The telomere length homeostasis is tightly coupled with replication process. Mutations in proteins involved in replication often result in increase in telomeres repeat sequences.

A temperature sensitive mutation in polα, swi7H4, results in lengthening of telomeric repeat sequences. This study for the first time clearly establishes the role of DNA polα and other heterochromatin proteins in maintenance of telomere structure.

In this study it is shown that mutation in polα and other heterochromatin proteins result in aberrant recombination at sub-telomeric sequences and this recombination is dependent on homologous recombination pathway mediated by Rad50/Rhp51.
4.1 Role of DNA polymerase α in telomere organization

4.1.1 Replication machinery and heterochromatin proteins in telomere length homeostasis

Heterochromatin plays an important role in silencing of mating type, centromere, rDNA and telomere regions in *S. pombe* (Elgin and Grewal, 2003; Klar, 2007). In recent past a close association has been shown between telomeric sequences and heterochromatin components. Accessibility experiments using expression of *E. coli* dam$^+$ methylase in budding yeast showed that the telomeric sequences exist in a heterochromatin state and become accessible to dam$^+$ methylase activity in strains that contained a mutation that suppressed the telomeric position effect (Gottschling, 1992). In *Saccharomyces cerevisiae*, it has been shown that the telomeres exert a position effect on mitotic recombination (Stavenhagen and Zakian, 1998). The chromodomain protein HP1/Swi6 was reported to localize at the telomeres of interphase polytene and mitotic chromosomes in Drosophila and mutation in HP1/Swi6 causes aberrant chromosome associations and multiple telomeric fusions (Fanti et al., 1998). A novel deacetylase enzyme (Sir2p) is required for the establishment and maintenance of telomeric heterochromatin in budding yeast (Shore, 2001). In *Drosophila melanogaster* the telomere-associated sequences (TAS) constitute distinct and non-overlapping domains in telomeres. SUUR (Suppressor of Underreplication), HP2, SU(VAR)3-7 and H3Me3K27 localize to the cap region of telomeres (Andreyeva et al., 2005). A histone H3 lysine 79 methyltransferase, also known as Dot1 (Disruptor of telomeric silencing-1), is required for the establishment of telomeric heterochromatin boundaries (Altat et al., 2007). RNA interference pathway was demonstrated to play an active role in telomere homeostasis in mouse. Dicer1 deficiency in *Mus musculus* leads to decreased DNA methylation, concomitant with increased telomere recombination and telomere elongation (Benetti et al., 2008). The Sir2 deacetylase family member, SIRT6 in human, is an NAD$^+$-dependent, histone H3 lysine 9 (H3K9) deacetylase which functions as specific modulator of telomeric chromatin. SIRT6 depletion results in telomere dysfunction, end-to-end chromosomal fusions and premature cellular senescence (Michishita et al., 2008).

The end-replication problem of telomeres and involvement of replication machinery in telomere homeostasis suggest a close relation between telomere synthesis and DNA replication machinery (Dahlen et al., 2003). It has been demonstrated that mutations in budding yeast *POL1* (Polα) result in telomere elongation (Adams and Holm, 1996; Carson and Hartwell, 1985). The role of *pol12*, the B subunit of the DNA polymerase α (Pol1)/primase complex, in telomere length...
control and capping in budding yeast has been well studied (Grossi et al., 2004). Due to inability to sequester and inactivate telomerase, recessive mutations in poll in fission yeast were shown to cause telomere elongation (Dahlen et al., 2003). The inhibition of DNA Pola and Polδ by aphidicolin leads to elongation of G-strand, suggests that G and C strand synthesis is tightly coordinated (Fan and Price, 1997). Similarly, addition of new telomeres by telomerase in S. cerevisiae requires both DNA polymerase-α and DNA polymerase-δ (Dies and Gottschling, 1999). The telomere elongation phenotype has been observed in DNA polymerase-α mutant in human cells, suggesting that coordination of telomerase with the replication machinery is a common feature in all organisms (Nakamura et al., 2005). In S. pombe, the TRF1- and TRF2-homologue, Taz1, is required for telomere replication. In absence of Taz1, replication forks are stalled at telomeric sequences even if they were present at the interior of chromosomes (Miller et al., 2006). This suggests that the Taz1-dependent telomere-replication phenotype is due to characteristics of the telomeric sequence itself and not to its position on the chromosome. 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.

Keeping the above information in mind, we have investigated the role of DNA replication and heterochromatin proteins in regulating the integrity of telomeres in S. pombe. We observe elevated level of subtelomeric duplication in pola/swi7H4 and some of the heterochromatin mutants; this event seems to be dependent on the Rhp51 and Rad50. In addition, we find that the silencing mutants pola/swi7H4, clr1-clr4 and swi6 also display some common recombinant subtelomeric fragments, indicating a role of heterochromatin structure in preventing subtelomeric recombination and maintaining telomere integrity and function.

