The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1

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It is well established that acetylation of histone and nonhistone proteins is intimately linked to transcriptional activation. However, loss of acetyltransferase activity has also been shown to cause silencing defects, implicating acetylation in gene silencing. The something about silencing (Sas) 2 protein of Saccharomyces cerevisiae, a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60) acetyltransferase family, promotes silencing at HML and telomeres. Here we identify a ∼450-kD SAS complex containing Sas2p, Sas4p, and the tf2fl-related Sas5 protein. Mutations in the conserved acetyl-CoA binding motif of Sas2p are shown to disrupt the ability of Sas2p to mediate the silencing at HML and telomeres, providing evidence for an important role for the acetyltransferase activity of the SAS complex in silencing. Furthermore, the SAS complex is found to interact with chromatin assembly factor Asf1p, and asf1 mutants show silencing defects similar to mutants in the SAS complex. Thus, ASF1-dependent chromatin assembly may mediate the role of the SAS complex in silencing.

[Key Words: SAS; silencing; MYST; acetyl transferase; ASF1; chromatin assembly factor]

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Transcriptional silencing is a form of position-dependent and gene-independent transcriptional repression in which chromatin structure is altered over large regions of the genome. In Saccharomyces cerevisiae, silenced loci include the HML and HMR mating-type loci, the telomeres, and genes within the ribosomal DNA. Many gene products have been identified that contribute to silencing (Sherman and Pillus 1997; Lustig 1998). Silent information regulator [Sir] proteins 1–4 are well-studied factors that play important functions in transcriptional silencing (Cockell et al. 1998). Sir2p, Sir3p, and Sir4p are components of a multiprotein complex, and mutations in any of these genes result in the complete derepression of the silent mating and telomere loci. In contrast to mutations in SIR2, SIR3, or SIR4, mutations in SIR1 only lead to a partial derepression of the silent-mating loci. This is because in sir1 mutants, there is an epigenetic phenomenon in which some cells have fully silenced HM loci, whereas others have fully expressed loci (Pillus and Rine 1989). Telomeric silencing, however, appears to be independent of Sir1p (Aparicio et al. 1991).

In a yeast genetic screen conducted to identify enhancers of sir1 epigenetic silencing defects, something about silencing [SAS] 2 was identified (Reifsnyder et al. 1996). The genetic experiments showed that SAS2 has opposite regulatory effects, depending on the silenced locus. Sas2p promotes silencing at HML and telomeres but weakens it at an HMR locus with mutations in HMR-E silencer elements (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). Recently, the SAS4 and SAS5 genes were also isolated as negative regulators of silencing at HMR-containing defective HMR-E silencer elements (Xu et al. 1999a,b). SAS4 and SAS5 were also found to be positive regulators of silencing at HML and telomeres, and similar to SAS2, each gene interacts genetically with SIR1 in silencing at HML. Strains containing null alleles of all combinations of sas2, sas4, and sas5 were no more...
defective for HML silencing than any of the single deletion strains [Xu et al. 1999b]. These genetic experiments suggest that SAS2, SAS4, and SAS5 function in the same genetic pathway.

Sas2p is a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60) family of acetyltransferases. MYST-related proteins have been identified from yeast to humans and include the following: human MOZ [Borrow et al. 1996], MORF [Champagne et al. 1999], TIP60 [Yamamoto and Horikoshi 1997], and HBO1 [Iizuka and Stillman 1999]. Drosophila MOF [Smith et al. 2000], and yeast Sas2p [Reifsnyder et al. 1996], Sas3p [Takechi and Nakayama 1999; John et al. 2000], and Esa1p [Smith et al. 1998]. These MYST-related proteins show a high degree of sequence conservation in the acetyl-coenzyme A (acetyl-CoA) binding and zinc finger regions. Most MYST proteins have been shown to possess histone acetyltransferase (HAT) activity [Sterner and Berger 2000].

Substrate specificity of the MYST acetyltransferases has also been investigated. For instance, in addition to HAT activity, TIP60 possesses autoacetyltransferase activity [Creaven et al. 1999]. Though Sas2p and MOZ contain the highly conserved acetyl-CoA binding motif, the histone acetyl transferase activity and substrate specificity of these proteins have not been identified.

