Sap1 is a replication-initiation factor essential for the assembly of pre-replicative complex in the fission yeast *Schizosaccharomyces pombe*

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A central step in the initiation of chromosomal DNA replication in eukaryotes is the assembly of pre-replicative complex (pre-RC) at late M and early G1 phase of the cell cycles. Since 1973, four proteins or protein complexes, including cell division control protein 6 (Cdc6)/Cdc18, minichromosome maintenance protein complex, origin recognition complex (ORC), and Cdt1, are known components of the pre-RC. Previously, we reported that a non-ORC protein binds to the essential element ∆9 of the *Schizosaccharomyces pombe* DNA-replication origin ARS3001. In this study, we identified that the non-ORC protein is Sap1. Like ORC, Sap1 binds to DNA origins during cell growth cycles. But unlike ORC, which binds to asymmetric AT-rich sequences through its nine AT-hook motifs, Sap1 preferentially binds to a DNA sequence of 5’-(A/T)n(G/C)(A/T)n(G/C)(A/T)n-3’ (n ≥ 1). We also found that Sap1 and ORC physically interact. We further demonstrated that Sap1 is required for the assembly of the pre-RC because of its essential role in recruiting Cdc18 to DNA origins. Thus, we conclude that Sap1 is a replication-initiation factor that directly participates in the assembly of the pre-RC. DNA-replication origins in fission yeast are defined by possessing two essential elements with one bound by ORC and the other by Sap1.

One of the central questions in eukaryotic DNA replication initiation is how pre-replicative complex (pre-RC) is assembled. The precise assembly of pre-RC, which is strictly regulated temporally and spatially, is critical to ensure that each replication origin fires once per S phase and each segment of chromosomal DNA is duplicated also once per cell division cycle. In the last 40 years, significant progress has been achieved in understanding the molecular mechanism of pre-RC assembly, and the progress is mainly reflected in the identification of four pre-RC components, Cdc6/Cdc18, minichromosome maintenance protein complex (MCM), origin recognition complex (ORC), and Cdt1, and delineation of the order of these proteins loaded onto DNA origins (1–7). However, some basic questions regarding pre-RC assembly still remain. For example, the molecular mechanism of pre-RC assembly in fission yeast and metazoans remains to be elucidated; the structures of DNA origins in fission yeast and metazoans remains to be determined; we do not know how cells in fission yeast to metazoans select replication-initiation sites and why DNA origins in these species are significantly larger than the budding yeast DNA origins; and the regulation of pre-RC assembly requires further study so that we have a better understanding of how cells avoid DNA re-replication within one cell cycle. All of these questions are long-standing ones in the field of eukaryotic DNA replication.

Pre-RC assembles at specific sites/DNA origins on chromosomal DNA, and these pre-RC sites act as DNA origins to initiate DNA synthesis with a different extent during S phase. The structures of DNA origins, as we have known, are not conserved from budding yeast to fission yeast and human cells. In the budding yeast *Saccharomyces cerevisiae*, origins have an average length of 100−150 bp, which includes an essential consen sus sequence called the A element and the non-conserved B region that is also important for origin activity (8–10). The A element is recognized and bound by ORC (3, 6). Together with

[5] The abbreviations used are: pre-RC, pre-replicative complex; MCM, minichromosome maintenance protein complex; ORC, origin recognition complex; EMM, Edinburgh minimal medium; TBZ, thiabendazole; HU, hydroxyurea; HSQC, heteronuclear single quantum coherence; IP, immunoprecipitation; ChiP-seq, DNA-chromatin immunoprecipitation for deep sequencing.

This article contains supplemental Tables 1 and 2 and Figs. 1–4.

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the A element, a part of the B region (close to the A element) is also used for assembly of pre-RC. In the fission yeast Schizosaccharomyces pombe, origin sizes range from ~500 to 1500 bp. Unlike in _S. cerevisiae_, a conserved sequence like the A element is not yet identified in _S. pombe_ origins, but they do contain two or more elements that are highly important or essential for origin activity. These elements often have highly asymmetric AT-rich sequences, with A in one strand and T in the other. _In vitro_ and _in vivo_ footprinting assays indicate that some of these elements are bound by _S. pombe_ ORC and are sites for pre-RC formation (11–15) and that other elements are associated with a non-ORC protein (12). Replication origins in metazoans are still not defined but appear similar to _S. pombe_ origins in certain ways. First, they also lack an apparent consensus sequence. Second, their sizes are also large (16). Third, some studies have shown that AT-rich sequences, as they are in _S. pombe_ origins, are also important for metazoan origin activity (17, 18). The nature of metazoan origins, the reason for their similarity to _S. pombe_ origins, and the reason why they are 5–10 times larger than _S. cerevisiae_ origins all remain elusive.

Because pre-RC assembles on DNA origins, the origin structure must be directly related to the mechanism of how pre-RC is assembled. DNA origins in fission yeast and metazoans are significantly larger and remarkably different from budding yeast origins. Thus, the mechanism for pre-RC assembly must be different in some aspects among these organisms. The autonomously replicating sequence ARS3001, an _S. pombe_ DNA-replication origin, contains two essential elements, Δ3 and Δ9 (19). Our previous studies of replication initiation at the _S. pombe_ origin, ARS3001, indicated that ORC binds mainly to the Δ3 element, one of the two essential elements in this origin, and assembles a pre-RC at the Δ2 and Δ3 sites. However, ARS3001 has an additional essential element, Δ9, that is bound by a non-ORC protein (12). Deleting the Δ9 element or increasing the distance between the Δ3 and Δ9 elements from the initial ~300 bp to 1.8 kb abolishes ARS3001 origin activity, indicating that the Δ9 region is an integral element of ARS3001 (12). To examine the function of the Δ9 element as well as the protein binding to Δ9 element, we isolated the Δ9 element-binding protein and identified it as _S. pombe_ Sap1 protein. Sap1 was initially identified as a protein bound to a sequence involved in mating-type switching (20). However, it has also been found to be essential for cell viability; thus, it must perform other function(s) essential for cell growth because mating-type switching is not an essential process for cell growth (21). In this study, we found that Sap1 binds to DNA origins and interacts with ORC. It was also found that Sap1 is essential for the transition from _G_1 to _S_ phase during cell-division cycles. We demonstrated that Sap1 directly participates in the assembly of pre-RC by its essential function in recruiting Cdc18 (the homologue of Cdc6 in _S. pombe_) to DNA origins. A genome-wide pre-RC and replication-initiation sites were determined at high resolution by a DNA-chromatin immunoprecipitation for deep sequencing (ChIP-seq) assay targeting ORC, Sap1, Cdc18, Cdt1, MCM, and RPA. Through comparison of DNA sequences in these pre-RC and replication-initiation sites, a relatively conserved sequence of 5′-(A/T)n(C/G)(A/T)n−10(G/C)(A/T)n−3′ (n ≥ 1) exists in _S. pombe_ DNA origins. This sequence is basically very similar to the Sap1-binding sequence of 5′-AAAATATTATTATTGGAAAA-3′ in the origin ARS3001 (12). A remarkable feature of this sequence is that there are two G:C pairs that bracket 9–10 A:T pairs. At the 5′- or 3′-flanking side of the two G:C pairs are one or more A:T pairs. We obtained the crystal structure of Sap1-DBD (DNA-binding domain) at a resolution of 1.04 Å. Further, we determined the biochemical interaction between Sap1 and DNA by resolving the structure of Sap1-DBD and DNA sequence of 5′-AAAAATATTATTATTGAAAA-3′ (the Sap1-bound sequence on origin ARS3001) by NMR. Thus, we conclude that Sap1 is a replication initiation protein and directly participates in the assembly of pre-RCs. The nature of DNA origins in fission yeast _S. pombe_ is defined by possessing two essential origin elements, one bound by ORC and the other by Sap1.