4.1.2 A novel telomeric pattern in DNA polα/swi7H4 mutant

In fission yeast mutation in DNA polymerase α have been shown to cause telomere elongation and elongated telomeres were restored to normal length on expression of normal Polα (Dahlen et al., 2003). Our study was based on a temperature sensitive polα mutant, swi7H4, which grows normally at 25°C and 30°C but not at 36°C (Murakami and Okayama, 1995). The swi7H4 mutation used in this study (G889D) is located within homology box VI, which is in the conserved nucleotide binding domain found in all DNA polymerases (Ahmed et al., 2001). To test the effect of swi7H4 on telomere length, the wild type and swi7H4 mutant strains were grown at 30°C. Genomic DNA from wild type and polα mutant was prepared according to Moreno et al.
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(1991). Genomic DNA was digested with EcoR1 and resolved by 1.2% agarose gel electrophoresis followed by Southern blotting. The Southern blots were hybridized with radioactively labeled probes prepared from pAMP001 plasmid containing a 1.1 kb EcoRI telomeric fragment (Gifted by A. Matsura). Digestion of pAMP001 with EcoR1+Apal yields a 0.8 kb fragment known as TAS1 and Apal-distal EcoRI fragment of 0.3 kb, which represents only telomeric repeat region (Figure 36A). Identical Southern blots were probed with 1.1 kb, 0.8 kb and 0.3kb radioactively labeled probes, as shown in Figure 36B. The results show extensive telomere elongation in polα/swi7H4 mutant strain (Figure 36B, lanes 2, 4 and 6). Furthermore, a surprising finding was the presence of a distinct 0.9 kb band in swi7H4 mutant (arrowhead) (Figure 36B, lanes 2 and 4). Interestingly the 0.9 kb band did not hybridize with the 0.3 kb repeat region probe (Figure 36B, lane 6) and seems to be absent in wt strain (Figure 36B, lanes 1, 3 and 5). This observation pointed towards two possibilities: i) either the 0.9 kb band represents a short telomere, which might have originated due to erosion of telomeric repeats, or ii) it could be an outcome of rearrangements between sub-telomeric sequences and arise from internal EcoR1 sites, which are 0.9 kb apart.

4.1.3 The 0.9 kb fragment is overrepresented in polα/swi7H4 mutant strain

To confirm whether the 0.9 kb band was actually absent in wild type strain or it is undetectable in EcoRI blots (Figure 36B, lanes 1, 3 and 5), genomic DNA was digested with EcoR1-Apal followed by agarose gel electrophoresis and Southern blotting. Southern blots were probed with 1.1, 0.8 and 0.3 kb probes as indicated (Figure 37). Results show that the 0.9 kb band is present at barely detectable level in wild type strain (Figure 37, lanes 1 and 3; arrowhead) and the amount of 0.9 kb band was almost 3-4 fold higher in swi7H4 mutant (Figure 37, lanes 2 and 4). Both wild type and swi7H4 strain taken in this experiment were of same genetic background. Thus, along with telomeric elongation, some additional events in of swi7H4/pola mutant result in either telomeric repeat erosion or overrepresentation of some of the sub-telomeric sequences.

4.1.4 The presence of 0.9 kb band is independent of genetic background

To check the effect of genetic background on generation of the 0.9 kb EcoRI fragment, genomic DNA was prepared from wt, h+ swi7/H4 and PΔ17 swi7H4 strains, digested with EcoRI and Southern blots were probed with 1.1 kb telomeric fragment. Identical pattern was observed in different genetic backgrounds h+ (Figure 38, lane 2) or PΔ17 (Figure 38, lane 3), ruling out a role of genetic background for the observed fragment pattern.
Fig. 36. Southern blot showing a novel telomeric pattern in polα/swi7H4 mutant. (A) The restriction map of the fission yeast telomere, showing Apal-distal telomeric repeat region, 0.8 kb EcoRI-Apal fragment (TAS1) and the 1.1 kb EcoR1 fragments derived from the plasmid pAMPO01. (B) Genomic DNA from wild type (lanes 1, 3 and 5) and swi7H4 mutant strain (lanes 2, 4 and 6) was digested with EcoRI and resolved by 1.2% agarose gel electrophoresis followed by Southern blotting. Hybridization was performed with radioactively labeled 1.1kb EcoRI fragment (lanes 1 and 2), 0.8kb EcoRI-Apal fragment (lanes 3 and 4) and 0.3 kb Apal-distal fragment (lanes 5 and 6). In comparison to wild type the swi7H4 strain shows novel telomeric pattern containing both elongated (lanes 2 and 4, smear around 1.2-1.8 kb region) as well as short telomere (lanes 2, 4, the 0.9 kb band marked by arrowhead). The shorter fragment does not hybridize to repeat region (0.3 kb) probe (lane 6). m, refers to DNA size markers.
Fig. 37. The novel 0.9kb band is barely detectable in wild type strain. Genomic DNA from wild type (lanes 1, 3 and 5) and swi7H4 mutant (7H4) strain (lanes 2, 4 and 6) was digested with EcoRI-Apal and Southern blots were probed with radioactively labeled 1.1 kb EcoRI fragment (lanes 1 and 2), 0.8 kb EcoRI-Apal fragment (lanes 3 and 4) and 0.3 kb Apal-diatal fragment (lanes 5 and 6). The swi7H4 strain shows extensive elongation of telomere when probed with repeat region probe (lane 6), while the 0.9 kb band, which was not visible in EcoRI blots (Fig. 1), was actually present at barely detectable amount in wild type strains (lanes 1 and 3, arrowhead) and it does not hybridize with (0.3 kb) repeat region probe (lane 5, arrowhead). m, refers to DNA size markers.
4.1.5 Role of normal meiosis in generation of novel telomeric pattern

During meiotic prophase in *S. pombe*, homologous chromosomes get clustered near the spindle pole body (SPB) and telomeres are located proximal to the nucleolus (Funabiki *et al.*, 1993). It has been shown that telomeric repeats are sufficient to induce telomeric clustering and the meiotic telomere-SPB complex consists of Taz1 protein (Hiraoka, 1998). The pairing of telomeres along the repeat region is likely to generate some rearrangement in telomeric sequences during meiosis. To check the possibility that the novel telomeric pattern might be a result of normal meiosis, wild type strain was subjected to random spore analysis. Genomic DNA from six colonies growing from spores was digested with *EcoRI* followed by Southern blotting and hybridization with the 1.1 kb *EcoRI* fragment. In all the six segregants an identical telomeric pattern was observed (Figure 39, lanes 1 to 6) ruling out the possibility of generation of the novel telomeric pattern during meiosis.

