SUMMARY

Heterochromatin represents transcriptionally silent domains of the genome wherein large tracts of DNA are assembled into a specialized chromatin structure that is inhibitory to transcription. Such repression is crucial for a variety of chromosomal processes, including gene regulation, nuclear organization, chromosome segregation. Regulation of gene expression by transcriptional silencing necessitates a precise establishment, maintenance and heritability of such silenced states. The faithful inheritance of such epigenetically maintained repression seems really intriguing. Transcriptionally silent loci of fission yeast (mating-type locus, centromeres and telomeres) have served as a paradigm for understanding the mechanism of this form of repression.

Heterochromatinization involves the concerted and coordinated actions of a plethora of components. Several trans-acting factors (Clr1, Clr2, Clr3, Clr4, Clr6, Rik1, Swi6) and cis-acting sequences (silencers) are required to mediate silencing in fission yeast. The list of factors implicated in this complex silencing process is really burgeoning. Work done in our lab has added DNA polymerase α, a replicative polymerase (Ahmed, 2000; Ahmed and Singh, 2001; Ahmed *et al.*, 2001) and a post-replication DNA repair protein Rhp6 (Singh *et al.*, 1998) to this burgeoning list.

As a follow up of these initial findings, the present study was on the topic:

Interaction of DNA polymerase α and Rhp6 with heterochromatin components in *Schizosaccharomyces pombe.*

DNA Polα-mediated role in silencing:

It has been suggested that DNA replication is coupled to transcriptional silencing and this ensures the proper establishment and propagation of silent chromatin states. Findings in our lab also support the existence of such a coupling. A temperature-sensitive mutant of DNA polymerase α, *swi7H4* (Murakami and Okayama, 1995) was found to be defective in silencing all the heterochromatic loci (mating-type loci, centomeres and telomeres) (Ahmed, 2000; Ahmed and Singh, 2001; Ahmed *et al.*, 2001) pointing to a probable role of this polymerase in silencing. It was seen that the chromodomain protein Swi6 (HP1 homolog), the major structural

component of *S. pombe* heterochromatin, is delocalised from the silent loci in the mutant. Following this up, the major findings of the present study were:

- DNA Polα interacts directly with Swi6 *in vivo* in wild-type cells suggesting that this polymerase may mediate the recruitment of this silencing protein to heterochromatic sites and thereby aid in the establishment of silencing.
- Swi6 does not interact with mutant *polα* and hence, is delocalised away from the silent loci in the *swi7H4* mutant.
- DNA Polα interacts with Clr4 (the histone methyltransferase that methylates histone H3 at Lys9 position, a modification upon which Swi6 docks). In view of this, it can be envisaged that this polymerase may interact with a complex of silencing proteins like Swi6/Clr4/Rik1 during DNA replication and initiate a chain of events involving recruitment of Clr4 and Swi6 to *mat*, *cen* and telomere loci, histone H3-K9 methylation and subsequent binding of Swi6. Interaction of DNA Polα with Clr4 may provide a potential mechanism for inheritance of repressive H3-K9 methylation states and thereby serve to integrate DNA replication with chromatin modifications.
- Present study demonstrates a direct physical interaction between Swi6 and Clr4, the fission yeast homologs of mammalian HP1 and SUV39H1. This interaction is vital for establishment of silencing. Though this interaction stands established in mammalian systems (Yamamoto and Sonoda, 2002), a direct demonstration of an interaction between the two components had been lacking in *S. pombe*.
- In *S. pombe*, silencing factors such as Swi6, Clr4 and histone deacetylases are required for proper chromosome segregation as proper heterochromatin assembly is required for the faithful propagation of genetic information through mitosis and meiosis. Consistent with a role of DNA Polα in heterochromatin assembly in *S. pombe*, *swi7H4* mutation influences chromosome dynamics during mitosis and meiosis.
- An analysis of mitotic chromosome segregation in *swi7H4* mutant showed that this mutant exhibit lagging chromosomes, a phenotype also exhibited by silencing-defective mutants like *clr4*, *swi6*, etc. This phenotype was observed with a frequency as high as 19% in *swi7H4* mutant. Apart from lagging chromosomes, unequal segregation of the chromosomes to the daughter nuclei was also observed suggesting that the process of chromosome segregation is not robust in this mutant.

- Moreover, it was observed that centromeric cohesion is defective in this mutant. In view of the direct physical interaction of DNA Polα with Swi6 and the requirement of Swi6 for cohesin recruitment (Bernard *et al.*, 2001; Nonaka *et al.*, 2002), it can be envisaged that DNA Polα -mediated Swi6 recruitment may attract the cohesin complex to the heterochromatic sites and thereby lead to the establishment of cohesion during the S-phase.
- *swi7H4* mutant also exhibits aberrant meiotic chromosome segregation with a high frequency (26%) suggesting that DNA Polα is required for the fidelity of chromosome segregation during meiosis as well. Though several aberrant phenotypes were observed in sporulating *swi7H4* mutant, the phenotypes resulting from missegregation events during Meiosis II were predominant. In view of this, it is speculated that DNA Polα may also influence Rec8-mediated centromeric cohesion during meiosis.

