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spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast

Junko Kanoh and Fuyuki Ishikawa

Telomeres are essential for genome integrity. scRap1 (*S. cerevisiae* Rap1) directly binds to telomeric DNA [1–3] and regulates telomere length and telomere position effect (TPE) [4–6] by recruiting two different groups of proteins to its RCT (Rap1 C-terminal) domain [7]. The first group, Rif1 and Rif2, regulates telomere length [8, 9]. The second group, Sir3 and Sir4 [10], is involved in heterochromatin formation [11–13]. On the other hand, human TRF1 and TRF2, as well as their fission yeast homolog, Taz1, directly bind to telomeric DNA [14–16] and negatively regulate telomere length [16–20]. Taz1 also plays important roles in TPE and meiosis [16, 20, 21]. Human Rap1, the ortholog of scRap1, negatively regulates telomere length and appears to be recruited to telomeres by interacting with TRF2 [7]. Here, we describe two novel fission yeast proteins, spRap1 (*S. pombe* Rap1) and spRif1 (*S. pombe* Rif1), which are orthologous to scRap1 and scRif1, respectively. spRap1 and spRif1 are independently recruited to telomeres by interacting with Taz1. The *rap1* mutant is severely defective in telomere length control, TPE, and telomere clustering toward the spindle pole body (SPB) at the premeiotic horsetail stage, indicating that spRap1 has critical roles in these telomere functions. The *rif1* mutant also shows some defects in telomere length control and meiosis. Our results indicate that Taz1 provides binding sites for telomere regulators, spRap1 and spRif1, which perform the essential telomere functions. This study establishes the similarity of telomere organization in fission yeast and humans.

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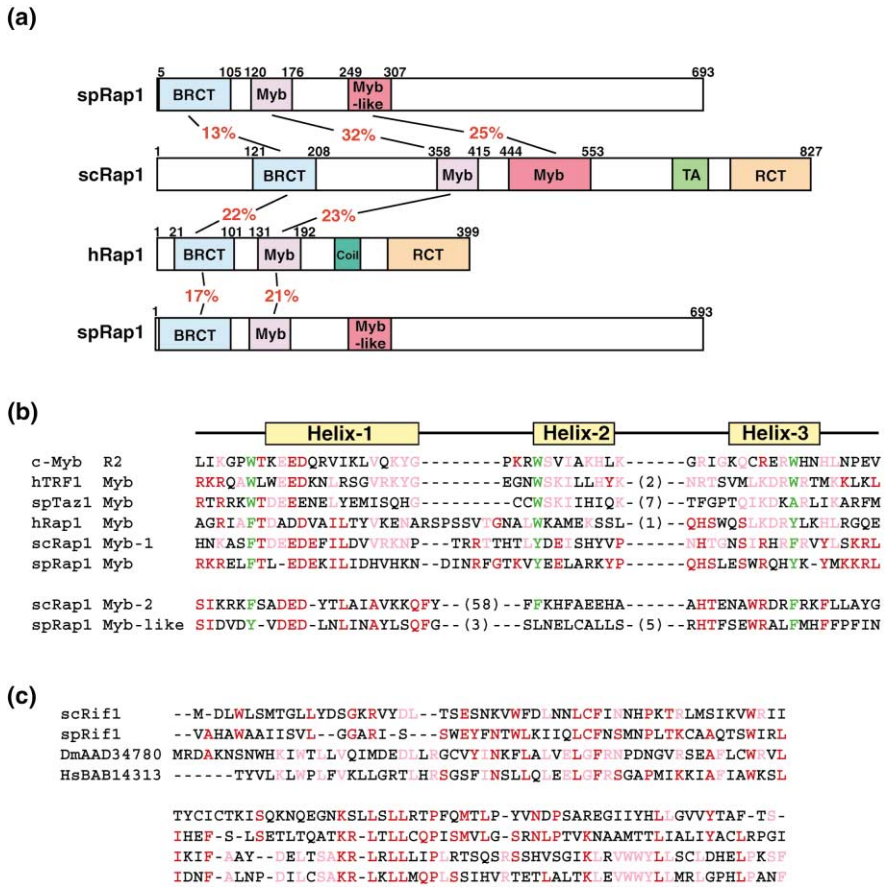
Results and discussion

