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ARTICLE *in* YEAST · AUGUST 2008

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Published online 10 July 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1603

Research Article

Role of heterochromatin in suppressing subtelomeric recombination in fission yeast

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Received: 20 March 2008
Accepted: 18 May 2008

Abstract

Telomere length is regulated by a complex interplay of several factors, including telomerase, telomere-binding proteins, DNA replication machinery and recombination. In yeast, DNA polymerase α is required for *de novo* synthesis of telomeres from broken ends of DNA, and it also suppresses the elongation of normal telomeric repeats. Heterochromatin proteins Clr1–Clr4 and Swi6 and DNA polα organize heterochromatin structure at mating type, centromere, rDNA and telomere regions that are refractory to transcription and recombination in *Schizosaccharomyces pombe*. Here, we have addressed the role of heterochromatin structure in regulating the integrity and organization of telomeric regions. Here, we show that subtelomeric duplication and rearrangements occur in *polα* and heterochromatin mutants and find that some of the putative duplication events are dependent on the Rad50 pathway. Thus, our study shows a role of heterochromatin in maintaining the integrity of the subtelomeric regions by suppressing their recombination in *Sz. pombe*.

Keywords: **Polα; heterochromatin; subtelomere; fission yeast; recombination**

Introduction

Heterochromatin plays an important role in silencing of mating type, centromere, *rDNA* and telomere regions in *Schizosaccharomyces pombe* (Elgin and Grewal, 2003; Klar, 2007). Maintenance of the structural integrity and function of telomeres is vital for cell viability and prevention of disease in higher organisms. Because of the end-replication problem of DNA *Polα*, telomeres get shortened after every generation, which is compensated by elongation with telomerase (Savitsky *et al*., 2006). Absence of the *trt1*$^{+}$ gene (encoding the catalytic subunit of telomerase) causes telomere shortening, which can be circumvented by chromosomal circularization or telomeric recombination (Bhattacharyya and Lustig, 2006; Nakamura *et al*., 1998). Similar events occur in the *rad3, tel1* double mutant (Naito *et al*., 1998). In *ku70Δ* strains, telomere shortening is compensated by the recombination of subtelomeric regions (Kibe *et al*., 2003; Shore, 2001). Heterochromatin may also play a role in telomere integrity in addition to silencing at mating type and centromeres; some heterochromatin proteins, such as Taz1, Rap1, Swi6, Chp2 and Clr4, also play a role in silencing at telomeres in *Sz. pombe* (Baur *et al*., 2001; Cooper *et al*., 1997; Nimmo *et al*., 1998; Thon and Klar, 1992). Interestingly, DNA Polα is also required for telomere silencing (Ahmed, 2001; Ahmed *et al*., 2001; Nakayama *et al*., 2001a). Recently, the SHREC complex, comprising Clr1–Clr2–Clr3, has been shown to be physically associated with the telomere to effect silencing (Sugiyama *et al*., 2007). Furthermore, deletion of Taz1, which binds to telomeric repeat sequences, can also elicit telomere fusions under meiosis and stress conditions through a Ku-dependent pathway (Ferreira and Cooper, 2001).

Studies in the budding yeast have shown that heterochromatin regulates the telomere position effect, but surprisingly has no effect on telomere

recombination; rather, aberrant recombination was reported in mutants in the Rad50 pathway (Stavenhagen and Zakian, 1998). In human cells, mutations in the histone methyltransferases Suv39h1 and Suv39h2 genes have been found to elicit telomere repeat elongation (Garcia-Cao *et al*., 2004). A recent study has reported widespread duplication and rearrangements in the subtelomeric regions of human telomeres, although the mechanism of these events is not clear (Linardopoulou *et al*., 2005). It should be stressed here that human cells contain chromatin machinery similar to that of *Sz. pombe* (Blasco, 2007).

The *Sz. pombe* system has become one of the best model systems to study the role of heterochromatin in the biology of the organism where a plethora of heterochromatin factors and their mutants have been identified. To exploit this resource, we have investigated the role of heterochromatin proteins in regulating the integrity of telomeres in *Sz. pombe*. We observe an elevated level of a subtelomeric fragment of 0.9 kb in *polα/swi7H4* and some of the heterochromatin mutants, which probably arises from an endoduplication by intrachromosomal recombination between the subtelomeric regions. This event seems to be dependent on Rhp51 and Rad50. In addition, we find that the silencing mutants *polα/swi7H4, clr1-clr4* and *swi6* also display some common recombinant subtelomeric fragments, indicating a role of heterochromatin structure in preventing the subtelomeric recombination to maintain the telomere integrity and function.

Materials and methods

Materials and reagents

Media components were purchased from SRL (Mumbai, India) or Difco (Detroit, MI, USA), nylon membranes from Advanced Microdevices (Ambala, India), [α-^{32}P] dCTP from BARC (Mumbai, India) and X-ray films from Hindustan Photo Films (India). Oligonucleotides were purchased from Biobasic (Canada).

Strains, media and growth conditions

All media were prepared according to Moreno *et al*. (1991). Strains used for the experiments were generated by suitable crosses between a set of closely related isogenic strains (a list of strains will be provided on request). In most experiments, strains were grown in YEA medium at 30 °C before DNA isolation. Growth of strains in nitrogen plus and minus media was performed according to Ferreira and Cooper (2001).

