Discussion

During this study, we studied the role of anaphase promoting complex in heterochromatin assembly. Earlier studies in lab have shown that APC affects silencing at all the three heterochromatic loci (Nakwal N., PhD thesis, 2007). Also, APC localizes to the heterochromatin in Swi6 dependent manner, in turn Swi6 as well as Clr4 (H3K9me2) localization is also defective in APC mutants suggesting the role of APC in the recruitment of Swi6 and Clr4 to the heterochromatin. So, we check the direct physical interaction of Swi6 and Clr4 with APC subunits mainly Cut4 and Cut9. Interestingly, we found out that APC subunits Cut4 and Cut9 physically interact with Swi6 as well as Clr4 (Figure 3.3, 3.4 and 3.5). Moreover, these interactions are abolished in case of*sng2*-1 (*cut4* mutant allele) and *cut9*-665 mutants (Figure 3.6). During heterochromatin assembly in yeast, Clr4 acts upstream by creating the binding site H3K9me for Swi6 which further self-associates and spreads over chromatin to form silent state of chromatin (Nakayama *et al.*, 2001). So, we further interested to check whether APC interact with Swi6 and Clr4 independent of each other or not. To check this, we created *swi6*Δ and *clr4*Δ mutant strains having HA-tagged Cut9 or HA-tagged Cut4. Surprisingly, we found out that APC subunits (Cut4 and Cut9) does not interact with Clr4 in the absence of Swi6 but APC (Cut9) interacts with Swi6 even in the absence of Clr4. This suggests that the interaction of APC with Clr4 is mediated by Swi6 (Figure 3.7 and 3.8). Further, Swi6 contains conserved N-terminal chromodomain and C-terminal chromo-shadow domain linked by a less conserved hinge region. It is interesting to find out the domain specific interaction of Swi6 with APC. So, we clone and express both the domains as well as hinge region of Swi6 as GST-fusions and check the domain specific interaction of Swi6 with Cut9. Surprisingly, we found that Cut9 specifically interacts with chromo-shadow domain (CSD) of Swi6 (Figure 3.9) which is already known for protein-protein interaction (Jones *et al.*, 2000; Nielsen *et al.*, 2001).

APC plays major role during G1 and mitosis (Amon *et al.*, 1994; King *et al.*, 1996; Yamano *et al.*, 1996) whereas heterochromatin formation takes places during S-phase. So, it is interesting to check in which stage of cell cycle APC (Cut9) interacts with Swi6. We did a cell synchronization experiment using *cdc25* mutant strain and found out that Swi6 specifically interacts with Cut9 in G1/early S-phase of the cell cycle (Figure 3.10). Thus, the activity of APC during mitosis may be regulated by Swi6.

Cyclins (Cdc13), Cohesin (Rad21) and Securin (Cut2) are the known targets of APC (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996b; Yamamoto *et al.*, 1996). Cdc13 is a mitotic cyclin forms an active complex with Cdk (Cdc2) which plays crucial role in mitotic exit (Booher and Beach, 1986; Hindley and Phear, 1984; Wuarin *et al.*, 2002). Cdc13 degradation by APC, in turn, inactivates Cdc2 which leads to mitotic exit (Wuarin *et al.*, 2002). We further studied the effect of Swi6 on Cdc13 degradation and found out that in the absence of *swi6* (in *swi6*Δ strain), Cdc13 is more stable and degrades late as compared with wild type *swi6*$^{+}$strain (Figure 3.11). Also, the activity of Cdc2/Cdc13 complex has been observed more in case of *swi6*Δ mutant (Figure 3.12). Hence, the results suggest that Swi6 plays important role in mitotic exit by regulating the degradation of Cdc13 and thus the activity of Cdc2/Cdc13 complex.

Earlier studies have shown that Swi6 recruits cohesin as well as Mis4 (a cohesin loading factor) to the chromatin and interacts with Psc3 subunit of cohesin complex (Bernard *et al.*, 2001; Furuya *et al.*, 1998; Nonaka *et al.*, 2002). Additionally, Nonaka *et al* (2002) suggest that cohesin complex and Swi6 work in the same pathway as the double mutant of Swi6 and cohesin subunits (Psc3 and Rad21) are not viable. Cohesin degradation is also crucial for sister chromatid separation during cell cycle (Uhlmann, 2001; Uhlmann *et al.*, 1999) and cohesin degradation is delayed during metaphase-to-anaphase in the absence of Swi6 (Nakwal N., PhD thesis, 2007). APC regulates the degradation of cohesin indirectly by the ubiquitination of Cut2 (securin) following its degradation by proteasome pathway (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996b). Cut2 (securin) forms a complex with Cut1 (separase) during the cell cycle and inhibits its activity. After degradation of Cut2 by APC, Cut1 degrades Rad21 subunit of cohesin complex and thus help in sister chromatid separation (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996a; Irniger *et al.*, 1995). Therefore, we hypothesized that Swi6 may play a central role in mitosis during metaphase-to-anaphase transition in which on the one hand it recruits APC (Nakwal N., PhD thesis, 2007) and on the other side, it recruits cohesin complex (Bernard *et al.*, 2001; Nonaka *et al.*, 2002). Therefore, Swi6 may interact with other substrates of APC and bring them in the close vicinity of APC to increase the fidelity of the sister chromatid separation leading to faithful cell cycle progression. To check our hypothesis, we check the interaction of cohesin subunit Rad21 with Swi6 and Clr4, and found out that Rad21 did not interact with Swi6 and Clr4 (Figure 3.17). Degradation of Rad21 is Cut1 mediated and the delayed in Rad21 degradation in *swi6*Δ