Many acetyltransferases are components of large multiprotein complexes in which associated subunits are often required for specific HAT activity and function in transcription [Howe et al. 1999; Brown et al. 2000]. The complexes containing Sas3p, Esa1p, dMOF, and hTIP60 have been purified and characterized [Allard et al. 1999; Ikura et al. 2000; John et al. 2000; Smith et al. 2000]. The human TIP60 complex contains additional subunits possessing ATPase, DNA helicase, and DNA-binding activities [Ikura et al. 2000]. Sas3p is the catalytic subunit of the HAT complex NuA3, which also contains the TBP-associated factor TAF130. Sas3p mediates the interaction between NuA3 and Spt16p, a component of the yeast CP complex [Cdc68/Pob3] that functions in transcription elongation and DNA replication [John et al. 2000]. NuA4 is ~1.3 MD in size, and five Esa1p-interacting subunits have been identified [Allard et al. 1999; Eisen et al. 2000; Galarneau et al. 2000]. Characterization of these subunits provides us with important information about the role of HAT complexes in gene expression.

Histone acetylation is important for the regulation of gene silencing. Deletion of GCN5 enhances telomere silencing [Sun and Hampsey 1999]. In contrast, deletion of SAS2 leads to derepression of HML and a telomere proximal reporter gene [Reifsnyder et al. 1996]. Mutations in HAT1, which encodes a subunit of a type B HAT, weaken telomeric silencing in the presence of certain histone tail mutations [Kelly et al. 2000]. Furthermore, loss of the Nat1p–Ard1p cytoplasmic N-terminal acetyltransferase leads to silencing defects [Mullen et al. 1989].

The significance of acetylation of histones and other proteins relevant to silencing remains unclear. Although SAS2 has been genetically associated with silencing, it is not known how the specific aspects of SAS2-mediated silencing are conferred. Here we identify and characterize the native Sas2p complex and investigate the effect of mutations in the Sas2p acetyl-CoA binding site on silencing in vivo. We also show that the Sas2p complex interacts with chromatin assembly factor Asf1p, and deletion of both ASF1 and SIR1 causes derepression of HML. Further, we show that mutations in ASF1, like those in SAS genes, improve silencing at mutated HMR loci.

Results

The native Sas2 complex is ~450 kD in size

To gain further insight into the function of Sas2p, we sought to investigate whether it was a component of a multiprotein complex. To determine the molecular size of the native Sas2p-containing complex, Sas2p was tagged at its C terminus with the 13-Myc epitope by homologous recombination at the SAS2 chromosomal locus. Whole cell extracts were prepared from a strain expressing Sas2p–Myc (YJW265) and separated by Superose 6 size exclusion chromatography. Fractions were monitored for the presence of Sas2p–Myc by Western blot analysis. Only one protein peak was detected, which eluted in fractions 26 to 30 and corresponded to a molecular mass of ~450 kD (Fig. 1, upper panel). To confirm these results, two other strains that expressed either a C-terminal 3-HA epitope tagged Sas2p (YJW214) or an

Figure 1. Detection of a 450-kD Sas2p-containing complex. Yeast extracts were separated by Superose 6 size exclusion chromatography, and fractions were analyzed for Sas2p by Western blot analysis (anti-Myc or anti-HA monoclonal antibodies). (Top panel), Superose 6-separated whole cell extracts prepared from strain YJW265 expressing Sas2p–Myc. (Middle panel) Whole cell extracts prepared from strain YJW214, expressing Sas2p–HA. The Sas2p–HA complexes were enriched by Mono Q anion-exchange chromatography before separation by Superose 6. (Bottom panel) Superose 6-separated whole cell extracts prepared from strain YJW213 expressing the GAL1-regulated HA–Sas2p. Arrows mark peak fractions for molecular weight standard proteins.
N-terminal 3-HA tagged Sas2p [YJW213] regulated by the GAL1 promoter were constructed. The Sas2p–HA complexes were enriched by Mono Q anion-exchange before separation by Superose 6 to improve detection of the native complex with the HA-antibody. Again, the Sas2p–HA signal eluted in fractions 26 to 30 as observed for Sas2p–Myc (Fig. 1, middle panel). Similarly, when extracts prepared from the GAL-regulated HA–Sas2 strain were separated by Superose 6, the HA–Sas2p also peaked in fractions 26 to 30 (Fig. 1, lower panel). These results indicate that the type and the position of the epitope tags do not affect the size of the Sas2 complex. Although the HA–Sas2p signal was spread out, this was likely caused by the overproduction of Sas2p by the strong GAL1 promoter. The results from the three chromosomally integrated Sas2p-tagged strains indicate that the size of the native Sas2 complex is ~450 kD.

