The fission yeast (S. pombe) is a single celled unicellular eukaryote, which shares many biochemical and physiological similarities with higher eukaryotic systems. The mating type switching and silencing in fission yeast is an interesting phenomena which provide an excellent system where the mechanism of replication initiation, replication pause, replication termination, genetic imprinting, recombination, gene silencing, histone modifications and RNAi processes can be studied simultaneously.
1.1 Fission yeast *Schizosaccharomyces pombe* (*S. pombe*) as model system

Yeast is an excellent model organism for fundamental biological research (Yanagida, 2002). The fission yeast (*Schizosaccharomyces pombe*) and the budding yeast (*Saccharomyces cerevisiae*) are among the most widely studied yeast species. Although both the species share common designation “yeast”, they have diverged from one another about 1000 million years ago (Heckman *et al*., 2001; Hedges, 2002). These single celled organisms are genuine eukaryotes and share fundamental similarities in cell processes with higher eukaryotic systems (Forsburg, 2005). Each yeast species can serve as a unique model system to study and to explore the cellular processes and functions of larger eukaryotes (Forsburg, 1999; Forsburg and Nurse, 1991; Russell and Nurse, 1986). Both yeasts are harmless, genetically tractable genetic systems. That can be easily manipulated in the laboratory using well developed molecular tools (Forsburg, 2001; Wixon and Wood, 2006).

*S. pombe* was isolated in 1890s from East African millet beer (*pombe* means *beer* in Swahili), and is the sixth model eukaryotic organism whose genome has been sequenced (Wood *et al*., 2002). This lower eukaryotic organism has only 4824 predicted genes, which is the lowest number recorded so far for any eukaryotic organism (Yanagida, 2002). Many *S. pombe* proteins appear to be more similar to their mammalian homologs than the *S. cerevisiae* (Sipiczki, 2000). The presence of introns in 43% of its genes (Wood *et al*., 2002), sequence requirements for the splicing of introns (Kaufe *et al*., 1985; Russell and Nurse, 1986), complex organization and the longer size of centromeres (35, 65 and 110 kb on chromosome I, II and III, respectively) (Nakaseko *et al*., 1986; Niwa *et al*., 1989; Takahashi *et al*., 1992), the compact centromere organization, the control of mitotic cell cycle (Russell and Nurse, 1986), the complexity of replication origins, lesser redundancy of the genome (Wood *et al*., 2002), all contribute to make it resemble higher eukaryotes more than *S. cerevisiae*. Moreover, the range of silencing proteins and the histone modification patterns of higher eukaryotes are conserved in *S. pombe*, thus making it an ideal system to study eukaryotic gene regulation and silencing.

*S. pombe* has proportionally more genes conserved in higher eukaryotes in comparison to its counterpart *S. cerevisiae*. Budding yeast has lost many genes associated with the signalosome, a proteosome-related complex required for diverse signaling pathways that are present in fission yeast (Aravind *et al*., 2000). Budding yeast lacks a number of genes of splicing machinery but these genes are present in fission yeast (Aravind *et al*., 2000). RNA interference (RNAi) pathway
is well developed in *S. pombe*. The proteins required for RNAi are Dcr1, Ago1 and Rdpl (Hall *et al.*, 2002; Schramke and Allshire, 2003; Volpe *et al.*, 2003; Volpe *et al.*, 2002) are absent in budding yeast, but present in fission yeast.

Chromosome organization of fission yeast is maintained by unique proteins which are present in metazoans but absent in budding yeast, including Clr4/SuVar3-9 histone methyltransferase (Bannister *et al.*, 2001; Nakayama *et al.*, 2001), the HP1-Heterochromatin proteins like Swi6 and Chp2 (Eissenberg and Elgin, 2000; Ekwall *et al.*, 1995; Halverson *et al.*, 2000; Thon and Verhein-Hansen, 2000), Telomere proteins Taz1, the first TRF1/TRF2 ortholog (Cooper *et al.*, 1997; Nimmo *et al.*, 1998), and the recently identified second TRF1/TRF2 ortholog Tbf1 (Pitt *et al.*, 2007), Pot1 (Baumann and Cech, 2001) and centromere associated CENP-A (Takahashi *et al.*, 2000) and CENP-B (Baum and Clarke, 2000; Irelan *et al.*, 2001). This difference extends beyond the sequence difference to chromosomal elements. A high degree of sequence and chromatin organizational similarity of fission yeast with metazoan system makes *S. pombe* an ideal model eukaryotic organism.

**1.2 Mating type switching in fission yeast**

Fission yeast undergoes a simple life cycle in which the haploid state is more prevalent (Figure 1). Fission yeast cells growing mitotically as haploid cells in rich medium and reproduce by means of fission to produce nearly equal-sized daughter cells. Such mitotically dividing cells do not express their cell types are, therefore, sterile and do not undergo mating. Under conditions of nutritional starvation, especially for nitrogen, the cells express their cell type (Kelly *et al.*, 1988). A particular cell type is determined by expression of genetic information present at the mat locus. The cell type is known as Plus if it contains mat-P allele, whereas those with the mat-M allele exhibit Minus cell type. The P- and M- specific regions are 1104 and 1128 bp long, respectively. The mat-P and mat-M loci each encode two divergently transcribed genes (Pc; 118 amino acids, Pi; 159 amino acids, Mc; 181 amino acids and Mi; 42 amino acids). Mutational analysis revealed that products of these genes are required for mating (Kelly *et al.*, 1988), meiosis and sporulation (Klar, 2007). The predicted amino acid sequence of one of the *P* genes shows the presence of a homeodomain motif, the characteristic of DNA binding proteins and one of the *M* genes contains the HMG (high-mobility group) domain. Molecular studies have established that mat1 genes encode regulatory factors that control many cell type-specific genes distributed throughout the genome (Kelly *et al.*, 1988).
Fig. 1. Life cycle of fission yeast. *S. pombe* predominantly exist in haploid stage. Cells of fission yeast exhibit one of two different mating types, called Plus and Minus. During starvation conditions cells with opposite mating types mate to form a zygote. Usually, the zygote undergoes meiosis and sporulation. Rarely, the zygote can enter mitosis, thereby creating a diploid strain.
When a mixture of cells of both plus and minus types are grown under starvation condition, the cells are arrested in G1 stage of cell cycle, express their cell type and mate with those of the opposite mating type to produce diploid zygote (Figure 1), which undergoes meiosis to produce four haploid meiotic products, called ascospores, enclosed within a sac-like structure called ascus. Each ascus contains two spores with mat1P allele and the remaining two with mat1M allele. Thus, mat1 allele follows Mendelian segregation pattern. Occurrence of meiosis and sporulation within a fission yeast colony can be determined by an easy staining assay with iodine. Sporulating colonies produce a starch-like compound that stains with iodine to give black color, while those incapable of sporulation do not stain (Gutz, 1985). The colonies containing cells of a single mating type cannot mate and therefore, do not undergo meiosis and sporulation and are called heterothallic strains. Such strains do not give staining with iodine vapors. Those capable of mating-type switching can do so and are, therefore, designated homothallic strains, or self-mating strains and give black color when exposed to iodine vapors.

1.2.1 Organization of mating type locus

The mating type region in S. pombe is located 19kb centromere distal on the long arm of chromosome II. It spans about 30 kb region, containing three cassettes as direct repeats (Figure 2), named as mat1-M/P, mat2-P and mat3-M, separated by two spacer regions. Region between mat1 and mat2 is about 15kb long, called “L-region” and region between mat2-P and mat3-M is about 11 kb long, known as “K-region” (Beach, 1983; Beach and Klar, 1984). Genetic studies mapped the mat2/3 loci to within 1.0 centiMorgan (cM) of mat1 locus (Egel, 1984). Each mat cassette is flanked by homology boxes that are termed H1, to the right, and H2 to the left. The H2 box of mat2-P and mat3-M is extended further to the left by an additional H3 box not present at mat1. The mat2 and mat3 regions always remain in silent state. Another unusual property of the mat2/3 region is that recombination, both mitotic and meiotic, is totally prohibited in the 11-Kb intervening “K-region” (Egel, 1984). Furthermore, marker genes, such as ura4, introduced within or around the mat2/3 region are highly repressed or expressed in a variegated fashion (Ayoub et al., 1999; Ayoub et al., 2000; Thon et al., 1994; Thon and Klar, 1992). Deletion studies revealed the existence of repression element (REII) on the centromere proximal side of mat2. Deletion of REII has been reported to affect silencing at mat2 region (Ekwall et al., 1991). Another repression element was located within 500 bp proximal side of mat3 (Thon et al., 1999). Sequence analysis of 10.9-kb “K-region” showed that a ~4.3-kb centromere homology region (cenH) is present between mat2 and mat3 loci. It shows 96% sequence identity to part of dh and dg centromeric repeat elements (Grewal and Klar, 1997) that are found in S. pombe centromeres.
Fig. 2. Mating Type region of fission yeast. Mating type region consists of three cassettes, the P Allele (white box) and M allele (black box). The homology boxes H1, H2, and H3 flanking the cassettes are indicated. The imprint is shown by a black arrow head; IR-L and IR-R, are left and right inverted regions, respectively. The RE2 and RE3 (Recombination enhancers) shown by Two circles. The cold region for transcription and recombination is shaded. The L and K regions and cenH are indicated. The deletions in the distal mat1 regions Msmt-0, PA17 and smt-s1, the regulatory elements within the H1 box and the SAS1 and SAS2 cis-activating sites are shown.
This was an interesting finding since auxotrophic markers inserted in centromeres are also silenced by many of the same factors identified for silencing the mating-type K-region (Allshire et al., 1995). Since transcriptionally silent mat2/3 region is located in close proximity of transcriptionally active mat1 region, the presence of boundary elements is most likely in this region. Availability of the genomic sequence also led to the identification of two identical inverted repeat 2.1-kb sequence elements, one present on the left (IRL) and one on the right (IRR) of the mat2/3 region (Noma et al., 2001; Thon et al., 2002). These IRR and IRL elements act as barriers for spreading silencing into adjacent regions.

Presence of replication pause sites at mating type region plays important role in mating type switching. One such site is known as replication terminator sequence, RTS1, present 700 bp to the left of mat1 cassette. RTS1 is a strong replication terminator. It blocks replication forks traversing from the “left” of mat1 and ensures that only the replication fork traversing from the right toward mat1 replicates the mat1 locus (Dalgaard and Klar, 2001a). Another replication pause site is located within the mat1-M cassette. This directional pausing site has been mapped at 340 bp to the left of the imprint at H1 and is termed as mat1 pause site, MPS1 (Dalgaard and Klar, 2000; Vengrova and Dalgaard, 2004). A similar pause site in the mat1-P cassette remains to be characterized. Farther out to the right of mat1, the essential “switch activating protein” (Sap1) binds to the bipartate switch-activating sites SAS1 and SAS2, located 130–160 bp to the right of the H1 box; deletion of Sap1-binding site reduces the efficiency of mating type switching (Arcangioli and Klar, 1991).

1.2.2 Rules of mating type switching

Mating-type switching in fission yeast follows similar rules as in budding yeast, but the underlying mechanisms are entirely different and more complex then budding yeast. In nutrient supplemented medium, *S. pombe* grows by mitotic division where the cell divides by fission (Egel et al., 1980; Leupold, 1950). However, the *P* and *M* mating types are unstable and the cell-types interchange spontaneously nearly every other generation (Egel, 1977). Consequently, a clonal population derived from a single cell contains a mixture of *P* and *M* cells in nearly equal proportions and the strain is designated as homothallic (*h*90) because 90% cells are engaged in mating and meiosis. Pedigree analysis at the single cell level shows that the mating-type switching follows strict rules (Egel and Eie, 1987; Klar, 1990; Miyata and Miyata, 1981) (Figure 3). A remarkable feature of this system is that switching occurs in a highly regulated fashion by the following rules:
1.2.2.1 A single-daughter switchable rule:

A recently switched cell, that is incompetent to switch produces two daughters, of which only one is switchable (Ps in Figure 3, s for switchable) and other is unswitchable (Pu in Figure 3, u for unswitchable) This accurs in ~80% of cell divisions. Both daughters never switch simultaneously (Egel and Eie, 1987; Klar, 1990; Miyata and Miyata, 1981). The switchable cell (Ps) produces one switched daughter (M), whereas the other daughter does not switch.

