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Published in:
Journal of Cell Biology
DOI:
10.1083/jcb.200312061
Publication date:
2004
Document Version
Early version, also known as pre-print
Citation for published version (APA):
The fission yeast heterochromatin protein Rik1 is required for telomere clustering during meiosis

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Telomeres share the ability to silence nearby transcription with heterochromatin, but the requirement of heterochromatin proteins for most telomere functions is unknown. The fission yeast Rik1 protein is required for heterochromatin formation at centromeres and the mating-type locus, as it recruits the Clr4 histone methyltransferase, whose modification of histone H3 triggers binding by Swi6, a conserved protein involved in spreading of heterochromatin. Here, we demonstrate 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.

Introduction

Telomeres are critical nucleoprotein structures that prevent degradation and fusion of chromosome ends and are required for faithful chromosome segregation. When cells progress from the mitotic cycle to sexual development and meiosis, telomeres acquire new functions, implying that their composition or structure must change. Telomere clustering at the nuclear periphery during early stages of meiosis is widespread among diverse eukaryotes (Dernburg et al., 1995) and has been shown to be crucial for successful meiotic chromosome segregation in fission yeast (Chikashige et al., 1994, 1997; Cooper et al., 1998; Nimmo et al., 1998).

Fission yeast cells lacking the telomere binding protein Taz1 exhibit multiple defects in the chromosome movements that accompany meiotic prophase (Cooper et al., 1998; Nimmo et al., 1998). In vegetatively growing wild-type cells, the centromeres localize to a single cluster adjacent to the spindle pole body (SPB) during interphase. Upon meiotic induction, this organization changes dramatically, with the telomeres gathering at the SPB in haploid cells responding to mating pheromone (Chikashige et al., 1997). Once haploids have mated, the centromeres separate from the SPB, leaving only the telomeres associated with it. This arrangement persists throughout the subsequent “horsetail” stage, during which the zygotic nucleus assumes an elongated shape and migrates back and forth across the zygote, with the SPB-telomere complex leading the nuclear movement, pulling the chromosomes in tow. Telomeres lacking Taz1 are unable to stably associate with the SPB, leading to a markedly disorganized zygotic nucleus, reduced homologue pairing, and defective meiotic chromosome segregation (Cooper et al., 1998; Nimmo et al., 1998). Taz1 must recruit a second telomere protein, Rap1, to organize these chromosomal rearrangements (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001), but the mechanism underlying associations between the Taz1–Rap1 complex and the SPB remains elusive.

Fission yeast telomeres share structural features with centromeres and the mating-type locus, including the ability to form repressive chromatin domains (Nimmo et al., 1994).
The Rik1 protein functions in an early step of heterochromatin formation at those loci, as it is required for methylation of histone H3 on lysine 9. This modification is mediated by the chromo- and SET-domain protein Clr4 and allows recruitment of Swi6, a chromodomain-containing HP-1 orthologue (Ekwall et al., 1996; Nakayama et al., 2001). Consequently, mutants in rik1, clr4, and swi6 are all compromised in centromere function. Each of these mutants also displays defects in telomere silencing but normal telomere length (Ekwall et al., 1996). Here, we characterize Rik1 function. Although it parallels Swi6 function in promoting mating, Rik1 acts together with Clr4 but independently of Swi6 to promote meiotic telomere clustering. These results establish that the heterochromatic nature of telomeres is specifically important for their meiotic function.

Results and discussion
Characterization of the rik1+/+ gene
When depleted of a nitrogen source, fission yeast cells of opposite mating type (P and M) undergo morphological changes that allow them to mate to form diploid zygotes, which subsequently undergo meiosis and sporulation (Nielsen, 2004; Yamamoto, 2004). The rik1 mutation causes both low sporulation efficiency and irregular asci of the diploid strain, and found by tetrad dissection that the disruption cosegregated with a sporulation defect identical to that of the original mutant. Furthermore, the disrupted allele was tightly linked to the original rik1-304 mutation. Thus, we conclude that the cloned fragment represents the rik1+/+ gene and not an exogenous suppressor and that rik1+/+ is not essential for cell viability.

