Fission yeast Arp6 is required for telomere silencing, but functions independently of Swi6

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ABSTRACT

The actin-related proteins (Arps), which are subdivided into at least eight subfamilies, are conserved from yeast to humans. A member of the Arp6 subfamily in Drosophila, Arp4/Arp6, co-localizes with heterochromatin protein 1 (HP1) in pericentric heterochromatin. Fission yeast Schizosaccharomyces pombe possesses both an HP1 homolog and an Arp6 homolog. However, the function of S.pombe Arp6 has not been characterized yet. We found that deletion of arp6+ impaired telomere silencing, but did not affect centromere silencing. Chromatin immunoprecipitation assays revealed that Arp6 bound to the telomere region. However, unlike Drosophila Arp4/Arp6, S.pombe Arp6 was distributed throughout nuclei. The binding of Arp6 to telomere DNA was not affected by deletion of swi6+. Moreover, the binding of Swi6 to telomere ends was not affected by deletion of arp6+. These results suggest that Arp6 and Swi6 function independently at telomere ends. We propose that the Arp6-mediated repression mechanism works side by side with Swi6-based telomere silencing in S.pombe.

INTRODUCTION

Heterochromatin is a transcriptionally inactive region in the eukaryotic chromatin found near centromeres and telomeres (1). Heterochromatin is constituted by non-histone chromosomal proteins such as heterochromatin protein 1 (HP1) that are found in a variety of eukaryotic organisms ranging from Schizosaccharomyces pombe to humans (2). Drosophila HP1 binds to centric heterochromatin and telomeric regions and participates in chromatin packaging and gene silencing (3). The swi6+ gene of the fission yeast S.pombe encodes an HP1 homolog and is required for silencing at centromeres, telomeres and the silent mating-type loci (4–6). Recently, the chromo-domain in the HP1/Swi6 protein family was shown to interact with methylated lysine 9 of histone H3 (7–11).

The mechanisms of the transcriptional silencing at the silent mating-type loci and centromere have been well characterized in S.pombe (12–14). Mutations in several genes, including clr1+, clr2+, clr3+, clr4+, clr6+, swi6+, rik1+ and hdf1+, cause partial derepression at the centromere and silent mating-type loci (14). In S.pombe, histone deacetylases and methylases are thought to act in a sequential process. Histone deacetylases, Clr6 and Clr3, deacetylate histone H3 at lysine 9 and lysine 14, respectively, and then histone H3 is methylated at lysine 9 by Clr4 (11). Clr4 is a member of the SU(VAR)3-9 protein family, which includes human SUV39H1 and Drosophila SU(VAR)3-9. Members of this protein family contain the SET domain, which is required for methyltransferase activity (15,16). A mutation in rik1+ completely abolishes methylation of H3 at lysine 9 and localization of Swi6 at the centromere and mating-type loci (11,17).

Genes required for silencing at the centromere and silent mating-type loci, such as clr1+, clr2+, clr3+, clr4+, clr6+, swi6+, rik1+ and hdf1+, are also required for telomere silencing in S.pombe (14). However, additional trans-acting factors including taz1+, rap1+ and rtf1+ are required for silencing at telomeres. Mutations in any of these genes specifically affect telomeric silencing (18–20). Taz1 is a telomere-binding protein that is a member of the TRF family, which negatively regulates telomere length. Rap1 and Rif1 localize to telomeres through Taz1 (19,21). Schizosaccharomyces pombe spHst4, which is a Sir2 homolog, is also required for silencing at telomeres (22). In addition to spHst4, there are two Sir2 homologs, spSir2 and spHst2, in S.pombe (23). Recently the S.pombe Sir2 was reported to be required for silencing at the donor mating-type loci, telomeres and the inner centromeric repeats, suggesting that Sir2 plays a conserved role in heterochromatin assembly in eukaryotes (24).