### Results

#### Identification of a new DNA-replication origin-binding protein

We previously found that a non-ORC protein binds to an essential sequence Δ9 in the origin ARS3001, because two DNase I-hypersensitive sites were detected in the Δ9 sequence (12). To identify the Δ9-binding protein, an EMSA was performed. As shown in Fig. 1A, a protein present in the chromatin extract of the wild-type _S. pombe_, _Sp-dk1_, bound to the Δ9 sequence but not to an unrelated DNA fragment obtained from pBluescript plasmid. The Δ9-binding protein was subsequently isolated by cation and anion-exchange chromatography, followed by selective Δ9-DNA affinity (Fig. 1B) and glycerol-gradient sedimentation (Fig. 1C). As shown in Fig. 1C (top), the Δ9-binding protein (indicated by the asterisk) was purified to apparent homogeneity (fractions 3–8). The distribution of the purified protein (top) and Δ9 DNA-binding activity (bottom) were coincident across the glycerol gradient. This protein has a molecular mass of ~30 kDa and was identified as Sap1 by mass spectrometry. Sap1 was localized selectively in the nucleus, and the majority of Sap1 is bound to chromatin based on analyses using GFP-tagged Sap1 and Western blotting (data not shown). The level of Sap1 on chromatin, similar to that of ORC, was constant throughout the cell cycle (Fig. 1D).

To determine the function of Sap1, we selected a temperature-sensitive (ts) mutant of Sap1, _Sp-dk1-sap1ts5_. _Sp-dk1-sap1ts5_ cells were able to grow at 26 °C but not at 36 °C (Fig. 1E). The length of the cell cycle in the mutant cells was ~30 min longer than _Sp-dk1_ at 26 °C, and some cells were elongated (Fig. 1F). At 36 °C, the _sap1ts5_ cells could grow for approximately two cell cycles (~6 h); subsequently, they stopped dividing and became elongated (Fig. 1F). DNA sequencing revealed that the _sap1ts5_ gene contained two point mutations that resulted in two amino acid changes (E178K and K187N) located in the coiled-coil region of Sap1 (22). To examine the effect of these two-point mutations on Sap1 function, we first assessed whether the association of Sap1 with chromatin was altered. Western blotting indicated that the total amounts of Sap1 in wild-type and mutant cells were equivalent at 26 and 36 °C but that the level of Sap1 associated with chromatin in mutant cells was reduced by ~30% at 26 °C and 10-fold at 36 °C (Fig. 1G). These results suggest that the association of Sap1 with chromatin may be required for its essential function in the cell growth cycle.
Using the sap1ts5 strain, we were able to confirm the in vivo binding of Sap1 to the Δ9 element but not to unrelated DNA in an EMSA. A and C, after anion- and cation ion-exchange chromatography, the Δ9-binding protein was further purified by Δ9 DNA-affinity chromatography (B) and glycerol-gradient sedimentation (C). The top panels in B and C show SDS-polyacrylamide gels stained with silver; the bottom panels show EMSAs used to detect the Δ9-binding protein. An asterisk in the top panel in C indicates the band corresponding to the Sap1 protein. D, the level of Sap1 associated with chromatin was constant during the cell growth cycle. The S. pombe strains JL197 ura4-D18cdc10-129 (G1), JL202 ura4-D18cdc22-M45 (S), and JL206 ura4-D18cdc25-22 (G2) were used to arrest cells at G1, S, and G2 phase, respectively. The cells were grown to an A595 = 0.3 at 26 °C and then shifted to 36.5 °C for an additional incubation of 3.5 h. To arrest cells at M phase, 100 μg/ml TBZ was added to the cell culture (A595 = 0.5), and the culture was incubated for an additional 3.5 h. Chromatin was prepared from these cells, and the amounts of Sap1 and Orc6 were analyzed by Western blotting. E, sap1ts5 mutant. The Sp-dk1 and Sp-dk1-sap1ts5 cells grown at 26 or 36 °C. Sp-dk1 and Sp-dk1-sap1ts5 cultures were first incubated in YE at 26 °C to A590 = 0.2 and then shifted to 36 °C for an additional 12 h. G, Sap1ts5 dissociated from chromatin at 36 °C. Sp-dk1 and Sp-dk1-sap1ts5 cultures grown to A590 = 0.2 at 26 °C were each split into two samples; one was incubated at 26 °C, and the other was incubated at 36 °C for an additional 5 h. Sap1 was detected by Western blotting. H, in vivo DNase I footprinting assay of the Δ9 region in Sp-dk1 and Sp-dk1-sap1ts5 cells. The cells were cultured as described in G. Lane 1, naked DNA; lanes 2 and 3, Sp-dk1 cells grown at 26 and 36 °C, respectively; lanes 4 and 5, Sp-dk1-sap1ts5 cells grown at 26 and 36 °C, respectively. *, the two hypersensitive DNase I sites.

Using the sap1ts5 strain, we were able to confirm the in vivo binding of Sap1 to the Δ9 element, because Sap1ts5 dissociates from chromatin at the restrictive temperature. Two hypersensitive DNase I sites were previously mapped to the Δ9 sequence (12). Therefore, an in vivo footprinting assay was performed to determine the binding of Sap1 to the Δ9. As shown in Fig. 1H, the two hypersensitive DNase I sites disappeared in Sp-dk1-sap1ts5 cells at 36 °C (lane 5), whereas they were readily detectable in Sp-dk1 cells at 26 and 36 °C and in Sp-dk1-sap1ts5 cells at 26 °C (lanes 2–4). This result confirms that Sap1 binds to the Δ9 element in vivo.

Sap1 binds to DNA regions/origins that are bound by ORC

To determine whether Sap1 binds to all DNA origins, ChIP-seq analysis was performed. As a positive control, genome-wide ORC binding sites were also examined with the same method.
The results presented in Fig. 2 give two conclusions: 1) Sap1 binds to DNA sequences that ORC associates with, indicating that Sap1 binds to DNA origins, and 2) Sap1 and ORC binding sites are overlapped across the entire genomic DNA, which suggests that Sap1 and ORC interact with one another after they bind to DNA origins. The interaction of Sap1 and ORC is further confirmed with reciprocal IPs and protein affinity chromatography. As shown in Fig. 2B, IP against Sap1 or the Orc1 subunit of ORC in chromatin extracts brought down Orc1 and Sap1, respectively. This indicates that Sap1 interacts with ORC in vivo. To examine Sap1 interacting with ORC directly, protein affinity chromatography was conducted with purified Sap1 and ORC. The result in Fig. 2C shows that the peak of ORC (Orc1) eluted from Sap1-conjugated agarose beads occurred at 0.3–0.4 M KCl, whereas ORC (Orc1) was eluted from the agarose beads at 0.1–0.15 M salt. This result indicates that Sap1 interacts with ORC directly.

We examined all of the ORC- and Sap1-binding sites and calculated that there were ~600 strong ORC/Sap1-binding sites (the height of binding peaks is 250; the size of the binding region is ~1 kb or more) and ~2000 medium-strength ORC/Sap1 binding sites (the peak height is 150–250; the size of the binding region is ~0.5 kb). These numbers are fairly close to the ~400 strong and ~500 weak origins previously identified in S. pombe (23). 50% of the 637 ORC binding sites determined with the ChIP-microarray assay by Hayashi et al. (24) are overlapped with the medium to strong ORC binding sites identified in this study; within the 0.5–2 kb range, the ORC binding sites identified by Hayashi et al. (24) and by us are overlapped. Some differences in identifying ORC binding sites are probably due to the different experimental approaches, ChIP-seq versus ChIP-microarray.

**Sap1 is required for the transition from G1 to S phase of the cell-division cycle**

The cell cycle of Sp-dk1-sap1ΔΔ cells is ~30 min longer than that of Sp-dk1 cells even at the permissive temperature of 26 °C. If Sap1 functions in DNA replication, a functionally defective
Sap1 would extend the length of S phase and increase the percentage of cells in this phase. Thus, the percentage of cells undergoing S phase was measured in an asynchronous population using BrdU incorporation and immunofluorescence at the permissive growth temperature of 26 °C. As shown in Fig. 3, there were 50, 42, and 46% more cells in S phase in the Sp-dk1-sap1ts5 cells compared with the Sp-dk1 cells after BrdU labeling for 5, 10, and 20 min, respectively. This result indicates that S-phase progression was affected by the Sap1 mutation in Sp-dk1-sap1ts5 cells.