4.1.6 *tazlΔ* mutant also exhibits novel telomeric pattern

It has been shown that, like *swi7H4* mutation, *tazlΔ* mutation also abrogates telomere silencing (Cooper *et al.*, 1997). We checked the telomere pattern of *tazlΔ* mutant. Interestingly, *tazlΔ* mutant also showed the 0.9 band in addition to a smear around 2-10 kb (Figure 40, lane 3). Interestingly, the smear in the 2-10 kb range, represents an extensive subtelomeric rearrangement in *tazlΔ* mutant (Ferreira and Cooper, 2001). The telomere elongation was more severe in case of *swi7H4/tazlΔ* double mutant (Figure 40, lane 4).

4.1.7 Heterochromatin mutants also exhibit the novel telomeric pattern

Since *swi7H4* and *tazl* mutations have similar effect on telomere silencing and the heterochromatin structure at telomeres, it is possible that the novel telomeric pattern could be an outcome of perturbation of heterochromatin structure Therefore, the effect of mutations in heterochromatin proteins in generation of novel telomeric pattern was checked. Southern blots of *EcoRI* digests of mutant strains probed with 1.1 kb telomeric probe showed that, like *swi7H4*, *clr2* and *swi6* mutants contain a distinct 0.9kb band (Figure 41, lanes 2, 4 and 7) while *clr1*, *clr3* and *clr4* mutants contain a much fainter 0.9 kb band (Figure 41, lanes 3, 5 and 6). This is in addition to the smear around 1.1-1.2 kb representing the repeats plus subtelomeric region. These results suggest a common molecular event among *swi7H4*, *clr1-clr4*, *swi6* and *tazl* mutants (Arcangioli and Klar, 1991; Blasco, 2007; Kanoh *et al.*, 2005; Thon *et al.*, 1994; Thon and Klar, 1992) and
Fig. 38. The novel telomeric pattern of swi7H4 strain is independent of strain background. Genomic DNA from wild type (lane 1) and swi7H4 mutant strain in (h+ and PA17 genetic background) (lanes 2 and 3) was digested with EcoRI and Southern blot was probed with radioactively labeled 1.1 kb EcoRI fragment. The results shows 0.9kb band is present in 7H4 mutant in both backgrounds.

Fig. 39. The novel telomeric pattern of swi7H4 is not a product of meiosis. To check whether the novel subtelomere is produced during meiosis, several wild meiotic spores were cultured and checked for the appearance of novel telomeric pattern. Genomic DNA from six independent colonies growing out of spores was digested with EcoRI and Southern blot was probed with radioactively labeled 1.1 kb EcoRI fragment (lanes 1 to 6). m, refers to DNA size markers.
Fig 40. Like *swi7H4* the *taz1Δ* mutant also exhibit novel telomeric pattern. Genomic DNA from wild type, *swi7H4*, *taz1Δ* and *swi7H4/taz1Δ* double mutant was digested with *Eco*RI and Southern blot was probed with radioactively labeled 1.1 kb *Eco*RI fragment. Like *swi7H4* mutant, *taz1Δ* mutant also showed both the 0.9 band in addition to a smear around 2-10 kb. The smear in the 2-10 kb range, represents an extensive subtelomeric rearrangement in *taz1Δ* mutant (Ferreira and Cooper, 2001) In case of *swi7H4/taz1Δ* double mutant the telomeric rearrangement was even more severe. m, refers to DNA size markers.
Fig. 41. The role of silencing factors in generation of novel telomeric pattern. Genomic DNA from wt (lane 1), swi7H4 (lane 2), clr1 (lane 3), clr2 (lane 4), clr3 (lane 5), clr4 (lane 6) and swi6 (lane 7) mutant strains was digested with EcoRI and Southern blot was probed with radioactively labeled 1.1 kb EcoRI fragment. m, refers to DNA size markers.
indicate that all silencing mutants involved in abrogation of telomere silencing may elicit the unique telomere pattern.

4.1.8 A dominant effect of \textit{swi7H4} mutation on novel telomere pattern

To check the effect of \textit{swi7H4} along with heterochromatin mutants, double mutants were generated by standard genetic cross. Southern blots of \textit{EcoRI} digests of DNA from single and double mutants were hybridized to 1.1 kb \textit{EcoRI} probe. Results show that the \textit{swi7H4/pola} mutation exerts a dominant effect on \textit{clr1-4} and \textit{swi6} silencing mutants. The appearance of elongated telomere as well as 0.9 kb band becomes more pronounced in double mutants (Figure 42, lanes 4, 6, 8, 10 and 12).