Actin-related proteins (Arps) compose the actin family together with conventional actins, and Arps are classified into subfamilies according to their sequence similarities (25). In addition to conventional actins, at least eight Arp subfamilies are conserved from yeast to humans, and some of the Arp subfamilies have been implicated in nuclear organization and function (26). Saccharomyces cerevisiae Act3/Arp4, a member of the Arp4 subfamily, was the first Arp shown to be localized in the nucleus (27,28). ACT3/ARP4 is an essential gene that is involved in transcriptional regulation (27,29).

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Act3/Arp4 binds to core histones in vitro and is thought to recruit chromatin remodeling and histone acetyltransferase complexes onto chromatin (30,31). Act3/Arp4 is part of the Esa1-containing NuA4 HAT complex and is recruited specifically to DNA double-strand breaks that are generated in vivo (32,33).

Members of the Arp6 subfamily have been reported in budding yeast, fission yeast, Drosophila, chicken and humans (34); however, the function of the Arp6 subfamily has not been well characterized. Drosophila Arp4/Arp6 co-localizes with HP1 at the centric heterochromatin, suggesting a physical interaction between these two proteins (35,36). However, the role of Arp6 in heterochromatin remains unclear. Here we characterized the function of S. pombe Arp6 in heterochromatin. We tested whether Arp6 was involved in silencing at centromeres and telomeres. We also investigated the localization of Arp6 and examined its functional link to Swi6 and Taz1.

**MATERIALS AND METHODS**

**Schizosaccharomyces pombe strains, media and genetic methods**

The S. pombe strains used in this study are listed in Table 1. All strains are derivatives of JY746 h⁴ or FY1862. Standard procedures and media were used for propagation and genetic manipulation (37). YPAD medium consisted of 1% yeast extract, 2% polypeptone, 2% glucose and 20 μg/ml adenine. YE medium consisted of 0.5% yeast extract, 3% glucose and 2% polypeptone, 2% glucose and 20 μg/ml adenine. All experiments were repeated at least twice with similar results.

An arp6 knockout plasmid, pT7arp6ura4, was constructed as follows. A 2.9-kb fragment containing part of the arp6 ORF, which was amplified by PCR using genomic DNA and primers 1 (5'-GTAAGAGGAGCATTCTCC-3') and 2 (5'-TCTGTATGATTGATCCTTCT-3'), was subcloned into pT7Blue T-Vector giving the plasmid pT7arp6. Next the 1.8-kb ura4 gene was inserted into the BsmI site in pNTarp6, giving the knockout plasmid pT7arp6ura4. The arp6:ura4 fragment, which was amplified by PCR using primers 1 and 2, was used for transformation of haploid strain FY1862 by using the lithium acetate method (37). Stable transformants were isolated, and gene disruption was confirmed by PCR.

To tag Arp6 with the Myc epitope at the C-terminus, we amplified the arp6+ ORF by PCR with primers 3 (5'-GCTACCATATAAAGCTGG-3') and 4 (5'-CCCCGGG-GTATTCCTCTTTTGTGTC-3'); the Smal site is underlined) from wild-type genomic DNA, and cloned into the EcoRV site of pT7Blue T-Vector. Then the DNA fragment containing the arp6+ gene was digested with Smal and cloned into the plasmid pFAa-13Myc-kanMX6, which had been cut with SmaI and PvuII. pFAa-13Myc-kanMX6 plasmid, which contains 13 copies of the Myc epitope and a kanMX6 marker, was provided by John R. Pringle (University of North Carolina) (38). The resulting plasmid was linearized with PvuII, and used for transformation. Other double mutants were constructed by genetic crosses.

**Assay of silencing**

The transcriptional silencing at centromeres and telomeres was examined as described previously (20). Ten-fold serial dilutions of cells were spotted onto YPAD, low adenine (YE) or SD (synthetic medium) –histidine plates and incubated for 3 days at 30°C.