Next, the stage at which cells arrest occurs was determined in the absence of Sap1. Two diploid strains with sap1−/ura4− and sap1+/− ura4−/+ genotypes were constructed. In S. pombe, both the sap1 and ura4 genes are located in chromosome III. For the diploid strain of sap1+/− ura4−/−, the sap1 gene is present in both copies of chromosome III, but the ura4 gene is deleted. In the diploid strain of sap1+/− ura4−/−, one copy of chromosome III harbors a normal sap1 gene, but the ura4 is gene absent; another copy of chromosome III has a normal ura4 gene, but the sap1 gene is deleted. Spores obtained from the two diploid strains were germinated in the appropriate medium with or without uracil and then examined by flow cytometry. As shown in Fig. 3C, the spores from the sap1+/+ strain germinated, replicated their DNA from 1C to 2C, and then went on to generate a population with normal cell growth cycles. In contrast, the spores with the sap1− genotype germinated, but the germinated spores could not initiate DNA synthesis, and the cells thus maintained a DNA content of 1C. The bottom panel in Fig. 3C shows the morphology of the germinated spores with or without the sap1 gene. Approximately 80% of the spores from the sap1+/+ ura4−/− strain germinated at 6–9 h after incubation and matured into healthy cells at ∼20 h. Fifty percent of the spores from the sap1− ura4−/+ strain had a genotype of sap1− ura4− and could not germinate in medium lacking uracil. Approximately 80% of the remaining spores (sap1− ura4−) also began to germinate after 6–9 h of incubation and grew into cells, some of which grew to longer than the...
normal size. This result confirms that Sap1 is required for the transition from G₁ to S phase of the cell cycle.

**DNA replication is inhibited in the sap¹ts⁵ cells**

The genome of YMF234 cells harbors several copies of the Cdc18 gene (the Cdc6 homologue in fission yeast) under the control of the nmt1 promoter (25). When Cdc18 is overexpressed in Edinburgh minimal medium (EMM) lacking thiamine, it causes cells to constantly reinitiate DNA replication within a cell cycle, which results in DNA re-replication and nuclear enlargement (25) (Fig. 4A). Thus, YMF234 cells provide a convenient experimental system to examine whether a protein functions in replication. It is expected that a functionally defective replication protein will affect the extent of re-replication and nuclear enlargement in YMF234 cells. To examine the function of Sap1 in replication, we constructed a YMF234-sap¹ts⁵ strain in which the wild type sap1 gene is replaced by the sap¹ts⁵ gene. The level of overexpressed Cdc18 was approximately the same in YMF234 and YMF234-sap¹ts⁵ cells in EMM at 26 °C (Fig. 5B), but the extent of DNA re-replication was significantly inhibited in YMF234-sap¹ts⁵ cells compared with YMF234 cells even at the permissive temperature of 26 °C, based on nuclear enlargement and DNA content measured by flow cytometry (Fig. 4, A and B). To examine whether DNA replication was further inhibited in YMF234-sap¹ts⁵ cells at 36 °C, the cells were grown at 26 °C for 12 h in EMM to allow the overexpression of Cdc18 (Cdc18 overexpression was maximal at 10–12 h after the cells were inoculated into EMM). At this point, the cells had not commenced DNA re-replication and had not elongated. The culture was then quickly shifted to 36 °C and incubated further. We found that almost all of the YMF234-sap¹ts⁵ cells were slightly elongated and able to grow in EMM for about 2 days (Fig. 4C). Subsequently, some of the elongated cells began to die, and the cells with a normal length began to accumulate (data not shown). In contrast, YMF234 cells were able to grow in EMM for only 12–15 h and then elongated and died. Flow cytometry indicated that DNA content in YMF234 cells was ~4- and 7-fold greater than that in YMF234-sap¹ts⁵ cells at 26 and 36 °C, respectively (Fig. 4, A–C, right panels). These 4- and 7-fold differences appear to underestimate the actual difference in DNA content between YMF234 and YMF234-sap¹ts⁵ cells at 26 and 36 °C because we found that DNA was significantly degraded particularly in YMF234 cells possessing very enlarged nuclei. These results indicate the following: 1) Cdc18 is a multicopy suppressor of the sap¹ts⁵ mutant, and 2) Sap1 functions in replication, because the partial loss of Sap¹ts⁵ function at 26 and 36 °C (the dissociation of Sap¹ts⁵ from chromatin) diminished Cdc18 overexpression-induced DNA re-replication.

**Replication initiation is significantly inhibited in Sp-dk1-sap¹ts⁵ cells at the restrictive temperature**

That Sap1 binds to DNA origins and Sap1 is required for the transition of G₁ to S phase and Cdc18 overexpression-induced DNA re-replication suggests that Sap1 functions at the replication initiation step. To demonstrate the initiation function of Sap1, the efficiency of replication initiation was examined on 30 origins in Sp-dk1 and Sp-dk1-sap¹ts⁵ cells at the permissive and restrictive temperatures of 26 and 36 °C. These 30 origins are located on chromosomes I, II, and III (23). As shown in Fig. 4D, the efficiency of replication initiation was inhibited by an average of 70% in Sp-dk1-sap¹ts⁵ cells compared with Sp-dk1 cells at 36 °C. In contrast, at 26 °C, the average inhibition was 7–10%. This result further indicates that Sap1 functions for replication initiation.

**Sap1 is required for recruiting Cdc18 to chromatin for the assembly of pre-RC**

To elucidate the molecular mechanism of how Sap1 functions in replication initiation, we examined the role of Sap1 in the assembly of pre-RC. We conducted this assay first in YMF234 cells when the cells were in S phase for constant pre-RC assembly and replication initiation under Cdc18 overexpression. YMF234 and YMF234-sap¹ts⁵ cells were grown in EMM at 26 °C until all of the cells just elongated and were in S phase for DNA re-replication (cell elongation was detected about 1 h after DNA re-replication started). The cultures were then divided into three fractions, which were incubated at 26, 32, or 36 °C for an additional 5 h. After the temperature shifted, all YMF234 and YMF234-sap¹ts⁵ cells continued to elongate and reached to a maximum length after 5 h of incubation. Flow cytometry analysis showed that all YMF234 and YMF234-sap¹ts⁵ cells were in S phase with greater than 2C DNA. Next, the assembly of pre-RC was examined by quantifying the amounts of Sap1, ORC, and Cdc18 associated with chromatin. We found that whole cellular amounts of Sap1 in YMF234 and YMF234-sap¹ts⁵ were approximately equivalent and did not change with different growth temperatures (Fig. 5A). However, the amount of Sap1 associated with chromatin was reduced by ~1.5-, 4-, and 10-fold in YMF234-sap¹ts⁵ cells compared with YMF234 cells at 26, 32, and 36 °C, respectively (Fig. 5A), which is consistent with the previous result (Fig. 1G). The levels of ORC (Orc6 subunit) present in YMF234 and YMF234-sap¹ts⁵ cells and associated with chromatin were approximately equivalent and were unaffected by growth temperature, which indicated that the binding of ORC to chromatin was not altered by Sap1 (Fig. 5A). In contrast to ORC, although the total amounts of Cdc18 in YMF234 and YMF234-sap¹ts⁵ cells were approximately equal, the levels of Cdc18 associated with chromatin were reduced by ~30%, 4-fold, and >10-fold in YMF234-sap¹ts⁵ cells compared with YMF234 cells at 26, 32, and 36 °C, respectively (Fig. 5B). It should be noted that most of the Cdc18 associated with chromatin was phosphorylated by CDK due to the activation of pre-RC (26, 27), which reduced its migration (Fig. 5B). This result indicates that Sap1 is required to recruit Cdc18 to chromatin for pre-RC assembly. Consistent with the reduced amount of Cdc18 associated with chromatin in YMF234-sap¹ts⁵ cells at 36 °C, the level of MCM associated with chromatin was also reduced by ~30% and 10-fold in YMF234-sap¹ts⁵ cells compared with YMF234 cells at 26 °C and 36 °C, respectively (Fig. 5C), confirming the requirement of Cdc18 for the recruitment of MCM to DNA.