The predicted Rik1 protein shows homology over its entire length to the evolutionarily conserved Ddb1 group of proteins implicated in cullin-mediated protein ubiquitination during S-phase and nucleotide excision repair (Groisman et al., 2003; Liu et al., 2003). Thus, Rik1 displays 19% identity and 40% similarity to human DDB1, and 20% identity and 44% similarity to fission yeast Ddb1. As we have submitted the rik1 sequence to GenBank (GenBank/EMBL/DDBJ accession no. AF136156), reference to it has already appeared in the literature, although the identity of the rik1+/+ gene has not been previously reported.

Rik1 shares functions required for mating with Swi6
We investigated the basis for the severe sporulation defect seen in rik1+/+ strains, which encompasses both reduced mating efficiency and aberrant sporulation of those zygotes that do form (Egel et al., 1989). Previously, it was reported that rik1+/+ cells are defective in the generation of heterochromatin at the silenced mat2-P and mat3-M donor cassettes (Nakayama et al., 2001). Consequently, the normally nonrecombinational domain between mat2-P and mat3-M is rendered accessible to meiotic recombination (Egel et al., 1989), and the silent donor cassettes are partly expressed (Ekwall and Ruusala, 1994). This gives rise to a low level of sporulation from the haploid state and reduces mating efficiency, thus providing a partial explanation for the rik1+/+ mating defect. Similar phenotypes have been observed in swi6 and clr1, 2, 3, and 4 mutants (Klar and Bonaduce, 1991; Lorentz et al., 1992; Thon and Klar, 1992; Thon et al., 1994).

Figure 1. Factors contributing to reduced sporulation of rik1Δ cells. (A) rik1Δ cells have a defect in directionality of mating-type switching. Sporulation of colonies with the indicated genotypes was visualized by iodine staining, which causes spores to appear black. The directionality mechanism that causes a pronounced reduction in sporulation of h+/Δ relative to h+Δ is lost in rik1Δ and swi6Δ cells. (B) The rik1Δ mutation gives rise to rearrangements in the mating-type region. Southern blot of HindIII-digested DNA hybridized to an M-specific probe. In addition to the mat1-M, mat3-M, and smt bands present in wild type, several rearrangement bands in the rik1Δ strain are indicated by arrows. White line indicates that intervening lanes have been spliced out. (C) Aberrant ascus formed by sporulating rik1Δ cells.
In wild-type strains, a directionality mechanism ensures that cells preferentially switch to the opposite mating type. Thus, cells harboring mat1-P switch to mat1-M by copying the information stored at the silent mat3-M locus, and similarly, mat1-M cells preferentially choose the content of the silent mat-2P locus when switching. This switching pattern is subverted if the contents of the donor loci are swapped (Thon and Klar, 1993). Colonies of these so-called h09 strains sporulate at a much-reduced frequency due to extended sectors of cells with the same mating type, suggesting that donor choice is specified by genome position rather than mating-type information.

In swi6 and clr4 mutants, the directionality mechanism is lost and switching becomes random (Thon and Klar, 1993; Ivanova et al., 1998), suggesting that the heterochromatin structure around mat2-P and mat3-M is important for this mechanism. To determine if rik1+ contributes to directionality, we compared the sporulation frequencies of h09 rik1 and h09 rik1 strains by iodine staining of sporulating colonies. As shown in Fig. 1 A, the h09 rik1 and h09 rik1 strains sporulate to a comparable extent, demonstrating that the directionality mechanism indeed requires rik1+.

swi6 mutations lead to frequent rearrangements in the mating-type region (Egel et al., 1984), which give rise to subclones of unswitchable cells, providing another avenue to reduced mating efficiency. We found that rik1 cells similarly accumulate aberrantly sized DNA fragments in the mating-type region (Fig. 1 B), demonstrating that rik1 mutants acquire rearrangements therein. Thus, leakiness of the silent donor cassettes, impaired directionality of mating-type switching, and rearrangements in the mat region all confer reduced mating in rik1 cells. Collectively, these phenotypes are shared with swi6 mutants and likely reflect the failure of rik1 cells to establish heterochromatin via Swi6 recruitment.