Sas4p and Sas5p are components of the Sas2 complex

Genetic experiments have shown that SAS2, SAS4, and SAS5 are positive regulators of silencing at HML and telomeres and that the role of these SAS genes in silencing of HML is not redundant [Xu et al. 1999b]. These results suggest that Sas4p and Sas5p function in the same pathway as Sas2p. To investigate the size of the Sas4 and Sas5 complexes, C-terminal 13-Myc–tagged strains were generated (YJW228 and YJW229). Separation of whole cell extracts from the Sas4–Myc and Sas5–Myc strains by gel filtration showed that the Sas4p- and Sas5p-containing complexes are similar in size to the Sas2 complex (Fig. 2A). This raises the possibility that Sas2p, Sas4p, and Sas5p may in fact be components of the same complex. Accordingly, deletion of either SAS4 or SAS5 might alter the size of the Sas2 complex. To examine this, whole cell extracts from wild type and sas4Δ, sas5Δ, and sas4Δ sas5Δ strains expressing Sas2p–Myc were fractionated by Superdex 200 size exclusion chromatography (Fig. 2B). The Sas2p–Myc signal from the three deletion strains was more spread out and ran at significantly lower apparent molecular weights, although the Sas2 complex was present in all of the strains. The peak fractions of the Sas2p–Myc were shifted from fraction 25 to fractions 27, 26, and 27 by the deletion of the SAS4, SAS5, and SAS4/SAS5 genes, respectively. These results show that mutations in SAS4 and SAS5 alter the size or conformation of the Sas2 complex and suggest that Sas4p and Sas5p are components of the Sas2 complex.

To confirm the presence of Sas4p and Sas5p in the Sas2 complex, double-tagged yeast strains containing GAL-regulated HA–Sas2p and Sas4p–Myc or Sas5p–Myc were generated [YJW230 and YJW231]. Coimmunoprecipitation experiments were performed with whole cell extracts and peak Superose 6 fractions from the double-tagged strains grown in dextrose or galactose medium. Sas2p was immunoprecipitated with HA-antibody, and coprecipitation of Sas4p and Sas5p was monitored by anti-Myc Western analysis. As shown in Figure 3, Sas4p and Sas5p coprecipitated with Sas2p from both the whole cell extracts and the peak fractions of Superose 6 only when HA–Sas2 expression was induced with galactose. Control experiments performed with extracts and Superose 6 fractions prepared from strains grown in dextrose medium show that efficient coprecipitation of Sas4p and Sas5p was dependent on their interaction with Sas2p. The low level of Sas4p–Myc detected in the bead fraction when the strain was grown on dextrose is likely caused by leakiness of the GAL1 promoter. The cofractionation of Sas2p with Sas4p and Sas5p and the immunoprecipitation experiments indicate that Sas4p and Sas5p are indeed components of the Sas2 complex. Thus, we named the Sas2 complex, which also contains Sas4p and Sas5p, simply the SAS complex.

Figure 2. Sas4p and Sas5p are components of the Sas2 complex. [A] Extracts were prepared from strains YJW265, YJW228, and YJW229 expressing the C-terminal Myc-tagged Sas2, Sas4, and Sas5 proteins, respectively. The sizes of the Sas2, Sas4, and Sas5 complexes were determined by analysis of elution profiles from Superose 6. [B] Comparison of the Sas2p–Myc elution profiles from Superdex 200 size exclusion chromatography following fractionation of whole cell extracts prepared from wild type [YJW265] and sas4Δ [YJW269], sas5Δ [YJW270], and sas4Δ sas5Δ [YJW271] mutant strains. Shown are Western blots of column fractions probed with Myc-antibodies.

Figure 3. Sas4p and Sas5p are components of the Sas2 complex. [A] Coimmunoprecipitation experiment with whole cell extracts and peak Superose 6 fractions from the double-tagged strains grown in dextrose or galactose medium. Sas2p was immunoprecipitated with HA-antibody, and coprecipitation of Sas4p and Sas5p was monitored by anti-Myc Western analysis. As shown in Figure 3, Sas4p and Sas5p coprecipitated with Sas2p from both the whole cell extracts and the peak fractions of Superose 6 only when HA–Sas2 expression was induced with galactose. Control experiments performed with extracts and Superose 6 fractions prepared from strains grown in dextrose medium show that efficient coprecipitation of Sas4p and Sas5p was dependent on their interaction with Sas2p. The low level of Sas4p–Myc detected in the bead fraction when the strain was grown on dextrose is likely caused by leakiness of the GAL1 promoter. The cofractionation of Sas2p with Sas4p and Sas5p and the immunoprecipitation experiments indicate that Sas4p and Sas5p are indeed components of the Sas2 complex. Thus, we named the Sas2 complex, which also contains Sas4p and Sas5p, simply the SAS complex.

GENES & DEVELOPMENT 3157
Osada et al.

Figure 3. Sas4p and Sas5p coimmunoprecipitate with Sas2p. Coimmunoprecipitation experiments were performed with both whole cell extracts (WCE) and Superose 6 fraction 28 prepared from double-tagged strains HA–Sas2p, Sas4p–Myc (YJW230) and HA–Sas2p, Sas5–Myc (YJW231; indicated at the bottom of the panel). Protein fractions were incubated with HA-antibodies, and 10% of input (in), 10% of supernatant (sup), and bead (be) fractions from the immunoprecipitates were probed for Myc-tagged proteins.