The assembly of pre-RC normally occurs at the G₁ phase of a cell cycle. Thus, we also examined how pre-RC assembly is affected in a normal cell cycle when Sap1 function is defective. Sp-dk1 and Sp-dk1-sap¹ts⁵ cells were first arrested in G₂/M...
phase with thiabendazole (TBZ) and then released to G1 phase at 36 °C. The G1-phase cells were then lysed to obtain the chromatin and the cytoplasmic and nucleoplasmic fraction (see the legend of Fig. 5D). The amounts of the ORC, Sap1, Cdc18, and Cdt1 proteins in these fractions were measured by Western-blotting analysis. As shown in Fig. 5D, the total amounts of

Figure 4. Inhibition of DNA re-replication and replication initiation in sap1ΔΔ cells. A, enlargement of nuclei as a consequence of DNA re-replication in YMF234 cells. Early log phase cells grown in YE medium at 26 °C were inoculated into EMM for an additional incubation of 1–1.5 days. The cells were then collected for imaging and flow cytometry analysis. The arrows indicate swelling due to enlarged nuclei. The same result was obtained when YMF234 cells were grown at 36 °C. B and C, inhibition of DNA re-replication in YMF234-sap1ΔΔ cells. Early log-phase YMF234-sap1ΔΔ cells grown in YE medium were inoculated into EMM and incubated for 12 h at 26 °C (at this point, the Cdc18 protein was already overexpressed to its highest level, but the cells remained short and had not entered the DNA re-replication stage). The culture was then split into two samples; one sample was incubated at 26 °C, and the other sample was incubated at 36 °C for an additional 10–12 h. The cells were then collected for imaging and flow cytometry analysis. D, replication initiation is inhibited in SpΔΔ-sap1ΔΔ cells at 36 °C. Sp-dk1 and Sp-dk1-sap1ΔΔ cells were first arrested at G2/M phase with TBZ at 26 °C and then released and grown in fresh YE supplemented with 12.5 mM HU at 26 or 36 °C. Ten minutes after the septa disappeared, cell growth and DNA synthesis were immediately stopped by the addition of ethanol up to 70%. Genomic DNA was then prepared, and the amount of DNA at the indicated DNA origin was measured by quantitative PCR.
ORC, Sap1, Cdc18, and Cdt1 were approximately equal in Sp-dk1 and Sp-dk1-sap1<sup>ts5</sup> cells at 26 and 36 °C. However, at 36 °C (Fig. 5D, right), the amounts of Sap1 and Cdc18 associated with chromatin were reduced by ~5- and 4-fold, respectively, in Sp-dk1-sap1<sup>ts5</sup> cells compared with Sp-dk1 cells. In contrast to Cdc18, the loading of Cdt1 onto chromatin was not affected...
by Sap1 (Fig. 5D, right). The left panel shows the results obtained when the cells were grown at 26°C. The loading of Cdc18 onto chromatin was slightly reduced in Sp-dk1-sap1<sup>ts5</sup> cells, as was the binding of Sap1 to chromatin. In Sp-dk1-sap1<sup>ts5</sup> cells (Fig. 5D), as in YMF234-sap1<sup>ts5</sup> cells (Fig. 5A), the amount of ORC (Orc1 subunit) bound to chromatin was not reduced at either 26 or 36°C. Consistent with the inhibition of Cdc18 loading onto chromatin, the loading of MCM onto chromatin was also inhibited in Sp-dk1-sap1<sup>ts5</sup> cells. The amount of MCM associated with chromatin was reduced by ~4-fold at 36°C but was only slightly reduced at 26°C in Sp-dk1-sap1<sup>ts5</sup> cells (Fig. 5E).

The requirement of Sap1 for recruiting Cdc18 to DNA origins suggests a physical interaction between Sap1 and Cdc18. To confirm that, a reciprocal IP assay was performed. As shown in Fig. 5F, IPs using antibodies against Sap1 or Cdc18 pulled down Cdc18 and Sap1, respectively, indicating the physical interaction between the two proteins.

The genome-wide identification of pre-RC and replication-initiation sites

The sequence that is bound by Sap1 and creates two hypersensitive DNase I sites in DNA origin ARS3001 is 5'-Aaaa-CaatatTattGaaa-3' (The capital A and T bases are the hypersensitive DNase I sites) (Fig. 1H) (12). To determine whether a similar sequence is present in all S. pombe DNA origins, the genomic sites where pre-RC assemblies and DNA replication initiates were precisely identified by ChIP-seq. To identify pre-RC sites, ChIP-seq targeting Cdc18, Cdt1, or MCM (Mcm2) was conducted, respectively, with the cells at G<sub>1</sub> phase. Cdc18, Cdt1, and MCM are known pre-RC components that are recruited to DNA origins by ORC and Sap1 for assembly of pre-RC at late M and MCM are known pre-RC components that are recruited to DNA origins by ORC and Sap1 for assembly of pre-RC at late M and MCM are known pre-RC components that are recruited to DNA origins by ORC and Sap1 for assembly of pre-RC at late M and G<sub>1</sub> phase. To determine replication-initiation sites, ChIP-seq targeting RPA (Rpa1) was performed with the cells at the transition of G<sub>1</sub> to S phase. RPA is a single-stranded DNA binding protein, and it functions at replication forks. To ensure that RPA is cross-linked to replication forks in which replication just initiates, formaldehyde was added to cultures approximately 5 min before replication was initiated. As shown in Fig. 6, the genomic binding sites of Cdc18, Cdt1, and MCM overlapped nicely with previously identified ORC and Sap1 binding sites, indicating that pre-RC sites are identified accurately. This result also indicates that the previously identified ORC and Sap1 binding sites are indeed used for assembly of pre-RC. Further, these pre-RC sites are indeed used as replication-initiation sites because RPA association sites also overlapped well with these pre-RC sites (Fig. 6).

Next, approximately 200 of these pre-RC/repliation-initiation sites were examined. We found that there exist two types of sequence in the majority of pre-RC sites. One is a stretch of AT-rich sequence of ~10–30 bp. The other is 5’-(A/T)<sub>n</sub>(C/G)(A/T)<sub>n</sub>−10(G/C)(A/T)<sub>n</sub>−3’ (n ≥ 1), similar to the Sap1 binding sequence of 5’-Aaaa-CaatatTattGaaa-3’ in origin ARS3001. The AT-rich sequence should be the ORC-binding site (11, 13), and 5’-(A/T)<sub>n</sub>(C/G)(A/T)<sub>n</sub>−10(G/C)(A/T)<sub>n</sub>−3’ (n ≥ 1) should be bound by Sap1. The putative Sap1-binding sequence of 5’-(A/T)<sub>n</sub>(C/G)(A/T)<sub>n</sub>−10(G/C)(A/T)<sub>n</sub>−3’ has the following features: 1) it is also an AT-rich sequence; 2) there are two G:C pairs in the sequence, which distinguishes it from ORC-binding sequence; and 3) between the two G:C pairs are 9 or 10 AT base pairs. Sap1 is a face-to-face homodimer (22), and the two G:C base pairs may have a critical role in the interaction between Sap1 and DNA.

The crystal structure of the DNA binding domain in Sap1

To determine the biochemical basis of the interaction between Sap1 and 5’-(A/T)<sub>n</sub>(C/G)(A/T)<sub>n</sub>−10(G/C)(A/T)<sub>n</sub>−3’, we tried to obtain the Sap1-DNA crystal structure. Based on the restricted trypsin-digestion mapping of Sap1 domains (supplemental Fig. 1) and sequence analysis, there are three obvious domains in Sap1: the N-terminal domain (amino acids 1 to 133–138), the middle coiled-coil region (amino acids 133–138 to 181–196), and the C-terminal domain (amino acids 181–196 to 254) (Fig. 7A). This result is consistent with the previous structural study of Sap1 (22). A DNA band-shifting assay with the Ψ9 element of ARS3001 confirmed that the N-terminal domain of Sap1 is the DNA-binding domain in Sap1 (data not shown) (22). We were unable to obtain a crystal structure of Sap1 or the Sap1-DNA complex but were able to obtain a crystal structure of the DNA-binding domain of Sap1 (Sap1-DBD) that contains the N-terminal 136 amino acids of Sap1. Subsequently, the crystal structure of this domain was resolved at a resolution of 1.04 Å (Fig. 7B and supplemental Table 2). This DNA-binding domain consists of four α-helices (α1, residues 34–45; α2, residues 56–71; α3, residues 78–99; α4, residues 107–134) and three connecting loops (Fig. 7B, top left). The α2 and α3 helices, together with the connecting loop between them, constitute a typical helix-turn-helix DNA-binding motif. Helix 3 is probably the DNA recognition helix and contacts with the DNA major groove. The N-terminal region (residues 1–31) is highly flexible, and it is invisible in the electron density map.