To investigate how Taz1 executes its role at telomeres in fission yeast, we identified two novel genes, *rap1*$^+$ and *rif1*$^+$, in the *Schizosaccharomyces pombe* genome sequence database as homologs of scRap1 (SPBC1778.02) and scRif1 (SPAC6F6.17 and SPAPJ736.01), respectively. spRap1 has a BRCA1 C-terminal (BRCT) domain and a Myb motif (Figures 1a and 1b). The Myb motifs of spRap1, scRap1, and hRap1 constitute a subfamily with respect to the residues at the hydrophobic core of the 3 helix bundle [22, 23] and in the length of turns connecting the first and second helices. We also found a region of spRap1 showing similarity to the second Myb domain of scRap1 (spRap1 Myb-like and scRap1 Myb-2 in Figure 1b). The PREDATOR program [24] predicted that this region has a helix-turn-helix motif. However, the region's significance is not known at present. Although scRap1 and hRap1 have the Rap1 C-terminal (RCT) domain at their C termini [7], we could not find any in spRap1, suggesting that protein-protein interactions are mediated in a different manner in fission yeast. The predicted product of the *rif1*$^+$ gene shows a significant similarity to scRif1 throughout the entire sequence (20% identity; E value = $5 \times e^{-4}$; see Supplementary material). We also identified the *Drosophila* and human expressed-sequence tag (EST) sequences, the predicted peptide sequences of which showed significant similarities to the limited N-terminal region of spRif1 (Figure 1c).

To analyze the functions of spRap1 and spRif1 in telomere maintenance, we first examined the telomeric DNA length (Figure 2a). The *rif1* strain had telomeric DNA that was approximately 200 bp longer than that of the wild-type strain (lane 2). It is known that telomeric DNA is markedly elongated in the *taz1* strain [16, 20] (lane 3). The *rap1* strain showed a similar level of telomere elongation (lane 4). The *taz1 rif1* and *taz1 rap1* strains had telomere length similar to that of the *taz1* strain (lanes 5 and 6), suggesting that spRif1 and spRap1 regulate telomere length in a Taz1-dependent manner. Interestingly, the *rap1 rif1* double-disruptant strain showed more elongated telomeres than the *taz1* or *rap1* strain (lane 7). This result suggests that spRif1 and spRap1 have independent roles in telomere maintenance.

We next examined TPE by using the strain that has the *ura4*$^+$ gene inserted within the telomeric region [20] (Figure 2b). The wild-type and *rif1* cells had efficient TPE and hardly grew on a selective medium lacking uracil. The *taz1* cells grew well on the same medium, which confirms that Taz1 is essential for an efficient TPE,

