SUMMARY

Schizosaccharomyces pombe contains large heterochromatic regions distributed on three chromosomes. The processes of formation of heterochromatin are conserved between fission yeast and higher eukaryotes. In addition, the trans-acting factors of the pathway are also found to be conserved in higher eukaryotes (Cam and Grewal, 2004; Elgin and Grewal, 2003). In *S. pombe*, the functional chromosomal domains: centromeres, the mating-type region and telomere require the formation of heterochromatin (Allshire et al., 1995). Among all heterochromatic regions, centromere, telomere and mating-type locus share a common feature. Each of these domains contains *dg/dh* repeat elements. These repeat elements are preferential target of RNAi-mediated heterochromatin formation. The heterochromatin at pericentromeric repeats and subtelomeric regions are important for chromosome segregation and maintenance (Shimada and Murakami, 2010). Post-translational histone modifications and various trans-acting factors are required for heterochromatin assembly. These factors comprise different regulatory proteins which are either directly involved in modification or interact with modified histones. In *S. pombe* several histone deacetylases (HDACs), histone methyl transferases (HMTs) are linked with initiation of heterochromatin assembly. Methylation of H3 lysine 9 (H3K9) residue is hall mark of heterochromatinization, carried out by the conserved methyltransferase Su(var)3-9 in *Drosophila,* SUV39H1 in human, and Clr4 in *S. pombe.* These methylated residues provides the binding site for the conserved chromodomain binding protein, known as HP1 in *Drosophila* and human and *Swi6* in fission yeast. The *Swi6* and HP1 proteins bind specifically to the histone H3 tails that are methylated at lysine 9 by Clr4/Suv39h enzyme in fission yeast and metazoans, respectively. Thus, Swi6 and Clr4 proteins are central player of heterochromatin assembly, but the signal that stimulates Clr4 to initiate with methylation and in turn silencing is still undeciphered. Recent reports suggest that hypoacetylation of chromatin triggers the Clr4 to methylate H3K9 histone. After binding of Swi6 to the K9 methylated H3 residues, a few complexes like RITS (RNA induced transcriptional silencing), RDRC (RNA dependent RNA polymerase) in combination with RNA-interference (RNAi) pathway, initiate heterochromatin assembly. Then, Swi6 by self-association property spread the heterochromatin to neighboring rgions. The proteins like Swi6 and Clr4 (chromodomain proteins) provide an interface to various effector complexes of silencing, post-translational modification of histones, chromosome segregation, cohesin localization, and DNA replication The two

chromodomain protein Clr4 and Swi6 influence position effect in *S. pombe* (Allshire et al., 1995; Thon et al., 1994). When either *clr4* or *swi6* is mutated or deleted silent genes at different heterochromatic regions become derepressed.

Clr4 is also a component of a multiprotein complex known as "Clr4 methyltransferase complex (CLRC)"(Zhang *et al.*, 2008). The CLRC complex is responsible for nucleation and spreading of heterochromatin, but the mechanism of its recruitment to heterochromatin repeats still remains elusive. The heterochromatinization in *S. pombe* takes place in RNAi-mediated and RNA-independent pathway. The RNAi-independent pathway involves the factors which on its own initiate the heterochromatin formation. In mating-region the Atf1/Pcr1 proteins (DNA-binding protein) are known to establish the heterochromatization on their own. In RNAi-dependent pathway, the HDACs deacetylate the chromatin followed by the H3K9 methylation and binding of HP1/Swi6 protein. Further other components of RNAi-pathway like RITS, RdRC, and other complexes also act to form heterochromatin, which again involves H3K9 methylation, RNAi pathway components, and DNA binding proteins.

In *S. pombe* there are four chromodomain proteins: Swi6, Chp1, Chp2, and Clr4. The chromodomain is an evolutionary conserved protein module and best recognized as the binder of methylated histone tail (Bannister *et al.*, 2001). But several lines of evidence suggest the chromodomain also possesses DNA and RNA binding property (Akhtar *et al.*, 2000; Bouazoune *et al.*, 2002; Ishida *et al.*, 2012; Keller *et al.*, 2012). In the present study we investigated the DNA binding property of Swi6 and Clr4 and its importance in gene silencing pathway. To understand the mechanism by which these proteins target the chromatin, we investigated the detailed nucleic acid binding property of Swi6 and Clr4. For this, we choose the DNA corresponding to the nascent transcripts from centromeric repeats, which are previously identified as siRNA by Reinhart group (Reinhart and Bartel, 2002). Both Swi6 and Clr4 show specific and strong binding towards single-stranded DNA. Moreover, Swi6 also showed strongest binding towards DNA-RNA hybrid, followed by the double-stranded DNA and single-stranded DNA. Swi6 is comprised of N-terminal chromodomain and C-terminal chromoshadow domain linked with flexible hinge region. The hinge region of Swi6 homologs is also known to bind with RNA (Muchardt *et al.*, 2002). We investigate the contribution of N-terminal, C-terminal and hinge region of Swi6 toward DNA binding ability. We observed that chromdomain as

well as the hinge region confer DNA binding property to Swi6, while chromoshadow domain failed to bind to DNA. The hinge region of Swi6 is marked by presence of three conserved lysine residues. Further, we studied this binding property with respect to hinge region mutant Swi6 (3K→3A) (positioned at 242, 243, and 244 amino acids, named as triple lysine mutant) of Swi6. Previous results from the laboratory showed that the triple lysine mutant is defective in directionality of mating-type switching as well as in silencing at telomere and centromere. We found that all three residues are important for binding with DNA as single and double lysine mutants still have ability to bind with DNA.