The overall structure of Sap1-DBD is stabilized by a hydrophobic core composed of residues from all four helices (Fig. 7B, bottom left): Leu<sup>40</sup>, Met<sup>41</sup>, Val<sup>44</sup>, and Leu<sup>61</sup> in helix 1; Phe<sup>59</sup>, Tyr<sup>60</sup>, and Ile<sup>63</sup> in helix 2; Ile<sup>85</sup>, Ile<sup>86</sup>, Ile<sup>88</sup>, and Met<sup>89</sup> in the helix 3; and Leu<sup>112</sup>, Leu<sup>115</sup>, Trp<sup>119</sup>, and Leu<sup>126</sup> in the helix 4. Leu<sup>72</sup> in the N-terminal flexible loop and Leu<sup>73</sup> in the loop between helix 2 and helix 3 also participate in the formation of the hydrophobic core. The 4-hydroxyphenyl groups in Tyr<sup>60</sup> (helix 2), Tyr<sup>92</sup> (helix 3), and the indole ring of Trp<sup>119</sup> (helix 4) define the border between the hydrophobic core and solvent-contacting surface, besides their contribution in forming the hydrophobic core. All of the above residues are highly conserved in Sap1 proteins in fission yeast, which indicates that these hydrophobic interactions are critical in stabilizing the overall folding of Sap1-DBD. Besides the hydrophobic interactions, there are four salt bridges in this domain. As shown in the two right-hand panels of Fig. 7B, the salt bridge between Asp<sup>53</sup> and Arg<sup>130</sup> connects the helix 4 and the loop between helix 1 and helix 2; the salt bridge between Glu<sup>30</sup> and Lys<sup>62</sup> connects helix 1 and 2; Arg<sup>95</sup> in helix 3 interacts with Glu<sup>109</sup> and Asp<sup>113</sup> in helix 4; and the ionic interaction between Arg<sup>24</sup> in helix 1 and Glu<sup>111</sup> in helix 4 makes a fourth bridge. These ionic interactions should also contribute to stabilize this domain. Like a typical DNA-binding protein,
Sap1-DBD has a very asymmetric charge distribution with positive charges on one side and negative charges on the other (Fig. 7C).

The biochemical basis for the interaction between Sap1 and DNA

Next, we took an NMR approach to examine how Sap1 interacts with the DNA sequence of (A/T)n(C/G)(A/T)n G/C(A/T)n 3’ (supplemental Fig. 2 and Table 1). The NMR experiments were first performed on a protein-DNA complex using Sap1-DBD and a double-stranded DNA sequence of two complementary oligonucleotides 5’-C1A2A3A4A5C6A7-A8T9A10T11T12-3’ and 5’-A31A32T33A34T35T36G37T38T39-T40T41G42-3’ (the nucleotide numbering is based on the double-strand sequence as shown in Fig. 7E). This DNA sequence represents half of the Sap1 binding sequence in DNA origin ARS3001, 5’-CAAAAATATTTATTGAAAAA-3’. A reason for using half of the Sap1 dimer-binding DNA sequence is that Sap1-DBD is a monomer; another reason is that a structural study by NMR is relatively easier if a small complex is used. The C6 and G37 nucleotides represent one of the two consensus CG pairs in the sequence 5’-(A/T)n(C/G)(A/T)n G/C(A/T)n 3’-A8T9A10T11T12-3’. The interacting sites between Sap1-DBD and DNA were initially identified based on NMR chemical shift perturbations, and the structure of this complex was subsequently built up based on the crystal structure of Sap1-DBD together with experimentally measured intermolecular nuclear Overhauser effects (NOEs) between the protein and DNA.

Figure 6. Genome-wide identification of pre-RC and replication-initiation sites. For ChIP-seq assays targeting ORC, Sap1, Cdc18, Cdt1, MCM, and RPA, the genes of orc1, cdc18, cdt1, mcm2, and rpa1 were tagged by 3HA, respectively, in an S. pombe strain with a genetic background of cdc25ts. For determining ORC and Sap1 binding sites on chromatin DNA, unsynchronized cells were used. For determining pre-RC and replication-initiation sites, the cells were first arrested at G2/M phase by incubating the log phase of cdc25ts cells (A590 0.3, first incubated at 26 °C) for 3.5 h at 36.5 °C, and then temperature was quickly reduced to 26 °C, and cells continued to incubate at 26 °C for 35 min (the cells at G1 phase) and 70 min in the presence of 12.5 mM HU (the cells at early S phase). A preliminary assay suggested that the earliest time point for the presence of RPA in chromatin is 70 min after the cells were released from G2/M arrest. The following ChIP-seq assays were conducted routinely. A polyclonal antibody against Sap1 was used for the Sap1 ChIP-seq assay. Monoclonal HA antibody was used for the other protein ChIP-seq assay. Shown are the aligned binding or associating peaks of proteins ORC, Sap1, Cdc18, Cdt1, MCM, and RPA on 75 kb of chromatin DNA from chromosome I, II, and III.
The Sap1-DNA complex is relatively dynamic, which results in not all of the NMR cross-peaks being observable. The dynamic nature of this complex is in accordance with the fact that crystallization of the Sap1-DBD and DNA complex has so far been unsuccessful. Due to this reason, we were not able to obtain spectra with enough NMR signals (peaks) and thus not a sufficient number of NOE signals for every region of the complex. In particular, quite a number of signals were missing in the 112–119 region (helix 4), and assignments were incomplete for several hydrophobic residues (e.g. residues Leu\textsuperscript{112} and Leu\textsuperscript{115} in helix 4) that largely contribute to the core packing of the protein even in the spectra with the highest quality that we could obtain.

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acquire. Therefore, a de novo calculation of the Sap1-DBD-DNA complex structure with high precision could not be obtained solely based on the NMR data.

Fortunately, we were able to assign most of the residues/atoms located on the binding interfaces and identified a sufficient number of intermolecular NOE distance restraints.