Purification and characterization of the SAS complex
To further characterize the SAS complex, it was purified to homogeneity. For this purpose, a double-tagged yeast strain harboring chromosomally integrated Sas4p–Myc and a plasmid expressing Sas2p tagged with an N-terminal 6xHis and a C-terminal Flag and regulated by the GAL10 promoter was used (YJW276). This SAS expression plasmid was also used for the mating assays and could fully complement the sas2 deletion (see Fig. 5B). The SAS complex was purified on the basis of the Sas4p–Myc signal, using the anti-Myc Western blot analysis as outlined in Figure 4A. The Flag affinity-purified SAS complex was fractionated by Superdex 200, and each fraction was analyzed by silver stain and Western blot analysis (Fig. 4B). The major Sas2p peak (fraction 23, 24) was analyzed by silver stain and Western blot analysis (Fig. 4B). The major Sas2p peak (fraction 23, 24) corresponds to a molecular mass of ~450 kD, similar to the complex characterized in Figures 1 and 2. Western blot analysis revealed that Sas4p–Myc (p100) was only found in the 450-kD Sas2 complex (Fig. 4B, middle panel). A significant portion of Sas2p was also detected at higher molecular weights (e.g., fraction 20). Whether this is a unique complex containing Sas2p or an artifact of overexpression of Sas2p is under investigation. As a control, we performed mock purification from whole cell extracts prepared from the wild-type parental strain containing an empty expression vector (YJW243). No protein bands were detected in the Flag eluate from the control strain by silver staining (data not shown).

Three major bands (p100, p72, and p30) cofractionated with the Sas2p (p40) peak fraction. To identify the co-eluting proteins, the p100, p72, p40, and p30 bands were excised and analyzed by mass spectrometry. Twelve, four, and three peptides obtained from p100, p40, and p30 corresponded to the Sas4p, Sas2p, and Sas5p, respectively (Fig. 4C). This finding confirmed that Sas2p, Sas4p, and Sas5p co-associate in a unique and distinct complex. Thirteen peptide sequences from p72 correspond to six different yeast proteins. Experiments are in progress to confirm which, if any, of these proteins are bona fide subunits of the SAS complex.

The conserved acetyl-CoA binding motif of Sas2p is required for HML and telomere silencing
The yeast Sas2, Sas3, and Esa1 proteins all show a significant degree of conservation in the MYST-related acetyl-CoA binding site (Fig. 5A). However, for some MYST proteins (including Sas2p), HAT activity has not been described yet. To test for Sas2p HAT activity, we performed HAT assays with the purified SAS complex. Partially and highly purified fractions of the SAS complex failed to acetylate free or nucleosomal histones in vitro (data not shown). Recombinant Sas2p could not be assayed because it was insoluble. It is possible that Sas2p might require other cofactors to acetylate histones or that it acetylates nonhistone proteins. Either possibility would suggest that the highly conserved acetyl-CoA binding motif in Sas2p should be essential for its function. Mutations within the predicted acetyl-CoA binding pocket in Sas3p (M1 429GYG to AAA; M2 426QR to AA) have been shown to greatly reduce HAT activity of the NuA3 complex (John et al. 2000). By comparison, a Sas3p (M3 434LM to AA) mutation in the hydrophobic pocket somewhat distal to the acetyl-CoA binding site did not affect NuA3 HAT activity (John et al. 2000). To investigate the role of the Sas2p putative acetyl-CoA binding site in vivo, we mutated conserved amino acids that are predicted to be important for acetyl-CoA binding and examined their effect on silencing at the silent mating-type loci and at a telomere. For this purpose, we constructed point mutants in SAS2, which corresponded to SAS3 mutants M1, M2, and M3 (Fig. 5A).

The SAS2 gene was originally isolated as an enhancer of the epigenetic silencing defects of yeast sir1 mutants. SAS2 also contributes to silencing at the telomeres (Reissnyder et al. 1996). The combined deletion of sir1 and sas2 genes causes a much more severe silencing defect at HML than does the deletion of sir1 alone. To investigate the relevance of the Sas2p putative acetyl-CoA binding site on HML silencing, wild-type and mutated SAS2 alleles were transformed into the sir1 sas2 double-mutant strain (Fig. 5B). We constructed three types of expression vectors. Two of them contain the native SAS2 promoter on either CEN or 2µ plasmids, and the other is regulated by the GAL10 promoter. As judged by mating assays, the sas2-M3 mutant restored HML silencing to wild-type levels when overexpressed and to intermediate levels when expressed from a CEN plasmid (Fig. 5B). However, mutant strains carrying the sas2-M1 mutation could no longer restore the mating-type silencing (Fig. 5B). Thus, residues within the acetyl-CoA binding domain (M1), not distal (M3), are absolutely required for the SAS2 silencing function. This result indicates that the con-
served acetyl-CoA binding motif in Sas2p is necessary for the HML silencing in vivo and strongly suggests that Sas2p possesses acetyltransferase activity in vivo.