The above results strengthen the role of DNA Polα in the formation of specialized heterochromatic structure at centromeres.

In light of the present results, it can be concluded that DNA Polα may play a role in integrating the processes of DNA replication with heterochromatin assembly and chromosomal cohesion in *S. pombe*. Since DNA Polα, Swi6 and Clr4 are evolutionarily conserved proteins across eukaryotes, the present study may have implications in eukaryotic gene regulation.

Studies on Rhp6-mediated role in silencing:

Rhp6 (the RAD6 homolog in *S. pombe*) that plays a role in post-replication DNA repair, ubiquitylation of proteins including histones, was earlier shown to be involved in mating-type silencing. Rhp6 is a 151 amino acids long protein possessing ubiquitin conjugating activity (E2) like Rad6 with a single cysteine residue at position 88 being essential for this E2 activity. It was shown earlier that *sng1-1*, a point mutation in the second intron of *rhp6* (the gene has 4 introns) leads to a switching-dependent loss of silencing suggesting a role of Rhp6 in chromatin remodelling during replication associated with switching (Singh *et al.*, 1998). A follow up of this led to the identification of a 22 kDa non-histone protein called Uhp1 (ubiquitinated histone like protein) as a target/mediator of Rhp6 in our lab (Naresh, 2000; Naresh *et al.*, 2003). It was found that Uhp1 (also called p22/p25/Obr1) levels are greatly elevated in the *sng1-1/rhp6-*

mutant and *rhp6Δ* strains compared to the wild-type. An overexpression or deletion of the gene encoding this protein leads to switch-dependent silencing defect akin to that of the *rhp6*⁻ mutant. Studies suggested this chromatin remodeling protein may be important for initiating assembly of silent chromatin states (Naresh, 2000; Naresh *et al.*, 2003).

It was speculated that Rhp6 may influence silencing pathways through its pleiotropic effects. Therefore, apart from Uhp1 as a mediator, it was envisaged that this protein with ubiquitin conjugating activity may impinge upon several substrates, including histones, that ultimately culminates in silencing.

Hence, the following two aspects of the role of Rhp6 were followed up:

a) **Uhp1-mediated role in propagating the silent chromatin state in fission yeast.**

b) **Involvement of Rhp6 in silencing by affecting the histone modifications.**

a) Uhp1-mediated role:

Following up this line of work, the major findings were

- *rhp6*⁻ exerts a modest effect on the expression of *uhp1* gene at the transcriptional level.
- Uhp1 undergoes cell-cycle dependent and Rhp6-mediated ubiquitylation *in vivo*.
- Uhp1 shows a transient chromatin localization at the silent mating-type loci coincident with S-phase.

In light of the above findings, it was envisaged that this mediator of Rhp6 functions transiently during the assembly of chromatin at the replication forks neighbouring the silent loci, following which, it is ubiquitylated and degraded.

- Uhp1 possesses ubiquitin binding property. The basis of this binding property may be the presence of a monoubiquitin binding motif, called CUE motif, in Uhp1. Residues 30-72 of Uhp1 show 80% consensus with CUE domain including the presence of critical residues for Ub binding.
- The present study demonstrates that Uhp1 interacts with histone methyltransferase, Clr4, *in vitro* and *in vivo*. This suggests that Uhp1 may mediate the recruitment of Clr4 to

heterochromatic regions that leads to H3-K9 methylation followed by Swi6 binding and hence, initiates the silencing process. Alternatively, Uhp1 may modulate/influence the activity of Clr4. This interaction justifies the role of Uhp1 as a crucial silencing factor at the heterochromatic regions that may act alone or in a complex to initiate the formation of repressive structures.

- Uhp1 influences the histone modification code as it was observed that the gross level of H3-K9 dimethylation is reduced in *uhp1Δ* strain. The reduction of this heterochromatic modification was also observed in strain overexpressing *uhp1*. This explains the silencing defects observed in both the *uhp1Δ* strain and in strain overexpressing *uhp1*, and lends support to the speculation that Uhp1 may be a critical component involved in recruitment of Clr4 and/or modulating the function of Clr4 during heterochromatin assembly.
- Uhp1 co-purifies with K9-methylated histone H3 suggesting that a possible mode of association of Uhp1 with heterochromatic regions *in vivo* may be through interaction with the specifically modified histone H3.
- In *dcrΔ* derivatives showing a partial silencing defect at the centromeres, an overexpression of *uhp1* leads to a complete derepression of the marker gene inserted at the centromeric *otr* repeats, indicating that Uhp1 may co-operate/interact with the RNAi pathway components in establishing heterochromatic states. *uhp1* overexpression in *ago1Δ* and *rdp1Δ* strains does not cause any apparent derepression of the reporter gene. Since, it has been suggested that in *ago1*$^-$ and *rdp1*$^-$, the spreading of heterochromatin may be blocked whereas in *dcr1*$^-$, the block is at the level of initiation step of heterochromatin assembly (Volpe *et al.*, 2002), the observed specific effect of Uhp1 in *dcr1Δ* strains indicates that an optimum level of Uhp1 may be essential for the nucleation/initiation step of heterochromatin assembly. In view of these results, it is envisaged that this protein may interact directly with the RNAi pathway or indirectly affect this pathway by influencing the dynamics of Clr4.
- The above results seem exciting keeping in view the earlier findings that the expression of *uhp1/p25* is regulated by AP-1 like transcription factor Pap1 (Toda *et. al.*, 1992) and recent microarray analysis showing that this gene (also called *obr1*) is an Atf1-dependent stress-responsive gene (Chen *et. al.*, 2003) suggesting that Uhp1 may be