**Figure 7. Interaction between Sap1 and a specific DNA sequence.** A and B, crystal structure of the Sap1 DNA-binding domain. A, a scheme of three domains in Sap1. The DNA-binding domain of Sap1 is located in the N-terminal 140 amino acids, four NM-XX repeat motifs are included in the C-terminal domain, and a long coiled-coil is located in the middle. B, crystal structure of the DNA binding domain of Sap1; the top left panel shows the location of four helices in this domain, the bottom left panel exhibits the amino acids that have hydrophobic interactions; and the right two panels exhibit the four salt bridges in this domain. C, Sap1 DNA binding domain has a very asymmetric surface charge distribution. Blue and red, positive and negative charges, respectively. D–H, the biochemical basis of the interaction between Sap1 and DNA. D, diagram of the structure model of Sap1-DBD-DNA complex from two sides with a 180° rotation. The structure model was calculated based on the crystal structure of Sap1-DBD domain, a B-form DNA model, and experimental NOE distance restraints from NMR data. The critical C6-G37 base pair is colored in red. Helix 3 in Sap1-DBD inserts into the major groove, while part of the N-terminal flexible loop (amino acid residues 1–31) resides at the immediately neighboring minor groove. The spatial locations of amino acid residues Arg29, Asn83, Thr30, Arg29, Ser87, Arg90, and Ile86 are indicated in the diagram. E, schematic representation of the interaction between the residues in Sap1 and the Sap1-binding DNA sequence in origin ARS3001. The dashed line indicates the interaction between amino acid residues Arg90, Asn83, Thr30, Arg29, Ser87, Arg90, and Ile86 are indicated in the diagram. F, model of two Sap1-DBDs binding to a conventional B-form DNA structure. The two consensus C-G pairs are colored in red and magenta. G and H, band shifting assays to examine the interaction between Sap1-DBD or Sap1 and the DNA sequence 5'-AAATATTTATTGAAAAG-3'. The oligonucleotides containing a methyl group on either the C5 position of underlined deoxycytidine (Me-dC) or the N2 position of underlined deoxyguanosine (Me-dG) were synthesized. After annealing, double-stranded oligonucleotides were purified using PAGE. The band-shifting assay was conducted according to a routine method. The amounts of oligo-DNA and Sap1-DBD or Sap1 in the reactions are indicated. The positions of shifted bands are indicated. I, the origin activity of ARS3001 was significantly reduced when the Sap1-binding sequence was mutated or the distance between the critical C and G bases was altered. The underlined AA, TA, TT, or TT bases were mutated to CC; the C or G bases was mutated to T; the position of TT deletion or insertion is indicated. The transformation frequency of wild type ARS3001 was assumed to be 100%. J, a scheme depicts the structure of DNA origins and the assembly of pre-RC in the budding yeast S. cerevisiae and the fission yeast S. pombe. In S. cerevisiae, DNA origins have two essential elements that are recognized and bound by ORC and Sap1, respectively. On DNA origins, ORC and Sap1 interact with each other to form a complex. At late M and G1 phase, S. cerevisiae ORC loads Cdc6 and Cdt1 to origins, and subsequently MCM is loaded onto origins for assembly of pre-RC. In S. pombe, both ORC and Sap1 are required to recruit Cdc18 to DNA origins, and Cdt1 is recruited by ORC alone. Similar to S. cerevisiae, the recruitment of MCM is executed by Cdc18 and Cdt1. Error bars, S.D.
between Sap1-DBD (e.g., residues Ala80, Ile86, Arg90, Val91) and DNA. By taking a similar approach used previously in the structural studies of protein-DNA complexes (28–30), we were able to reveal the interaction surface between Sap1-DBD and DNA based on the high-resolution crystal structure of Sap1-DBD and the NMR-derived intermolecular contacts. As expected, the helix α3 in Sap1-DBD fits into the major groove of the DNA duplex and forms the major binding site (Fig. 7D). Residues Ala80, Ile86, Arg90, and Val91 in α3 were observed to have close NOE contacts with the DNA molecule. For example, the Ala80 side chain packs with the sugar ring of A7 via hydrophobic and van der Waals interactions. The hydrophobic side chain of Ile86 interacts with the sugar ring of T33 and the base group of A34 and particularly packs with the methyl group in the base of T35. The hydrophobic region of the Arg90 side chain interacts with the methyl group of the T35 base, whereas the Val91 side chain interacts with A4-A5 backbone sugar rings. Inspection of the complex structure reveals that the terminal amino groups of the Arg90 side chain are in proximity with the N7 atom of A5 and the O4 atom of T36, both of which are hydrogen-bond acceptors and may form hydrogen bonds with the Arg90 side chain. In addition, Ser87 interacts with the sugar ring and the base of C6 via hydrophobic and polar interactions; Arg82 interacts with the A32-T33 backbone via electrostatic interactions; Glu85 side chain interacts with the A8 base via polar interactions and may also act as a hydrogen-bond acceptor for the amino group of A34. A schematic summary of these interactions is shown in Fig. 7E.

Furthermore, the Lys26-Thr30 segment in the flexible N terminus interacts with the adjacent minor groove and forms a minor binding site (Fig. 7, D and E). This segment contains several positively charged and polar residues and mainly interacts with the negatively charged backbones of nucleotides A5, T40, and T41, shown in blue dashed lines in Fig. 7E. Therefore, the major binding site formed by the helix α3 and the minor binding site formed by the Lys26-Thr30 segment fit into the major groove and minor groove, and they clamp the C6 nucleotide in the highly conserved C/G pair in the 5′-(A/T)9(C/G)(A/T)3–10(G/C)(A/T)3′-(Fig. 7D).

Next, we examined whether Sap1 (dimer) and Sap1-DBD (monomer) bind to the same kind of DNA sequence with a similar or identical mechanism. Thus, two more assays were conducted. First, we used an NMR analysis to examine whether the overall structure of the DNA-binding domain in Sap1 and Sap1-DBD is the same. The result shown in supplemental Fig. 3 indicates that the overall structure of the DNA binding domain in Sap1-DBD remains unchanged compared with that in Sap1 because the well-dispersed signals overlay well. Second, using a band-shifting assay, we directly examined the interaction between Sap1 or Sap1-DBD and the sequence 5′-CAATATTTATTGAAAAG-3′. As the above NMR analysis indicated, the G:C base pair that is underlined is critical for the interaction between Sap1-DBD and DNA. Therefore, we introduced a small chemical modification, a methyl group, on either the C5 position of deoxycytidine (Me-DC) or the N2 position of deoxyguanosine (Me-dG). The methyl group on the C5 posi-
tion of dC faces the major groove of DNA (31), and thus, it should affect the binding of Sap1-DBD to the sequence due to a steric hindrance. But the methyl group on the N2 position of dG faces the minor groove, and it should not affect Sap1-1DBD interacting with the sequence. The same assumption should also apply to Sap1 if Sap1 and Sap1-DBD bind to DNA with an identical biochemical mechanism. The results shown in Fig. 7 (G and H) show that 1) both Sap1-1DBD and Sap1 bound to the sequence; 2) the affinity of Sap1 to the sequence was about 3–4-fold higher than that of Sap1-DBD (Fig. 7G), which is expected because Sap1 possesses two DNA-binding domains; 3) at maximum, two Sap1-DBD molecules bound to the sequence, which is also expected because this DNA sequence contains two Sap1-DBD-binding sites (Fig. 7G); 4) when one G:C pair is methylated on the C5 position of deoxy-cytidine (Me-dC), only one Sap1-DBD molecule bound to the sequence (Sap1 also bound to the sequence, but its affinity to the sequence was reduced (Fig. 7G)); 5) when both G:C pairs are methylated on the C5 position of deoxycytidine (Me2-dC), the affinity of both Sap1-DBD and Sap1 to this sequence was further reduced (Fig. 7H); and 6) as expected, the methylation on the N2 position of deoxyguanosine (Me-dG) at the two G:C pairs did not affect the interaction between Sap1-DBD and Sap1 and the sequence (Fig. 7H). Thus, the band shift assay indicates that Sap1 and Sap1-DBD bind to the sequence 5’-CAAAA-CAATATTTATTGAAAAA-3’ with a similar biochemical mechanism.

The results obtained from the structural examination of Sap1-DBD-DNA complex by NMR suggest that the consensus C-G pair and its immediate 5’- and 3’-neighboring A-T pairs form the most intense contacts with Sap1, and these contacts may create an important foundation for Sap1 to bind to DNA (Fig. 7D). As addressed above, the amino groups in the side chain of residue Arg90 lie close to the A/T bases (A5 and T36) on the two sides of the C6-G37 pair, and hydrogen bonds could form between them. Although we were not able to directly manifest the existence of the hydrogen bonds, even when two distance restraints between Arg90 and A5/T36 nucleotides were added in the procedure of calculating the complex structure, it still shows that these two additional restraints are fully compatible with the existing experimental data and obtain the same structure of the complex except that the side chain of Arg90 becomes relatively fixed by simultaneously forming two hydrogen bonds with the A5 and T36 nucleotides. To further test whether the 5’-(A/T)(C/G)(A/T)-3’ trinucleotide sequence underlies the DNA sequence-recognition specificity of Sap1, we built four additional structural models with DNA sequences bearing mutations in these three sites using the 3D-DART web server (32). These structural models were aligned with the Sap1-DNAwt structure to see if these mutations would affect the interaction of Sap1 and DNA. As shown in supplemental
Fig. 4 (A and B), interchanging the C-G bases between two strands (DNA-m1) does not cause steric clashes or interfere with hydrogen bonds, suggesting that the binding of Sap1 to DNA is not affected. However, if the C-G base pair is changed to either A-T or T-A (DNA-m2 and m3), it results in steric clashes between the bulky methyl group of thymine and the protein, which would drastically destabilize the interaction (supplemental Fig. 4, C and D). On the other hand, interchanging the A/T nucleotides on the two sides of the C-G pair (DNA-m4) would not affect the hydrogen-bonding pattern, because the hydrogen acceptor now becomes the O4 atom of T5 and N7 atom of A36 (supplemental Fig. 4, E and F). Taken together, it appears that the 5'-H11032-(A/T)(C/G)(A/T)-3' sequence has a critical role in determining the binding of Sap1 to DNA.

