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

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By

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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

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-Pradeepa MM

Contents

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 β3 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 ($TP2_{NLS}$) 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-β1 as the template. Molecular docking simulations of $TP2_{NLS}$ with importin-4 structure led to the identification of a $TP2_{NLS}$ 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 with in 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 with in the nucleus before deposited on to chromatin. Phosphorylation temporarily inhibit 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.