**RNA analysis**

Total RNA was prepared from wild-type cells (FY1862) and arp6Δ cells (TM001) by using an RNasy Mini kit (Qiagen). The amount of mRNA was quantitated by using LightCycler RNA Master SYBR Green I (Roche) with LightCycler Instrument (Roche). The following primers were used in RT–PCRs to amplify the His3 mRNA (top, 5'-TGC-TATACATAGAGATGCTATT-3'; bottom, 5'-TATAGTTTCTGGCAATACATACATATATT-3') and Eno1 mRNA (top, 5'-CCCGGGTTTAAAACTTAGCAGCCTT-3'; bottom, 5'-CTTTCAACGTCTTGAACG-3').

**Chromatin immunoprecipitation**

The chromatin immunoprecipitation (ChIP) assay described by Takahashi et al. (39) was adopted with modification. Cells grown in 100 ml of YPAD medium at 30°C were fixed with formaldehyde. For immunoprecipitation, anti-Swi6 antibody (40), anti-dimethyl-histone H3-K9 antibody (Upstate) or anti-Myc antibody (Cell Signaling Technology) and protein-G-coated dynabeads (Dynal) were used. Immunoprecipitated DNA was extracted and suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA). The following primers were used in PCRs.

### Table 1. Schizosaccharomyces pombe strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>JY746</td>
<td>h⁴ leu1-32 ura4-D18 ade6-M210</td>
<td>M. Yamamoto</td>
</tr>
<tr>
<td>FY1862</td>
<td>h⁰ leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:−:ade6 TAS-his3-tel 1(L) TAS-ura4-tel2(L)</td>
<td>R. Allshire</td>
</tr>
<tr>
<td>FY612</td>
<td>h⁰ leu1 ura4-D18 ade6-M216 swi6:GFP:kanMX6</td>
<td>Y. Watanabe</td>
</tr>
<tr>
<td>FY614</td>
<td>h⁰ leu1 ura4-D18 ade6-M216 swi6::kanMX6</td>
<td>Y. Watanabe</td>
</tr>
<tr>
<td>TM001</td>
<td>h⁰ leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:−:ade6 TAS-his3-tel 1(L) TAS-ura4-tel2(L)</td>
<td>This study</td>
</tr>
<tr>
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<td>h⁰ leu1-32 ura4-D18 ade6-M210 arp6::Myc:kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>TM003</td>
<td>h⁰ leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:−:ade6 TAS-his3-tel 1(L) TAS-ura4-tel2(L) arp6::ura4+</td>
<td>This study</td>
</tr>
<tr>
<td>TM004</td>
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<td>This study</td>
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<tr>
<td>TM005</td>
<td>h⁰ leu1 ura4-D18 ade6-M210 arp6::Myc:kanMX6 taz1::ura4+</td>
<td>This study</td>
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<tr>
<td>TM006</td>
<td>h⁰ leu1 his3-D1 ura4-D18 ade6-M210 TAS-ura4-tel2(L) arp6::ura4+ swi6::GFP:kanMX6</td>
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<tr>
<td>KT21-UHA</td>
<td>h⁰ leu1 ura4-D18 ade6-M210 otr1Rsp1:−:ade6 TAS-his3-tel 1(L) TAS-ura4-tel2(L) taz1::ura4+</td>
<td>This study</td>
</tr>
<tr>
<td>TK017</td>
<td>h⁰ leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:−:ade6 TAS-his3-tel 1(L) TAS-ura4-tel2(L) swi6::kanMX6</td>
<td>This study</td>
</tr>
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</table>
to amplify the telomere DNA (top, 5'-CGGCTGACGGTGTTGGCCACTA-3', bottom, 5'-GTGTGGAATTGAGTATGGTTGA-3'), subtelomere DNA (top, 5'-CTACTTTGTTGACTGCACCCCTAAACG-3', bottom, 5'-AAAGTGAGAAACTGGAAGAAGTAATC-3'), enol1+ DNA (top, 5'-TGCCCGGGTTTTAAAACAGCTTCTTGTTGAG-3', bottom, 5'-CTTGTTACACCTGTGGAGTAACG-3') and actl+ DNA (top, 5'-GGAATTCCCTACGTTGGTGATGA-3', bottom, 5'-GGAGGAGTTGACGACAGGT-3').