Southern hybridization

Restriction digests of DNA samples were subjected to agarose gel electrophoresis, Southern blotting and hybridization, as described previously (Singh *et al*., 1998). DNA was isolated according to Moreno *et al*. (1991). For hybridization, probes were prepared by digestion of the vector pAMP001 to generate the following DNA fragments: *Eco*RI fragment (1.1 kb); *Eco*RI–*Apa*I subtelomeric fragment TAS1 (0.8 kb) and *Apa*I–*Eco*RI (0.3 kb) telomeric repeat fragment. The right *Eco*RI site is present in the vector (Figure 1A). The DNA fragments were radiolabelled with [α^{32}-P]-dCTP and used for hybridization, followed by autoradiography.

Telomere tailing, PCR and cloning

Telomere tailing, PCR, cloning and sequencing were performed according to Dreesen and Cross (2006). DNA was eluted from the gel and subjected to poly (dC) tailing with terminal deoxy transferase (Bangalore Genei), followed by PCR using the oligo Apa out (CCACTATTGGGCCCACC) and the poly dG oligo $(GGGGGG)_3$. The PCR conditions were: 95 °C for 2 min; 45 cycles of (95 °C for 1 min, 55 °C for 45 s, 72 °C for 40 s); and then 72 °C for 5 min. The PCR products were resolved by agarose gel electrophoresis. Bands of ~200 bp were eluted and cloned into the vector pGEM-T Easy system-I by TA cloning (Promega), followed by sequencing using the T7 primer on an ABI3130XL analyser.

Results

An altered telomere pattern in the *polα* mutant *swi7H4*

Because, like heterochromatin proteins, Polα also plays a role in telomere silencing (Ahmed, 2001; Ahmed *et al*., 2001; Nakayama *et al*., 2001b), we compared the telomere pattern of isogenic strains