Unexpectedly, the sas2-M2 mutant was also able to restore HML silencing (Fig. 5B). By analogy to the Esa1p crystal structure, residues corresponding to M1 and M2 interact with acetyl-CoA. The crystal structure of Esa1p/CoA indicates that the M1 mutation, which includes two Gly to Ala mutations, would likely disrupt the conformation of the α3 helix and loop observed to interact with acetyl-CoA [Yan et al. 2000]. In contrast, the M2 mutation residues are predicted to make modest interactions with acetyl-CoA. Removing side-chains of Esa1p Gln312 and Arg313 would be expected to weaken the Esa1p/CoA interactions only slightly. We therefore predict that the mutant Sas2-M2 might retain weak acetylation activity that is sufficient for restoring silencing. This evidence is also supported by telomere silencing experiments (described below).

It was formally possible that the sas2-M1 mutant did not rescue silencing because of a lack of protein expression or a failure to become incorporated into the SAS complex. To rule this out, the SAS complex was partially purified from strains [YJW275, 278–280] carrying wild-type and mutant His–Sas2p–Flag expression constructs. Anti-Flag Western blot of fractions from anion exchange and size exclusion columns indicated that the abundance and the size of the mutant SAS complexes were unchanged (data not shown).

To investigate the effect of the mutations in the con-
Figure 5. The conserved acetyl-CoA binding motif of Sas2p is required for HML and telomeric silencing. (A) Sequence comparison of acetyl-CoA binding motifs of Sas2p, Sas3p, and Esa1p. The positions of the mutated amino acid residues are shown. The residues of Esa1p involved in acetyl-CoA binding are indicated in gray, and underlined amino acids represent buried residues (Yan et al. 2000). (B) The effect of the SAS2 acetyl-CoA binding site mutations on silencing at HML was assayed by measuring mating efficiencies. Strains expressing wild-type and mutant alleles of SAS2 from CEN-based plasmids containing the SAS2 promoter (YJW281, YJW282, YJW283, YJW284, and YJW285) were patched onto plates lacking uracil and replica plated onto minimal medium containing 5-FOA. The silencing efficiency was measured by 5-fluoroorotic acid (5-FOA) sensitivity. 5-FOA is toxic to Ura+ cells. Lack of telomeric silencing, and thus expression of the URA3 gene, results in sensitivity to 5-FOA, whereas strains that repress URA3 transcription are resistant to 5-FOA. The wild-type strain containing the empty expression vector grew on 5-FOA plates because the URA3 gene was repressed. In contrast, the sas2 deletion strain was sensitive to 5-FOA, in agreement with a previous report by Reifsnyder et al. (1996). Although the plasmid containing the wild type and the innocuous mutant sas2-M3 gene fully complemented the deletion of SAS2, the sas2-M1 plasmid showed no detectable complementation activity and behaved like the empty vector. These results were obtained from both SIR1 wild-type and sir1 deletion strains. Interestingly, the mutant sas2-M2 showed only partial restoration of silencing in SIR1 cells but restored wild-type levels of silencing in sir1 deletion strains. This is surprising because Sir1p does not play a major role in the silencing of telomeric reporter genes (Aparicio et al. 1991). Perhaps deletion of SIR1 releases Sir2p, Sir3p, and Sir4p from the HM loci and thus improves telomeric silencing.

Taken together, the mating-type loci and telomere silencing experiments indicate that the conserved acetyl-CoA binding site in Sas2p is required for HML and telomeric silencing. The severity of the effect of different mutations correlates with their predicted effect on acetyl-CoA binding. These results suggest that the acetyltransferase activity of Sas2p is necessary for its function in the silencing in vivo.