Rik1 has a function in meiotic telomere clustering not shared by Swi6

Although Rik1 appears to act in concert with Swi6 in regulating mating-type switching and centromere function, a pronounced departure from the behavior of swi6 mutants is revealed by observing the products of rik1Δ meiosis. Although swi6Δ zygotes sporulate to produce ascii of normal appearance containing four uniformly sized round spores apiece, rik1Δ ascii often contain fewer than four spores, and the spores vary markedly in size and shape (Fig. 1 C and Table I).

The aberrant rik1Δ ascii are reminiscent of those seen in strains lacking Taz1 (Table I). Observation of rik1Δ ascii by FISH with telomere-adjacent probes and immunofluorescent localization of SPBs reveals that meiotic telomere clus-

**Figure 2.** Telomeres fail to associate with the SPB during meiotic prophase in rik1 and clr4 mutants. (A) Indirect immunofluorescence against the Sad1 component of SPB and FISH to the telomere-adjacent rDNA were performed on zygotes in the horsetail stage. Examples are shown as merged images of anti-Sad1 (red), telomere-adjacent rDNA (green), and chromatin (blue). (B) The experiments in A were quantified for homozygous crosses of each strain indicated.
tering is severely disrupted. Fig. 2A shows examples of typical wild-type horsetail nuclei in which a single telomere signal colocalizes with the SPB, and rik1Δ horsetail zygotes in which the nuclear shape is lumpy rather than smoothly elongated and telomere signals are clearly separate from the SPB. Quantitation of telomere clustering in horsetail nuclei (Fig. 2B) shows that telomere–SPB association is reduced in rik1Δ zygotes to the same extent as in taz1Δ zygotes (~22% of taz1Δ zygotes and ~25% of rik1Δ zygotes exhibit rDNA-SPB colocalization). We also found that endogenously tagged Taz1-GFP, a marker for the telomere, fails to localize to the leading edge of meiotic horsetails in live rik1Δ cells (Fig. 3). Although the images in Fig. 3 show only one spot of Taz1-GFP, roughly one in three rik1Δ horsetail nuclei show more than one spot (not depicted). However, the weakness of the Taz1-GFP signal prevents us from using it to rigorously quantify telomere–telomere associations, as detection may require high local Taz1 concentrations found only at clustered telomeres. In taz1Δ horsetail nuclei, a residual level of telomere–telomere association is observed even when the telomeres are separated from the SPB (Cooper et al., 1998), and mitotic telomere clustering persists to a large extent in taz1Δ cells. Presumably, the tight clustering of telomeres seen during meiosis stems from the SPB association of telomeres rather than from meiosis-specific connections between telomeres, and the residual telomere clustering seen in mutants with disrupted telomere–SPB association is not stable enough to confer proper homologue pairing. Centromeric dissociation from the SPB upon meiotic induction occurs efficiently in

Table 1. Defects in asci morphology and number

<table>
<thead>
<tr>
<th>Asci with aberrant size and number spores</th>
<th>Asci with four normal spores</th>
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<tr>
<td></td>
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<tr>
<td>clr4-681G486D</td>
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<td>16</td>
</tr>
</tbody>
</table>

The frequency of zygotic asci having 1, 2, 3, and 4 spores was scored by light microscopy. Four-spored asci were further categorized on the basis of spore morphology and size to delineate normal and aberrant spores (“normal” means the four spores within an ascus are round and of approximately equal size). n, number of asci counted. clr4-W487* contains a nonsense mutation.

