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

Masaru Ueno*, Tadashi Murase, Tatsuya Kibe, Noriyuki Ohashi, Kazunori Tomita, Yota Murakami2, Masahiro Uritani, Takashi Ushimaru1 and Masahiko Harata3

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 in eukaryotes is transcriptionally inactive region in the euchromatic 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).

*To whom correspondence should be addressed. Tel: +81 54 238 4762; Fax: +81 54 237 3384; Email: scmueno@ipc.shizuoka.ac.jp

Nucleic Acids Research, Vol. 32 No. 2 © Oxford University Press 2004; all rights reserved
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 FY1862. Standard procedures and media were used for propagation and genetic manipulation (37). YPAD medium consisted of 1% yeast extract, 3% glucose, 2% polypeptone, 2% glucose and 20 g/ml adenine. YE medium consisted of 0.5% yeast extract, 2% polypeptone, 2% glucose. All procedures and media were used for propagation and genetic manipulation (37). YPAD medium consisted of 1% yeast extract, 3% glucose, 2% polypeptone, 2% glucose and 20 μg/ml adenine. YE medium consisted of 0.5% yeast extract, 3% glucose. 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′-GTAGAGGAGCCAATCC-3′) and 2 (5′-TCTGATATCATGATTCTC-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′-GCTTATATATATGAGTG-3′) and 4 (5′-CCCCGGG-TATTCCTCCTTTTCGTC-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 Smal 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 RNeasy 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′-TGGATACATATAGAGATGTATT-3′; bottom, 5′-TATAGTATTTCCTGCAATACAAAGTT-3′); the SmaI 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 Smal 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.

### 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>
</tr>
</thead>
<tbody>
<tr>
<td>JY746</td>
<td>h* leu1-32 ura4-D18 ade6-M210</td>
<td>M. Yamamoto</td>
</tr>
<tr>
<td>FY1862</td>
<td>h* leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:::ade6 TAS-his3-tel 1(L) TAS-ura4-\text{-}tel2(L)</td>
<td>R. Allshire</td>
</tr>
<tr>
<td>FY612</td>
<td>h* leu4 ura4-D18 ade6-M216 swi6:GFP:kanMX6</td>
<td>Y. Watanabe</td>
</tr>
<tr>
<td>FY614</td>
<td>h* leu4 ura4-D18 ade6-M216 swi6:kanMX6</td>
<td>Y. Watanabe</td>
</tr>
<tr>
<td>TM001</td>
<td>h* leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:::ade6 TAS-his3-\text{-}tel 1(L) TAS-ura4-\text{-}tel2(L) arp6::ura4*</td>
<td>This study</td>
</tr>
<tr>
<td>TM002</td>
<td>h* leu1-32 ura4-D18 ade6-M210 arp6::Myc:kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>TM003</td>
<td>h* leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:::ade6 TAS-his3-\text{-}tel 1(L) TAS-ura4-\text{-}tel2(L) arp6::Myc:kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>TM004</td>
<td>h* leu4 ura4-D18 ade6-M210 arp6::Myc:kanMX6 swi6:kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>TM005</td>
<td>h* leu4 ura4-D18 ade6-M210 3 days at 30° C</td>
<td>This study</td>
</tr>
<tr>
<td>TM006</td>
<td>h* leu4 his3-D1 ura4-D18 ade6-M210 TAS-ura4-\text{-}tel2(L) arp6::ura4* swi6:GFP:kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>KT21-UHA</td>
<td>h* leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:::ade6 TAS-his3-\text{-}tel 1(L) TAS-ura4-\text{-}tel2(L) taz1::ura4*</td>
<td>This study</td>
</tr>
<tr>
<td>TK017</td>
<td>h* leu1-32 his3-D1 ura4-D18 ade6-M210 otr1Rsp1:::ade6 TAS-his3-\text{-}tel 1(L) TAS-ura4-\text{-}tel2(L) swi6::kanMX6</td>
<td>This study</td>
</tr>
</tbody>
</table>
to amplify the telomere DNA (top, 5'-CGGCTGACG-GGTGGGGGCAATA-3', bottom, 5'-GTGTGGAATTGA-GTATGGTTGAA-3'), subtelomere DNA (top, 5'-CTACTT-ACTGCCACTTCATACG-3'; bottom, 5'-AAGTAGGAGAA-TGAAGAATTGAAT-3'), eno1+ DNA (top, 5'-TGCCCC-GGGTTCACAACTTAGCAGCGT-3'; bottom, 5'-CTTC-TCAACGCTTTGAAG-3') and act1+ DNA (top, 5'-GGA-TTCCTACGTGTTGTA-3'; bottom, 5'-GAGAGGAGAT-TGGACGAGCATT-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) (Molecular 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+ wild-type allele. Telomere silencing in wild-type (FY1862), arp6Δ (TM001) and taz1Δ (KT21-UHA) cells was studied. (B) Quantitative RT-PCR was performed on RNA prepared from wild-type (FY1862) and arp6Δ cells (TM001) using primers to amplify His3 mRNA and to amplify Enol1 mRNA as a control. The RT±PCR products, His3 cDNA (his3) and Enol1 cDNA (eno1), separated by agarose gel are shown. (C) Serial dilution assay on low adenine and non-selective (YPAD) plates. Centromere silencing in wild-type (FY1862), arp6Δ (TM001) and swi6Δ (TK017) was studied.

**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.
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 telomere DNA (~2 kb away from telomere ends) was immunoprecipitated in both the wild-type strain and arp6Δ cells. In contrast, the localization pattern of Swi6 in subtelomere DNA was not affected by deletion of arp6Δ. Cells expressing swi6Δ-GFP in the wild-type strain (PY612) or in arp6Δ cells (TM006) were fixed with methanol and stained with DAPI. The positions of Swi6-GFP are indicated by arrows.

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.

S.cerevisiae Arp6 is distributed throughout the nucleus, when Arp6-GFP was expressed from the GAL1 promoter (26). Therefore, we examined the cellular localization of Arp6-Myc by using indirect immunofluorescence microscopy. As shown in Figure 4, the localization of Arp6 coincided with the nuclear 4′,6′-diamino-2-phenylindole (DAPI) staining. This result indicates that the localization of Arp6 is not confined to the telomere, but rather is distributed throughout the nucleus. In Drosophila, the localization pattern of Arp4/Arp6 is altered in cells expressing mutant forms of HP1 (36). In contrast, the localization pattern of S.pombe Arp6 was not affected by deletion of swi6Δ (Fig. 4). This result further suggests that Swi6 and Arp6 function independently.

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.

ACKNOWLEDGEMENTS
We thank Elaine Nimmo and Robin Allshire for providing strains, Kohta Takahashi, Shigeaki Saitoh and Mitsuhito Yanagida for the ChIP assay protocol, Masayuki Yamamoto and Yosinori Watanabe for providing strains and John R. Pringle for providing plasmids. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan to M.U., and by a grant from the Yokohama City Collaboration of Regional Entities for the Advancement of Technological Excellence, JST, to M.U.

REFERENCES