Asf1p interacts with the SAS complex

Sas4p [amino acids 339-481] was found in a two-hybrid screen with chromatin assembly factor Asf1 (anti-silencing function 1; Sutton et al. 2001). ASF1 was originally identified as a gene that derepresses transcriptional silencing when overexpressed (Le et al. 1997; Singer et al. 1998). Drosophila ASF1 has been shown to be a component of the replication-dependent chromatin assembly factor (RCAF) complex (Tyler et al. 1999). Human Asf1p associates with acetylated histone H3 and H4, functioning as a histone chaperone for chromatin assembly in vitro (Munakata et al. 2000). To determine whether Asf1p interacts with Sas4p in vitro, GST pull-down assays were performed (Fig. 6A). 35S-labeled Sas4p interacted with GST–Asf1p but not with GST alone. By comparison, 35S-labeled Sas2p and Sas5p did not bind to GST–Asf1p. These results suggest that only Sas4p interacts with Asf1p in the absence of the other two subunits. To provide evidence that Asf1p interacts with Sas4p in vivo, immunoprecipitation experiments were performed (Fig. 6B). Whole cell extracts from strains expressing HA-tagged or nontagged Asf1p with Sas4–Myc protein were incubated with HA-antibody and precipitated. Immuno-
precipitated Sas4–Myc protein was only detected on the beads from the strain expressing HA-tagged Asf1p, indicating that Asf1p interacts with Sas4p at physiological levels. We have shown that Sas4p is a component of the SAS complex (Figs. 2–4). If Asf1p interacts with the SAS complex, Sas2p and Sas5p should also coprecipitate with Asf1p–HA. Immunoprecipitation experiments using Asf1p–HA and nontagged Asf1p showed that Asf1p–HA interacts with both the Sas2p–Myc and Sas5p–Myc [Fig. 6B]. To provide further support for the Asf1–SAS complex interaction, reciprocal immunoprecipitation experiments were performed [Fig. 6C]. For these experiments, a plasmid expressing Asf1p, containing a C-terminal Myc tag regulated by the native promoter, was transformed into strains harboring chromosomally integrated HA–Sas2p, HA–Sas4p, and HA–Sas5p [YJW458, YJW459, and YJW460]. As a control, Asf1p–Myc expression plasmid was transformed into the untagged strain [YJW457]. Extracts from strains expressing untagged Sas proteins or HA-tagged Sas proteins were precipitated with HA-antibody, and coprecipitation of Asf1p was detected by anti-Myc Western blot analysis. Consistent with our previous findings, all of HA-tagged Sas proteins coprecipitated with Asf1–HA (Fig. 6B,C). This indicates that only a fraction of Asf1p is associated with the SAS complex and is consistent with the fact that Asf1p is also found in other complexes [e.g., with Rad53] that function in processes independent of silenc-
ing [e.g., DNA repair; Le et al. 1997; Tyler et al. 1999; Emili et al. 2001; see below].

To rule out the possibility that the interaction between the SAS complex and ASF1 is mediated via DNA/chromatin, extracts were treated before immunoprecipitation with either ethidium bromide (EtBr) or micrococcal nuclease (MNase). These reagents disrupt DNA-protein interaction by either distorting DNA structure by intercalation (EtBr) or by degrading DNA (MNase). The efficiency of interaction between Asf1 and Sas proteins was not influenced by the addition of either EtBr or MNase [Fig. 6D], indicating that the interaction between the native SAS complex and Asf1p is not mediated via DNA/chromatin.

The effect of ASF1, SIR1, and SAS2 disruption on sensitivity to mutagens

Asf1 mutants show multiple phenotypes, including slow growth, a weak sensitivity to ultraviolet light, a sensitivity to the DNA alkylating reagent methyl methane sulphonate (MMS), and a sensitivity to hydroxyurea (HU), a chemical inhibitor of ribonucleotide reductase that blocks DNA replication by impairing dNTP synthesis [Le et al. 1997; Tyler et al. 1999; Emili et al. 2001]. The functional interaction between ASF1 and RAD53 [DNA damage checkpoint protein gene] may play a role in the control of DNA repair [Emili et al. 2001; Hu et al. 2001].

ASF1 also contributes to gene silencing. Overexpression of ASF1 reduces silencing at mating-type loci, telomeres, and ribosomal DNA [Le et al. 1997; Singer et al. 1998]. Though the asf1 mutant showed no significant defect in the silencing at mating-type loci and telomeres, deletion of ASF1 in combination with a null allele of CAC1 or CAC2, which encode subunits of chromatin assembly factor 1 [CAF-1], weakens gene silencing [Tyler et al. 1999; Sutton et al. 2001].

To test for a role of the SAS complex and Sir1p in DNA repair, we examined the effects of disruption of ASF1 in combination with a null allele of SIR1 and/or SAS2 on sensitivity to mutagens [Fig. 7A]. Although asf1 mutants grow slowly, no additional effects on growth by disruption of ASF1 in sas2 and/or sir1 deletion strains were observed. Mutation of SIR1 and/or SAS2 in both ASF1 and asf1 strains did not enhance the sensitivity to DNA damaging agents or the DNA replication inhibitor. These results indicate that SIR1 and SAS2 are not involved in ASF1-mediated DNA repair and DNA replication and suggest that interaction between the SAS complex and Asf1p is important for different ASF1 functions.