4.1.9 Enhanced rate of mating type rearrangements in \textit{swi7H4} mutant

Since \textit{pola} mutation was reported to be associated with Swi6 delocalization and abrogation of heterochromatin structure (Ahmed \textit{et al.}, 2001), and the elevated level of rearrangements in mating type organization was previously reported in cohesin and \textit{swi6} mutants (Nonaka \textit{et al.}, 2002), we checked the organization of mating type in a homothallic \textit{h90/swi7H4} strain. Most of the colonies of the strain give dark staining with iodine. However, the mutant also produced light staining colonies at a high rate (9.3%; 21 out of 225). The results of Southern blot hybridization showed that light colonies carried a rearrangement in the mating type similar to the \textit{h}^+ rearrangement reported by (Beach and Klar, 1984) (Figure 43, L1, L2).

4.1.10 The Trt1 protein level remain unaltered in \textit{swi7H4/Pola} mutant

The physical interaction of \textit{Pola} and Trt1 has been shown earlier and Trt1 has been shown to be responsible for the aberrant telomere extension in the \textit{pola/primase} mutants. In mutant form \textit{Pola} and Trt1 interaction was compromised leaving Trt1 to cause telomere repeat expansion in \textit{pola} mutant (Dahlen \textit{et al.}, 2003). To check the level of Trt1 in \textit{swi7H4} mutant, \textit{trt1} gene was tagged with 3HA epitope in wild type and \textit{swi7H4} strains. Protein extracts from different strains were western blotted with anti-HA, anti-pola and anti-tubulin antibodies. Results show that the level of Trt1 remains unaltered in \textit{swi7H4} strain (Figure 44) implying that the \textit{swi7H4} mutation does not affect the stability of Trt1.

4.1.11 The 0.9 kb band does not originate from erosion of telomeric repeats

It has been shown that under nitrogen starvation conditions \textit{taz1A} mutant undegoes progressive repeat erosion followed by chromosome circularization (Ferreira and Cooper, 2001).
Fig. 42. The dominant effect of *swi7H4* mutation. The genomic DNA from wild type, *swi7H4*, *clr1-clr4* and *swi6* single and double mutants of *swi7H4* with *swi6* and *clr1-4* was digested with *EcoRI* and Southern blots was probed with radioactively labeled 1.1 kb *EcoRI* fragment. The results show the dominant effect of *polα* *swi7H4* mutation with respect to silencing mutants. The appearance of elongated telomere as well as 0.9 kb band becomes more pronounced in double mutants (lanes 4, 6, 8, 10 and 12).
Fig. 43. **Enhanced rate of mating type rearrangements in swi7H4 mutant.** The genomic DNA from \( h^{90} wt, h^{90} swi7H4 \) dark (D) and light strains (L1 and L2) was digested with \( HindIII \) and Southern blots was probed with radioactively labeled 10.4 kb \( mat1M \) fragment.

Fig. 44. **The level of Trt1 remains unaltered in swi7H4 mutant strain.** The \( trt1 \) gene was tagged with HA epitope and cell extracts from wild type HA \( trt1 \) and \( swiH4/HAtrt1 \) along with untagged control were resolved by SDS polyacrylamide gel electrophoresis followed by western blotting with anti HA antibody, anti pol\( \alpha \) antibody and antitubulin antibody (loading control).
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To clarify whether the 0.9kb EcoRI fragment originates from the telomeric end due to telomere repeat erosion or it results from an internal EcoRI site, swi7H4 strain was grown in media lacking nitrogen for 8 days. Genomic DNA was prepared from wild type and swi7H4 strain digested with EcoRI followed by Southern blotting and probing with 1.1 kb EcoRI fragment. Results show that the EcoRI digestion pattern of the swi7H4 mutant remains unaltered even after 8 days of growth under nitrogen starvation condition (Figure 45, right panel). These results argue against the possibility that the 0.9kb band may have originated from telomeric repeat erosion.

To further confirm this result, the NsilI digested samples were probed with 0.8 kb TAS1 (Figure 46, left panel) and 0.3 kb repeat region probe (Figure 46, right panel). Both blots show identical pattern, which again rules out the possibility of repeat erosion from some of the telomeric ends. In case of repeat erosion the signal with repeat region probe was significantly reduced like in case of taz1Δ mutant, but we did not observe such change in our samples, which indicates that all the telomeric ends are intact in swi7H4 and heterochromatin mutants. Further, The NsilI digested samples show some extra bands around 4-5 kb position (Figure 46, lanes 2 to 7) which are absent in wild type strain (Figure 46, lane 1). These extra bands might have originated by rearrangements in subtelomeric region in swi7H4 and in heterochromatin mutants.

4.1.12 Characterization of 0.9 kb subtelomeric band

Since the 0.9 kb band was present at barely detectable amount in wild type samples and it seems to be 3-4 fold enriched in some of the heterochromatin mutants, we planned to determine the origin of 0.9 kb band. Sequences of all telomeric cosmid clones available at Sanger center website (http://www.sanger.ac.uk/Projects/S_pombe/telomeres.shtml) were analyzed (Figure 47A and 47B). Results were quite surprising. The presence of a 0.9kb (903bp) EcoRI fragment can be predicted from telomeric cosmids pNSU21, 64 and 65. This suggests that 0.9 kb band possibly originates from subtelomeric region. All the available telomeric cosmids were derived from AZ6 strain (h², leu, 1-32, gifted by D. Beach) by Neil Sugawara (Sugawara, 1989) and the collection of cosmid clones show fairly good representation of 0.9 kb fragment (3 out of 6). However, in our experiments we tested different wt strains in different genetic (h², h⁻ and h⁺) backgrounds and found that the level of 0.9 kb band was always 3-4 fold lower than that in heterochromatin mutants.