**Indirect immunofluorescence microscopy**

Indirect immunofluorescence microscopy was performed according to the protocol previously published by Caspari et al. (41) with the following change: Anti-Myc 9B11 monoclonal antibody (Cell Signaling Technology) was diluted 1:100. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Moleculer Probes) was used at a dilution of 1:2000.

**RESULTS**

**Arp6 is required for transcriptional silencing at telomeres, but not centromeres**

In *S. pombe*, one Arp6 homolog has been found in the genome database (34), but the function of this protein has not been studied. The *Drosophila* Arp6 subfamily colocalizes with HP1 at the centric heterochromatin (36). The *S. pombe* HP1 homolog, Swi6, is required for transcriptional silencing at both telomeres and centromeres (4–6). These facts prompted us to test whether *S. pombe* Arp6 was involved in transcriptional silencing at telomeres and centromeres. To study the in vivo function of *S. pombe* arp6+, we first made a heterozygous strain (*arp6+*) using a one-step gene replacement procedure, in which one of the chromosomal *arp6+* genes was replaced with a *ura4*+ cassette. Spores derived from the heterozygote were viable regardless of auxotrophy for uracil, indicating that *arp6* is not essential (data not shown). Deletion of *arp6*+ did not affect growth rate, suggesting that *arp6*+ is not required for mitotic growth (data not shown). Next we disrupted the *arp6*+ gene in the FY1862 strain and examined the transcriptional silencing at telomeres and centromeres. The FY1862 strain carries his3+ and *ura4*+ ~300 bp from the left telomeres of chromosomes I and II, respectively, and ade6+ within cen1 (20). Wild-type FY1862 cells did not grow on SD−histidine plates, while in contrast *arp6*Δ cells were able to grow on SD−histidine plates (Fig. 1A), indicating that Arp6 is required for telomere silencing. We also performed RT−PCR experiments and found that a significant amount of His3 mRNA was expressed in *arp6*Δ cells, but not in wild-type cells (Fig. 1B). This result further suggests that Arp6 is required for telomere silencing. We next examined silencing at centromeres. As centromere silencing is impaired in *swi6*Δ cells, the *ade6*+ gene inserted at the centromere was expressed. As a result, *swi6*Δ cells do not become red on low adenine (YE) plates (Fig. 1C). In contrast to *swi6*Δ cells, *arp6*Δ cells became red on low adenine plates, indicating that silencing at the centromere was normal in *arp6*Δ cells (Fig. 1C).

**Arp6 binds to telomeres independently of Swi6 and Taz1**

As Arp6 was required for telomere silencing, but not centromere silencing, we next tested the binding of Arp6 to telomeres by the ChIP assay. We tagged the C-terminus of Arp6 with Myc-tag (38). Telomere silencing was not affected by tagging of Arp6 with Myc-tag (data not shown). Anti-Myc antibody was used for immunoprecipitation and the precipitated DNA was amplified by PCR with primers for the telomeric region or ade6+as a control. Telomere DNA was significantly amplified in cells that expressed Myc-tagged Arp6 protein from their own *arp6* promoter (Fig. 2). These results indicate that Arp6 binds to telomere DNA. The binding of Arp6 to telomeres was not affected by deletion of *swi6*+ or *taz1*+ (Fig. 2). These results indicate that Arp6 binds to telomeres independently of Swi6 and Taz1.
Localization of Swi6 to telomeres is not affected in arp6Δ cells

We next examined whether the localization of Swi6 to telomeres was affected by deletion of arp6Δ by using the ChIP assay. Telomere DNA was immunoprecipitated in both the wild-type strain and arp6Δ cells when anti-Swi6 antibody was used for the immunoprecipitation (Fig. 3A). These results indicate that arp6Δ is dispensable for the binding of Swi6 to telomere DNA.

