly basic protein with a molecular mass of 6.5 kDa. TP2 can be divided into an N-terminal zinc finger domain (residues 1-86) and a C-terminal basic domain (residues 87-114). Earlier work by Meetei *et al.* (2000) led to the identification two novel zinc finger motifs in the N-terminal domain of rat TP2, which are implicated in the preferential binding of TP2 to GC-rich DNA *in vitro*. TP1 and TP2 knock out mice show reduced fertility and abnormal chromatin condensation during spermiogenesis emphasizing the role of TP2 in spermiogenesis. TP2 has also been shown to be phosphorylated immediately after its synthesis (Green *et al.*, 1994). Previous reports show that the import of TP2 into haploid spermatid nucleus requires the components of cytosol and ATP. The knock out mice for TP1 and TP2 have shown that these proteins are an essential component of the spermatid nucleus and that they play a crucial role in the chromatin remodeling and chromatin condensation. However, the *in vivo* mechanism of the appearance of these proteins on chromatin and their replacement has not been obtained at a molecular level by any of the earlier studies. The present investigation

was aimed at: a) To understand the nuclear import machinery of TP2 in haploid germ cells. b) To identify the nuclear chaperone involved in interacting with TP2. c) To identify the novel post-translational modifications in Transition protein 2 and their role in TP2 function

In this thesis a detailed analysis was carried out to characterize the molecular components underlying the nuclear translocation of TP2. Real-time PCR analysis of the expression of different importins in testicular germ cells revealed that importin-4 and importin  $\beta$ 3 are significantly up-regulated in tetraploid and haploid germ cells. Physical interaction studies as well as an *in vitro* nuclear transport assay were carried out using recombinant TP2 and the nuclear localization signal of TP2 (TP2<sub>NLS</sub>) fused to glutathione S-transferase in digitonin-permeabilized, haploid round spermatids. All these results show importin-4 to be involved in the import of TP2. A three dimensional model of importin-4 protein was generated using the crystal structure of importin- $\beta$ 1 as the template. Molecular docking simulations of TP2<sub>NLS</sub> with importin-4 structure led to the identification of a TP2<sub>NLS</sub> binding pocket spanning the three helices (helices 21-23) of importin-4, which was experimentally confirmed by *in vitro* interaction and import studies with different deletion mutants of importin-4. In contrast to TP2, TP1 import was through a passive diffusion process. Importins are known to have chaperonic function in safe transport of basic proteins from cytosol. Another interesting aspect of the present study is the identification of NPM3 as a novel TP2 interacting protein, which could perform a role in safe storage and transport of TP2 within the nucleus till it appears on chromatin.

Hyperacetylation of histone H4 is linked to their replacement by transition proteins. Present work shows that TP2 is acetylated *in vivo* as detected by anti acetylated lysine antibodies and mass spectrometric analysis. Further, recombinant TP2 is acetylated *in vitro* by acetyltransferase KAT3B (p300) more efficiently than KAT 2B (PCAF). p300 but not PCAF was also detected in elongating spermatids. p300 acetylates TP2 in its C-terminal domain, which is highly basic in nature possessing chromatin condensing properties. Mass spectrometric analysis showed that p300 acetylated 4 lysine residues in the C-terminal domain of TP2. Acetylation of

TP2 by p300 leads to significant reduction of its DNA condensation property as studied by Circular Dichroism and Atomic Force Microscopy analysis. Other interesting effect of acetylation of TP2 is the impediment of interaction of TP2 with NPM3, a putative histone chaperone, whose expression is elevated in haploid spermatids. Thus acetylation of TP2 adds a new dimension towards its role in the dynamic reorganization of chromatin during mammalian spermiogenesis.

Based on these findings and earlier results from our laboratory a model has also been proposed in which immediately after synthesis TP2 gets phosphorylated, and then gets translocated into the nucleus which is mediated by importin-4. Once TP2 enters the nucleus, a nuclear chaperone NPM3 binds and helps in safe storage and transport within the nucleus before deposited on to chromatin. Phosphorylation temporarily inhibits the condensation property of the basic C-terminal domain, thus allowing lateral diffusion of TP2 along the chromatin to facilitate the process of its zinc finger modules searching and docking onto the GC-rich CpG island sequences. Subsequent dephosphorylation unmasks the C-terminal basic domain and triggers the initiation of chromatin condensation. TP2 gets acetylated by p300 and this event could have a role either in local decondensation of chromatin for DNA repair activity that is associated with repair of several strand breaks in the genome occurring at the stage of histone removal. Alternatively it is also possible that the acetylation helps in eviction of TP2 from chromatin in the late stages of spermiogenesis before the appearance of protamines.