1.2.2.2 The recurrent switching rule:

The sister of the switched cell inherits the switching competence as it produces a switched and nonswitched pair of cells in about 80% cell divisions. Thus, chains of switching pedigrees, similar to stem cell lineage in higher eukaryotes, can be observed where one daughter at each cell division is switched but the other one is not.

1.2.2.3 The directionality rule:

As ~80% of switchable cells switch to the opposite allele, there must be a nonrandom donor locus choice, such that P cells favor mat3-M as donor, whereas M cells favor mat2-P as donor locus (Thon and Klar, 1993).

The same rules apply when M cells switch to P type. Collectively, these rules suggest that two different kinds of consecutive developmentally asymmetric cell divisions are required to produce only one-in-four switched granddaughters in the pedigree of a Pu or Mu cell. Such a pattern of cell divisions is analogous to the asymmetric stem-cell division pattern observed in higher eukaryotes, where one daughter is always identical to the parental cell, whereas the other daughter is differentiated to a specific cell lineage (Klar, 2007).

1.2.3 Molecular mechanism of Switching

Mating-type switching in fission yeast is an interesting phenomenon and is executed by an entirely different mechanism as compared to budding yeast. The switch initiating double-strand break in Saccharomyces cerevisiae requires recombinational repair for survival while, the initial damage in Schizosaccharomyces pombe only affects a single strand and remains unrepaired for one complete cell cycle.

In budding yeast, mating type switching is initiated with a double-strand break (DSB) made by HO endonuclease at a ~20 bp target sequence. The DSB is usually repaired by
Fig. 3. Asymmetric segregation of switching competence in fission yeast. The Plus (P) and minus (M) mating-types are indicated. When a newly switched cell divides, the two daughter cells exhibit different developmental programs. One of the cells is switchable (Ps), as it gives rise to a switched and a switchable cell in the next division, whereas the other cell divides to produce a switchable (Ps) and an unswitchable (Pu) cell. Thus only one out of four cousins ever switches its mating type.
directional gene conversion from a partly homologous donor cassette before replication, resulting in mating-type switching in both daughter cells. The HO endonuclease of \textit{S. cerevisiae} belongs to the so-called homing endonucleases (Bakhrat \textit{et al.}, 2004). No homolog of the HO endonuclease has been found in the entire genome sequence of \textit{S. pombe} (Wood \textit{et al.}, 2002).

In fission yeast instead of double strand break, only one strand is marked with a fragile modification at \textit{matl} locus. This initial event is referred to as imprint. The nature of imprint and the enzyme activity responsible for it remain to be confirmed. Unlike budding yeast the imprint as such is not lethal to cells without donor cassettes (Klar and Miglio, 1986). On the other hand, the overall DNA sequence required for switching at the \textit{matl} locus extends \textasciitilde 300 bp away from the imprint at H1 box within \textit{matl} cassette in either direction. Several recent findings have not only explored this DNA modification, but also shed light on its relation to replication forks in general with particular relevance to lagging-strand processing in one cell cycle and stalling of leading-strand progression in the next round of cell cycle (Dalgaard and Klar, 2000; Holmes \textit{et al.}, 2005; Kaykov and Arcangioli, 2004; Kaykov \textit{et al.}, 2004; Lee \textit{et al.}, 2004; Vengrova and Dalgaard, 2004).

1.2.4 Chemical nature of the imprint

Southern blot analysis of DNA isolated from homothallic cells showed that there is a site-specific DSB at \textit{matl} locus (Beach, 1983; Beach and Klar, 1984). The frequency of the break remains constant throughout cell cycle and is found in \textasciitilde 25\% of extracted DNA (Beach, 1983). However, Southern blots performed under mild conditions of DNA extraction, showed only a nick or alkali-labile modification on one strand of DNA which was converted to DSB under vigorous DNA extraction procedure (Arcangioli, 1998; Vengrova and Dalgaard, 2004).

The nature of imprint is a matter of debate (Arcangioli and Kaykov, 2005; Vengrova and Dalgaard, 2005). While one school of thought claims that it is a single-strand break (SSB) (Arcangioli, 1998), another showed that imprint is an alkali-labile, RNase sensitive modification within a contiguous chain of nucleotides (Vengrova and Dalgaard, 2004). The former assumption has been supported by detection of a novel nick, which is unphosphorylated at either end, containing 3'OH and 5'OH ends, so that it cannot be ligated directly (Kaykov and Arcangioli, 2004). This conclusion was based on a series of reconstitution experiments, characterizing the conditions that allow the exact regeneration of the parental DNA sequence from the imprint fragments nicked \textit{in vivo}. The original sequence could be perfectly restored by phosphorylation.
and ligation not only for wild-type, but also for a CTGCAG PstI restriction target covering the site of the imprint. However, the alternative claim (Vengrova and Dalgaard, 2004) of 1–2 interspersed ribonucleotides in the DNA chain at the site of the imprint has been supported by ligation-mediated PCR (LMPCR) experiments showed that the imprint actually consists of two ribonucleotides (Vengrova and Dalgaard, 2006).

1.2.5 Genes involved in switching event

Switching events can be divided into three main steps: Imprinting, utilization of imprint for gene conversion and resolution step. Three classes of genes have been shown to participate in different steps of switching event; mutation in these genes result in slow switching phenotype and therefore called swi mutants (Egel et al., 1984)

Class Ia: These genes are required for setting up the imprint at mat1 and include swi1, swi3 and swi7. Mutation in any one of these genes results in reduced level of imprint.

Class Ib: These genes are required for the initial step of gene conversion, reflected by normal level of imprinting but reduced switching. This class includes swi2, swi5 and swi6 genes.

Class II: These genes are required for the final resolution step of gene conversion and include swi4, swi8, swi9, swi10 and rad22. Mutations in these genes result in rearrangements of mating-type region.

1.2.6 Role of swi1 and swi3 in setting up imprint

The products of class Ia genes are involved in setting up the imprint: mutations in swi1, swi3 and swi7 all result in reduced level of imprinting and mat-P/M interconversion. Available literature suggests that swi1 and swi3 play an indirect role in setting the imprint. A DNA replication pause site was reported at the site if imprint even in nonswitching strains (Dalgaard and Klar, 1999) and this pause site was abolished in swi1 and swi3 mutants (Dalgaard and Klar, 2000). Inversion of mat1 region also results in abrogation of this pause site (Dalgaard and Klar, 1999). These studies suggest that swi1 and swi3 along with the imprinted sequence in proper orientation makes a suitable combination so that the imprint can be generated at the right site. This observation was further supported by one recent observation that the Swi1 and Swi3 protein interact with each other and this protein complex binds to the replication pause site MPS1 inside the mat1M cassette and to the RTS1 site (Dalgaard and Klar, 2000; Lee et al., 2004). The Swi1-Swi3 protein complex has been implicated as stabilizer of stalled replication forks in general (Noguchi et al., 2003; Noguchi et al., 2004). Screening for loss of terminator function at RTS1 yielded a missence allele, swi1-rtf3, which was defective in replication termination at RTS1 not at
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MPS1 (Dalgaard and Klar, 2000). All these observations suggest an indirect role of swi1 and swi3 in setting up the imprint at mat1 locus. The Swi1-Swi3 complex acts as a general replication fork protection complex RFC (Noguchi et al., 2004), and acts by stabilizing the replication fork in the critical area to give time to generate the imprint.

1.2.7 Role of swi7 (pol α) in setting up imprint

The swi7 gene was classified as Class Ia gene. A unique mutation in swi7 (swi7-1) results in reduced level of imprinting at mat1 locus (Egel et al., 1984), and was mapped to the catalytic subunit of DNA polymerase α (Singh and Klar, 1993). These findings functionally implicate Pola/primase in setting up the imprint at mat1 locus. As the chemical nature of imprint suggests that it is either a nick or one or two ribonucleotides (Arcangioli and Kaykov, 2005; Kaykov and Arcangioli, 2004; Vengrova and Dalgaard, 2005; Vengrova and Dalgaard, 2006), but not even a single observation has been made to answer how such site-specific modification is generated on mat1 DNA. The imprinting event occurs only on the newly made lagging strand during S phase (Holmes et al., 2005) and the replication pause site is not affected in swi7-1 mutant, suggesting that swi7 function is distinct from that of swi1 and swi3 (Dalgaard and Klar, 2000). In light of these observations pola seems to be a suitable candidate for involvement in setting up imprint at mat1 locus, especially because only Polα can synthesise an RNA moiety on a DNA template as Polα/primase complex, and its activity is required more often for replicating the lagging strand. On the other hand, if one assumes that imprint is actually a nick, not an RNA moiety, even then Polα seems to be a suitable candidate for imprinting, because certain DNA polymerases are known to code for Intein homing endonucleases (Matsumura et al., 2006; Nishioka et al., 1998; Stoddard, 2005).

Homing endonucleases are a class of site-specific DNA endonucleases encoded by open reading frames within introns and inteins. They initiate the mobility of their host element by recognizing intronless or inteinless alleles of their host gene and making a double-strand break. The homing endonucleases are notable for their long target sites and a tolerance for sequence polymorphisms in their substrates (Kowalski and Derbyshire, 2002). The vast majority of homing endonucleases fall into four families that are defined by the presence of conserved sequence elements, the LAGLIDAG, the GIY–YIG, the His-Cys Box, and the HNH families (Kowalski and Derbyshire, 2002). Sequence search and alignment indicates that fission yeast DNA polymerase α contains a motif, which shows similarity with the LAGLIDAG homing endonuclease motif. In addition, fission yeast DNA Polα also seems to contain a sequence motif
that resembles the Restriction endonuclease (REase) signature sequence, called PD (D/E) XK. Nearly all structurally characterized REases belong to the PD (D/E) XK superfamily Restriction-endonuclease-like proteins. Such PD(D/E)XK motif-containing proteins constitute a large and diverse superfamily of enzymes that are involved in numerous nucleic acid cleavage events important for various cellular processes. The SCOP database currently groups 23 families of known structure in the restriction endonuclease-like superfamily, including, among others, 15 different restriction endonucleases (Bujnicki, 2003), Holliday junction resolvases (endonuclease I, Hjc) (Hadden et al., 2001; Nishino et al., 2001), lambda exonuclease (Kovall and Matthews, 1997) and very short patch repair (Vsr) endonuclease (Tsutakawa et al., 1999). Their function varies from repairing damaged DNA (Vsr), resolving Holliday junctions (endonuclease I, Hjc), performing additional cleavage events in DNA recombination (lambda exonuclease), to protection of host organisms against foreign DNA invasion (restriction endonucleases).