Figure 1



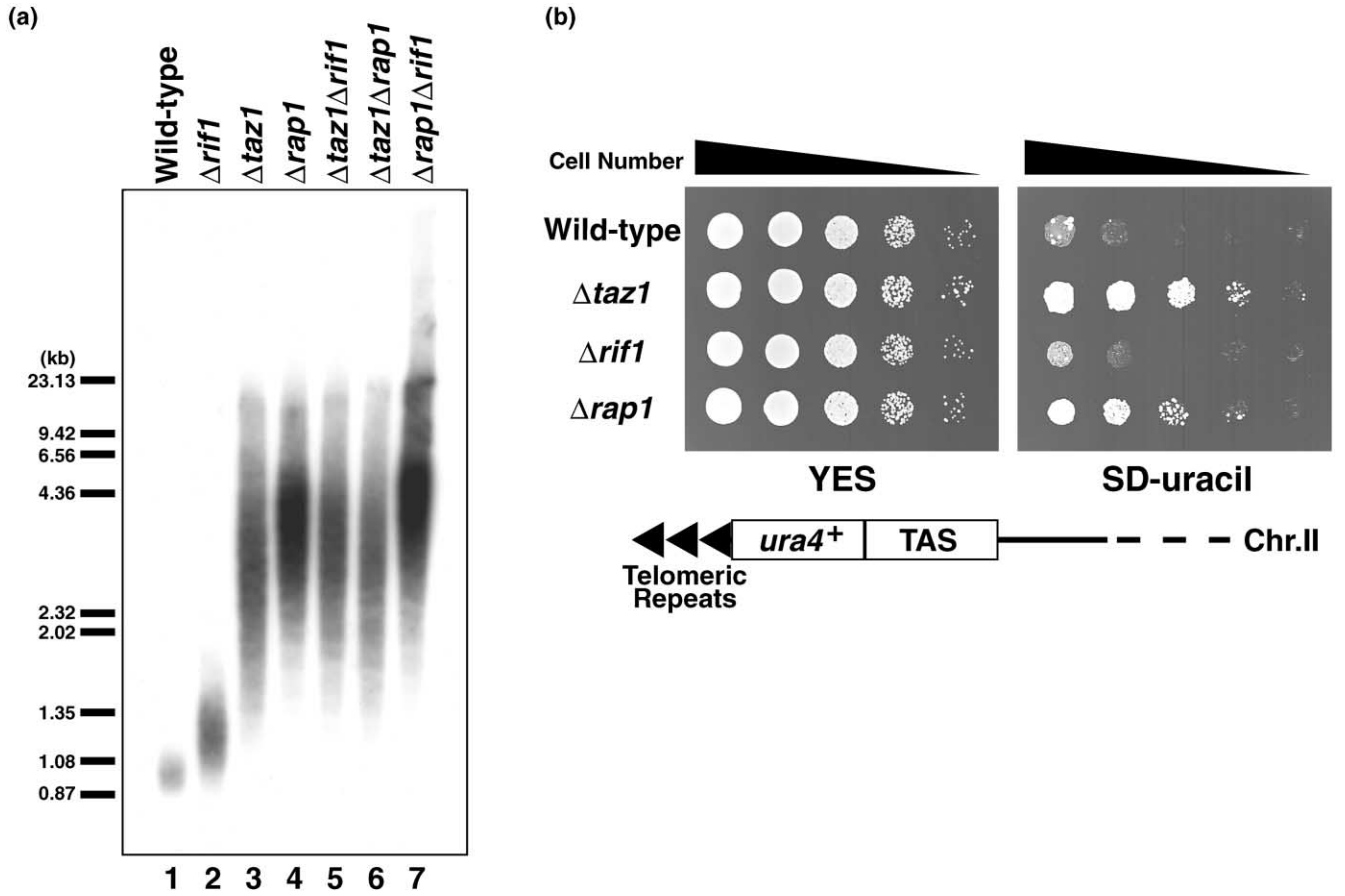
Structures of spRap1 and spRif1. **(a)** Overall structural similarities among spRap1, scRap1, and hRap1. Abbreviations are as follows: BRCT, BRCA1 C terminus; Myb, Myb-related HLH motif; TA, transactivation domain; RCT, Rap1 C terminus; Coil, putative coiled-coil motif. Percentage identities of amino acid sequences are shown for each domain. **(b)** Alignment of the Myb domains of Rap1 proteins and other proteins. The position of three α helices in human c-Myb are shown at the top [30]. The highly conserved residues forming hydrophobic cores are in green. Amino acids conserved in spRap1 are in red, and those conserved in other proteins but not in spRap1 are in pink. Abbreviations are as follows: c-Myb R2, the second Myb motif of human c-Myb; scRap1 Myb-1 and Myb-2, the first and second Myb motifs of scRap1; spRap1 Myb and Myb-like, the first and second Myb(-like) domains of spRap1. **(c)** Alignment of the N-terminal conserved sequences of scRif1, spRif1, the candidate *D. melanogaster* homolog (Dm, GenBank AAD34780), and the candidate *H. sapiens* homolog (Hs, GenBank BAB14313). Amino acids conserved in spRif1 are in red, and those conserved in other proteins but not in spRif1 are in pink.

as previously reported [16, 20]. The *rap1* cells also grew well on the selective medium, indicating that spRap1 is required for an efficient TPE. Both the *rap1* and *rif1* cells showed normal position effects on the *ade6*$^+$ gene inserted within the centromere (data not shown). Therefore, spRap1 has a role specifically in TPE.