Furthermore, the A-T base pairs located further away from the consensus C/G pair also have several interactions with Sap1, but they are mostly backbone interactions. Because Sap1 is a homodimer via the C-terminal coiled-coil region (22), the 9–10 A-T base pairs between the two C-G sites in the 5'-H11032-(A/T)n(C/G)(A/T)n-10(G/C)(A/T)n-3' sequence may offer optimal spacing for simultaneous interactions with two Sap1-DBDs from a Sap1 homodimer, as depicted in Fig. 7F. We speculate that if the A-T is changed to C-G among the spacing A-T base pairs, it would introduce an additional 5'-H11032-(A/T)(C/G)(A/T)-3' site preferable for the binding of one Sap1-DBD but would have incorrect spacing to the second 5'-H11032-(A/T)(C/G)(A/T)-3' site for binding with another Sap1-DBD, thus disrupting a proper interaction with the Sap1 dimer. In testing these ideas, we performed a series of mutations on the Sap1-binding sequence. As shown in Fig. 7I, when the AT bases were changed to CC, the critical C and G bases changed to T, or the spacing distance between the two critical GC base pairs was altered from 10 base pairs to 8 or 12 base pairs by deleting or inserting two T bases, the origin activity of ARS3001 was basically eliminated. These results are in support of the data from the NMR examination of Sap1 and DNA interaction.
Sap1 is an essential replication-initiation factor

Discussion

It has been speculated for a long time that an additional protein may bind to DNA origins and participate in pre-RC assembly in the fission yeast S. pombe. The speculation is based on the fact that DNA-replication origins in the fission yeast are 5–10 times larger than the replication origins in the budding yeast. In our previous study, the in vivo footprinting assays indicate that a non-ORC protein binds to the essential Δ9 element of the fission yeast origin ARS3001 (12). In this study, this non-ORC protein is identified as Sap1 protein. Subsequently, Sap1 is demonstrated to be a new pre-RC component. The major experimental evidence includes the findings that 1) ORC and Sap1 interact with each other and bind to the same DNA regions/origins across entire genomic DNA (Fig. 2); 2) Sap1 is required for the transition of G1 to S phase in cell-division cycles (Fig. 3); 3) when Sap1ts5 dissociates from chromatin at restrictive temperature, replication initiation is significantly inhibited in the 30 origins examined (Fig. 4D); 4) Cdc18 overexpression-induced DNA re-replication is significantly inhibited in the sap1ts5 cells at both permissive and restrictive temperatures (Fig. 4, A–C); 5) Sap1 is required for loading Cdc18 and subsequently MCM to DNA origins (Fig. 5); and 6) the biochemical interaction between Sap1 and DNA is determined, and Sap1 preferentially binds to a DNA sequence of 5’-(A/T)n(C/G)(A/T)n-10(G/C)(A/T)n-3’ (Fig. 7). The above evidence establishes that Sap1 is a replication-initiation protein that binds to DNA-replication origins and directly participates in pre-RC assembly.

In S. cerevisiae, an in vitro biochemical analysis indicates that incubating purified ORC, Cdc6, Cdt1, and MCM complex together with DNA can lead to loading MCM as a double hexamer onto DNA (33–35). This result shows that ORC, Cdc6, Cdt1, and MCM are sufficient for pre-RC assembly in the budding yeast. Thus, a question arises as to why an additional protein, Sap1, in the fission yeast is required for pre-RC assembly. The reason should be due to the fact that ORC interacts with DNA differently in the two yeasts. In S. pombe, the Orc4 subunit has an extra domain (~60 kDa) of nine AT-hook motifs located in its N-terminal portion, and these nine AT-hook motifs are solely responsible for ORC binding to AT-rich DNA sequences (11–13), whereas in S. cerevisiae, ORC uses its five subunits (except for Orc6) to contact with DNA (36). The different ORC/DNA interaction in the budding and the fission yeast results in the different mechanism to load Cdc6 and Cdc18 to DNA. In the budding yeast, ORC alone loads Cdc6 to the DNA origin, whereas in the fission yeast, an additional protein, Sap1, besides ORC, is required to load Cdc18 to DNA origins properly for subsequent loading of MCM to DNA origins by Cdc18 and Cdt1. It is found that Sap1 and ORC interact with one another to form a complex on DNA origins (Fig. 2). This interaction brings ORC and Sap1 together, which provides the molecular basis for their joint action in loading Cdc18 onto DNA origins (Fig. 7).

This study demonstrates that Sap1 is a second origin-recognition protein. Another origin-recognition protein is ORC. Thus, DNA origins in the fission yeast S. pombe are defined by possessing two essential origin elements; one is bound by ORC and the other by Sap1. S. pombe ORC binds to asymmetric AT-rich sequences (11–14, 37), whereas Sap1 binds to 5’-(A/T)n(C/G)(A/T)n-10(G/C)(A/T)n-3’ (n ≥ 1). Although the Sap1-binding sequences are also AT-rich sequences, they have a specific feature that sets them apart from the ORC-binding sequences. The Sap1-binding sequences contain two G:C base pairs that are critical for the interaction of Sap1 and DNA (Fig. 7), and the G:C pairs are separated by nine to ten AT base pairs. For S. pombe ORC, it preferentially binds to totally asymmetric AT sequence (11). By examining those pre-RC or replication-initiation sites identified in this study (Figs. 2 and 6), the average distance between a Sap1-binding site and an ORC-binding site is ~300–600 bp, which makes the size of S. pombe DNA origins in a range of ~500–800 bp. Therefore, the existence of a second origin element bound by Sap1 explains why S. pombe DNA origins are 5–10 times larger than S. cerevisiae DNA origins.

Experimental procedures

Purification of Sap1 protein and preparation of polyclonal antibodies against Sap1, Cdt1, Orc2, Cdc18, Cdc6, and Mcm2, among others

The His6-Sap1 protein was overexpressed in Escherichia coli at 26 °C. The cell extract containing Sap1 was first applied to an SP-Sepharose column, and Sap1 remained in the flow-through fraction. This fraction was directly applied to Q-Sepharose, and Sap1 was eluted with 0.25–0.30 M KCl. The fractions containing Sap1 were then applied to an Ni2+ column, and Sap1 was eluted with 250 mM imidazole. After dialysis to remove the imidazole, gel filtration was used to further purify Sap1. The purified His6-Sap1 was stored at −80 °C.

A polyclonal antibody against Sap1 was obtained by immunizing rabbits with purified recombinant Sap1 protein. Polyclonal antibodies against S. pombe Orc2, Orc6, Cdc18, and Cdt1, among others, were also obtained in this way. SpOrc1 in Sp-dk1 cells was tagged with 3HA, and the 3HA-Orc1 protein was able to function normally in cells.

Selection of a sap1mut

The sap1 gene was cloned into the pFA6a-kanMX6 plasmid to obtain pFA6a-kanMX6-sap1. The cells of the mutator strain XL1-Red (endA1 gyr96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (Tetr)) harboring pFA6a-kanMX6-sap1 were incubated for several generations to acquire random mutations in the sap1 gene. The sap1 gene was further randomly mutated with Tag DNA polymerase-catalyzed PCRs. The randomly mutated sap1 gene was then transformed into Sp-dk1 cells, and sap1 temperature-sensitive temperature-sensitive mutants were selected by growing transformed colonies at 26 and 36 °C. The sap1 gene in the putative sap1 temperature-sensitive mutants was further sequenced and verified.

Preparation of S. pombe chromatin for chromatin-bound protein assays

S. pombe cells were grown to log phase, collected, and resuspended in a 0.3% culture volume of lysis buffer (1.2 m sorbitol, 20 mM potassium phosphate, 5 mM DTT, 2 μg/ml each of aprotinin, leupeptin, pepstatin, and PMSF, pH 7.0) contain-
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ing 2 mg/ml each of lyticase (Cellgen Biolabs, Beijing, China) and lysing enzyme (Sigma). Next, the cells were incubated at 30 °C with gentle shaking. When >98% of the cells could be lysed with 0.8% Triton X-100, they were collected and washed once with lysis buffer and then resuspended in a 0.6% culture volume of buffer A (50 mM HEPES-HCl, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 5 mM DTT, 0.04% Nonidet P-40, 1 mM EDTA, 1 mM EGTA). The cells were lysed with 0.8% Triton X-100. Low-speed centrifugation was used to remove unlysed cells and cell debris. Chromatin was collected by high-speed centrifugation (30,000 × g, 12 min) and washed twice with buffer A. The supernatant contained a mixture of cytoplasm and nucleoplas.