1.2.8 Role of DNA sequence elements in switching

Imprinting at matl locus in fission yeast seems to be sequence-independent because imprinting remains unaffected even on replacing the target sequence with the PstI site sequence (Kaykov and Arcangioli, 2004). The imprint escapes the cell’s DNA repair mechanism and remains unrepaired throughout one cell cycle. Interestingly, repairing of the imprint by forced transcription trough matl has been achieved by inserting a thiamine repressible nmtl promoter outside the SAS1 boundry (Holmes et al., 2005), which suggests that the imprint is not entirely inaccessible to repair machinery. The transcription-mediated repair of the imprint implies that either a shielded chromatin structure or strictly phased immobilized nucleosomes might be responsible for protecting the imprint from DNA repair machinery (Egel, 2005). Substitution scanning mutagenesis where PstI sites have been introduced at various distances from the imprint (Figure 2), revealed three cis acting sites controlling the efficiency of imprinting at regular 20bp intervals, which is equal to two turns of DNA double helix; PstI sites located at 20, 40 and 60bp to the right side of the imprint exerted more effect on imprinting, in comparison to 10, 30 and 50bp intervals (Kaykov et al., 2004). The sites located at 20 bp intervals may have interfered with DNA bending at regular intervals important for nucleosome positioning.

1.2.9 Directionality of Mating-Type Switching

Mating type switching in fission yeast is a remarkable system in which cells predominantly (72-90%) switch to the opposite matl allele, such that mat2P is the preferred donor in mat1M cells, whereas mat3M is the donor choice in mat1P cells (Miyata and Miyata, 1981)
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(Figure 3). This process of non-random donor choice is referred to as directionality of switching. The location (or chromosomal context) of the donor loci, not the DNA sequence is responsible for directionality of switching. This fact was proved by one elegant experiment performed in $h^{90}$ strains (i.e. the reverse of $h^{50}$), such strains, where the donor information was swapped, as $mat2M$ and $mat3P$, (Thon and Klar, 1993) showed very much reduced level (18%) of switching, presumably because the $mat1P$ locus still switched to $mat3$, which now contains the homologous $P$ allele instead of normal M allele and likewise, $mat1M$ switches using $mat2M$ allele as the donor. It has also been shown that heterochromatin within the $mat2/mat3$ interval including the K-region is essential for the directionality of switching (Grewal and Klar, 1997; Noma et al., 2001; Thon and Klar, 1993). A replacement of 7.5 kb of the K-region with $ura4$ (designated $K\Delta::ura4$) restricts the donor choice to $mat3M$ cassette (Grewal and Klar, 1997), suggesting that the chromatin structure of the K-region may help in bringing the $mat2P$ donor cassette in vicinity of the $mat1$ locus. Similarly, a mutation in $swi6$ severely reduces the efficiency of mating-type switching in $h^{90}$ cells and the switching defect is attributed to their inability to utilize $mat2$ as a donor (Grewal and Klar, 1997; Thon and Klar, 1993). It was proposed that a higher order chromatin structure preferentially brings one donor close to $mat1$ in a cell-type specific manner by intrachromosomal interaction (Grewal and Klar, 1996; Klar and Bonaduce, 1991; Thon and Klar, 1993). It has also been shown that a recombination-promoting complex (RPC) containing Swi2 and Swi5 exhibits cell-type specific localization patterns at the silent mating-type region and this differential localization modulates donor preference during mating-type switching. Swi2 binds specifically to a cis-acting DNA element, called Swi2-dependent recombination enhancer (SRE), located 0.5 kb to the right of $mat3$ locus. In $P$ cells, Swi2 binding is restricted to SRE, but it spreads throughout the entire 20-kb heterochromatic $mat2/3$ region in $M$ cells. The spreading of Swi2 from the SRE site across the $mat2/3$ interval depends on the Swi6-containing heterochromatin complexes. Moreover, the localization of the recombination repair protein Swi5 is determined by Swi2, which also displays cell type-specific distribution at the $mat$ locus. Together these proteins activate donors for recombination during mating-type switching (Jia et al., 2004). Thus, the orchestration of two components, the heterochromatin and RPC ensures switching to the opposite mating-type in each cell-type (Jia et al., 2004). Since $swi6$ mutants show an inability to utilize $mat2$ as a donor (Thon and Klar, 1993), in view of the recent results, it is proposed that the altered directionality control in this mutant results from defective heterochromatin organization at $mat2/3$ interval. Mutation in $swi6$ abolishes higher-order organization of the mating-type region and hence, prevents the spreading of RPC from the
recombination enhancer element (SRE) located adjacent to mat3. However, the restricted presence of RPC at SRE makes mat3 the preferred donor in swi6 mutant cells (Jia et al., 2004).

1.2.10 Strand segregation model of asymmetric cell division

This model suggests that the switching pattern results from inheritance of specific parental DNA strands and explains asymmetric cell division of fission yeast cell and the one-in-four granddaughter switching rule. The strand-segregation model (Figure 4) suggests that both the complementary DNA strands, indicated as “Watson” and “Crick” strands of the parental mat1 locus, are differentially modified in a strand-specific fashion, such that their replication produces “differentiated” chromatids (Klar, 1987). The hypothetical modification (imprinting) may be a precursor to generating the DSB of the imprinted chromosome involving only one specific sister chromatid. The model suggests that the DNA replication itself can set the developmental program for asymmetric cell divisions to generate switching of only one-in-four granddaughter cells.

1.2.11 The current Uni-directional replication model of mating type switching

In fission yeast, a perfectly designed and rigid cascade of events ensures that only one-in-four grand-daughter cells switch their mating type and that the sister of a switched cell switches in the next division. The ‘Unidirectional replication model’ (Dalgaard and Klar, 2001b) relies on the inherently asymmetrical nature of DNA replication. It proposes that during DNA replication of a newly switched cell, a fork moving in from the telomeric end usually replicates the mat1 locus, the fork moving from centromereric direction is blocked by RTS1 some 700 base pairs to the left of the mat1 cassette (Dalgaard and Klar, 2001a) (Figure 5). The replication fork approaching mat1 from the telomere direction pause within the mat1-M cassette at MPS1 site at 340 base pairs to the left of the imprint at H1 box (Dalgaard and Klar, 2000). These stalled replication forks are protected by the Swi1-Swi3 complex, also called the RFC “replication fork protection complex” (Noguchi et al., 2004). This transient cessation of the replication fork at this pause site is critical for setting the imprint at H1. At the same time an external boundary is defined by the SAS1 binding site for the essential Sap1 protein, whose function is still unknown (Arcangioli and Klar, 1991) (Figure 6).

The overall topology of replication at mat1 indicates that the imprinting event occurs on the newly synthesized lagging strand, perhaps during maturation of a critical Okazaki fragment. In this manner only one DNA strand gets imprinted and replication is completed further after a transient halt. After completion of S phase, the cell divides and the imprinted chromosome is
Fig. 4. **The strand-segregation model.** The asymmetric cell division of fission yeast cells and the one-in-four granddaughter-switching rule is explained on the basis of strand segregation model. The top strand (*green*) and the bottom strand (*red*) are called is called the Watson (W) strand and the Crick (C) strand respectively. The *matl* DNA is replicated by a replication fork that approaches it from the left. Thus, only the newly synthesized W strand at *matl* is imprinted during the lagging-strand replication, and in next generation only one in four granddaughters switches its mating type.
Fig. 5. Unidirectional Replication of *matl* and the trans-acting factors important for *matl* imprinting. The imprint is shown as a red arrowhead. The DNA polymerase $\alpha$ (the *swi7* gene encodes polymerase $\alpha$), Sap1p, Swi1p and Swi3p are main trans-acting factors. The protein Sap1p of unknown function binds to SAS1 and SAS2. The *matl* is replicated by a distal origin. The gray arrow indicates that *matl* is replicated unidirectionally.
Fig. 6. The organization of the *S. pombe mat1* cassette at the imprinting stage during S phase. The left termination site (RTS) and *mat1* pause site (MPS1), Homology boxes H1 and H2 and SAS sites are depicted. Interactions between the external boundary SAS1 and the internal replication pause site MPS1 are necessary for placing a protected nick in the middle of a longer than usual Okazaki fragment.
inherited by one daughter cell, called "switchable cell" and the unimprinted chromosome by the other daughter cell, called "unswitchable cell". During the next cell division, the leading strand replication of the switchable cell proceeds to the last base at the imprint and is disrupted by the imprinted template. The 3' end of the arrested leading strand can serve as a primer for bypass conversion (Klar and Miglio, 1986). This homology search appears to be guided by the newly discovered protein complex Swi5-Swi2, which is specific for the mat region. The specificity of the Swi5-Swi2 complex for the silent mat2-mat3 region is likely based on strong affinity of Swi2 for Swi6 protein (Jia et al., 2004). These interactions account for involvement of class Ib genes, swi2, swi5 and swi6 in mating-type switching. In this manner the information of donor cassette gets copied, imprint is repaired and replication proceeds further (Figure 7), while the reactivated fork is again stalled at the MPS1 inside the mat1 cassette (Dalgaard and Klar, 2000) and the new imprint is generated in the neo-synthesized lagging strand as if the template were in the virgin state for imprinting. In this way one switchable cell divides into one switched and one switchable cell, whereas the other cell from previous cell division divides to produce a switchable cell and one unswitchable cell.

1.3 Centromeres

Fission yeast centromeres are highly specialized chromosomal structures that resemble the centromeres of higher eukaryotes (Chikashige et al., 1989; Takahashi et al., 1992). There are three centromeres in fission yeast known as cen1, cen2 and cen3 which are 40, 65 and 110 kb long and located on chromosome I, II and III, respectively (Nakaseko et al., 1986; Niwa et al., 1989; Takahashi et al., 1992). These contain a ~15 kb central region composed of a ~4-7kb central core domain (cnt) flanked by inverted repeats (imr). This central region is surrounded by 10-100 kb of repeated sequences (called otr) containing highly repeated motifs (dg and dh) common to all centromeres (Takahashi et al., 1992) (Figure 8). These centromeres, like those of humans, show characteristics of heterochromatin, where the central core is weakly silenced and the outer flanking repeats are more strongly silenced. Marker genes inserted at the otr, imr or cnt regions are subject to position-effect control (Allshire et al., 1994; Allshire et al., 1995). Like mating-type region, centromeres also show linked recombinational suppression along with transcriptional silencing (Allshire et al., 1994; Allshire et al., 1995; Nakaseko et al., 1986).

1.3.1 Factors mediating centromere silencing

The trans-acting factors Swi6, Clr1-Clr4, Rik1 and Clr6 also mediate silencing at centromeres (Allshire et al., 1995; Grewal et al., 1998; Thon and Verhein-Hansen, 2000). In
Fig. 7. The mechanism of switching via unidirectional replication of mat1. The unidirectional replication of a newly switched P cell results in imprinting of single strand, which is converted into double-strand break during the next round of replication. Through DSB mediated recombination, information from donor cassette is copied. Arrows indicate inheritance of a specific strand. Subscript ‘u’, unswitchable cell; subscript ‘s’, switchable cell; black, leading-strand synthesis; red, lagging strand synthesis. Switching (green arrows) is initiated when the leading-strand replication is stalled at the imprint.

Dalgaard and Klar, 1999
particular, Clr4, Rik1 and Swi6 are required for silencing within the outer repeats of centromeres but not within the central core region. Silencing at centromeres also involves additional centromere-specific factors, including a chromodomain protein Chp1 (Doe et al., 1998). Chp1 along with Swi6 is mainly restricted to the otr region (Figure 8) (Partridge et al., 2000). In addition, mutations in 12 csp (centromere suppression of position effect) loci, that affect silencing in the outer repeats have been identified (Ekwall et al., 1999). Two other factors, Mis6 and Mis12, are required for silencing within the central core and inner repeats (Partridge et al., 2000; Saitoh et al., 1997). Histone deacetylases and homologs of the CENP-B protein also influence centromeric heterochromatin (Grewal et al., 1998; Nakagawa et al., 2002). Centromeric silencing is also affected by mutations in the proteasome subunit 19S, suggesting that some silencing component may be regulated by proteolytic degradation (Javerzat et al., 1999). Some temperature sensitive lethal mutants have been shown to specifically alter central core silencing, named sim I to 7 (silencing in middle of the centromere (Allshire, Website www.igh.cnrs.fr). Out of seven three sim2 alleles have been shown to carry missense mutations in the histone-fold domain of Cnp1 (Takahashi et al., 2000), which encodes the histone H3-like counterpart of CENP-A and CSE4 found at vertebrate and budding yeast kinetochores, respectively.