Figure 3. Telomeres fail to associate with the SPB in live rik1Δ or clr4Δ cells. (A) Telomere clustering monitored via localization of endogenously tagged Taz1-GFP at the leading end of horsetail nuclei in live cells. (B) Quantitation of the experiments in A.
The Rik1 protein is required for telomere clustering | Tuzon et al. 763

both strains, indicating that centromeres separate from the SPB independently of Rik1, Taz1, or telomere status (unpublished data).

Consistent with the normal ascus morphology seen in swi6Δ crosses, meiotic telomere clustering occurs at wild-type levels in swi6Δ zygotes (Figs. 2 B and 3 B). Hence, Rik1 promotes meiotic telomere–SPB association in a Swi6-independent manner.

Rik1 is not required for protection of telomeres against fusions

Not only do taz1Δ cells suffer from a lack of meiotic telomere clustering, but they also accumulate end-to-end chromosome fusions during the nitrogen starvation-induced G1 arrest period that precedes mating (Ferreira and Cooper, 2001). These fusions are formed by the nonhomologous end-joining (NHEJ) pathway of DNA double strand break repair, which acts predominantly in G1 cells and joins telomeres that have become unprotected via Taz1 loss. As Rik1 is required for the telomeric function of meiotic SPB association, we investigated whether or not it might also be required to protect chromosomes from NHEJ-induced end-fusions. To address this possibility, we used pulsed field gel electrophoresis to analyze chromosomes isolated from both nitrogen-starved taz1Δ cells and spores of taz1Δ meiosis (Fig. 4), rik1Δ deletion resulted in no such telomere fusion bands. Thus, Rik1 is dispensable for the telomere function of preventing chromosome end-to-end fusion.

Consistent with a role for Rik1 in meiotic telomere clustering, rik1Δ strains display reduced levels of spore viability intermediate between those of wild-type and taz1Δ strains (Fig. 5). The very low spore viability of taz1Δ cells is partially suppressed when NHEJ-mediated fusion is prevented by simultaneous deletion of the pku70 gene (Fig. 5), yielding a spore viability value similar to that of rik1Δ cells, which is in line with our observation that meiotic rik1Δ cells do not sustain telomere fusions. The spore viability of swi6Δ cells is also reduced relative to wild type due to centromeric cohesion defects (Kitajima et al., 2003), yet it is still higher than rik1Δ, which is consistent with meiotic rik1Δ cells suffering from both centromeric defects and telomere clustering defects.

Clr4 function is also required for meiotic telomere clustering

To address the possibility that Rik1 action is mediated by the Clr4 histone methyltransferase at meiotic telomeres as it is at centromeres and the mating-type loci, we examined meiosis in zygotes harboring various clr4 mutations (Fig. 2
B, Fig. 3, Fig. 5, and Table I). Although ascus morphology, telomere clustering, and spore viability were only mildly affected in strains harboring the R320H, G378S, or W487* mutations, the G486D or null mutants show defects, yielding similar levels of aberrant asci, telomere clustering, and spore viability to rik1Δ mutants. Interestingly, the clr4+/clr4-G486D diploid undergoes a defective asygotic meiosis (Table II), indicating that the G486D mutation is dominant. This finding suggests that Clr4 is partially redundant with some other protein for meiotic function, and that the Clr4-G486D protein interferes with the ability of this other protein to promote proper meiosis. Because the G486D mutant severely reduces histone H3 methylation activity (Nakayama et al., 2001), these observations suggest that telomere–SPB associations are mediated at least in part by Clr4-dependent histone methylation.

Conclusions

Previous studies have established an important role for Rik1 in recruiting the Clr4 histone methyltransferase to DNA regions targeted for Swi6 binding and subsequent heterochromatic silencing. Consequently, there is a substantial overlap between phenotypes displayed by rik1Δ and swi6 mutant cells, and our data on mating-type switching defects in rik1Δ cells reinforce this scheme. However, visual inspection of sporulating cells clearly reveals that Rik1 has a meiotic function not shared by Swi6, and we demonstrate that this function is required for telomere clustering during meiotic prophase.