These observations raise some pertinent questions about the origin of the 0.9kb band, If the observed 0.9kb band has actually originated from the telomeres represented by pNSU21, 64
Fig. 45. Stress conditions do not elicit telomere repeat erosion in *swi7H4* mutant. DNA was isolated from wild type and *swi7H4* mutant strains after growth in medium containing nitrogen (left panel) or lacking nitrogen (right panel) for 8 days and digested with *EcoRI* followed by agarose gel electrophoresis, Southern blotting and hybridization with 1.1 kb radiolabelled *EcoRI*.

Fig. 46. Rearrangements of telomeric regions in *swi7H4* and silencing mutants. DNA was isolated from wild type, *swi7H4*, *clr1*-4 and *swi6* mutant strains and digested with *NsiI* followed by agarose gel electrophoresis, Southern blotting and hybridization with 0.8 kb TAS1 (left panel) and 0.3 kb repeat region (right panel) probe.
**Fig. 47. The restriction map of telomeric cosmids.** (A) The restriction patterns of the telomere cosmid clones pNSU21, pNSU64 and pNSU65. (B) The restriction patterns of the clones pNSU70, pNSU71 and pNSU77. The sizes of the various restriction fragments generated by *Eco*RI and *Apa*I are indicated. Regions of identity of the sequences with TAS1 probe are indicated by filled bars. The red colored arrows pointing right represent the regions of homology with the Apaout oligo. The fragments of 1534bp, 1979bp and 5173bp, corresponding to the sizes of the bands observed are boxed and also indicated along with other restriction fragments by double arrows. Empty boxes depict subtelomeric regions of partial TAS1 homology.
and 65 cosmids, then why its amount is so less in our wt strains? Why some of the telomeres represented by pNSU21, 64 and 65 cosmids are overrepresented in heterochromatin mutants? These questions prompted us to characterize the 0.9 kb band. The origin of 0.9 kb band was being unclear, keeping in mind that this band could have originated from rearrangements between different telomeric sequences we applied an unbiased approach to sequence the 0.9kb by telomere tailing PCR (Figure 48) (Dreesen and Cross, 2006). The genomic DNA isolated from different strains was digested with EcoRI, resolved by 1.2% agarose gel electrophoresis and the DNA around 0.9kb region was eluted from multiple lanes for each strain. The eluted DNA was denatured and tailed in the presence of terminal deoxynucleotidyl transferase (TdT) and dCTP. The polydC-tailed products were amplified by PCR using a reverse polydG oligo and a forward Apaout oligo. The forward Apaout was selected such that it can hybridize to multiple locations in subtelomeric region (Figure 47A and 47B, red colored right pointing arrows) so that it can amplify all the possible products originated from subtelomeric region. The PCR products thus obtained were cloned and sequenced. The number of clones sequenced for each strain is indicated (Figure 48).

4.1.13 Subtelomeric rearrangements in heterochromatin mutants

Interestingly, the sequencing results revealed a common sequence in all mutants ending with the EcoRI site followed by dC tail (Figure 49B), except in wild type and clr3 where we could not get any PCR product (probably because of very low level of the 0.9kb EcoRI band). The sequence obtained aligned with two subtelomeric regions in the cosmid pNSU64 (dashed arrows, Figure 49A, top panel), namely, fragment-1: Apal (4813) to EcoRI (5131) and Fragment-2: GGCCCCC (5714) to EcoRI (6034) in the clone pNSU64 (Figure 49A, dashed arrow, top panel), which shares significant restriction pattern similarity with the cosmids pNSU65 and pNSU21 (Figure 47A, named pNSU64 family). In addition, it also showed incomplete alignment (with gaps) with the region starting from (5786) in the clone pNSU70 (Figure 49A, dashed arrow, top panel), which show significant similarity with the cosmids pNSU71 and pNSU77 (Figure 47B, named pNSU70 family). Alignments of the sequence with two subtelomeric locations in “pNSU64 family” suggest a duplication of subtelomeric region. Such a hypothetical duplication is lacking in the “pNSU70 family’ of cosmids.
Fig. 48. Mapping of 0.9 kb in swi7H4 and heterochromatin mutants. The 0.9 kb region was eluted from multiple lanes of EcoRI digested samples of Swi7H4, clr1-4 and swi6 followed by telomere tailing, PCR amplification using polydG and ApaI out oligos, cloning and sequencing (Dreesen and Cross, 2006). Results revealed a common sequence in all mutants ending with the EcoRI site followed by dC tail except in wt and clr3 where we could not get any PCR product (probably because of very low level of the 0.9kb EcoRI band).
Fig. 49. The 0.9 kb band originates from the pNSU64 series of cosmids. (A) The sequence obtained aligned with two subtelomeric regions in the cosmid pNSU64 (dashed arrows), namely, Fragment-1: *ApaI* (4813) to *EcoRI* (5131) and Fragment-2: GGCCCCC (5714) to *EcoRI* (6034) in the clone pNSU64, In addition, it also showed incomplete alignment (with gaps) with the region starting from (5786) in the clone pNSU70 (dashed arrow) (B) The ClustalX alignment of the sequences related with Fragment-1 and 2 of pNSU64. The position of alignment and oligos used for PCR are indicated.
4.1.14 Variable representation of telomeric cosmids clones in Heterochromatin mutants