We next examined the localization of spRap1 and spRif1 by the chromatin immunoprecipitation method (Figure 3a). Precipitated DNA was amplified by PCR with the primers for the telomeric region, the K-region, or *ade6*$^+$. The K-region is a heterochromatin present between the silent mating-type loci. Telomeric DNA was detected in the Taz1-HA precipitates, and the Taz1-telomere association was not significantly reduced in the *rif1* and *rap1* strains, indicating that Taz1 does not require spRap1 or spRif1 for its association with telomeres. Telomeric DNA, but neither the K-region nor *ade6*$^+$ DNA, was present in the spRap1-HA and spRif1-Myc precipitates. However, telomeric DNA was hardly detected in the *taz1* strain, indicating that spRap1 and spRif1 are specifically bound to telomeres in a Taz1-dependent manner. Furthermore, spRap1 associated with telomeric DNA in the absence of spRif1, and spRif1 did so in the absence of spRap1, indicating that spRap1 and spRif1 associate with telomeres independently.

Localization of Taz1-HA, spRap1-HA, and spRif1-Myc to telomeres was also examined by indirect immunofluorescence (IF) and fluorescence in situ hybridization (FISH) methods (Figure 3b). Taz1-HA was colocalized with telomeres in the wild-type, *rap1*, and *rif1* cells. In contrast, spRap1-HA was colocalized with telomeres in the wild-type and *rif1* cells but did not appear as discrete spots in most of the *taz1* cells, indicating that spRap1 is bound to telomeres in a Taz1-dependent manner. spRif1-Myc was localized heterogeneously throughout the nucleus in the wild-type and *taz1* cells, but it appeared as discrete spots that were colocalized with telomeres in the *rap1* cells. These observations suggest that only a small fraction of the spRif1-Myc molecules may be bound to telomeres in the wild-type cells. spRif1 may have some function that is not related to telomere maintenance, or the nucleoplasmic spRif1 may indirectly regulate telomeres.