We find that clr4 mutant cells have a similar meiotic defect, suggesting that Rik1 regulates telomere behavior via Clr4 histone methyltransferase activity. Presumably, this methylation recruits some as yet unidentified factor distinct from Swi6. In addition to Swi6 and Clr4, the fission yeast genome encodes the two chromodomain proteins Chp1 and Chp2. However, our preliminary results suggest that neither Chp1 nor Chp2 are required for normal ascus formation (unpublished data). Recently, it was reported that Clr4 also has a Swi6-independent role in dicer-mediated degradation of centromeric transcripts into siRNAs (Schrack and Allshire, 2003). Cells lacking the RNAi machinery display markedly milder defects in meiotic telomere clustering than those observed in rik1Δ meiosis (Hall et al., 2003). Nevertheless, it will be interesting to see if Rik1 participates in the RNAi process, and if the Swi6-independent functions of Clr4 in centromeric RNAi processing and telomeric clustering involve a common target.

The telomeric protein Taz1 is required during sexual differentiation both to prevent NHEJ-mediated telomere fusions and to promote clustering of the telomeres at the SPB. The rik1Δ mutation allowed us to separate these two functions genetically, as Rik1 is required only for the latter. Hence, rik1Δ may provide a tool for studying the consequences of losing meiotic telomere clustering without the complication of simultaneous telomere fusion.

Materials and methods

Genetic procedures

Standard genetic procedures were performed as described previously (Gutz et al., 1974; Moreno et al., 1991). The mat1-M-specific RNA probe used for Southern blotting was transcribed from a 1016 bp BclI-TaqI fragment. Analysis of chromosome fusions was performed according to Ferrera and Cooper (2001). The swi6 deletion strain was obtained from H. Schmidt (Technical University of Braunschweig, Braunschweig, Germany), whereas strains carrying point mutations in the clr4 gene were obtained from G. Thon (University of Copenhagen, Copenhagen O, Denmark).

Cloning of the rik1Δ gene

A homothallic h² ura4-D18 rik1-304 strain was transformed with an S. pombe partial Sau3A genomic library (Welshy et al., 1991). Approximately 10,000 ura⁺ transformants were selected on sporulation medium and screened for rescue of the sporulation defect.

FISH, indirect immunofluorescence, and live analysis

Zygotes were prepared for indirect immunofluorescence using an anti-Sac1 antibody and FISH using a probe to the telomere-adjacent rDNA on chromosome III as described previously (Cooper et al., 1998). Images were captured on a microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) with an attached CCD camera (Hamamatsu). For live analysis, the endogenous taz1Δ gene was tagged at its COOH terminus using the Bahler gene-targeting method and plasmid pFA6a-GFP(S65T) (Bahler et al., 1998). Correct integration was confirmed by PCR and Western blot analysis. Taz1-GFP is fully functional, although strains harboring this allele show slight telomere elongation. Horsetail nuclei were visualized by Hoechst 33342 staining. Images were obtained using a Delta Vision microscopy system (Applied Precision, Inc.).

We thank Henning Schmidt and Genevieve Thon for strains, Yoshinori Watanabe for comments on the manuscript, and Judith Jaehning, Paul Mege, and our lab members for discussions and support.

This work was supported by the Danish Cancer Society (O. Nielsen), the National Institutes of Health (C.T. Tuzon and J.P. Cooper), and Cancer Research UK (C.T. Tuzon and J.P. Cooper).

Submitted: 8 December 2004
Accepted: 13 May 2004

References


Table II. The clr4-G486D allele is dominant

<table>
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<th>Asci with four normal spores</th>
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Stable diploid strains of the indicated genotype were sporulated, and the frequency of ascis with aberrant size and number spores was scored by light microscopy as in Table I.