### 1.3.2 Heterochromatin assembly at centromeres

Centromeres, like the mating-type region, are characterized by hypoacetylation of histones H3 and H4 and methylation of Lys9 of histone H3 (Ekwall et al., 1997). Mutations of Lys9 or Lys14 or Ser10 of histone H3 cause Swi6 delocalization and impairment of centromere function (Mellone et al., 2003). The underacetylated chromatin is specifically methylated at Lys9 of histone H3 by Clr4 (Nakayama et al., 2001; Rea et al., 2000) allowing binding of Swi6 and Chp1 via their chromodomains which, in turn, promote the assembly of silent chromatin (Bannister et al., 2001; Partridge et al., 2000; Partridge et al., 2002). RNAi components also mediate centromeric silencing. Deletion of dcr1, rdp1 or ago1 leads to transcriptional derepression of transgenes inserted into either the imr or otr region of the centromere. Furthermore, forward and reverse transcripts corresponding to the dg-dh repeats were detected in each of the mutant strains. Nuclear run-on experiments indicated that the reverse transcript was always transcribed but is presumably rapidly turned over by RNAi in wild-type cells. A forward transcript, which is not present in wild-type strains but is detected in RNAi mutants and clr4 mutants, likely initiates the RNAi process (Volpe et al., 2002). Strong support for this interpretation came from another study (Reinhart and Bartel, 2002), which demonstrated that a large percentage of the small RNAs cloned from S. pombe, which were thought to be Dicer cleavage products, showed perfect
Fig. 8. Schematic representation of fission yeast centromeres. The basic structure of Centromeres in fission yeast consists of a central core (cnt) of non-repetitive sequence flanked by innermost repeats (imr) and outer repeats (otr). The centromere is divided into two domains: the central core domain (cnt and imr) and the outer repeat domain. Different classes of mutants affect silencing in each domain, and each is associated with a distinct set of proteins. Short vertical lines represent tRNA genes. The central core region has a unique chromatin structure. CENP-A replaces histone H3 in the central region and upon this chromatin platform, the kinetochore is assembled. The outer repeats are packaged in nucleosomes which are underacetylated and di-methylated at H3K9 provides binding site for the chromodomain proteins Swi6 (HP1) and Chp1. This Swi6-containing heterochromatin is responsible for the recruitment of a high density of cohesin to the outer repeat. The assembly of heterochromatin is dependent on the RNAi machinery and siRNAs derived from centromeric transcripts. This involves the RNAse III-like endonuclease Dicer, the RITS complex (Chp1, Tas3, Ago1 and siRNAs), the RNA dependent-RNA polymerase Rdpl.
homology with the portions of the centromere affected by the RNAi pathway (Volpe et al., 2002). Moreover, the observed derepression was accompanied by the loss of methylation of Lys9 of histone H3 at dh repeats, as well as at the inserted transgenes, concomitant with Swi6 delocalization and disruption of centromere function (Volpe et al., 2002). Also, by Co-immunoprecipitation (Co-IP) it was shown that Rdpl is chromatin-bound and interacts with the dg repeats suggesting that the RNA processing events may occur in cis. These results led to the proposal that the transcription of the two strands of the centromeric repeats generates dsRNA molecules, which may be amplified by chromatin-bound Rdpl. Processing of the dsRNA by Dcr1 produces siRNA that would then guide histone methyltransferases, which would in turn recruit Swi6 that causes repression (Volpe et al., 2002). In addition, it has been shown that the dg ectopic silencer (a subfragment of dg that induces silencing at euchromatic sites) fails to assemble heterochromatin in RNAi mutants as well as in mutants defective in centromere silencing/structure. Recently, it has been shown that the CENP-A (Cnp1) chromatin, once established, is propagated by epigenetic means in the absence of heterochromatin but its establishment requires the RNAi machinery and heterochromatin both. The RNA interference (RNAi)-directed heterochromatin flanking the central kinetochore domain at fission yeast centromeres is required to promote CENP-A (Cnp1) and kinetochore assembly over the central domain (Folco et al., 2008).

Moreover, csp mutants are defective in the regulation of non-coding centromeric RNAs (Volpe et al., 2003). RITS, the RNAi effector complex, contains siRNA homologous to dg and dh repeat sequences in addition to Ago1, the chromodomain protein Chp1 and Tas3 (Verdel et al., 2004), suggesting that this complex mediates heterochromatin formation by RNA-RNA interaction with the nascent RNA being transcribed from the dg-dh region or by RNA-DNA interactions with dg and dh DNA. Moreover, like at the mat locus, RITS is tethered to silenced region by H3-Lys9 methylation and this tethering is essential for silencing and the continued production of siRNAs (Noma et al., 2004). It has been shown that Rdpl is associated with two conserved proteins, Hrr1, an RNA helicase and Cidl2, a member of the polyA polymerase family, in a complex that has RNA-directed RNA polymerase activity (RDRC, RNA-directed RNA polymerase complex) (Motamedi et al., 2004). RDRC physically associates with RITS in a manner that requires Dcr1 and Clr4. Moreover, both complexes are localized to the nucleus and associate with non-coding centromeric RNAs in a Dcr1-dependent manner. In cells lacking Rdpl, Hrr1 or Cidl2, RITS complexes are devoid of siRNA and fail to localize to the repeat sequences to initiate heterochromatin assembly. Recently heterochromatin dynamics during the cell cycle
and its effect on RNAPII transcription was studied in great detail and a brief period during the S phase of the cell cycle in which RNAPII preferentially transcribes centromeric repeats have been reported (Chen et al., 2008).

1.3.3 Replication dependence of centromeric heterochromatin

Centromeric Heterochromatin shows a unique structure in *S. pombe* which is arranged in two distinct domains, the central core domain (*cnt* and *imr*) and outer repeat domain (*otr*). The *otr* domain is marked by underacetylated histones H3 and H4 and methylated lys9 (K9), Swi6 and Chin (Figure 8). The central domain consists of CENP-A (Cnp1) and some histone H3-containing nucleosomes that are dimethylated on lysine 4 of histone H3. In fission yeast, two distinct CENP-A deposition phases across the cell cycle have been reported. It has been found that CENP-A deposition takes place during S and G2 phase of cell cycle and the S phase deposition is dependent on Ams2 GATA factor, which promotes histone gene activation, while the G2 deposition pathway is regulated by Hip1, a homologue of HIRA histone chaperone. CENP-A is normally localized to centromeres in S phase in an Ams2-dependent manner and the G2 pathway may salvage CENP-A assembly to promote genome stability (Takayama et al., 2008). Recently a direct role of replication proteins in maintenance of CENP A at the core region has also been shown. The Mci1, a DNA polymerase α (Pola) accessory protein, and DNA Pol α are required for the localization of CENP A to the central core region (Natsume et al., 2008). These studies in fission yeast clearly indicate that the recruitment of CENP A is a replication-dependent process.

1.4 Telomeres

Linear eukaryotic chromosomes are terminated at specialized nucleoprotein complexes called telomeres. Muller and McClintock in early 1930’s described the telomere as a protective structure at the terminal ends of the chromosome. In absence of telomeres chromosomes become unstable and end to end fusion takes place (Muller and Herskowitz, 1954). In the 1970s, Watson described the "end-replication problem" (Watson, 1972). During DNA replication, DNA-dependent DNA polymerase does not completely replicate the extreme 5' terminal end of the chromosome, leaving a small region of telomere uncopied. He also noted that a compensatory mechanism was needed to fill this terminal gap in the chromosome. The end-replication problem of chromosomal ends was an interesting puzzle of eukaryotic biology (Cech and Lingner, 1997; Lundblad, 1997). Removal of the terminal RNA primer by nucleases leaves an 8 to 12 nucleotide gap at the 5' end of the newly replicated DNA that cannot be refilled by conventional DNA replication. The discovery of the uniqueness of telomeric DNA In 1978 by Blackburn and Gall...
showed that in *T. thermophila*, telomeres consist of short repeats of GGGTT (Blackburn and Gall, 1978). Following this discovery, similar results were reported from several organisms, such as yeast (Shampay *et al.*, 1984), *Arabidopsis thaliana* (*A. thaliana*) (Richards and Ausubel, 1988), and humans (Moyzis *et al.*, 1988). A decade after the discovery of telomeric DNA by Blackburn (1985), she also formulated the solution to the end-replication along with Carol Grieder by the discovery of the enzyme terminal transferase (telomerase) in *T. thermophila* (Greider and Blackburn, 1985). They showed that telomerase is a ribonucleoprotein complex with an RNA template called telomerase RNA, later called TER (Greider and Blackburn, 1987), with sequences that are complementary to the respective telomere sequence (Shippen-Lentz and Blackburn, 1990). Telomerase uses RNA as the template to synthesize novel telomere repeats. The synthesis of telomeres is conducted by Telomerase Reverse Transcriptase (TERT), which forms the enzymatic core of the telomerase, TERT has been characterized in several species, including *T. thermophila* (Collins and Gandhi, 1998) and humans (Lingner *et al.*, 1997; Nakamura *et al.*, 1997) and fission yeast (Haering *et al.*, 2000; Leonardi *et al.*, 2008; Nakamura *et al.*, 1997)

1.4.1 Telomeric DNA

Blackburn showed that in *T. thermophila*, unlike normal DNA sequences, telomeres consists of short repeats of GGGTT (Blackburn and Joseph, 1978). Studies have shown that telomeric repeats do not vary much between different species, such as *Aspergillus nidulans* (Bhattacharyya and Blackburn, 1997). Although telomeric sequences are similar between several species, they can also vary in size and type. The number of telomeric repeats ranges from several thousands, as in humans, which consist of ~60,000 TTAGGG sequences, to a few hundred GGGGT repeats (Kirk and Blackburn, 1995). Another significant variation observed in telomeric tracts is the existence of more than one type of repeat in the same chromosome (McEachern *et al.*, 2000a). For instance, micronuclear telomeres of *T. thermophila* have an internal G4T3 telomeric tract (0.6 -1.0 kb) along with the external G4T2 tract (1.4 - 2.1 kb; Kirk and Blackburn, 1995). Similar examples exist in *S. cerevisiae, S. pombe* (Shampay *et al.*, 1984) and Chlorarachinophyte algae (Gilson and McFadden, 1995). The telomeric sequences in fission yeast *Schizosaccharomyces pombe* are peculiar among eukaryotes. The consensus sequence of T1–2ACA0–1C0–1G1–6 or T1–3ACA0–2C0–1G1–8 seems to be irregular (Konig and Rhodes, 1997; Zakian, 1995), but the basic unit is GGTACA, which is the most simpler and representative consensus sequence of fission yeast telomeres (Hiraoka *et al.*, 1998). The nucleotide sequences of *S. pombe* telomeres was determined by N. Sugawara (Sugawara, 1989). The nucleotide sequence GGTTAC (A) (C) G0-6 is the consensus sequence at fission yeast
telomeres, 5-GGTTACA-3 being the most commonly found, with a variation in the numbers of G residues in each repeating unit being the most common variant form. The RNA component of telomerase has been identified in S. pombe and the role of synthetic actions of telomerase in telomeric sequence variation has been reported recently (Leonardi et al., 2008; Webb and Zakian, 2008).