DOI: 10.1002/yea

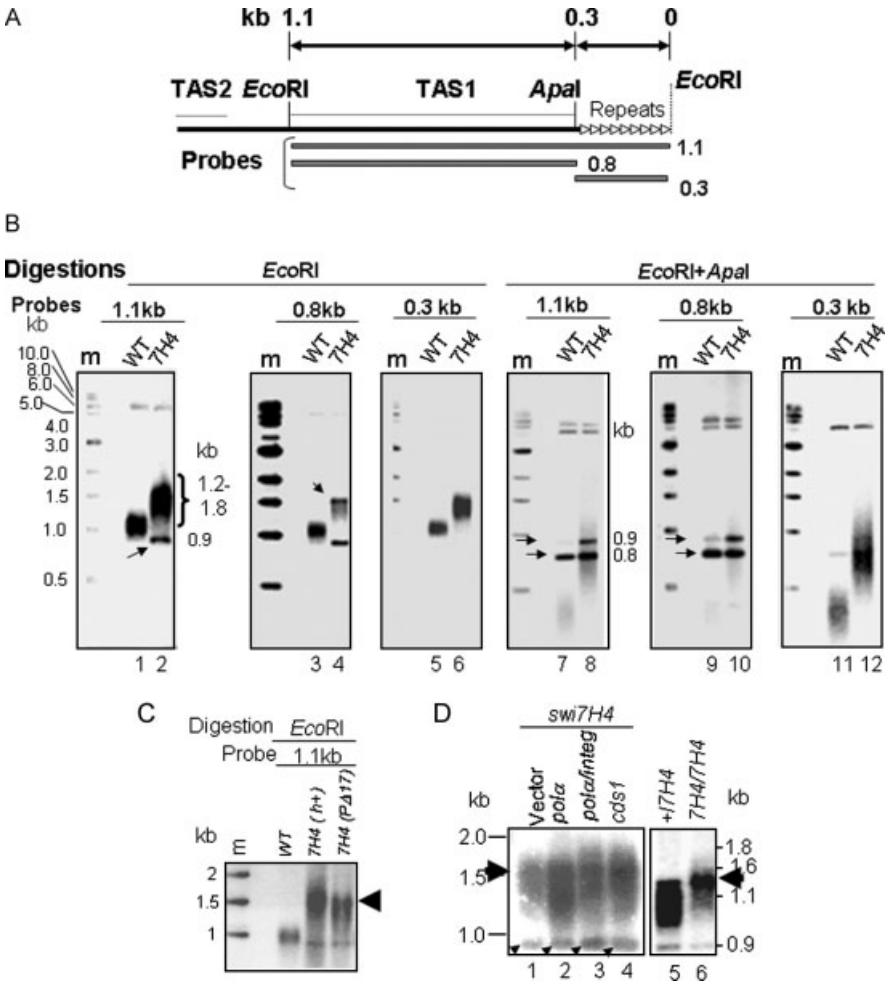


Figure 1. *swi7H4* mutation elicits a novel telomere pattern. (A) Restriction map of the telomere showing the 0.3 kb *Apa*I-distal telomeric repeat region, 0.8 kb *Eco*RI − *Apa*I fragment representing the TAS1 region and the 1.1 kb *Eco*RI fragment, obtained from plasmid pAMP001. The right *Eco*RI site is located within the vector. (B) Southern analysis of DNA from wt (lanes 1, 3, 5, 7, 9 and 11) and *swi7H4* mutant (lanes 2, 4, 6, 8, 10 and 12) strains. DNA was digested with *Eco*RI (lanes 1–6) or *Eco*RI + *Apa*I (lanes 7–12). In lane 4, the arrow indicates the position of the 1.5 kb *Eco*RI fragment, while in lanes 7–10 the arrows indicate the positions of the 0.8 and 0.9 kb *Eco*RI − *Apa*I fragments. (C) Southern analysis of *Eco*RI digests of DNA from wt, *swi7H4* in (h^+) and *PΔ17::Leu2* (Arcangioli and Klar, 1991) backgrounds. (D) Southern analysis of *Eco*RI digests of DNA isolated from *swi7H4* mutant transformed with vector alone (lane 1), *polα* gene on a low copy plasmid pWH5 (lane 2), carrying an integrative duplication of *polα* gene (lane 3) or *cds1* gene (lane 4) as well as from heterozygous (lane 5) and homozygous *swi7H4* mutant diploid (lane 6) strains, was digested with *Eco*RI and subjected to agarose gel electrophoresis. (C, D) The arrowhead indicates the position of the 1.5 kb *Eco*RI fragment. After Southern blotting, hybridization was performed with radioactively labelled probes as indicated

of the wild-type (wt) and the *swi7H4/polα* mutant. Interestingly, the *swi7H4* mutant showed telomere elongation when blots of *Eco*R1 and *Eco*RI + *Apa*I digests were probed with the telomere repeat fragment of 0.3 kb (Figure 1B, lanes 5, 6, 11, 12). Additional bands of ~0.9 and ~1.5 kb were observed in *Eco*RI blots of *swi7H4* mutant probed with the 0.8 kb *Eco*RI − *Apa*I subtelomeric probe (arrow, Figure 1B, lane 4) or the 1.1 kb *Eco*RI probe (arrow, Figure 1B, lane 2), but not with the telomere repeat probe (Figure 1B, lane 6). The blots of *Eco*RI + *Apa*I digests probed with the 1.1 kb *Eco*RI probe or the 0.8 kb *Eco*RI − *Apa*I fragment revealed two bands, one of which appears to co-migrate with the 0.9 kb band in the *swi7H4* mutant and is present at barely detectable level

DOI: 10.1002/yea

in wt (arrows, Figure 1B; compare lanes 7 and 9 with lanes 8 and 10), and the 0.8 kb band which is present in both wt and the *swi7H4* mutant (arrows, Figure 1B, lanes 7 and 9) and may represent the 0.8 kb subtelomeric *Eco*RI − *Apa*I fragment (Figure 1A), which remains unaltered. Thus, the 0.9 kb band is either absent or present at a very low level in wt (observed only on overexposure; Figure 1B, lane 1) and may originate either from the erosion of repeats from the chromosomal ends or from an internal subtelomeric duplication. An identical pattern was observed in different genetic backgrounds, h^+ or *PΔ17* (Arcangioli and Klar, 1991; Figure 1C), ruling out a role of genetic background for the observed fragment pattern.

Dominance of the *swi7H4* mutation in generating the telomere pattern

It has been shown that *αts11* and *αts13* mutations in the DNA *polα* gene cause telomere repeat expansion (Dahlen *et al*., 2003). This effect is recessive and the repeat expansion is restored to wt level by expression of the wt *polα* gene (Dahlen *et al*., 2003). However, Southern blot analysis showed that the telomere pattern of the *swi7H4* mutant was not restored to normal upon overexpression of the *polα* and *cds1* genes (Figure 1D, lanes 2–4), both of which have been shown to suppress the temperature-sensitive phenotype of the *swi7H4* mutant (Ahmed *et al*., 2001). Thus, unlike *αts11* and *αts13*, the effect of the *swi7H4* mutation on telomeric repeat expansion and subtelomeric rearrangement is dominant. The dominant nature of the effect was also indicated by the presence of a pattern in the heterozygous *swi7H4* diploid (Figure 1D, lane 5), which was similar to that of the haploid *swi7H4* mutant, with the homozygous *swi7H4* diploid showing a greater effect (arrowhead, Figure 1D, lane 6). Possibly these results suggest that the mutant Pol*α* protein interferes with the assembly of complexes that may regulate the repeat length and subtelomere structure (see below).

Since, like *swi7H4* mutation, *taz1Δ* mutation has also been shown to abrogate telomere silencing, we checked the telomere pattern of the *taz1Δ* mutant. Interestingly, like the *swi7H4* mutant, the *taz1Δ* mutant also showed both the 0.9 and the 1.5 kb bands in addition to a smear around 2–10 kb (Figure 2A). Moreover, the smear in the 2–10 kb range, representing an extensive subtelomeric rearrangement in *taz1* mutant (Ferreira and Cooper, 2001), is not observed in the *swi7H4* mutant even after 60 generations (data not shown).