To check the correspondence of the subtelomeric pattern observed in the mutants to the cosmids we carried out Apal digestion followed by Southern blot hybridization with the 0.8kb EcoRI probe, as depicted in Figure 36A. In addition to the 5kb band, which may originate from the cosmid pNSU77 (Figure 50B, the 5173bp band in pNSU77), bands of ~2kb and ~1.5 kb were also observed (Figure 50C), which may correspond to the 1979bp band of “pNSU64 family” (Figure 50A) and the 1534bp band of “pNSU70 family” of cosmids (Figure 50B), respectively. A closer examination indicates that the 0.9kb band can only originate internally from EcoRI digestion of the Apal band of 1979bp of pNSU64 family of cosmids (Figure 50A), which shows homology to the 0.8kb probe (additional sites of TAS1 homology are indicated by empty boxes, (Figure 50A and 50B). The much lower intensity of the 1979bp band in Apal digests of wt, clr1 and clr3 mutants (Figure 50C) now appears to be consistent with the barely detectable levels of the 0.9kb band on EcoRI digestion in wt, clr1 and clr3 mutants (Figure 41).

The representation of the cosmids clones in heterochromatin mutant strains is highly variable. The “pNSU64 family” subtelomeres having the 1534bp Apal fragment are overrepresented in swi7H4, clr2, clr4, swi6 and tazl mutants, while subtelomeres of wt, clr1 and clr3 are mainly represented by “pNSU70 family” having the 1979bp Apal fragment. Interestingly, the changes in the copy number are in nearly integral ratios (Figure 50C, right panel), arguing for endoduplication event/s among different telomeres in the heterochromatin mutants. Since telomeres have been shown to cluster together in S. pombe, which promotes homolog interactions and inhibits ectopic recombination in wild type strains (Chikashige et al., 2006; Davis and Smith, 2006; Tang et al., 2006), possibilities of inter and intra-chromosomal recombination are likely in the mutants. Notably, clr1, clr3, clr4 and swi6 mutants also share a common band of 1.3kb (Figure 50C), which may originate from recombination event at a common site in the subtelomeric region. Thus, Clr1, Clr3, Clr4 and Swi6 may act together in organizing a structure that prevents such recombination event. Indeed, a complex comprising Clr1-Clr2-Clr3, called SHREC complex, is localized at telomere and subtelomere regions to cause silencing (Sugiyama et al., 2007). Similarly, the presence of bands of 1.2 and 0.9 kb in swi7H4 and clr2 mutants, respectively (Figure 50C) may suggest a role of the encoded proteins in protecting distinct regions of subtelomeres from recombination.
Fig. 50. The underrepresentation of pNSU 64 series of cosmids in wild type strain and subtelomeric rearrangements in hetrochromatin mutants. (A) The restriction pattern of the telomere cosmid clones pNSU21, pNSU64 and pNSU65. (B) The restriction pattern of the clones pNSU70, pNSU71 and pNSU77. The sizes of the various restriction fragments generated by EcoRI and ApaI are indicated. Regions of identity of the sequences with TAS1 probe are indicated by filled bars. The fragments of 1534bp, 1979bp and 5173bp, corresponding to the sizes of the bands observed are boxed and also indicated along with other restriction fragments by double arrows. Empty boxes depict subtelomeric regions of partial TAS1 homology (C) DNA from wild type, swi7H4, clr1-clr4, swi6 and taz1 mutants was digested with ApaI, Southern blotted and hybridized with the 0.8 kb EcoRI-ApaI (TAS1) probe. Right panel shows a schematic representation of the approximate ratios of the intensities of the 2 and 1.5 kb bands depicted as boxed bar in Figure 3A and B and labeled as bands a and b.
4.1.15 Role of Ku70/80 and Rhp6 repair pathways in subtelomeric recombination

Since recombination events appear to occur in swi7H4 and heterochromatin mutants. To further investigate the role of repair pathways involved in subtelomeric rearrangements/recombination in swi7H4 and heterochromatin mutants, we decided to check the role of different recombination pathways in these events. In Schizosaccharomyces pombe, the Ku protein involved in the repair of DNA double-strand breaks and protects chromosome termini from nucleolytic and recombinational activities (Baumann and Cech, 2000). In absence of Ku activity, Rhp51 plays important roles in the maintenance of telomere ends (Kibe et al., 2003). Human subtelomeres are polymorphic patchworks of interchromosomal segmental duplications at the ends of chromosomes and non-homologous end-joining pathway is responsible for segmental duplication (Linardopoulou et al., 2005). The Rhp6 a structural and functional homolog of the RAD6 in budding yeast (Reynolds et al., 1990) plays its role in postreplication DNA damage repair. To check the involvement of Ku80, non-homologous end joining (NHEJ) and rhp6, postreplicative repair pathway in subtelomeric rearrangement in S. pombe, genomic DNA from ku80Δ, rhp6Δ single mutant and swi7H4/ku80Δ, swi7H4/rhp6Δ double mutants was digested with EcoRI and Southern blots were probed with radiolabeled 1.1 kb EcoRI telomeric fragment. Results show that the 0.9 kb band remains intact in swi7H4/ku80Δ (Figure 51, lanes 7 and 8) and swi7H4/rhp6Δ double mutant (Figure 51, lanes 4 and 5) indicates that NHEJ and post-replicative repair pathway play no role in the generation of the 0.9kb band.