1.4.2 Telomere Protein Components

Telomeric proteins form part of the telomeric nucleoprotein complex and are well characterized in humans, mouse, yeast and ciliated protozoa (McEachern et al., 2000b). They are differentiated on the basis of their ability to bind to telomeric sequences. The two main classes of telomeric proteins are the single and double-stranded DNA binding proteins. They are well characterized in S. cerevisiae (Klobutcher et al., 1981; Larson et al., 1994; Shore and Nasmyth, 1987), S. pombe (Cooper et al., 1997; Vassetzky et al., 1999) and humans (Broccoli et al., 1997; Chong et al., 1995). A significant role of these proteins in the stability of telomere structures (Conrad et al., 1990), telomere length regulation (Lustig et al., 1990; van Steensel and de Lange, 1997) and telomere maintenance (van Steensel et al., 1998) has been well demonstrated.

Three principal DNA-binding telomeric proteins, which bind to repeat sequence GGTTAG, have been found in human and other organisms. Two structurally related proteins, called telomeric repeat binding factors 1 (TRF1) (Chong et al., 1995) and telomeric repeat binding factors 2 (TRF2) (Broccoli et al., 1997), bind to double-stranded telomeric DNA, whereas POT1 (protection of telomeres 1) binds to telomeric single-stranded 3’ extensions. Recombinant S. pombe SpPot1 protein binds to single-stranded telomeric DNA (G strand) in vitro and the deletion of SpPot1 causes immediate loss of telomeric DNA, indicating that SpPot1 plays a critical role in protecting chromosome ends. The SpPot1 may also play a role in recruiting telomerase to the chromosome ends, like Cdc13 in budding yeast (Baumann and Cech, 2001; Baumann et al., 2002; Lei et al., 2002; Lei et al., 2003; Lei et al., 2004). Tazlp was the first TRF1/TRF2 ortholog to be discovered in S. pombe (Cooper et al., 1997). A second TRF1/TRF2 ortholog was recently identified and characterized as telomeric repeat binding factor 1 (Tbf1) (Pitt et al., 2007). The association of TRF1 and TIN2 (a TRF1 binding partner) with SA1 ortholog of the cohesin Scc3 subunit has been shown recently in human system (Canudas et al., 2007). Association of telomeric cohesin with Tazlp or Tbf1 in S. pombe has not been explored till date.
Taz1 protein contains a Myb motif at its C terminus and a TRFH (TRF homology) domain of approximately 200 amino acids in the central region. It binds directly to telomeric DNA, both in mitotically growing cells and in meiotic cells. The deletion of *taz1* was found to cause severe defects in telomere function (Cooper *et al.*, 1997; Nimmo *et al.*, 1998). *taz1* mutant contains extensively elongated telomeric DNA (up to ~5 kb) and is defective in telomere silencing like that in *swi6* mutant. Furthermore, *taz1* mutant shows defect in telomere clustering in the premeiotic horsetail stage, which causes abnormal spore formation. Taz1, therefore, plays important roles in at least three major telomere functions: regulation of telomeric DNA length, telomere silencing and telomere clustering in meiosis. Recently, Taz1 has been implicated in efficient replication fork progression through the telomere. Loss of Taz1 leads to stalled replication forks at telomeres and internally placed telomere sequences, regardless of whether the telomeric G-rich strand is replicated by leading or lagging-strand synthesis (Miller *et al.*, 2006). Another Myb motif-containing telomeric protein in *S. pombe* is Rap1 (SpRap1). It also has a BRCT domain (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). SpRap1 interacts with Taz1 in a two-hybrid system and is localized at the telomeres by its binding to Taz1 in mitotically growing cells. Therefore, SpRap1 does not bind directly to telomeric DNA and is basically bound to telomeres by interaction with Taz1. The deletion of *sprap1* causes telomere deficient phenotypes as severe as those observed in the *taz1* mutant (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). Collectively, three telomere functions, namely telomere length control, telomere silencing and telomere clustering at the horsetail stage are severely impaired in *sprap1* cells, in which Taz1 remains associated with the telomeres. Therefore, telomere-associated Taz1 by itself cannot fulfill the basic telomere functions and the primary role of Taz1 is to recruit telomere regulators to the telomeres.

Similar to yKu70/yKu80 in budding yeast, the fission yeast homologues of pKu70 and pKu80 are involved in telomere maintenance (Baumann and Cech, 2000; Manolis *et al.*, 2001; Miyoshi *et al.*, 2003; Nakamura *et al.*, 2002) as active mediators of nonhomologous end-joining (NHEJ) repair pathway. The *pku70* or *pku80* strains have slightly shorter telomeric repeat DNA than the wild type, and deletion of *pku70* or *pku80* causes rearrangement of telomere-associated sequences (TASs) that are located proximal to the telomeric repeat sequences (Kibe *et al.*, 2003), pKu70/80 inhibits the degradation and recombination of chromosome ends. Taz1 has been shown to protect pKu-dependent telomere fusion under nitrogen starvation conditions (Ferreira and Cooper, 2001). In contrast to yKu, the SpKu is not involved in telomere silencing, (Manolis *et al.*, 2001; Miyoshi *et al.*, 2003).
In human cells, homologous recombination (HR) provides an accurate mechanism for the repair of DNA double-strand breaks caused by replication fork breakdown at telomeres; cells defective in the recombinational repair proteins RAD51 or RAD54 exhibit telomere shortening and end-to-end chromosome fusions (Tarsounas and West, 2005). The Ku70-Ku80 heterodimer is required for telomere length regulation. Lack of pku70+ results in telomere shortening and rearrangements of telomere-associated sequences. The rearrangements of telomere-associated sequences in pku80+ mutants are Rhp51-dependent, but not Rad50-dependent. Rhp51 is bound to telomere ends when the Ku heterodimer was not present at telomere ends. Deletion of pku70+ increased the single-stranded overhang in both G2 and S phase (Kibe et al., 2003). The shortening of telomeres and increased recombination in subtelomeric sequences are also observed in pku70+Δ cells (Shore, 2001). This implies that Rhp51 binds to the G-rich overhang and promotes homologous pairing between two different telomere ends in the absence of Ku70/80 heterodimer. It was suggested that that, Ku heterodimer sequesters Rhp51 from telomere ends to inhibit homologous recombination activity but in absence of the Ku heterodimer, Rhp51 plays important role in the maintenance of telomere ends (Kibe et al., 2003).

Telomerase (trt1+) is the main protein, which maintains the telomeric ends by its reverse transcriptase activity (Haering et al., 2000). Unlike other organisms, fission yeast telomeres are heterogenous. The recent discovery of TER1 telomerase RNA in fission yeast explains the heterogeneous nature of telomeric sequences in fission yeast (Leonardi et al., 2008; Webb and Zakian, 2008). The trt1+Δ elicits telomere shortening and frequently leads to chromosomal circularization, or less frequently, to recombination (Naito et al., 1998; Nakamura et al., 1997). Likewise, the double mutant rad3, tell undergoes loss of telomeric repeats and chromosomes circularization by end-to-end joining (Nakamura et al., 1998). Telomerase-mediated telomere addition was studied by an elegant experiment in S. cerevisiae that creates a chromosome end immediately adjacent to a short telomeric DNA tract. The de novo end acts as a telomere and protected from degradation in a CDC13-dependent manner. Addition of telomeric sequences to this template has shown to be dependent on DNA polymerases α and δ (Diede and Gottschling, 1999).

1.4.3 Telomere silencing

The extreme ends of S. cerevisiae chromosomes containing TG-rich portion of the telomeres (called the telosome) is free of nucleosomes, whereas the subtelomeric repeats are bound with nucleosomes (Wright et al., 1992). Cdc13, Tel2, Rap1 recognize the TG repeats.
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However, only Rap1 is absolutely required for the budding yeast telomeric-position effect (TPE). Rap1 has been suggested to bind specifically to the TG-repeats and TG-bound Rap1 recruits SIR4, which in turn recruits SIR2 and stabilizes the Rap1-SIR3 interaction. Finally, the full SIR complex would be able to spread along the TG-rich sequences because of affinity of SIR3 and SIR4 for the hypoacetylated tails of H3 and H4. This spreading may require the active deacetylation of histone tails adjacent to the SIR complex by SIR2. The spreading appears to be counteracted by a histone acetylase called Sas2 (Kimura et al., 2002; Suka et al., 2002). The action of SIR2 deacetylase activity on adjacent nucleosomes may in fact be necessary for the cooperative, self-propagating nature of the linear and nucleosome-mediated spreading of SIR complexes along the chromosome. Another catalyst for SIR4 recruitment to telomeres is the yKu70/80 heterodimer, which is associated with termini, both through its end-binding function and its affinity for SIR4 (Gravel et al., 1998; Martin et al., 1999). Strains deficient in ku70 or ku80 lose telomeric repression. In addition, the Ku70/80 plays an important role is anchoring telomeres to the nuclear periphery (Hediger et al., 2002; Laroche et al., 1998), a localization that helps to create subnuclear compartments enriched for Rap1 and SIR factors (Maillet et al., 1996).

The telomeres of fission yeast are also subject to transcriptional silencing, which results in the variegated expression of integrated reporter genes (Nimmo et al., 1994; Nimmo et al., 1998). While TPE is only slightly affected by mutations affecting centromeric trans-acting factors, i. e., Clr1-4, Clr6, Swi6 and Rik1 (Grewal, 2000), another group of factors, rat1, lot2, lot3/taz1 and rap1, specifically affect repression in telomeric sites (Kanoh and Ishikawa, 2001; Nimmo et al., 1998). It has been shown that, mutations in taz1, rap1, swi6, chp2 and clr4 abrogate telomere silencing in fission yeast (Cooper et al., 1997; Nimmo et al., 1998; Thon and Verhein-Hansen, 2000). Deletion of taz1 also elicits telomere fusions, under meiosis or stress conditions, through a Ku-dependent pathway (Ferreira and Cooper, 2001). In lot2 and taz1 mutants, telomeres are no longer associated with the spindle pole body at the premeiotic horsetail stage, although it is not known whether these mutations significantly impair telomere clustering in interphase of vegetatively growing cells (Cooper et al., 1998; Nimmo et al., 1998).

In S. pombe, the chromatin changes associated with the TPE machinery are more closely related to those observed in Drosophila and mammals. Firstly, it requires the methylation of H3K9 residues by the Clr4 histone methyltransferase (Nakayama et al., 2001), which allows the binding of Swi6 (the ortholog of HP1). Secondly, specialized repeats (dg and dh) present at subtelomeres and other heterochromatin loci contribute to telomeric silencing through the...
formation of the RITS complex. This RNAi-induced transcriptional silencing machinery uses siRNAs and specific factors (i.e. Argonaute) to initiate the targeting of H3K9 methylation and heterochromatin assembly at repetitive DNA and stabilize the compaction of higher-order chromatin structure to silence specific chromosomal regions (Volpe et al., 2002). Recently, a newly identified complex termed as SHREC (Snf2/Hdac-containing Repressor Complex) composed of Clr1, Clr2, the Clr3 histone deacetylase and the SNF2 chromatin-remodeling factor homolog Mit1 has been identified (Sugiyama et al., 2007) and found to be associated with telomeres in fission yeast. SHREC interacts with Ccq1, a telomere binding protein capping and protecting telomeres from degradation (Flory et al., 2004) that, along with Taz1 and Swi6/HP1, acts in parallel to the RNAi pathway to restrict polymerase II and stabilize heterochromatin structures (Figure 9). The proper functioning of *S. pombe* telomeres requires the SET domain-containing Clr4 histone methyltransferase (Nakayama et al., 2001), which is also involved in the methylation of H3K9 at pericentric heterochromatin, both the telomere-repeat-binding protein Taz1 and the siRNA machinery have been shown to be important for the establishment of telomeric heterochromatin in fission yeast (Kanoh et al., 2005).