It is not clear whether the 0.9 kb *Eco*RI fragment originates from the telomere repeat erosion or results from subtelomeric duplication. It has been shown that the *taz1Δ* mutant undergoes repeat erosion with increasing number of generations (Ferreira and Cooper, 2001). However, unlike the *taz1* mutant, the *swi7H4* mutant did not show any alteration in the pattern even after 60 generations (not shown). Further, while

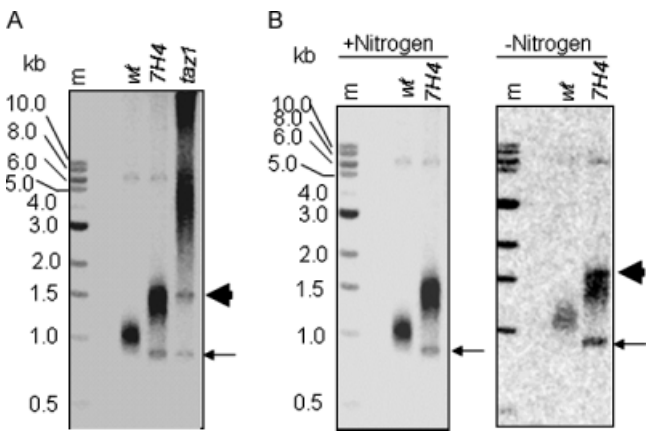


Figure 2. Stress conditions do not elicit telomere repeat erosion in *swi7H4* mutant. (A) Southern analysis of *Eco*RI-digested DNA from wt, *taz1Δ* and *swi7H4* mutant strains. (B) Southern analysis of *Eco*RI-digested DNA from wt and *swi7H4* mutant strains after growth in medium containing nitrogen (left) or lacking nitrogen (right) for 8 days. Blots were hybridized with radiolabelled *Eco*RI fragment as described in the legend to Figure 1. Arrows indicate the 0.9 kb band. The arrowhead indicates the position of the 1.5 kb *Eco*RI fragment

DOI: 10.1002/yea

growth of the *taz1Δ* mutant under nitrogen starvation conditions also causes progressive repeat erosion followed by chromosome circularization (Ferreira and Cooper, 2001), we observed no change in the *Eco*RI digestion pattern of the *swi7H4* mutant even after 8 days of growth under nitrogen starvation conditions (Figure 2B). These results argued against the possibility that the 0.9 kb band may have originated from telomeric repeat erosion.

New subtelomeric fragments in heterochromatin mutants

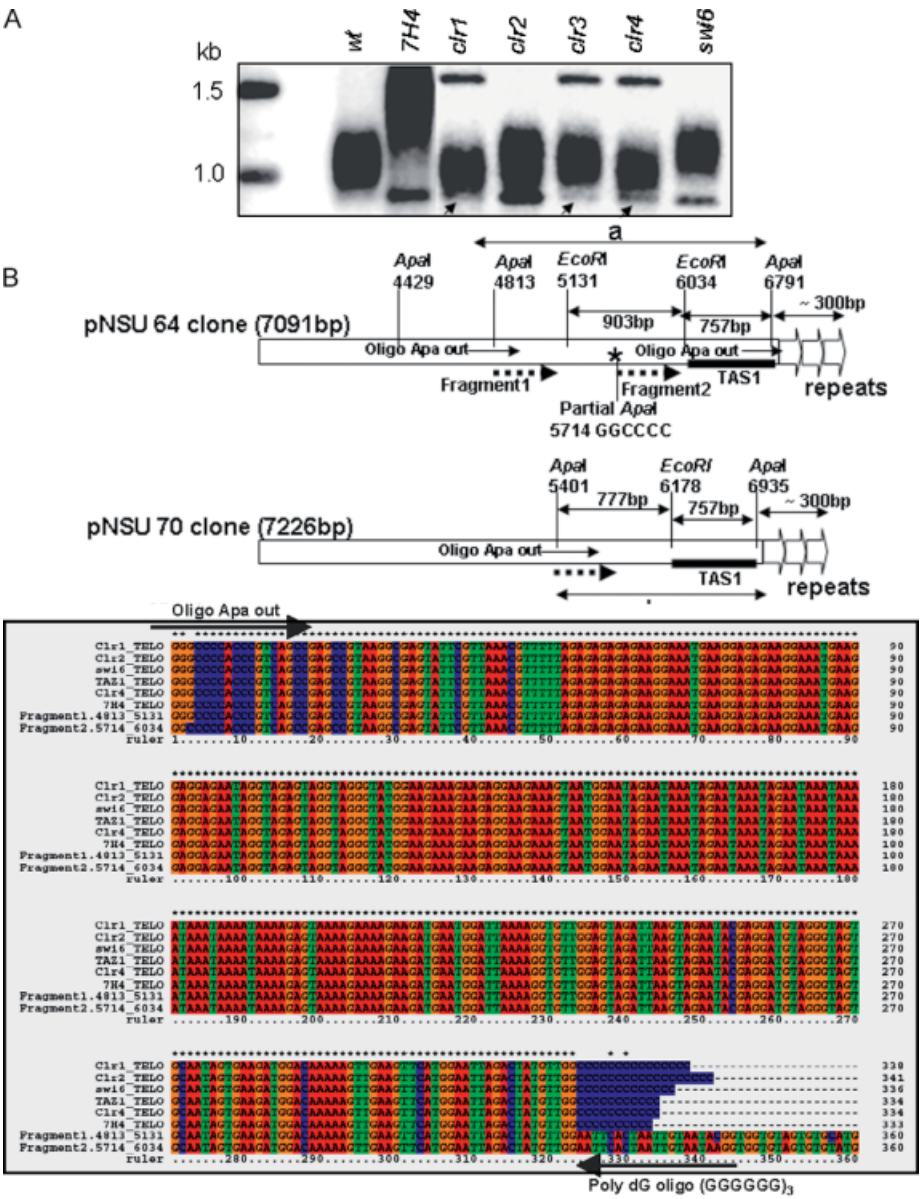
One way in which the *swi7H4* and *taz1Δ* mutations may impact telomere integrity is by their effect on telomere silencing through heterochromatin structure. Therefore, we checked whether other heterochromatin mutations affect the generation of the 0.9 and 1.5 kb bands. Indeed, the *clr2* and *swi6* mutants contain a distinct 0.9 kb band, while *clr1, clr3* and *clr4* mutants contain a much fainter band of 0.9 kb and a prominant band of 1.5 kb (Figure 3A; see below) in addition to the smear around 1.1–1.2 kb, representing the repeats plus subtelomere sequences. Thus, all *swi7H4, clr1–clr4, swi6* and *taz1* mutants (Arcangioli and Klar, 1991; Blasco, 2007; Kanoh *et al*., 2005; Thon *et al*., 1994; Thon and Klar, 1992), all of which abrogate telomere silencing, elicit the unique telomere pattern.