To get better structural insight into binding of Swi6 with DNA and Swi6 (3K-3A), we performed Small–angle X-ray scattering with wild type as well as mutant protein in collaboration with Dr. Ashish. This provided us the global structural features of Swi6, triple lysine mutant and their complex with DNA or RNA. Interestingly, SAXS analysis indicated that the triple lysine mutant showed a drastic collapse in "Z" shaped structure of wild type Swi6. The model constructed for Swi6 revealed that the protein structure changed from two-sided to a more compact one in Z-shape, indicating the conformational changes in proteins that brought both chromdomain and hinge region in close contact with DNA/RNA (Figure 26B).

The presence of Swi6 over the K-region between *ma2-mat3* intervals regulates the directionality of switching (Grewal and Klar, 1997). It has been shown that spreading of Swi6 over K-region facilitates the recruitment of recombination promoting complex (RPC) (Swi2-Swi5 complex) and further ensures the directionality of switching (Jia *et al.*, 2004). But the mechanism underlying this facilitation by Swi6 was undeciphered. So we questioned that whether Swi6 has any direct association with SRE3 element (as we already found the DNA binding ability of Swi6). SRE3 elements are present juxtaposed to the *mat3M* locus. Interestingly, EMSA results showed a strong association of Swi6 with SRE3, while triple lysine mutant failed to bind. This result also indicates strict role of lysine residues in interaction.

In the second part of the study we explored the binding property of another chromodomain protein Clr4. The Clr4 showed stronger binding towards the single stranded DNA than towards double stranded DNA. In addition, the binding of Clr4 was sequence-specific. Importantly, both Clr4 and Swi6 could bind to same DNA stretch, but

it is not clear whether they bind to same sequence. They also differ in their selectivity, as Swi6 binds more strongly to double stranded DNA than to single stranded DNA, while Clr4 binds more strongly to single stranded DNA compared to double stranded DNA. In a parallel, the RNA binding property of Clr4 and Swi6 was studied. As discussed earlier, Clr4 is comprised of amino terminal chromdomain and carboxy terminal SET domain. SET domain is the catalytic domain and has methyltransferase activity. The mutants in chromodomain (W31G and W41G) showed binding comparable to wild type Clr4. Similarly, the other pre-SET (R320A), SET (G339S, G341S), and post SET (G486D, G487STOP) were studied. Of these two SET domain mutants (G339S, and G341S) in evolutionarily conserved residues were defective in binding. Very interestingly, these two mutants were also defective in mating type switching.

In mating-region of *S. pombe*, there are other parallel pathways acts such as Atf1/Pcr1 pathway. Moreover, the mating-region is comprised of three linked *mating* loci (as discussed earlier). This is the debatable issue that whether Swi6 acts downstream or upstream to Clr4. In mating-region there is the region where H3k9 methylation takes place in Swi6 dependent manner as well as in Swi6 independent manner cenH region. This pictorial representation also indicates the co-ordinates of *cenH* where the peak of lysine methylation could be seen even in *swi6* mutant. In present study we detected the DNA binding property of Swi6 and Clr4. Furthermore, the previous results from lab indicates that Swi6 and Clr4 function in concert with each other (Haldar *et al.*, 2011). Thus, we decided to investigate that if Swi6 and Clr4 show autonomous DNA binding. Results showed that both Swi6 and Clr4 have multiple binding sites. Swi6 binds with subfragments K4a, K6b, K7a, and K7b, while Clr4 binds to K3a, K4a, K8b and 9a. The K4a fragment was common to both proteins and it would be very interesting to see the *in vivo* role of this binding in future studies.

Furthermore, we found that both Swi6 (present study) and Clr4 (Sumit Arora, PhD thesis, 2005) showed strongest binding towards DNA-RNA hybrid. In addition to *in vitro* studies, recent evidences also indicate the existence of DNA-RNA hybrid at heterochromatic region (Nakama *et al.*, 2012). Thus, we investigated that whether destabilization of DNA-RNA hybrid causes any effect on silencing and switching. Consistently, over-expression of RNaseH1 aborgates silencing at outer most repeats of centromere and mating type loci.

An important test of the role of nucleic acid binding by Swi6 and Clr4 in silencing and mating-type switching will involve testing the effect of RNaseH1 overexpression on localization of Swi6 an Clr4 at these regions. These experiments will be done in future studies.