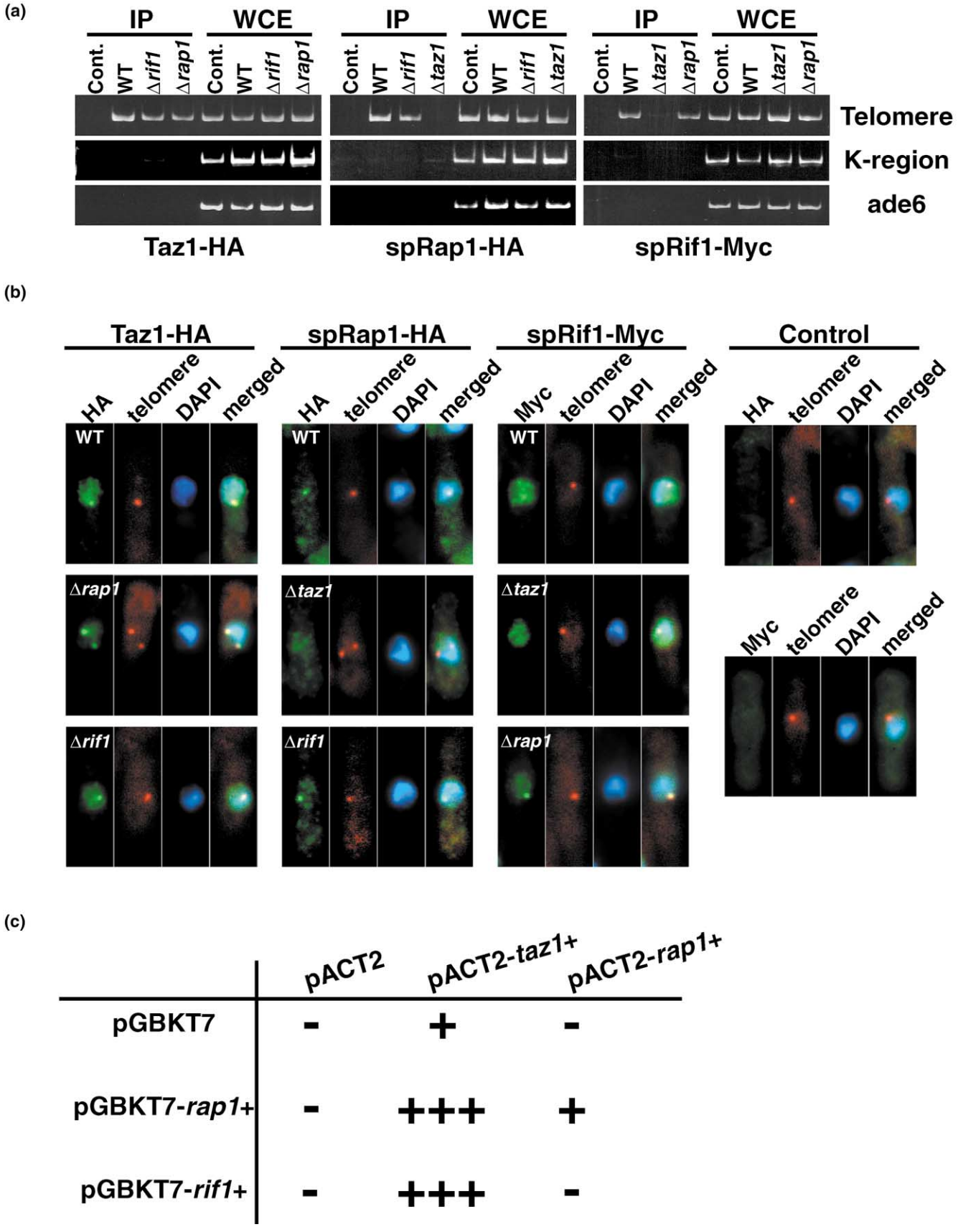
Figure 2



Phenotypes of Δ*rap1* and Δ*rif1* strains. **(a)** Telomere length in Δ*rap1* and Δ*rif1* strains. Genomic DNA of each strain was digested with EcoRI and subjected to Southern blotting. Approximately 300 bp of fission yeast telomeric repeats was used as a probe. Lane 1, wild-type (JK320); lane 2, Δ*rif1* (JK690); lane 3, Δ*taz1* (JK702); lane 4, Δ*rap1* (JK774); lane 5, Δ*taz1*Δ*rif1* (JK737); lane 6, Δ*taz1*Δ*rap1* (JK782); lane 7 Δ*rap1*Δ*rif1* (JK786). Note that telomere lengths shown here did not change after extensive subculturing. **(b)** Telomere position effects in Δ*rap1* and Δ*rif1* strains. Wild-type (FY1862), Δ*taz1* (JK703), Δ*rif1* (JK706), and Δ*rap1* (JK807) strains carrying the *ura4*$^+$ marker gene between the telomeric repeats and TAS (telomere-associated sequence) at the left arm of chromosome II were grown on a YES plate (complete) and an SD plate lacking uracil. Serial dilutions of the cells were spotted on each plate.