Subtelomeric recombination in heterochromatin mutants

Although the presence of the 0.9 kb *Eco*RI band can be predicted from a restriction map of pNSU21, pNSU64 and pNSU65 cosmids (Figure 4), available at the Sanger Center website (**http://www.sanger.ac.uk/Projects/S_pombe/telomeres.html**), it was barely detectable in wt strains (Figure 1A). To clarify the situation, we proceeded to characterize it. This was attempted by gel elution, telomere tailing, PCR amplification using polydG and *Apa*I out oligos, cloning and sequencing (Dreesen and Cross, 2006). The results revealed a common sequence in all mutants ending with the *Eco*RI site followed by dC tail (Figure 3B), except in wt and *clr3*, where we could not get any PCR product (probably because of the very low level of the 0.9 kb *Eco*RI band; see below). The number of clones sequenced for each mutant is mentioned in Figure 3C. The sequence obtained is aligned with two subtelomeric regions in the cosmid pNSU64 (dashed arrows, Figure 3B, top panel), viz. fragment 1 *Apa*I (4813) to *Eco*RI (5131) and fragment 2 GGCCCCC (5714) to *Eco*RI (6034) in the clone pNSU64, which share significant restriction pattern similarity with pNSU65 and pNSU21 (Figure 4A, named pNSU64 family). In addition, it also showed incomplete alignment (with gaps) with the region starting from (5786) in the clone pNSU70 (Figure 3B, dashed arrow, top panel), which shows significant similarity with pNSU71 and pNSU77 (Figure 4B, named pNSU70 family). Alignments of the sequence with two subtelomeric locations in 'pNSU64 family' suggest a duplication of subtelomeric region. Such a hypothetical duplication was not observed in the 'pNSU70 family' of cosmids.

To check the correspondence of the subtelomere pattern observed in the mutants to the cosmids, we carried out *Apa*I digestion followed by Southern blot hybridization with the 0.8 kb *Eco*RI probe, as depicted in Figure 1A. In addition to the 5 kb band, which may originate from the cosmid pNSU77 (Figure 4B; the 5173 bp band in pNSU77), bands of ~2 b and ~1.5 kb were also observed (Figure 4C), which may correspond to the 1979 bp band of the 'pNSU64 family' (Figure 4A) and the 1534 bp band of the 'pNSU70 family' of cosmids (Figure 4B), respectively. A closer scrutiny indicates that the 0.9 kb band can only originate internally from *Eco*RI digestion of the *Apa*I band of 1979 bp of the pNSU64 family of cosmids (Figure 4A), which shows homology to the 0.8 kb probe (additional sites of TAS1 homology are indicated by empty boxes in Figure 4A, B). The much lower intensity of the 1979 bp band in *Apa*I digests of wt, *clr1* and *clr3* mutants (Figure 4C) now appears to be consistent with the barely detectable levels of the 0.9 kb band on *Eco*R1 digestion in wt, *clr1* and *clr3* mutants (Figure 1B). Thus, the representation of the cosmid clones in heterochromatin mutant strains is highly variable. The 'pNSU64 family' subtelomeres having the 1534 bp *Apa*I fragment are overrepresented in *swi7H4, clr2, clr4, swi6* and *taz1* mutants, while subtelomeres of wt, *clr1* and *clr3* are mainly represented by the 'pNSU70 family' having the 1979 bp *Apa*I fragment. Interestingly, the changes in the copy number are in nearly integral ratios

DOI: 10.1002/yea



Mutant	# clones
swi7H4	3
clr1	1
clr2	4
clr4	3
swi6	4
taz1	4

Figure 3. Role of silencing factors Clr1–Clr4, Swi6 and Taz1 in maintaining the telomere integrity. (A) Southern analysis of *Eco*RI-digested DNA from *swi7H4, clr1, clr2, clr3, clr4* and *swi6* mutants. After Southern blotting, hybridization was performed with radiolabelled *Eco*RI fragment as described in the legend to Figure 1. Arrows indicate the fainter 0.9 kb bands in the *clr1*, *clr3* and *clr4* mutants. (B) Generation of a subtelomeric duplication in *clr1–clr4, swi6, swi7H4* and *taz1* mutants. (Top) A schematic of the restriction map of the telomere cosmid clones pNSU64 and pNSU70. *The sequence GGCCCCC. Dashed arrows labelled 'Fragment 1' and 'Fragment 2' indicate the regions of identity in cosmid pNSU64. The lower panel shows the alignment of the conserved sequences obtained in all the mutants with fragments 1 and 2. The sites of tailing at the 3′ end is shown to end at the *Eco*RI site and the oligos Apa out and poly dG used for PCR are indicated. (C) The number of clones sequenced in different mutants. a and b in (B) denote the 1979 and 1534 bp *Apa*I fragments, respectively, originating from cosmids pNSU21/64/65 and pNSU70/71/77