Asf1 mutants affect HML and HMR silencing

To investigate whether Asf1p and the SAS complex function together, we tested the mating efficiencies of strains

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**Figure 7.** DNA damage sensitivity and mating efficiencies of asf1, sir1, and sas2 mutants. (A) Effects of disruption of ASF1 and SIR1 and/or SAS2 on sensitivity to mutagens. Serial dilutions of wild type and mutant strains were spotted and grown on complete media in the absence (−), or presence of ultraviolet radiation [100 J/m²], MMS [0.006%], or HU [25 mM]. Strains analyzed were W303-1a [wild type], YJW433 [asf1Δ], YJW252 [sir1Δ], YJW435 [asf1Δ sir1Δ], YJW253 [sas2Δ], YJW436 [asf1Δ sas2Δ], YJW258 [sas2Δ sir1Δ], and YJW437 [asf1Δ sas2Δ sir1Δ]. [B] ASF1 contributes to silencing at HML. Mating assays are shown with quantitative mating assay results given below. Strains shown are W303-1a [wild type], YJW252 [sir1Δ], YJW253 [sas2Δ], YJW258 [sas2Δ sir1Δ], YJW433 [asf1Δ], YJW435 [asf1Δ sir1Δ], YJW436 [asf1Δ sas2Δ], and YJW437 [asf1Δ sas2Δ sir1Δ]. [C] Deletion of ASF1 restores silencing to strains with mutations in HMR-E. Quantitative mating assay results are expressed relative to a value of 1.0 for wild-type W303-1b. Strains shown are YAB53 [Δsir1Δ], YAB197 [Δsir1Δ], YCM7S [Δsir1Δ], SY587 [asf1ΔΔΔ sas2Δ], SY558 [asf1ΔΔΔ sir1Δ], and SY560 [asf1ΔΔΔΔΔ].
harboring a null allele of ASFI in combination with null alleles of SIR1 and/or SAS2 (Fig. 7B). Similar to deletion of either SIR1 or SAS2, deletion of ASFI results in a very slight reduction in silencing at HML, as indicated by quantitative mating analysis. On the other hand, the mating ability of a asf1 sir1 double mutant was three orders of magnitude less than that of wild type or single deleted strains and was almost the same as that of double sas2 sir1 or triple asf1 sas2 sir1 deletion strains. A strain containing null alleles of both ASFI and SAS2 was only slightly more defective than strains individually deleted for these genes. These results suggest that both Asf1p and the SAS complex function in a pathway that enhances the epigenetic silencing defects of sir1 mutants.

In contrast to their effect on silencing at HML, deletions of SAS2, SAS4, and SAS5 improve silencing at an HMR locus that has mutations in E silencer elements [Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997; Xu et al. 1999a]. We tested the effect of deletion of ASFI on HMR silencing by measuring the mating ability of three MATa strains with mutated HMR-E elements [Fig. 7C]. In all three cases, loss of ASFI resulted in a substantial improvement of mating, as has been previously reported for the SAS genes.

Discussion

We identified and characterized a SAS complex that is ~450 kD in size and contains Sas2p, Sas4p, and Sas5p. There are several lines of evidence that lead us to this conclusion. First, size exclusion chromatography showed that complexes containing Sas4p and Sas5p are similar in size to the protein complex containing Sas2p (Fig. 2A). Second, deletion of SAS4 and/or SAS5 alters the size of the Sas2 complex (Fig. 2B). Third, Sas4p and Sas5p coimmunoprecipitated with Sas2p [Fig. 3]. Fourth, a highly purified Sas2 complex contains Sas4p and Sas5p [Fig. 4B]. Finally, the mass spectrometry data confirmed that Sas4p and Sas5p are components of the Sas2 complex [Fig. 4C]. The existence of Sas2p, Sas4p, and Sas5p in the same complex explains recent studies, which have showed that the SAS2, SAS4, and SAS5 have similar functions in silencing of HML [Xu et al. 1999b].

In addition to the characterization of the subunits of the SAS complex, we have shown that the conserved acetyl-CoA binding site in Sas2p is required for HML and telomeric silencing (Fig. 5). This strongly suggests that acetyltransferase activity of the SAS complex is critical for silencing in vivo. However, the substrate of the SAS complex is currently unknown. We could not detect any HAT activity in partially or highly purified preparations of the SAS complex using free or nucleosomal histones (data not shown). Although these are negative results, they do suggest that the substrate of Sas2 might be proteins other than histones. We have provided strong evidence that the acetyl-CoA binding domain is essential for the silencing function of Sas2p in vivo. The identification of the substrate of the SAS complex will enable us to address the silencing mechanism mediated by the Sas2-dependent acetylation.