Regarding telomere length regulation in fission yeast, various mutations that alleviate telomeric silencing and heterochromatin formation have no or limited effects on telomere length, including the inactivation of genes encoding Swi6, Clr4 and other components of the RNAi machinery (Ekwall et al., 1996; Hall et al., 2003; Ueno et al., 2004). In contrast, results from *D. melanogaster* show that the heterochromatin HP1 protein and components of the RNAi machinery negatively regulate the transposition-based mechanism of telomere elongation (Perrini et al., 2004; Savitsky et al., 2006) suggesting that the role of heterochromatic activities in telomere length regulation is not necessarily conserved during evolution. Finally, in fission yeast the deletion of *set1*, which encodes a histone modifier that is involved in gene activation and of the deletion of SUMO E3 ligase gene *plil* alleviate telomere silencing and result in telomerase-mediated telomere elongation (Kanoh et al., 2003; Xhemalce et al., 2007; Xhemalce et al., 2004). These results raise the question of whether absence of histone H3 lysine4 (H3K4) methylation and sumoylation cause telomere elongation by directly modifying telomere structure or by modulating the expression of telomere-length regulators. Altogether, these findings suggest the existence of complex links between telomere components, chromatin factors and telomere length regulators, might exerts both direct and indirect effects.
Fig. 9. The regulation of telomeric position effect in Fission yeast. In *S. pombe*, the telomeric protein Taz1 and methylation of histone H3-K9 residues by the Clr4 histone methyltransferase recruit Swi6 to the telomeric-associated sequences and spread silencing toward the centromere to cover the subtelomeric region over 45-75 kb in cooperation with the RNAi-RITS machinery. The SHREC complex containing the Clr3 histone deacetylase and the Mit1 chromatin remodeling factor associates with Ccq1 and Swi6 and cooperates with the Taz1 and RITS pathway to facilitate chromatin condensation and telomeric silencing.
1.4.4 Role of Replication proteins in telomere maintenance

Telomere replication is achieved through the combined action of the conventional DNA replication machinery and the reverse transcriptase, telomerase. Conventional semi-conservative replication initiates at origins internal to the telomeric repeats. In yeast, forks near telomeres move towards the chromosome ends and there is no evidence that replication can initiate at the very end of a chromosome (Stevenson and Gottschling, 1999; Wellinger and Zakian, 1989). As a result, most telomeric repeats, at least those in double-stranded form, are replicated by semi-conservative mode of replication, keeping the C-strand as lagging strand and the G-strand as leading strand. Recently, in fission yeast Taz1 protein has been reported to be indispensible for semi-conservative replication at telomeres (Miller et al., 2006). Telomerase adds long G tails at 3’end of telomere. The interesting question is whether telomerase acts before or after completion of semi-conservative replication of telomeric DNA. In yeast, telomerase-dependent G-strand elongation occurs in late S phase, precisely at the same time as semi-conservative replication replicates chromosomal end-regions (Diede and Gottschling, 1999; Marcand et al., 2000; Wellinger et al., 1993b). Recent results suggest that telomerase acts as a dimer in yeast and human cells (Beattie et al., 2001; Prescott and Blackburn, 1997). This might be interpreted to mean that dimeric telomerase acts in collaboration at both the leading and the lagging strand, thus implying that it cannot act before semi-conservative replication. However, direct evidence that a telomerase dimer is associated with both daughter-ends of the same chromosome in vivo is lacking. Dissecting the timing of telomerase dependent replication with respect to semi-conservative replication requires further investigation. Concomitantly with these events, both leading and lagging strand ends of yeast chromosomes acquire G-tails longer than 25 bases (Dionne and Wellinger, 1998; Wellinger et al., 1996; Wellinger et al., 1993a). The question then arises as to how these longer G-tails are generated and subsequently processed to the short constitutive ones during the replicating phase. Are the mechanisms underlying these dynamic changes the same for both leading and lagging strand chromosome ends?

Since end-replication problem is closely associated with DNA replication, there must be a close connection between DNA replication machinery and telomere homeostasis (Dahlen et al., 2003). In budding yeast Pol12, the B subunit of the DNA polymerase α (Poll)-primase complex, has been shown to be involved in telomere length control and capping (Grossi et al., 2004). Recessive mutations in poll in fission yeast were shown to cause telomere elongation. This effect was shown to result from inability of mutant pola to sequester and inactivate the telomerase (Dahlen et al., 2003). Further, during synthesis of new telomeres the G-strand was extended by
telomerase and the corresponding C-strand was synthesized by DNA Polα and Pol δ; inhibition of the polymerases by aphidicolin leads to elongation of G-strand, thereby showing that G and C-strand synthesis is tightly coordinated (Fan and Price, 1997). Similarly, addition of new telomeres by telomerase in S. cerevisiae requires not only extension of the 3’ G-rich end by telomerase but also fill-in synthesis of the C strand by DNA primase, DNA polymerase-α and DNA polymerase-δ. G-strand polymerization by telomerase is inhibited in S. cerevisiae if DNA polymerase-α and DNA polymerase-δ are inactive, suggesting that telomerase needs to interact with the lagging strand synthesis machinery to be active (Diede and Gottschling, 1999). An excellent candidate for regulating the coordination between telomerase and the conventional DNA polymerases is the Cdc13 complex, which attracts telomerase to the chromosome ends in S. cerevisiae. Cdc13 binds to single-stranded G-rich telomeric DNA and then recruits telomerase (Bianchi et al., 2004; Pennock et al., 2001). Several studies have suggested that, subsequently, lagging-strand synthesis fills in the C strand, and then inhibits telomerase in a Cdc13-dependent manner (Chandra et al., 2001; Grossi et al., 2004). Little is known about coordinated C and G-strand synthesis in mammalian cells, but activation of a temperature-sensitive allele encoding DNA polymerase-α causes elongation of the G tail and of the telomere overall, suggesting that coordination of telomerase with the replication machinery is a common feature in all organisms (Nakamura et al., 2005). The G-rich and repetitive nature of telomeric DNA complicates replication as well, because it potentially allows the formation of secondary structures, such as G quartets (Rhodes and Giraldo, 1995). Consequently, telomeric proteins support the progressing replication fork, allowing efficient telomere synthesis. In human cells, the RecQ-like helicase WRN contributes to efficient telomere replication. Overexpression of a helicase-defective WRN allele causes the occasional loss of telomeres generated by the lagging-strand machinery, and telomerase expression compensates for this loss of telomeric sequence, implicating defective telomere maintenance in the pathology of Werner’s syndrome (Crabbe et al., 2004). Accordingly, targeted deletion of Wrn in mice leads to phenotypes that resemble the human Werner’s syndrome only when telomerase is also deleted (Chang et al., 2004). In summary, it is becoming increasingly clear that telomere replication and telomerase-dependent telomere elongation are highly coordinated processes and that telomeric proteins have essential roles in the regulation of these processes.

1.5 DNA Replication

Error-proof and precise replication of DNA in S-phase is prerequisite for faithful transmission of genome into progeny. DNA replication is a highly error-proof and coordinated
process performed by special molecular machines known as DNA polymerases. Apart from replication DNA polymerases are required DNA repair, DNA recombination, and bypassing of DNA damage. DNA polymerases Polα, Polβ, γ, δ and ε, are the key enzymes required to maintain the integrity of the genome under all these circumstances.

1.5.1 DNA Polymerases and genetic studies of yeast DNA polymerases

1.5.1.1 DNA Polymerase \( \alpha \)

In all eukaryotic organisms, Polα is the initiator polymerase. It is a heterotetrameric enzyme. The largest subunit of this multi-subunit enzyme of about 165-180 kD is called the catalytic subunit. In addition, Polα contains, one 70-kD subunit, also known as the B subunit or p70 subunit, with no detectable enzymatic activity (Collins et al., 1993; Foiani et al., 1994) and two subunits of 49 kD and 58 kD that contain the DNA primase activity (Copeland and Wang, 1993a; Wang, 1991). Mammalian DNA Polα/primase \((\text{pol} \alpha)\) consist of four subunits (A, B, C, D) with calculated molecular weights of 166 kDa, 66 kDa, 59 kDa and 50 kDa, respectively (Copeland and Wang, 1993b). Three separate domains have been reported in the catalytic p180 subunit (Wang, 1991).

\( (a) \) An N-terminal domain (amino acids 1–329), which appears dispensable for both the catalytic activity and the assembly of the tetrameric complex.

\( (b) \) A central domain (amino acids 330–1279), which contains all the conserved regions responsible for DNA binding, dNTP binding, and phosphoryl transfer.

\( (c) \) A C-terminal domain (amino acids 1235–1465), which is dispensable for catalysis but necessary for the interaction with the other subunits.

The catalytic subunit of human Polα is glycosylated (Hsi et al., 1990) and shows cell-cycle-dependent phosphorylation (Nasheuer et al., 1991). Due to posttranslational modifications, the apparent molecular mass of catalytic subunit of DNA polymerase \( \alpha \) is 180 kD (Copeland and Wang, 1991; Hsi et al., 1990; Nasheuer et al., 1991). The fission yeast Polα catalytic subunit is also phosphorylated in a cell-cycle-dependent manner (Park et al., 1995).

On the basis of sequence homology and structural similarities, polymerases have been grouped into five different families: A, B, C, X, and Y (Braithwaite and Ito, 1993; Ohmori et al., 2001). The eukaryotic replicative polymerases (Polα, Polδ, and Polε) belong to the B family of DNA polymerases and the mitochondrial poly belong to the family A.
On the basis of the Polα sequence, six highly conserved regions have been identified among eukaryotic, prokaryotic, and viral polymerases, named as regions I-VI. Their relative position along the primary sequence is also conserved; region IV is at the N terminus, followed by regions II, VI, III, I, and V (Brautigam and Steitz, 1998; Joyce and Steitz, 1994). The highly conserved region I is located in the palm, close to the thumb domain and contains one of the conserved aspartic acid residues that form the catalytic dyad of all known polymerases in family B (-YGDTDS- motif). The other invariant aspartic acid belongs to region II and is located at the tip of a β-sheet that is part of the palm subdomain (-DxxSLYS-, II region). Included in this region is the highly conserved SLYPS-II region, which is important for deoxynucleoside triphosphate (dNTP) binding. Although these motifs are absolutely conserved in Polα and δ subfamilies, Polε has considerably diverged from the consensus, so that the region I motif of Polε is -ELDTDG- and region II has become -DxxAMYPN-. Other residues important for dNTP binding are located in region III and they fold into a α-helix located in the fingers subdomain. Region IV is located at the N terminus and extends into a domain that is part of the 3'-5' exonuclease active site. The other two conserved regions, V and VI, are located in the thumb and fingers subdomains, respectively (Hubscher et al., 2002).

Genetic studies of budding and fission yeast DNA polymerases have revealed some novel and interesting functions of cellular DNA polymerases. S. pombe pola has a role in generation of DSB at matl locus. This finding also explains how developmental fate of a cell can be linked to DNA replication. Disruption of the swi7/pola gene is lethal both in switching and non-switching strains, as expected for an essential replication gene (Singh and Klar, 1993). Genetic studies in fission yeast (Kelly et al., 1993; Saka and Yanagida, 1993) have suggested that proteins or enzymes involved in the formation of an initiation or replication complex have a role in mitotic entry checkpoint operation. Germinating spores derived from S. pombe with a disrupted DNA pola gene have a heterogeneous phenotype. This is different from germinating spores derived from a disrupted polδ gene, which has a cell cycle division (cdc) phenotype (elongated phenotype) (Francescon et al., 1993). Further analysis has shown that germinating spores derived from spores lacking the DNA pola gene showed abnormal nuclear morphology with 1N DNA content (Bhaumik and Wang, 1998). A multicopy suppressor of a temperature-sensitive DNA pola mutant named cds1+ (check DNA synthesis) encoding a protein with a typical protein kinase motif has been reported. Genetic data suggest that the primary role of cds1+ is to monitor DNA synthesis by interacting with DNA pola and sending a signal to restrain the onset of mitosis during the progression of S phase (Murakami and Okayama, 1995). The observations of
germinating spores with pola gene disruption or deletion, together with the genetic results of cds1⁺, suggest that pola may be involved in cell-cycle checkpoint control.