4.1.16 Role of the Rad50/rhp51 recombination pathway in generating the 0.9kb fragment

To check whether one or more recombination pathways were involved in this process we carried out Southern blot hybridization of EcoRI and Apal digests of DNA from mutants affecting the NHEJ pathway (ku80Δ), the Rad50 homologous recombination pathway and the post replication repair pathway of Rad6/rhp6. Results showed that no distinct band of 0.9kb band could be observed in the EcoRI blots (Figure 52, left panel) in any mutant except in rhp6Δ (Figure 52, lane 5, left panel), This was quite expected because Rhp6 is reported to be involved in gene silencing in fission yeast (Naresh et al., 2003; Singh et al., 1998) and deletion of Rad6, the budding yeast homolog of rhp6 (Reynolds et al., 1990) has been reported to cause telomere elongation (Gatbonton et al., 2006). The Apal blot revealed a clear absence of the ~2kb band, the progenitor of the 0.9kb band, in both rad50Δ and rhp51Δ mutants strains (Figure 52, lanes 2 and 3 right panel) but not in the ku80Δ and rhp6Δ mutants (Figure 52, lanes 4 and 5, respectively, right
Fig. 51. Role of non-homologous recombination and postreplicative repair pathway in subtelomeric recombination. DNA was isolated from wild type, swi7H4, rhp6Δ, ku80Δ, swi7H4/rhp6Δ an swi7H4/ ku80Δ mutant strains, digested with EcoRI followed by agarose gel electrophoresis, Southern blotted and hybridized with radiolabelled 1.1 kb EcoRI fragment.
DNA was isolated from wild type, rad50Δ, rhp5Δ, rhp6Δ, and rad50Δ, and rhp5Δ strains, digested with EcoRI (left panel) and Apal (right panel) resolved by agarose gel electrophoresis. Southern blotted and hybridized with the 1.1 kb EcoRI and 0.8 kb EcoRI-Apal probe, respectively.
These results suggest that the 0.9kb band may indeed be generated by the Rad50/Rhp51 recombination pathway. Notably, rhp51Δ strain show an additional Apal band of 3kb (Figure 52, lane 2, right panel), which may represent an additional recombination event, whose cause is not known. In addition, all the mutants also showed smaller bands around the 1.1-1.2 kb size range, which may originate from some other recombination events.

### 4.1.17 A recombination-dependent endoduplication model

In one possible scenario, we speculate that such a recombination event may be initiated by DNA damage near or upstream of the Apal site (upstream of the repeat region), rendered accessible by the loss of a complex like SHREC. The Rad50/Rhp51 pathway may recognize partial homology in the region upstream of the Apal site within the telomeres corresponding to the pNSU70/71/77 family of cosmids and carry out replication/recombination of the downstream region, thus generating an endo-duplication event (Figure 53). The presence of an additional recombination product in the form of a 3kb Apal fragment (Figure 52A, lane 2, right panel), which is lacking in case of rad50Δ strain, suggests some additional recombination event in rhp51Δ mutant. A similar explanation could explain the generation of heterogeneity in the subtelomeric region observed among different heterochromatin mutants. On the basis of different observations a hypothetical model was proposed, which speculates that such a recombination event could explain the generation of the subtelomeres belonging to the pNSU21/64/65 family of cosmids from those belonging to the pNSU70/71/77 family of cosmids. Occurrence of such events may be facilitated in the subtelomere-telomere repeat junction region without affecting the repeat region in heterochromatin mutants because of enhanced chromatin accessibility. Alternatively or additionally, other Rad50/Rhp51-dependent recombination events may be occurring at elevated rate at upstream regions of homology (Kibe et al., 2003), and the TAS1 homology regions represented by empty boxes in (Figure 47 and 50), which may generate the additional bands observed in the Apal blots. The occurrence of the bands of identical size in clr1, clr3, clr4 and swi6 mutants may also reflect a role of the encoded wild type proteins in creating a distinct chromatin structure that prevents damage at specific sites or regions: in mutants, the components of the Rad50/Rhp51 recombination machinery may be recruited at the conserved sites to bring about distinct the recombination/duplication events.
Fig. 53. Role of Homologous recombination repair pathway in subtelomeric recombination. A speculative model depicting a hypothesized role of Rad50 and Rhp51 in generating subtelomeric duplication. Creation of distinctive hot spots of recombination near and upstream of the Apal site in the telomeres represented by the pNSU70 family of cosmids in the wild type and heterochromatin mutant strains may lead to an Rhp51/Rad50 mediated intrachromosomal duplicative recombination to generate the restriction pattern observed in the pNSU64 family of cosmids.
4.2 Discussion

Studies in different organisms have unearthed the importance of a complex interplay of several factors including telomerase, DNA replication machinery, and recombination and DNA-end protection mechanisms in telomere length homeostasis. In budding yeast and fission yeast, DNA replication plays a regulatory role in maintaining the length of telomeric repeats, mutants of DNA replication enzymes exhibit elongation of repeats (Carson and Hartwell, 1985; Dahlen et al., 2003; Diede and Gottschling, 1999). In budding yeast, Polα has been shown to interact with Cdc13, that also interacts with Est1 to coordinate the synthesis of G and C strand (Qi and Zakian, 2000). In *S. pombe*, Polα was shown to sequester telomerase into an inactive form; mutations in *pola* release telomerase, allowing it to extend the telomeric repeats (Dahlen et al., 2003). Various *pola* mutants showed a variable elongation of telomeres. However, the effect of these *pola* mutations was recessive and expression of *pola* gene in the mutants restored the wild type telomere pattern (Dahlen et al., 2003). In contrast, the effect of *swi7H4* mutation on telomere elongation is dominant. Earlier, it has been shown that the *swi7H4* mutation also abrogates silencing at the *mat*, *cen* and telomere loci (Ahmed et al., 2001; Ahmed and Singh, 2001). Interestingly, the effect of *swi7H4* mutation on mating type silencing was also dominant (Ahmed et al., 2001).