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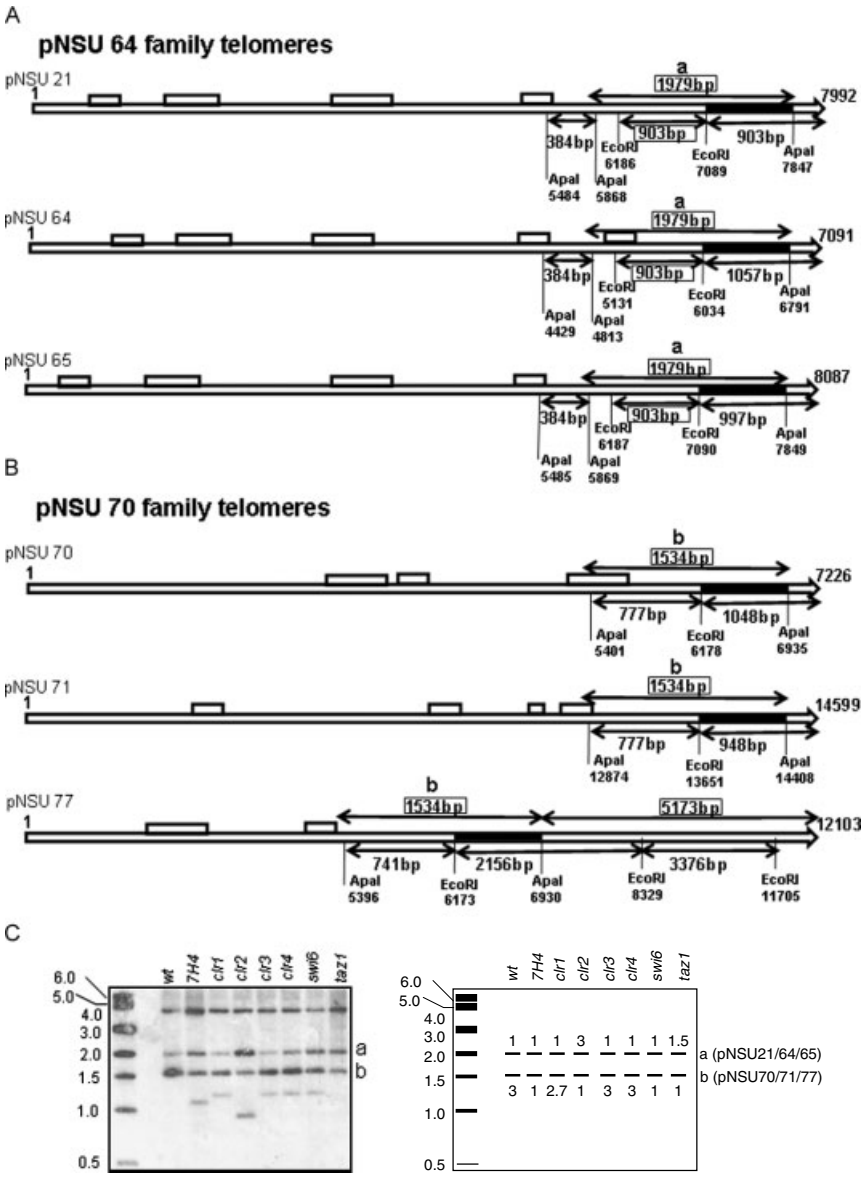

Figure 4. Mapping of subtelomere duplication and rearrangements in *swi7H4* and heterochromatin mutants. (A) The restriction patterns of the telomere cosmid clones pNSU21, pNSU64 and pNSU65. (B) The restriction patterns of the clones pNSU70, pNSU71 and pNSU77. The sizes of the various restriction fragments generated by *Eco*RI and *Apa*I digestion are indicated. Regions of identity of the sequences with TAS1 probe are indicated by filled bars. The fragments 1534, 1979 and 5173 bp, 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) Southern analysis of ApaI-digested DNA from wt and mutants. Southern blots were hybridized with the 0.8 kb *Eco*RI – *Apa*I (TAS1) probe, as depicted in the legend to Figure 1. (Right) Schematic representation of the approximate ratios of the intensities of the 2 and 1.5 kb bands depicted as a boxed bar in Figure 3A, B, and labelled as bands a and b

(Figure 4C, right panel), arguing for endoduplication event(s) among different telomeres in the heterochromatin mutants. Since telomeres have been shown to cluster together in *Sz. pombe* (Chikashige *et al*., 2006; Davis and Smith, 2006; Tang *et al*., 2006), which promotes homologue 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 occurring is likely in

DOI: 10.1002/yea

the mutants. Notably, *clr1, clr3, clr4* and *swi6* mutants also share a common band of 1.3 kb (Figure 4C), which may originate from a 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 a recombination event. Indeed, a complex comprising Clr1–Clr2–Clr3, called the 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 4C), may suggest a role of the encoded proteins in protecting distinct regions of subtelomeres from recombination.