In vivo footprinting assay

In vivo DNase I genomic footprinting assays investigating the association of Sap1 with chromatin were performed as described previously (12, 38) with one modification in Sp-dk1 and Sp-dk1-sap1ts5 cells grown at 36 °C. Sp-dk1 and Sp-dk1-sap1ts5 cells were grown in YE medium at 26 °C to A590 ~0.3, and then the cultures were shifted to 36 °C for an additional 3 h. The preparation of Sp-dk1 and Sp-dk1-sap1ts5 spheroplasts with lyticase and lysing enzyme was also performed at 36 °C to prevent the re-association of Sap1 with chromatin in Sp-dk1-sap1ts5 cells. DNase I digestion was conducted for a few minutes at room temperature (26 °C). All of the other footprinting assay conditions were the same as described previously (12).

Electrophoretic mobility-shift assays

The Δ9 DNA used in this assay was a fragment of 240 bp (5’-TCGTGCAGAAA...GAGTCAGAGA-3’) containing the 66-bp Δ9 element in ARS3001 (19). This fragment was radiola- beled at the 5’-end using T4 polynucleotide kinase. The 20-μl reaction mixture contained 5 ng of [32P]DNA, 0.5 μg of dG-dC DNA (an average length of 500 bp), 50 mM HEPES-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.5 mg/ml bovine serum albumin, 5 mM dithiothreitol, and the indicated amounts of chromatin extract. The reaction mixtures were incubated at room temperature for 10 min before fractionation by electrophoresis on a 1.5% agarose gel (Tris borate-EDTA buffer) for 3 h at 5 V/cm and 4 °C. The gel was then dried, and [32P]DNA was detected by autoradiography.

DNA-chromatin immunoprecipitation for deep sequencing (ChIP-seq) assays to determine the binding sites of S. pombe ORC and Sap1 on genomic were performed essentially as described previously (39) with some modifications. Briefly, Sp-dk1 cells were grown to log phase (A590 = 0.8) at 26 °C, and then 1% formaldehyde was added to cross-link the chromatin-bound proteins to DNA for 25 min. The formaldehyde-treated cells were collected, washed twice with water, and subjected to lycase and lysing enzyme treatment in a buffer containing 1.2 M sorbitol, 20 mM potassium phosphate (pH 6.8), 5 mM DTT, and 20 mM EDTA. When >98% cells could be lysed in 0.2% SDS, they were collected, washed with 1.2 M sorbitol, and lysed with PBS buffer containing 0.2% SDS. Chromatin was obtained by high-speed centrifugation and washed two times with PBS containing 0.2% SDS, followed by two washes with PBS. Chromatin was sheared to 150–350 bp with sonication and sub- jected to ChIP with monoclonal and polyclonal antibodies against 3HA-Orc1 and Sap1, respectively. The subsequent steps were similar to those described by Pidoux et al. (39). The obtained DNA fragments were sequenced.

Deletion of the sap1 gene and germination assay for sap1 spores

A copy of the sap1 gene (the entire ORF) was deleted from a diploid strain (ura4+ his3 leu2 adeM-210/206) and replaced with the ura4+ gene to obtain the sap1+/-/ura4+/- his3 leu2 adeM-210/206 diploid strain. The sap1+/-/ura4+/- and sap1+/-/ura4+/- diploid strains were first grown in YE and then plated on EMM for sporulation. Some of the unreleased spores were released by a brief digestion of the sporulated cells with lyticase. Concomitantly, the unsporulated cells were destroyed with lyticase. The spores were purified by glycerol- gradient centrifugation three times to remove any unsporulated cells. Any unsporulated cells remaining were killed by maintaining the spores in water for at least 1 week. The obtained sap1+ ura4+ his3 leu2 adeM-210/206 and sap1- ura4+ his3 leu2 adeM-210/206 spores were germinated in EMM + Leu + His supplemented with or without uracil.

Assay to evaluate the initiation efficiency of DNA replication

Sp-dk1 and Sp-dk1-sap1ts5 cells were grown in YE to A590 ~0.2 at 26 °C. The cultures were then supplemented with TBZ and shifted to 36 °C for an additional incubation of 3 h to arrest the cells in G2/M phase and inactivate Sap1ts5. The cells were then released from TBZ arrest and grown in fresh YE supplemented with 12.5 mM hydroxyurea (HU) at 36 °C. Ten minutes after the septa disappeared (~190 min of incubation after TBZ release), cell growth and DNA synthesis were immediately stopped by the addition of ethanol up to 70%. To examine the inhibition of replication initiation in Sp-dk1-sap1ts5 cells grown at 26 °C, Sp-dk1 and Sp-dk1-sap1ts5 cells were grown in YE to A590 ~0.3. The cultures were then supplemented with TBZ to arrest the cells at G2/M phase for 3.5 h. Subsequently, the cells were released from TBZ arrest and grown in fresh YE medium plus HU for 240 min (10 min after septa disappearance). Genomic DNA was prepared from these cells, and the amount of DNA at the indicated DNA origin region was measured by quantitative PCR. Equal amounts (50 ng) of genomic DNA from Sp-dk1 and Sp-dk1-sap1ts5 cells were used in all of the PCRs. The differences in the levels of PCR products formed with Sp-dk1 and Sp-dk1-sap1ts5 DNA were converted into a measurement of the efficiency of replication initiation inhibition. The inhibition rate of replication initiation was calculated by dividing the differences in the levels of PCR products formed with Sp-dk1 and Sp-dk1-sap1ts5 DNA by the amount of newly synthesized DNA in Sp-dk1 cells. The amount of newly synthesized DNA was calculated according to the efficiency of the specific origin as determined by Heichinger et al. (23).

Protein expression, purification, and oligo-DNA preparation

His6-tagged Sap1 or His6-Sap1-DBD was overexpressed in E. coli cells. Cell extracts containing Sap1 or Sap1-DBD were first applied to an Ni²⁺ column. The eluted His6-tagged Sap1 or His6- Sap1-DBD was cleaved with thrombin to remove the His6 tag.
Further purification steps were carried out using Resource S (GE Healthcare) cation exchange chromatography and Superdex200 (10/300 GL) (GE Healthcare) gel filtration. The final protein fractions were pooled, concentrated, and buffer-exchanged for subsequent crystallization. Sap1-DBD sample was buffer-exchanged to 10 mM HEPES (pH 7.2), 50 mM NaCl, 0.5 mM tris-(2-carboxyethyl)phosphine for crystal screen trials or concentrated to 0.7–1 mM and buffer-exchanged to 20 mM sodium phosphate (pH 6.0), 5 mM DTT for NMR.

Two complementary oligonucleotides, 5′-C₆A₉A₄₈A₃₄C₆₋₋₋₆A₈₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋Ngu thinner waist, a standard cell phone case, and a long-sleeved sweatshirt. The overall look is casual and comfortable, with a hint of urban streetwear style. The colors and fabrics chosen are versatile and easy to mix and match with other items in the wardrobe. The outfit is suitable for a variety of occasions, from casual outings with friends to more structured settings like office meetings. The combination of black and white creates a timeless and classic look that is always in style. The look is further enhanced by the addition of the bright orange details, which add a pop of color and make the outfit more interesting and eye-catching. The overall effect is a chic and stylish ensemble that is both fashionable and practical.
Sap1 is an essential replication-initiation factor

database potential of base-base positional interactions) for conventional B-form DNA were added (47). A total of 57 intermolecular NOE restraints were used in the final refinement. 200 structures were calculated, and the 20 lowest-energy conformers were selected as the representative structures. The complex structure showed a less-than-perfect clash score as presented in the validation report, which may arise from the fact that all protein backbone atoms in the helices were fixed during the simulated annealing procedure, whereas upon forming the complex, subtle structural adjustments may need to take place to circumvent these clashes.

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