spRap1 and spRif1 are localized at telomeres by interacting with Taz1. **(a)** ChIP assay of Taz1, spRap1, and spRif1 bound to telomeric DNA. (Left) Wild-type strain (JK800, control) and Taz1-HA-integrated strains in a wild-type background (TN327), in Δ*rif1* (JK764), and in Δ*rap1* (JK801) were used. "IP" lanes are the PCR products after immunoprecipitation. "WCE" lanes are the PCR products from the whole-cell extracts. (Middle) Wild-type strain (JK800) and spRap1-HA-integrated strains in a wild-type background (JK768), in Δ*rif1* (JK772), and in Δ*taz1* (JK770) were used. (Right) Wild-type strain (JK800) and spRif1-Myc-integrated strains in a wild-type background (JK710), in Δ*taz1* (JK713), and in Δ*rap1* (JK805) were used. **(b)** Localization of Taz1-HA, spRap1-HA, and spRif1-Myc proteins. Strains used in panel (a) were grown in the EMM medium, fixed, and subjected to indirect IF and FISH analyses. Taz1-HA and spRap1-HA proteins were detected with anti-HA antibodies, and spRif1-Myc was detected with anti-Myc antibodies. The telomeres of chromosomes I and II were detected by hybridization with a Cy3-labeled cos212 probe. DNA was stained with DAPI. Wild-type strain (JK800) was used for the negative control for the indirect IF. **(c)** spRap1 and spRif1 interact with Taz1 in a yeast two-hybrid system. *Saccharomyces cerevisiae* Y190 strain was transformed with pACT2-*taz1*$^+$, pACT2-*rap1*$^+$, or pACT2 (GAL4-AD), together with pGBKT7-*rif1*$^+$, pGBKT7-*rap1*$^+$, or pGBKT7 (GAL4-DBD). Transformants were assayed for β-galactosidase activity. A hyphen (-) indicates no activity; a plus sign (+) indicates weak activity (the pale blue color of the cells could be observed after the long incubation); and three plus signs (+++) indicate strong activity (the intense blue color could be observed in 1 hr).

Figure 3


(a)
IP
WCE
Cont.
WT
Δrif1
Δrap1
Δtaz1
Telomere
K-region
ade6
Taz1-HA
spRap1-HA
spRif1-Myc
(b)
Taz1-HA
spRap1-HA
spRif1-Myc
Control
HA
Myc
telomere
DAPI
merged
WT
Δrap1
Δtaz1
Δrif1
(c)
pACT2
pACT2-taz1+
pACT2-rap1+
pGBKT7
pGBKT7-rap1+
pGBKT7-rif1+
-
+
+++

To examine the physical interactions among spRap1, spRif1, and Taz1, we carried out the two-hybrid assay in budding yeast (Figure 3c). Taz1 interacted with both spRap1 and spRif1. Importantly, spRif1 did not interact with spRap1, which is significantly different from the case of scRif1, which interacts with the RCT domain of scRap1. Furthermore, spRap1 appears to weakly interact with itself.

In fission yeast, telomeres cluster toward the spindle pole body (SPB) at the premeiotic horsetail stage [25, 26]. Telomere clustering facilitates the pairing of homologous chromosomes in meiosis I [27, 28]. We examined the relative localization of SPB and telomeres in the horsetail stage cells (Figure 4a). In all examined wild-type and *rif1* cells, all the telomere signals clustered adjacent to SPB. As previously reported, telomere clustering was impaired in the *taz1* cells [20, 21]. However, approximately 50% of the *taz1* cells showed at least one telomere signal localized near the SPB. In contrast, in most (90%) of the *rap1* cells, all the telomere signals were localized apart from the SPB. It is known that telomeres cluster at the nuclear periphery in mitotically growing cells [29]. Telomere clustering was apparently normal in the mitotic *rap1* and *rif1* cells (data not shown), indicating that spRap1 has a critical role in positioning telomeres near the SPB specifically at the premeiotic horsetail stage.

We next investigated the localization of Taz1 and spRap1 at the horsetail stage (Figure 4b). In the wild-type cells, Taz1-HA and spRap1-HA were colocalized with telomeres (rows 1 and 3). Taz1-HA was also colocalized with telomeres even in the *rap1* cells (row 2). In contrast, in approximately 70% of the *taz1* cells, the spRap1-HA signal was diffused (row 4). In the rest of the *taz1* cells, spot signals of spRap1-HA that colocalized with one of the telomeres could be observed (row 5). Notably, these telomere-spRap1-HA spot signals in the *taz1* cells were frequently colocalized with SPB (row 5). These results have two implications. First, spRap1 may have an intrinsic tendency to be localized near SPB. Second, spRap1 may bind to telomeres, although inefficiently, in a Taz1-independent manner.