Swi6 interacts with methylated lysine 9 of histone H3. Thus, we next tested whether histone H3 at lysine 9 was still methylated in arp6Δ cells by using the ChIP assay. Figure 3B shows that subtelomere DNA (~2 kb away from telomere ends) was immunoprecipitated in both the wild-type strain and arp6Δ cells when anti-dimethyl-H3-K9 antibody was used for the immunoprecipitation. These results indicate that Arp6 is not involved in the modification of histone H3 at lysine 9.

Swi6 also localizes to the centromere and silent mating-type loci. Therefore, we examined the nuclear localization of Swi6-GFP in arp6Δ cells by using fluorescence microscopy (42). As reported previously (6), wild-type cells expressing Swi6-GFP fusion protein contained 2–4 foci at the nuclear periphery (Fig. 3C). These foci were also observed in arp6Δ cells, indicating that arp6Δ is dispensable for localization of Swi6 to the silenced heterochromatin (Fig. 3C).

DISCUSSION

Drosophila Arp4/Arp6 has been shown to play roles in heterochromatin. However, the exact roles of the Arp6 subfamily in chromatin organization have not been studied in detail. Here we found that S.pombe arp6Δ is required for transcriptional silencing at telomeres, but not at centromeres (Fig. 1). The binding of Arp6 to telomeres was not affected by deletion of swi6Δ (Fig. 2). Moreover, the localization of Swi6 to telomeres was not affected by deletion of arp6Δ (Fig. 3). These two facts suggest that Swi6 and Arp6 function independently at telomere ends. In Drosophila, a physical interaction between Arp4/Arp6 and HP1 has been suggested. Therefore, we examined the interaction between Swi6 and Arp6-Myc by co-immunoprecipitation assays. Although we could detect both Swi6 and Arp6-Myc using antibodies against Swi6 and Myc-tag, respectively, we could not detect an interaction between these two proteins by co-immunoprecipitation assays using soluble yeast cell extracts (data not shown). These data are consistent with our genetic data suggesting that Swi6 and Arp6 function independently.
Schizosaccharomyces pombe has another HP1 homolog, Chp2, and the chp2 mutant shows a larger silencing defect at telomeres than the swi6 mutant (43). Therefore, we examined the interaction between Chp2-myc and Arp6-TAP by co-immunoprecipitation assay. However, we could not detect an interaction between these two proteins (data not shown).

As telomere silencing is markedly affected by deletion of taz1+, we also examined a functional link between Taz1 and Arp6. However, the localization of Arp6 to telomeres was not affected by deletion of taz1+ (Fig. 2). Moreover, the localization pattern of Taz1-GFP was not affected by deletion of arp6+ (data not shown). These results suggest that Arp6 functions independently of Taz1. As Arp6 binds to telomere ends, it seemed possible that deletion of arp6+ might affect telomere length. However, the telomere length was not affected by deletion of arp6+ (data not shown). This finding suggests that deletion of arp6+ does not affect the localization of telomere-binding proteins such as Taz1, Rap1 and Rif1 that are required for both telomere length regulation and telomere silencing (18,19,21).

Our results clearly demonstrate that Arp6 is required for telomere silencing. However, how arp6+ is involved in telomere silencing is still unknown. In addition to Swi6-based telomere silencing, several factors, including Rad3 and Pof3, are involved in telomere silencing in S.pombe (44,45). Rad3 and Pof3 are also required for DNA repair and/or DNA damage checkpoint (44,46). However, unlike pof3Δ and rad3Δ cells, arp6Δ cells were not sensitive to a DNA-damaging agent (methylmethane sulfonate), suggesting that Arp6 is not required for DNA repair or DNA damage checkpoint (data not shown).

In summary, the data presented here indicate that an Arp6-mediated repression mechanism works side by side with Swi6-based telomere silencing in S.pombe. As the presence of the Arp6 subfamily is conserved from yeast to humans, it is tempting to speculate that the Arp6-mediated repression mechanism is conserved in other eukaryotes. Further studies of S.pombe Arp6 will help to clarify the mechanism of Swi6-independent telomere silencing in S.pombe, and possibly also in other eukaryotes.

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