Role of the Rad50 recombination pathway in generating the 0.9 kb band

The above results suggested that some duplication and/or recombination events may cause the generation of the aberrant restriction pattern of telomeres in the heterochromatin mutants. To check whether one or more recombination pathways were involved in this process, we carried out Southern blot hybridization of *Eco*RI and *Apa*I digests of DNA from mutants affecting the NHEJ pathway (*ku80Δ*) the Rad50 homologous recombination pathway and the post-replication repair pathway of Rad6/rhp6. The results showed that while no distinct 0.9 kb band could be observed in the *Eco*RI blots (Figure 5) in any mutant, the *Apa*I blot revealed a clear absence of the ~2 kb band, the progenitor of the 0.9 kb band, in both *rad50Δ* and *rhp51Δ* mutants strains but not in the *ku80Δ* (Figure 5) and *rhp6Δ* mutants (data not shown). These results suggest that the 0.9 kb band may indeed be generated by the Rad50–Rhp51 recombination pathway. Notably, the *rhp51Δ* strain shows an additional *Apa*I band of 3 kb (Figure 5A, right), 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.

A recombination dependent endoduplication model

The observation that at least one major band of ~2 kb in the *Apa*I blot is absent in *rhp51Δ* and *rad50Δ* strains is highly interesting and suggests that Rad50 and Rhp51 may be actively involved in generating the band (corresponding to the cosmid family pNSU21/64/65) by an intrachromosomal recombination event. In one possible scenario, we speculate that such a recombination event may be initiated by DNA damage near or upstream of the *Apa*I site (upstream of the repeat region), which may be rendered accessible by the loss of a complex such as SHREC. The Rad50–Rhp51 pathway may recognize partial homology in the region upstream of the *Apa*I site within the telomeres corresponding to the pNSU70/71/77 family of cosmids to carry out replication/recombination of the downstream region, thus generating an endoduplication event (Figure 5B). The presence of an additional recombination product in the form of a 3 kb *Apa*I fragment (Figure 5A, right), 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. We are tempted to speculate that such a recombination event could have been responsible for the generation of the subtelomeres belonging to the pNSU21/64/65 family of cosmids, differing from those belonging to the pNSU70/71/77 family of cosmids. The 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 an elevated rate in upstream regions of homology, which may include the sites reported by Kibe *et al.* (2003) and the TAS1 homology regions represented by empty boxes in Figure 4A, B, which may generate the additional bands observed in the *Apa*I 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: enhanced accessibility of specific regions in heterochromatin mutants may lead to damage, followed by recruitment of the components of the Rad50/Rhp51 recombination machinery to these sites, leading to distinct recombination/duplication events.

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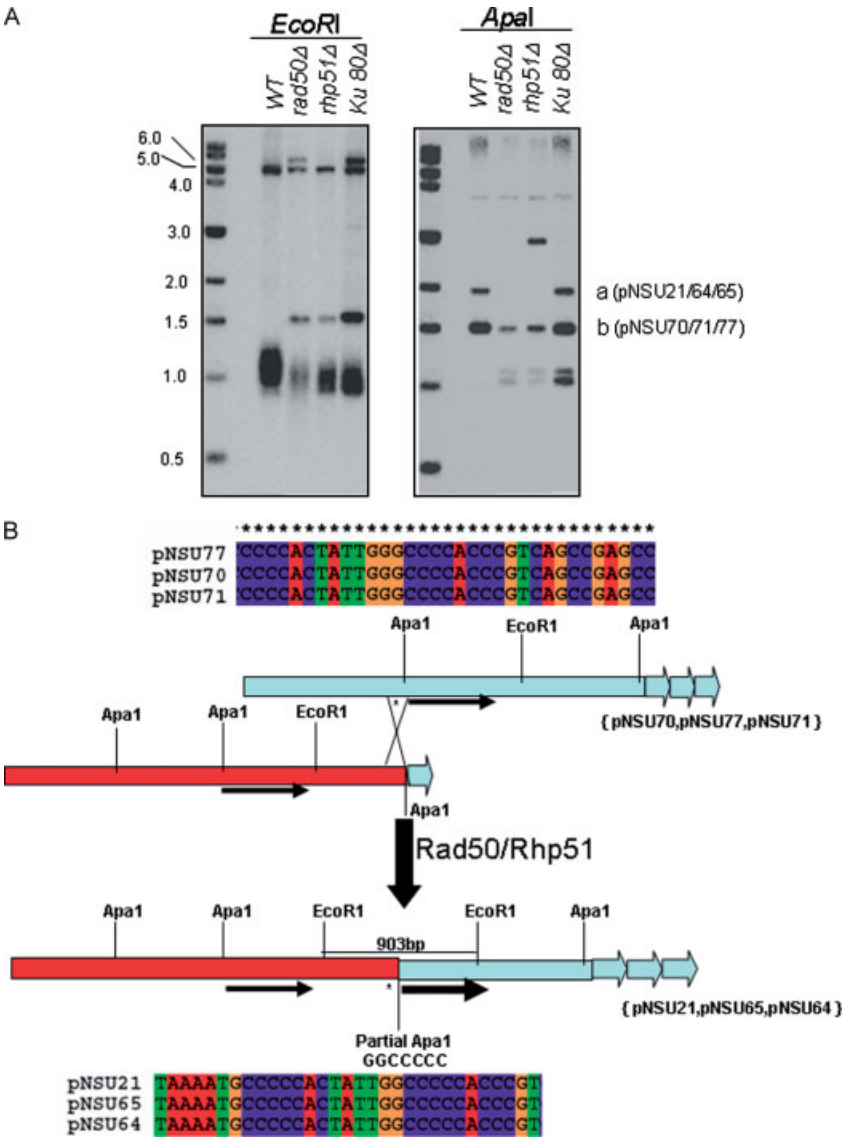