1.5.1.2 Other DNA polymerases

DNA polymerase δ is the other major replicative DNA polymerase. Five subunits have been identified in S. pombe Polδ (p125, p55, p54, p40 and p22), four in mammalian enzyme (p125, p66, p50, p12) and three in S. cerevisiae (p125, p58 and p55) (Hubscher et al., 2002). The mammalian subunits p66, p50 and p12 are homologous, respectively, to the p55, p40 and p22 subunits of the S. pombe enzyme. Polδ is present within eukaryotic cells as high molecular weight complexes and the small subunits may be critical for their maintenance. The largest catalytic subunit in S. cerevisiae is encoded by CDC2 gene. A temperature-sensitive mutation in this gene can arrest cells in the S-phase at non-permissive temperature (Boulet et al., 1989). DNA Polδ is a high processivity polymerase that is required for DNA synthesis in both the lagging and leading strands. It is dependent on Proliferating Cell Nuclear Antigen (PCNA) for its processivity. PCNA, a 36 kDa protein forms a circular clamp to which Polδ is loaded, increasing the processivity of DNA Polδ (Fairman et al., 1988; Tan et al., 1986). Along with PCNA, two other proteins are required for DNA Polδ function: Replication Protein-A (RP-A) for binding ssDNA and Replication Factor-C (RF-C) for binding the primer terminus (Kenny et al., 1989). This polymerase possesses a 3'-5' exonuclease activity (Lee et al., 1980). DNA polymerase ε, also known as DNA polymerase II, is a four subunit enzyme (p256, p80, p23, p22) that is essential in S. cerevisiae. Yeast Polε has a 3'-5' exonuclease activity and high processivity (Morrison et al., 1990). The polymerase activity of this protein is independent of PCNA. However, it requires PCNA and RF-C for its activity under very stringent conditions. In contrast to yeast Polε, human Polε, which is comprised of p261, p59, p17, p12 subunits, shows a limited processivity in vitro. Several studies have indicated that Polε is required for the basic DNA synthesizing function. The N-terminal part, which includes the conserved core domains with all the catalytically important residues, is dispensable for viability in both S. pombe and S. cerevisiae, whereas the C-terminal region is essential for checkpoint function (Feng and D'Urso, 2001; Kesti et al., 1999).

1.5.2 Coordinated Leading and Lagging Strand Synthesis mechanism

Pola/primase associates with the initiation complex at the DNA origin (Bell and Dutta, 2002) and starts to synthesize a short RNA/DNA hybrid of approximately 10 RNA nucleotides followed by 20 to 30 DNA nucleotides. This oligonucleotide is then utilized by Polδ or ε for processive elongation on both the leading and the lagging strand (Waga et al., 1994). Replication
on the lagging strand is characterized by small DNA pieces called Okazaki fragments, with a length of about 200 bases. In mammalian cells an initiation event has to happen $4 \times 10^4$ times on the leading strand (approximately the number of origins of DNA replication in a mammalian cell), but it has to be repeated at the beginning of each Okazaki fragment (about $2 \times 10^7$ times in mammalian cells).

The substitution of Polα/primase by the more processive Polδ holoenzyme is called polymerase switch and is dependent upon the synthesis of the RNA/DNA primer by Polα. The polymerase switch is coordinated and regulated by an ATP switch catalyzed by the auxiliary protein RF-C (Maga et al., 2000) and involves a complex network of interactions among Polα/primase, Polδ, RF-C, and the protein that binds single-stranded DNA, replication protein A (RP-A) (reviewed in 5). Both Polα/primase and Polδ are perfectly suited for their respective roles: Polα/primase can initiate synthesis de novo, whereas Polδ, through its interaction with proliferating cell nuclear antigen (PCNA, a processivity factor), has the ability to synthesize long stretches of DNA. The proposed dimerization of Polδ (Burgers and Gerik, 1998; Zuo et al., 2000) might play a role in the coordination of leading and lagging strand synthesis (like in the pol III holoenzyme in *E. coli*) and in establishing an asymmetric replication fork, possibly through association of Polα/primase to one of the two halves of the dimeric Polδ. Genetic analysis in budding yeast has shown that Polε is required for DNA replication (D'Urso and Nurse, 1997). In addition, UV cross-linking studies with replicating chromatin in mammalian cells detected Polε along with Polα and Polδ (Zlotkin et al., 1996). Experiments in human cells (Pospiech et al., 1999) and in *Xenopus* egg extracts (Waga et al., 2001) further suggested that Polε is involved in DNA replication. The N-terminal part of yeast Polε, including the conserved core domains with all the catalytically important residues, is dispensable for viability, whereas the C-terminal part, which is involved in protein-protein interactions and checkpoint control, is essential in *S. cerevisiae* (Kesti et al., 1999). *S. pombe* mutants with N-terminal deletions in Polε are viable, as in *S. cerevisiae*, but show accumulation of DNA damage and need expression of the checkpoint genes *rad3, hus1*, and *chk1* (Feng and D'Urso, 2001). These data do not necessarily mean that the catalytic activity of Polε is not involved in DNA replication, but rather suggest than it can be substituted for the basic synthetic function and not for the specialized checkpoint function. During evolution Polε could have acquired specialized function as a “sensor” polymerase for quality control of DNA replication, whereas the function of Polδ remained as a DNA synthesizing machine exclusively.
**1.5.3 Replication-Silencing Coupling**

In *S. cerevisiae*, the mating-type region comprises an expressed MAT locus flanked by silent donor loci HMLa and HMRa. Transcriptional silencing at HML and HMR requires cis-acting regulatory sites called silencers (*E* and *I*), which flank these silent regions. Silencers contain binding sites for the Origin Recognition Complex (ORC), ARS binding factor 1 (Abf1) and a Repressor Activating Protein (RAP1) (Loo and Rine, 1995). In addition, four proteins called Silent Information Regulators-SIR1, SIR2, SIR3 and SIR4 constitute the structural components of heterochromatin (Cheng and Gartenberg, 2000; Hecht *et al.*, 1995; Loo and Rine, 1995). SIR proteins form a complex, and the SIR3 and SIR4 proteins in this complex can bind to deacetylated histone tails *in vitro*. The SIR2 subunit is a NAD⁺-dependent deacetylase and its deacetylase activity is required for heterochromatin assembly. The principal role of SIR1 is the establishment rather than maintenance of silencing (Loo and Rine, 1995). The ORC component, besides initiating DNA replication by binding to replication origins in the DNA, serves to recruit SIR1 to the silencer sequence which, in turn, recruits SIR2, SIR3 and SIR4 and thereby, aids in silencing (Gardner *et al.*, 1999; Triolo and Stengelanz, 1996). Hence, this initiator of DNA replication has a silencing role as well, though different regions carry out separate functions as some ORC mutants are defective in replication initiation, not silencing (Dillin and Rine, 1997; Fox *et al.*, 1997). Besides this, coupling of the processes of DNA replication and silencing is supported by a number of findings. The establishment of silencing at HMR requires passage of yeast cells through S-phase (Miller and Nasmyth, 1984). In *S. cerevisiae*, two of the four silencers are chromosomal origins of replication (Rivier *et al.*, 1999). Mutations in ORC suppress position-effect variegation (a hallmark of silenced regions wherein genes inserted near to silenced regions exhibit variegated expression patterns) in *Drosophila* (Pak *et al.*, 1997). Mutations that disrupt silencing at rDNA and telomeres have been identified in DNA replication proteins and DNA-replication-related proteins such as DNA helicase Dna2p, PCNA loading factor Rfc1p (Singer *et al.*, 1998; Smith *et al.*, 1999). Also, mutations potentially suppressing silencing defects are found in DNA replication proteins including Cdc45, PCNA and RF-C (Ehrenhofer-Murray *et al.*, 1999).

**1.6 A Role of DNA polymerase α in Silencing**

An analysis of several temperature-sensitive (ts) mutants of DNA polymerase α revealed a ts mutant called *swi7H4* (Murakami and Okayama, 1995) that exhibits silencing defects at *mat2* and *mat3* apart from its centromere and telomere loci silencing defects (Ahmed *et al.*, 2001; Ahmed and Singh, 2001) pointed to a possibly direct role of DNA polymerase α in silencing and
hence, suggested a replication-silencing coupling. However, the silencing defects of such a mutant are independent of its replication and checkpoint defects. Also, it was observed that at the mat locus, the silencing defect of such a mutant is accentuated by the deletion of the cis-acting silencer flanking the mat2 locus. Such silencer-dependent phenotype is similar to that exhibited by swi6 mutants (Thon et al., 1994) suggesting that Pola may act in the same silencing pathway as Swi6.

1.7 Trans-acting factors:

Silencing at the mating-type loci involves several trans-acting factors including Clr1-Clr4 (Ekwall and Ruusala, 1994; Grewal et al., 1998; Thon and Klar, 1992), Swi6 (Lorentz et al., 1992), Rik1, Rhp6 (Choi et al., 2002; Nielsen et al., 2002; Singh et al., 1998). Mutations in some of these factors also affect silencing and chromatin structure at the mating type, centromeres and to some extent at the telomeres (Allshire et al., 1995).

1.7.1 Clr1

clr1 is cryptic loci regulator 1. It encodes a zinc finger C2H2 type protein and is located on the right arm of chromosome II. Mutations in clr1 result in expression of the ura4 reporter gene inserted at the mat2 and mat3 loci and aberrant haploid meiosis phenotype. The clr1 mutations also permit meiotic recombination in the 15-kb mat2-mat3 interval (Thon and Klar, 1992). Recently, Clr1 was also reported to be part of a multiprotein effector complex (SHREC) that mediates Transcriptional gene silencing at major heterochromatin loci in fission yeast. SHREC is also recruited to the telomeres by multiple independent mechanisms involving telomere binding protein Ccq1 cooperating with Taz1 and the RNAi machinery (Sugiyama et al., 2007). It has also been shown that Clr1 may act in concert with Clr3, a trans-acting factor with histone deacetylase (HDAC) activity (Hansen et al., 2005).

1.7.2 Clr2

clr2 gene encodes a 62 kD protein with no obvious sequence homologs. Deletion of clr2 affects transcriptional repression at the mating type region, centromere and silencing of a Pol II-transcribed gene inserted in the rDNA repeats, indicating that Clr2 is a general mediator of transcriptional silencing in S. pombe. Clr2 is necessary for histone deacetylation at the mating-type region, suggesting that Clr2 acts upstream of histone deacetylases to promote transcriptional silencing (Bjerling et al., 2004). Mutation in clr2 results in asymmetric expression of mat genes: the mat3-M expression is about two-fold higher than mat2-P expression in clr2 mutant (Ekwall
and Ruusala, 1994). *clr2* is also required for maintenance of recombinational block over *mat2/3* interval along with *clr3, clr4, rik1* and *swi6* (Thon *et al.*, 1994). Localization of Clr2 was also reported at telomere as a component of SHREC complex, which was reported to be present at major heterochromatic loci in fission yeast (Sugiyama *et al.*, 2007).