We next examined whether the *rif1* and *rap1* strains produce normal spores (Figures 4c and 4d). The wild-type cells produced four spores with an equal amount of DNA per zygote (spore viability, 60%). As reported previously, the *taz1* cells produced spores in abnormal numbers and/or shapes with variable amounts of DNA (spore viability, 22%) [16, 20, 21]. Some *rif1* cells produced aberrant spores (spore viability, 30%). In the *rap1* cells, the number of spores per zygote was greatly reduced, and most of the *rap1* spores showed unevenly segregated DNA (spore viability, 9%). Therefore, we conclude that both spRif1 and spRap1 are involved in productive spore formation.

In summary, three telomere functions, namely, telomere length control, TPE and premeiotic telomere clustering toward SPB, were severely impaired in the *rap1* cells, in which Taz1 was still associated with telomeres. Therefore, it is suggested that telomere-associated Taz1 by itself cannot fulfill the telomere functions and that these telomere functions are accomplished for the most part by spRap1. Therefore, the primary role of Taz1 is to provide binding sites for telomere regulators. However, there still remains a possibility that spRap1 associates with telomeres in a Taz1-independent manner, as suggested from our observation (Figure 4b). This binding mechanism may function as a backup system only when the major spRap1-Taz1-telomere interaction is impaired. In fact, we found that the recombinant spRap1 protein did not show any activity of binding to telomeric DNA in the gel-shift assay (data not shown). spRap1 may have an intrinsic affinity for the SPB, and the SPB-telomere association at the horsetail stage may be mediated by an unidentified molecule(s) that is closely associated with the SPB. From the analogy of Rif and Sir proteins in budding yeast, it is speculated that spRap1 has associating molecules that are more directly involved in telomere length control and TPE (Figure 4e).

GenBank accession numbers

The GenBank accession numbers for the *rap1*$^+$ and *rif1*$^+$ cDNAs are AY034032 and AY034033, respectively.

Supplementary material

Supplementary materials and methods plus a supplementary figure can be found with this article on the internet at http://images.cellpress.com/supmat/supmatin.htm.