Figure 5. Role of the Rad50 pathway in generating the duplicative recombination in the subtelomeric region. (A) Southern analysis of *Eco*RI (left) and *Apa*I digests (right) of DNA from wt, *rad50Δ, rhp51Δ* and *ku80Δ* strains. Southern blots were hybridized with the 0.8 kb *Eco*RI − *Apa*I probe. (B) 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 *Apa*I site in the telomeres represented by the pNSU70 family of cosmids in the wt 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

Discussion

Subtelomeric recombination events have not been widely reported. Our proposal is that heterochromatin may play an important role in suppressing telomeric recombination. In budding yeast, heterochromatin structure at *HM* loci reduces their accessibility to HO endonuclease (Klar *et al*., 1981), DNA repair (Terleth *et al*., 1989) and *E. coli dam* methylase (Gottschling, 1992; Singh and Klar, 1992); accessibility is greatly enhanced in silencing mutants (Bhattacharyya and Lustig, 2006; Klar *et al*., 1981; Singh and Klar, 1992; Terleth *et al*., 1989). Similarly, *rhp6* and *swi6* mutants exhibit

DOI: 10.1002/yea

enhanced accessibility of silent mating type loci in *Sz. pombe* to *dam* methylase (Singh *et al.*, 1998). More pertinently, the silencing mutants *clr1–clr4* and *swi6* abrogate the recombination block acting over the *mat2–mat3* 10.5 kb interval, referred to as the *K* region (Ahmed *et al.*, 2001; Klar and Bonaduce, 1991; Thon and Klar, 1992), with *swi6* mutant exhibiting an enhanced rate of aberrant recombination (Nonaka *et al.*, 2002). Thus, heterochromatin proteins may impose a similar recombination block on telomeres of *Sz. pombe*. In contrast, *sir* mutations in budding yeast, which abrogate the position-effect control of transcription at the telomere (Gottschling *et al.*, 1990) and enhance the accessibility of telomeres to *dam* methylase (Gottschling, 1992), do not abrogate the recombination block at telomeres; rather, it is abrogated by mutations in recombination pathways such as Rad51 (Stavenhagen and Zakian, 1998).

Although similar recombination events have not been reported in other organisms, the possibility of heterochromatin playing a role in suppressing recombination in other systems is not ruled out. For example, the telomere position effect (TPE) has been reported in budding yeast (Gottschling *et al.*, 1990) as well as in humans (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 interactions play an important role in initiating the assembly of heterochromatin (Luo *et al.*, 2002). Similarly, subtelomere proteins have been shown to negatively regulate telomere elongation (Berthiau *et al.*, 2006), and SIR3 and SIR4 are required for the integrity of telomeres in budding yeast (Palladino *et al.*, 1993).

Apart from the 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 the 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 *Sz. pombe*, defective meiosis in telomere silencing mutants suggests a role of silencing factors in maintaining telomere integrity (Cooper *et al.*, 1997). It is relevant to note that, independently of Taz1, Swi6 can also bind to telomeres and subtelomeres in *Sz. pombe* (Nimmo *et al.*, 1998). An important role of epigenetic factors is further highlighted by a study showing a role of SUMO E3 ligase Pli1p in centromere and telomere maintenance in *Sz. pombe* (Xhemalce *et al.*, 2004). In *Drosophila*, heterochromatin protein HP1 regulates telomere capping, telomere elongation and silencing (Perrini *et al.*, 2004). Also, an RNAi-based mechanism regulates telomere elongation (Savitsky *et al.*, 2006). Likewise, human subtelomeres exhibit elevated rates of intrachromosomal recombination and segmental duplication, although the mechanism of such events is not yet understood (Linardopoulou *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 *Sz. pombe* (Kanoh *et al.*, 2003) have also been shown to cause repeat expansion.

In the light of our results, we propose that heterochromatin structure generated by epigenetic factors such as Clr4/Suv39 and Swi6/HP1 may render the telomeres refractory to aberrant recombination as well as for transcription comprising a mechanism to maintain telomere structure and integrity. Because of the conservation of Clr4/Suv39 and Swi6/HP1 (Klar, 2007; Nakayama *et al.*, 2001b) in eukaryotes, the role of heterochromatin in structural and functional integrity of telomeres may be evolutionarily conserved.

Acknowledgements

We are grateful to the Council of Scientific and Industrial Research (CSIR), India, for intramural support. We thank A. Klar and G. Thon for the gift of the mutant strains *clr1–clr4* and *swi6*, J. Cooper for the gift of the *taz1Δ* strain, H. Okayama for the *swi7H4* strain and the *cds1* plasmid, M.

Ueno for the *rad50Δ, rhp51Δ* and *ku80Δ* strains, and Louis Prakash for the *rhp6Δ* strain. We are grateful to M. R. S. Rao and U. Varshney for the generous gift of terminal transferase. Kamlesh K. Bisht is the recipient of a Senior Research Fellowship from CSIR, India.

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DOI: 10.1002/yea