mutant strain (Nakwal N., PhD thesis, 2007) may be through Cut1, where Swi6 might interact with Cut1/Cut2 complex and bring it in the close proximity of APC which degrades Cut2 releases Cut1 which, in turn, degrades Rad21. We further checked the interaction Swi6 with Cut2 as well as Cut1 but found out that Cut1 as well as Cut2 did not interact with Swi6 (Figure 3.15 and 3.16). Thus, the effect of Swi6 on Rad21 degradation may be defective localization or activation of APC.

As shown in this study, Swi6 affects the Cdc13 degradation as well as the kinase activity of Cdc2/Cdc13 complex. Therefore, Swi6 may interact Cdc2/Cdc13 complex to regulate the Cdc13 degradation through APC localization, leading to mitotic exit. We studied the interaction of Cdc13/Cdc2 complex with Swi6 and no interaction was observed (Figure 3.13). Cdc13 is a mitotic cyclin whose level oscillates during the cell cycle resulting in the activation or deactivation of Cdc2 (Stern and Nurse 1996). Consequently, Swi6 may interact with Cdc13 transiently in a cell cycle dependent manner. We did a cell cycle synchronization experiment to check the cell cycle dependent interaction of Cdc13 with Swi6. Unfortunately, we found no interaction between Cdc13/Cdc2 complex and Swi6 even transiently during the cell cycle (Figure 3.14). Although, Swi6 affects Cdc13 and Rad21 degradation and APC localization but it might not directly involve in the coordination of cell cycle progression through degradation of cyclins and Rad21 by APC.

Swi6 is known to interact with various proteins involved in different cell cycle processes including DNA replication, RNAi machinery, chromatin remodelling, centromere functioning and silencing (Ahmed *et al.*, 2001; Fischer *et al.*, 2009; Motamedi *et al.*, 2008). We were interested in finding out how Swi6 coordinates all the processes or whether two different function of Swi6 are interdependent. So, we generated *swi6* mutants by random mutagenesis using hydroxyl amine. To generate *swi6* mutants we follow the strategy described in detail above (Figure3.18) in which we mark the *swi6* gene with *hph* gene (hygromycin resistance gene) at its 3'UTR and generate *swi6* mutants using hydroxyl amine. The mutated *swi6* gene marked with *hph* (hygromycin resistance gene) was transformed in the strain having HA-tagged cut9 with silencing background (*otr1R::ade6*). The transformants obtained were screened further for defective in silencing, chromosome segregation, APC functioning, showing *cut* phenotype and finally in interaction with Cut9 (APC subunit) (Table 3.1).

1) One of the main conclusions of this study is that Swi6 interacts with APC. This inference is based on co-immunoprecipitation of APC subunits Cut4 and Cut9 with Swi6. On the other hand, interaction of Clr4 with APC is Swi6-dependent. The interaction with APC involves the chromoshadow domain of Swi6. It is, however, not clear whether the interaction of Swi6 with Cut4 and Cut9 is direct or indirect, since Cut4 and Cut9 are part of multi-protein complex APC. Therefore, it would be of interest to determine with which subunit of APC does interact directly.

2) The interaction between Swi6 and APC has important physiological consequences. Their mutually cooperative recruitment to heterochromatin assembly, which plays a role in silencing, has been shown in our lab earlier (Dubey R.N., PhD thesis, 2001; Nakwal N., PhD thesis, 2007; Dubey *et al.*, 2009). These findings led us to speculate that Swi6 may influence the action of APC or its substrates, like Cut2/securin, Cdc13 and Rad21 (indirectly through Cut1). In accordance, we find that Cdc13 degradation (present work) and Rad21 degradation (Nakwal N., PhD thesis, 2007) is delayed in *swi6*Δ mutant. This delay could be correlated with results implying an inhibitory role of Swi6 towards Cdc2-Cdc13 kinase.

3) Because of lack of direct interaction of Swi6 with Cdc2-Cdc13, Rad21, Cut2 and Cut1, we speculate that the primary role of Swi6 may be at the level of activation of APC. Delayed activation of APC in *swi6*Δ may cause delay in degradation of Rad21, Cdc13 and possibly Cut2, leading to the physiological effects of *swi6*Δon delay in anaphase and mitotic exit, increased chromosome segregation defects and aneuploidy.

4) Above studies raise the question as to mechanism by which Swi6 may activate APC. APC activation is known to require association with Cdh1/Ste9 during G1 and with Cdc20/Slp1during mitosis. These broad issues will be addressed in future investigations.

5) The delay in degradation of Cdc13 in *swi6*Δ mutant may have consequences on DNA replication. The replication origins located near *mat* and *cen* loci are known

to be early replicating but are rendered late replicating in *swi6* mutant. This may be because the relatively prolonged presence of Cdc13 in *swi6*Δ mutant may exert negative effect on initiation of DNA replication by inhibiting the Cdc18/Cdc6 licensing or other factor, and its association with replicating origins. These possibilities need to be investigated further.