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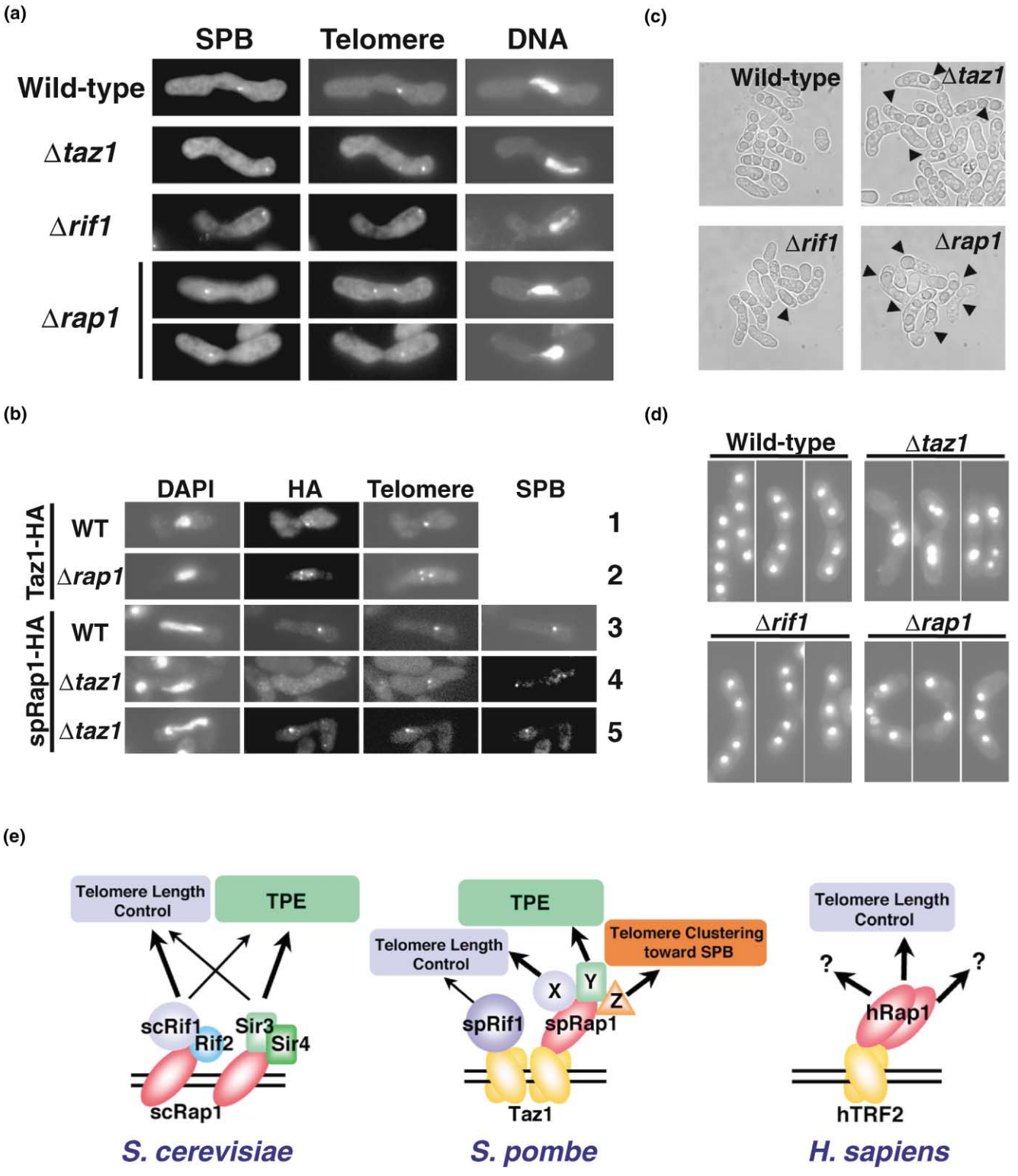
Δ*rap1* and Δ*rif1* strains are defective in meiosis. **(a)** The *rap1* disruptant has a defect in telomere clustering at the horsetail stage. We induced homothallic cells to initiate meiosis by incubating them on a MEA plate for 16 hr. Images of wild-type (JK800), Δ*taz1* (TN266), Δ*rif1* (JK698), and Δ*rap1* (JK778) cells are shown. **(b)** Localization of telomeres, SPB, Taz1-HA, and spRap1-HA in horsetail stage cells. Row 1 shows the wild-type strain expressing Taz1-HA (TN327); row 2 shows the Δ*rap1* strain expressing Taz1-HA (JK801); row 3 shows the wild-type strain expressing spRap1-HA (JK768); rows 4 and 5 show the Δ*taz1* strain expressing spRap1-HA (JK770). **(c)** Abnormal spore formation in the Δ*rap1* and Δ*rif1* strains. Sporulation was induced on a MEA plate for 48 hr. Arrowheads indicate abnormal spores. **(d)** Spores were stained with DAPI to examine the morphology of nuclei and DNA contents. Unevenly segregated DNA masses are observed in the Δ*rap1*, Δ*rif1*, and Δ*taz1* strains. **(e)** Model for the telomere binding proteins in eukaryotes. In budding yeast, scRap1 directly binds

Figure 4



(*continued from page 1628*)
to telomeric DNA. In fission yeast, spRap1 and spRif1 are bound to telomeres by interacting with Taz1. In this model, hypothetical spRap1-interacting proteins (X, Y, and Z) are assumed to play their specific roles at telomeres. Additional interactions not shown here would be required to explain why spRap1 deletion has more severe effects than Taz1 deletion in several assays. In humans, hRap1 is bound to telomeres by interacting with hTRF2.

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