### 1.7.3 Clr3

*clr3* is responsible for the deacetylation of lysine residues in the N-terminal tail of the core histones (H2A, H2B, H3 and H4). It plays an important role in transcriptional regulation, cell cycle progression and developmental events. Clr3 protein, when mutated, alleviates recombination block at *mat* region (Thon *et al.*, 1994) as well as silencing at donor loci and at centromeres and telomeres (Grewal *et al.*, 1998). Hypoacetylated histones are a hallmark of heterochromatin in organisms ranging from yeast to humans (Jenuwein and Allis, 2001). Clr3 belong to the class II family of HDACs. It is similar to the *S. cerevisiae* Hdal enzyme, binds directly to the mating-type region and preferentially deacetylates Lys14 of histone H3 (Bjerling *et al.*, 2002). Clr3 acts cooperatively with Sir2 throughout the genome, including rDNA, centromeres, *mat2/3* and telomeres. The most significant acetylation sites are H3K14Ac for Clr3 and H3K9Ac for Sir2 at their genomic targets. Clr3 also affects subtelomeric regions, which contain clustered stress and meiosis-induced genes (Wiren *et al.*, 2005). Localization of Clr3 containing SHREC complex at telomeres further supports the role of Clr3 in telomeric chromatin maintenance (Sugiyama *et al.*, 2007). Clr3, acts in a distinct pathway parallel to RNAi-directed heterochromatin nucleation to recruit Clr4 and mediate H3K9 methylation at the silent mating-type region and centromeres. At the *mat* locus, Clr3 is recruited at a specific site through a mechanism involving ATF/CREB family proteins. Once recruited, Clr3 spreads across the 20 kb silenced domain that requires its own HDAC activity and heterochromatin proteins including Swi6/HP1. It has also been demonstrated that Clr3 contributes to heterochromatin maintenance by stabilizing H3K9 trimethylation and by preventing histone modifications associated with active transcription, and that it limits RNA polymerase II accessibility to naturally silenced repeats at heterochromatin domains (Yamada *et al.*, 2005)

### 1.7.4 Clr4

*clr4* gene encodes the *S. pombe* homolog of the SU(VAR)3-9 protein family, which includes human Suv39H1, murine Suv39h1 and *Drosophila* SU(VAR)3-9 (Cavalli and Paro, 1998; Ivanova *et al.*, 1998; Jenuwein *et al.*, 1998; Koonin *et al.*, 1995). Members of this protein family have been identified as histone H3 methyltransferases that specifically methylate histone
H3 at lysine 9 position (Rea et al., 2000), thereby creating a binding site for heterochromatin-associated proteins like HP1 (Jenuwein, 2001; Lachner et al., 2001). Clr4p contains an amino-terminal chromodomain and a carboxyl-terminal SET domain [(Suvar)3-9, Enhancer of Zeste, Trithorax domain]. It has been shown that both the chromo and SET domains are required for H3-K9 methylation and Swi6 binding in vivo. However, in vitro studies show that only the SET domain is required for Clr4 HMTase activity (Nakayama et al., 2001). Taken together, these results indicate that the chromodomain is presumably required for targeting Clr4 to the mat2/3 region and centromeres, whereas the SET domain constitutes the catalytic site. The structure reveals an overall fold rich in beta-strands, a potential active site consisting of a SAM-binding pocket (Min et al., 2002). Chromo domain is required for proper silencing and directional switching of the mating type. Surprisingly, RNA differential display experiments demonstrated that clr4+ can mediate transcriptional activation of certain other loci (Ivanova et al., 1998). Mutation in clr4 results in asymmetric expression of mating type (Ekwall and Ruusala, 1994). In fission yeast, Clr4 methyltransferase was reported to exist as a complex termed as CLRC (Clr4-Rik1-Cul4) complex. It has also been proposed that Clr4-Rik1-Cul4 complex act concertedly at an early step in heterochromatin formation (Hong et al., 2005). Recently, Clr4 was reported to regulate the subnuclear localization of the mating-type region in fission yeast (Alfredsson-Timmins et al., 2007).

1.7.5 Rik1

Rik1, a protein related to DNA damage binding protein DDB1 and required for H3K9me, also interacts with Cul4, the association of which might serve to target Clr4 to heterochromatic loci (Jia et al., 2005). The Rik1 protein functions in close association with the Clr4 histone methyltransferase at an early step in heterochromatin formation. It has been found that subunits of a cullin-dependent E3 ubiquitin ligase are associated with Rik1 and Clr4 and Rik1-TAP preparations exhibit robust E3 ubiquitin ligase activity. Two novel proteins, Rafl and Raf2, which copurify with Rik1, are required for H3-K9 methylation and for transcriptional silencing within centromeric heterochromatin. Furthermore, expression of a dominant-negative allele of the Pcu4 cullin subunit disrupts regulation of K4 methylation within heterochromatin. These studies provide evidence for a novel Rik1-associated E3 ubiquitin ligase that is required for heterochromatin formation (Horn et al., 2005). Fluorescence in situ hybridization (FISH) shows that centromeres lag on late anaphase spindles in clr4 and rik1 mutant cells like in swi6 mutants. The Swi6 protein was found to be delocalized from all three silent chromosomal regions and dispersed within the nucleus in both clr4 and rik1 mutant cells. This finding implies that both the
Clr4 and Rik1 are required to recruit Swi6p to the centromere and other silent regions. Mutations in \textit{clr4}, \textit{rik1} and \textit{swi6} also result in elevated sensitivity to reagents, which destabilize microtubules and show a synergistic interaction with a mutation in the \(\beta\)-tubulin gene (\textit{nda3}). Further, the \textit{clr4-S5} and \textit{rik1-304} mutations cause the derepression of reporters inserted at \textit{cen1} and show highly elevated rates of chromosome loss. We propose that the products of these genes are integral in the assembly of a heterochromatin-like structure, with distinct domains, enclosing the entire centromeric region that reduces or excludes access to transcriptional machinery. These observations suggest that Clr4 and Rik1 must play a role in the assembly of Swi6p into a transcriptionally silent, inaccessible chromatin structure at fission yeast centromeres, which is required to facilitate interactions with spindle microtubules and to ensure normal chromosome segregation (Allshire \textit{et al.}, 1995; Ekwall \textit{et al.}, 1996). It has been demonstrated that Rik1 and Clr4, but not Swi6, are required along with the telomere protein Taz1 for crucial chromosome movements during meiosis. However, Rik1 is dispensable for the protective roles of telomeres in preventing chromosome end-fusion. Thus, a Swi6-independent heterochromatin function distinct from that at centromeres and the mating-type locus operates at telomeres during sexual differentiation (Tuzon \textit{et al.}, 2004).

1.7.6 Swi6

Swi6 is a fission yeast ortholog of highly conserved \textit{Heterochromatin Protein 1} (HP1), a class of chromobox genes that encode the structural adapters for assembly of macromolecular complexes involved in genome organization and expression. HP1 was first described in Drosophila as a heterochromatin-associated protein with dosage-dependent effects on heterochromatin-induced gene silencing (Eissenberg and Elgin, 2000). The switching gene \textit{swi6} ('swi' for switching) in \textit{S. pombe} is a non-essential gene that is required for the recombination-suppression and silencing at the mating-type region. In addition, this factor is also required for silencing at the centromeres, telomeres and rDNA loci (Ekwall \textit{et al.}, 1995; Thon and Verhein-Hansen, 2000). In agreement with this, FISH analysis has shown that Swi6 is localized at centromeres, telomeres and mating-type loci in \textit{S. pombe} (Ekwall \textit{et al.}, 1995). The \textit{swi6} gene encodes a 37 kDa protein that possesses an amino-terminal highly conserved chromodomain (chromatin organization modifier domain; abbreviated as CD) and a carboxy-terminal chromo shadow domain (CSD) linked by a variable intervening hinge region. It has been shown that CD from a mammalian HP1-like protein M31, can functionally replace that of Swi6, showing that CD function is conserved from yeast to humans (Wang \textit{et al.}, 2000). Swi6 has been shown to be present at the \textit{mat} loci at a relatively constant level throughout the cell cycle, wherein it may serve
as a molecular bookmark to propagate the silenced chromatin state during cell division (Nakayama et al., 2000). An increased dosage of Swi6 was shown to shift the metastable derepressed epigenetic state, generated by the deletion of the K-region to the repressed state suggesting an imprinting function of Swi6 in establishing the repressed chromatin state (Nakayama et al., 2000). The localization of Swi6 has been shown to be disrupted in cells bearing clr4-55, rikl-304 or swi6-115 mutations, indicating that the recruitment of Swi6 to the silenced regions is Clr4- and Rik1-dependent (Ekwall et al., 1996). Nakayama et al., (2003) have shown that the localization of Swi6 to the silent loci is mediated through its binding to histone H3 methylated at Lys9 (H3-K9 methylation), a modification that requires Clr4, another chromodomain protein possessing intrinsic histone methyltransferase activity. This is similar to the selective recognition of Lys9-methylated histone H3 by HP1 chromodomain (Bannister et al., 2001). The CSD of HP1 family is a protein-protein interaction domain that has been implicated in mediating interaction with other proteins like chromatin assembly factor, CAF1 subunit p150 (Murzina et al., 1999), Suv39 (Yamamoto and Sonoda, 2003) and also for self-association (self-association is a property of HP1 class that may explain the spreading effect of heterochromatin (Nielsen et al., 2001). Swi6 has been reported to interact with the cohesin subunit Psc3 and play a role in recruitment of cohesin to heterochromatic regions, promoting the proper segregation of chromosomes (Nonaka et al., 2002). Recently, it has been shown that the fission yeast Epel, a JmjC domain-containing protein, which is a negative regulator of heterochromatin, is recruited to heterochromatic loci by the heterochromatin protein Swi6/HP1. Moreover, Epel acts in a heterochromatin-specific context to promote Pol II accessibility by counteracting repressive chromatin (Zofall and Grewal, 2006b). Thus Swi6/HP1 is emerging as a platform to recruit both silencing and antisilencing activities in a balanced way which is crucial for heterochromatin maintenance (Verdel, 2006; Zofall and Grewal, 2006a).

1.7.7 Rhp6

The RAD6 gene of *Saccharomyces cerevisiae* encodes an ubiquitin conjugating enzyme and is required for DNA repair, DNA-damage-induced mutagenesis and sporulation. The *rhp6* gene in *S. pombe* share a high degree of structural and functional homology with RAD6. Null mutations of the *rhp6* gene confer a defect in DNA repair, UV mutagenesis and sporulation, and the RAD6 and *rhp6* genes can functionally substitute for one another (Reynolds et al., 1990). The *rhp6* gene product is required for postreplication DNA repair and ubiquitination of histones H2A and H2B. In fission yeast its role has been implicated in mating-type silencing by chromatin remodeling (Singh et al., 1998). Rhp6 was reported to exist as a component of the multisubunit
protein complex (termed HULC) that also contains two RING finger proteins Rfp1 and Rfp2, sharing homology with budding yeast Bre1 protein and a unique serine-rich protein Shfl. HULC is required for ubiquitination of histone H2B at lysine 119 (H2B-K119) and it localizes to heterochromatic sequences and Rhp6-induced changes in heterochromatic silencing are mediated predominantly through H2B ubiquitination (ubH2B). Interestingly, heterochromatic derepression caused by Rhp6 occurs independently of the involvement of HULC subunits and ubH2B in methylation of histone H3 at lysine 4 (H3K4me) (Zofall and Grewal, 2007). Both mating-type silencing and chromatin remodeling by Rhp6 involves an ubiquitinated histone-like protein Uhp1, which serves as an in vivo target or mediator of Rhp6 in silencing (Naresh et al., 2003).