another stress response element at work in bringing about heterochromatin assembly. In addition, the presence of Pap1 recognition motifs at the *cen* and *mat* loci may represent potential nucleation sites for heterochromatin assembly.

- Interestingly, Uhp1 levels are reduced in histone deacetylase mutants, *clr3Δ* and *clr6-1*, suggesting that these HDACs may exert transcriptional control on *uhp1*. This result seems intriguing as decreased deacetylation of histones have been linked to transcriptional activation, rather than repression.
- Expression of *uhp1* cannot alleviate the silencing defects of *clr3Δ* and *clr6-1* mutant strains. In view of this result, and Uhp1-Clr4 interaction, it may be envisaged that Uhp1 may be regulated by the HDACs but it acts at the step subsequent to the deacetylation of histones.
- Uhp1 influences the directionality of mating-type switching. This effect may be through the role of Uhp1 in affecting the heterochromatin organization of the mating-type region.

b) Role of Rhp6 in silencing –Histones as targets:

- Histone H2B is monoubiquitylated in *S. pombe in vivo*. H2B ubiquitylation is mediated by the ubiquitin conjugating activity of Rhp6 like in *S. cerevisiae*. However, Rhp18 is not required for the Rhp6-mediated H2B ubiquitylation in *S. pombe*.
- The putative ubiquitylation site of H2B in *S. pombe* is the conserved residue, K119. To study the effect of H2B ubiquitylation on silencing pathways, we tried to mutate this site. H2B(K119R) mutation was introduced into the plasmid-borne *htb1*. However, this mutation could not be incorporated into the chromosomal copy suggesting that mutation of this H2B ubiquitylation site may be deleterious in *S. pombe*. The ubiquitylation of this residue may be impinging on an essential gene regulatory pathway/pathways. However, the apparent essentiality of this residue is an impediment to the elucidation of the role of Rhp6 mediated H2B ubiquitylation in *S. pombe*.
- Rhp6 influences K9-dimethylation on histone H3 suggesting that Rhp6 may affect this heterochromatin mark directly or indirectly.

In view of results showing that Rhp6 affects H2B ubiquitylation and H3 methylation, it is speculated that Rhp6-mediated H2B ubiquitylation drives the histone modifications at

histone H3 and thereby affects silencing. Ubiquitylation of histone H2B by Rhp6 may target the silencing factors like Clr4 (directly or indirectly) to chromatin sites and direct the assembly of silent chromatin states.

- Uhp1 may serve as a transducer for Rhp6-mediated effect on histone H2B ubiquitylation and H3 methylation. Uhp1 may serve as a ubiquitin receptor, recognizing the monoubiquitin tag on H2B. Uhp1 may serve to transduce the signal from Ub-H2B to H3 by binding to monoubiquitylated H2B on one hand and recruiting the HMTase, Clr4, concomitantly to bring about K9 methylation on histone H3 within the same nucleosome. The ubiquitin binding property of Uhp1 and the presence of a putative CUE domain in Uhp1 support such a role of Uhp1. In addition, the possible *in vivo* binding of Uhp1 to K9-methylated histone H3 and interaction of Uhp1 with histone H3 HMTase, Clr4, also lend support to this speculation.
- *rhp6/sng1-1* mutant is sensitive to the microtubular destabilizing drug, TBZ and is also cold-sensitive. This suggests that Rhp6 plays an important role in heterochromatin organization at the centromeres which is required to facilitate interactions with microtubules and hence, is required for centromeric functions like the other silencing factors Swi6, Clr4 etc.
- Rhp6 is essential for meiosis. *rhp6/sng1-1* mutant has a very low level of sporulation like the *rhp6* deletion mutant (Singh *et al.*, 1998). The meiotic/sporulation defects of this mutant were further analysed. A small proportion of cells that undergo meiosis show aberrant chromosome segregation.

Thus, Rhp6 profoundly influences chromatin organization. In view of the conservation of Rhp6/RAD6 and the essentiality of mammalian/human RAD6 for the normal developmental programs, the present study may have implications in mammalian biology.

Apart from the above, it was fortuitously observed that Snf1, a *S. cerevisiae* histone H3-Ser10 kinase, shows a very strong interaction with Clr4. Since Snf1 is highly conserved across mammalian species, Snf1-like kinases in higher systems may interact with and modulate the activity of Suv39H1/Clr4. This interaction may aid in the elucidation of precise relationships between histone modifications associated with expressed and repressed states, an area that has remained elusive.