Heterochromatin structure perturbation can produce global effect on chromatin organization. In budding yeast, inaccessibility of Heterochromatin render the *hm* loci resistant to HO endonuclease cleavage (Klar et al., 1981), DNA repair (Terleth et al., 1989) and methylation by *E. coli dam* methylase (Gottschling, 1992; Singh et al., 1998) and the accessibility is greatly enhanced in silencing mutants (Bhattacharyya and Lustig, 2006; Klar et al., 1981; Singh and Klar, 1992; Terleth et al., 1989). In fission yeast, mutations in *rhp6* and *swi6* enhanced the accessibility of silent mating type loci to *dam* methylase (Singh et al., 1998). The silencing mutants *clr1-clr4* and *swi6* abrogate the recombination block acting over *mat2-mat3* interval, referred to as the *K* region (Ahmed et al., 2001; Klar and Bonaduce, 1991; Thon and Klar, 1992) and *swi6* mutant exhibits enhanced rate of aberrant recombination (Nonaka et al., 2002). Despite a number of such observations where it has been clearly established that loss of heterochromatin structure results in increased chromatin accessibility, occurrence of such aberrant recombination events in absence of heterochromatin structure has never been reported in context to telomeric regions. The possibility that heterochromatin protein may impose a similar recombination block on telomeres in *S. pombe* seems to be compelling assumption. The supporting evidence of this hypothesis comes from the


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*sir* mutants in budding yeast, which abrogate the position effect control at telomere (Gottschling *et al.*, 1990) and enhance the accessibility of telomeres to dam methylase (Gottschling, 1992).

Although enhanced recombination in subtelomeric region in absence of heterochromatin structure has not been reported yet, the possibility of heterochromatin structure playing a role in telomere structure maintenance has been shown in other systems. For example, telomere position effect (TPE) has been reported in budding yeast (Gottschling *et al.*, 1990) as well as human (Baur *et al.*, 2001; Klar and Bonaduce, 1991); heterochromatin plays an important role in regulating TPE and chromatin accessibility at telomeres (Gottschling, 1992; Klar and Bonaduce, 1991; Koering *et al.*, 2002). In fact, a role of recombination in telomere maintenance has been shown in human cells (Dunham *et al.*, 2000). Furthermore, an underlying role of epigenetic mechanisms in regulating mammalian telomere length and recombination is beginning to be recognized (Blasco, 2007; Garcia-Cao *et al.*, 2004; Gonzalo *et al.*, 2006). In budding yeast, deregulation of telomere size, stability and function is observed in *rap1* mutants (Kyrion *et al.*, 1993) and Rap1-Sir4 interaction plays an important role in initiating assembly of heterochromatin (Luo *et al.*, 2002). Similarly, subtelomere proteins have been shown to negatively regulate telomere elongation (Berthiau *et al.*, 2006), while, SIR3 and SIR4 are required for position and integrity of telomeres in budding yeast (Palladino *et al.*, 1993).

Apart from telomerase, telomere-telomere recombination serves as a bypass mechanism for telomere maintenance in budding yeast (Teng and Zakian, 1999). In fact, analogous to this study, RAD50 and RAD51 dependent pathways have been recognized to maintain telomeres in absence of telomerase in yeast (Le *et al.*, 1999). Furthermore, a Rad51-dependent pathway of telomere repeat amplification is observed in *ku70, cdc13*+ senescent cells (Grandin and Charbonneau, 2003), while Rad50-dependent and Rif1-independent recombination causes telomerase-independent lengthening of yeast telomeres (Teng *et al.*, 2000). In *S. pombe*, defective meiosis in telomere silencing mutants suggests a role of silencing factors in maintaining telomere integrity (37). It is relevant to note that independently of Taz1, Swi6 can also bind to telomeres and subtelomeres in *S. pombe* (Nimmo *et al.*, 1998). An important role of epigenetic factors is further highlighted by study showing a role of SUMO E3 ligase Pli1p in centromere and telomere maintenance in *S. pombe* (Xhemalce *et al.*, 2004). In Drosophila, heterochromatin protein HP1 regulates telomere capping, telomere elongation and silencing (Perrini *et al.*, 2004). Likewise, human subtelomeres exhibit elevated rates of intrachromosomal recombination and segmental duplication, although the mechanism of such events is not well understood (Linardopoulos *et al.*,...
2005). A role of homologous recombination in human telomeres is also indicated by the generation of t-loop deletions (Wang et al., 2004). Recently, mutations in suv39h1 and suv39h2 in human (Garcia-Cao et al., 2004) and set1Δ in S. pombe (Kanoh et al., 2003) have also been shown to cause repeat expansion.

In light of above information, it is the first time that a role of heterochromatin in suppressing Rad50/Rhp51 mediated subtelomeric recombination has been shown in any system. We propose that heterochromatin structure generated by epigenetic factors likeClr/Cuv39 and Swi6/HP1 may render the telomeres refractory to aberrant recombination as well as transcription and thus maintain telomere structure and integrity. Because of the conservation of Clr4/Suv39 and Swi6/HP1 (Klar, 2007; Nakayama et al., 2001), the role of heterochromatin in structural and functional integrity of telomeres may be evolutionarily conserved.