SAS complex interacts with ASF1

SAS complex links DNA replication and silencing to chromatin modification

It has been shown that the restoration of silencing at the HMR locus with a mutated silencer by a sas2 mutant required Orc2p and Orc5p, which are subunits of the origin recognition complex (ORC; Ehrenhofer-Murray et al. 1997). ORC is an initiator complex for DNA replication but also affects transcriptional silencing [Dutta and Bell 1997]. HBO1 [histone acetyltransferase binding to ORC] is a member of the MYST acetyltransferases family and has been identified as an interaction partner of the human ORC1 protein [Iizuka and Stillman 1999]. HBO1 also interacted with the androgen receptor and a transcriptional repression domain of HBO1 was identified [Sharma et al. 2000]. Genetic interactions between SAS2 and ORC-encoding genes suggest that HBO1 might be functionally related to the Sas2 protein. Thus, the Sas2 complex might play a key role in linking DNA replication and gene silencing to chromatin modification.

Sas5p is a tf2f domain-containing protein

It remains unclear how SAS4 and SAS5 mediate SAS2-dependent silencing. Sas4p and Sas5p might be essential for the structural integrity of the SAS complex, because deletion of the SAS4 and/or SAS5 genes altered the size of the SAS complex. Sas4p has no obvious homology with other known proteins. A BLAST search [Altschul et al. 1997] reveals that an N-terminal stretch of ~100 amino acids in Sas5p has homology with proteins that have been identified in S. cerevisiae, S. pombe, Caenorhabditis elegans, Drosophila, Arabidopsis, and humans. This domain is ~60% homologous in all these proteins and is registered as the tf2f domain in the NCBI Conserved Domain Database. These tf2f domain-containing proteins include yeast TAFI3p30. The prototype of this family, TAFI30p, is a component of at least five yeast transcription-related complexes, NuA3, TFIID, TFIIF, SRF-mediator complex, and SWI/SNF [Henri et al. 1994; Cairns et al. 1996; Mochtader et al. 1996; Gustafsson et al. 1998; John et al. 2000]. It is likely that all members of the tf2f family are involved in chromatin modification/remodeling processes. However, the exact role of these proteins in chromatin modification/remodeling is unclear. Deletion of TAFI30p does not alter the structural integrity or the catalytic activity of the SWI/SNF complex [Cairns et al. 1996]. Similarly, the deletion of TAFI30 results in modest decreases in NuA3 HAT activity and does not significantly alter the size of NuA3 [John et al. 2000]. The Sas5p tf2f domain is similar to that in the human GAS41, ENL, and AF-9. GAS41 has been found to be amplified in low-grade gliomas [Fischer et al. 1997]. Recently, GAS41 was also identified as a binding partner of NuMA, a component of the nuclear matrix in interphase cells [Harborth et al. 2000]. The interaction between NuMA and GAS41 may provide a link between nuclear architecture and gene expression. Human ENL and AF-9 proteins are implicated in human acute leukemia, because ENL and AF-9 were found fused
### Yeast Strains

| Strain   | Genotype                                                                 | Source          |
|----------|---------------------------------------------------------------------------|-----------------|
| W303-1a  | MAT a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100                 | A. Sachs        |
| YJW213   | aHis3MX6:PGAL1-3HA-SAS2                                                   |                 |
| YJW214   | aSAS2-3HA::His3MX6                                                       |                 |
| YJW219   | akanMX6:PGAL1-3HA-SAS4                                                   |                 |
| YJW220   | akanMX6:PGAL1-3HA-SAS5                                                   |                 |
| YJW228   | aSAS4-13Myc:kanMX6                                                       |                 |
| YJW229   | aSAS5-13Myc:kanMX6                                                       |                 |
| YJW230   | aHis3MX6:PGAL1-3HA-SAS4-SAS4-13Myc::kanMX6                               |                 |
| YJW231   | aHis3MX6:PGAL1-3HA-SAS4-SAS2-SAS5-13Myc::kanMX6                          |                 |
| YJW243   | W303-1a [pESC/URA (pS99)]                                                |                 |
| YJW252   | JRY4622 [a sir1Δ::LEU2]                                                  | D. Rivier       |
| YJW253   | DRY1655 [a sas2Δ::TRP1]                                                  | D. Rivier       |
| YJW254   | DRY1656 [a sas4Δ::kan]                                                   | D. Rivier       |
| YJW258   | DRY1657 [a sas5Δ::HIS3]                                                  | D. Rivier       |
| YJW258   | DRY1658 [a sir1Δ::LEU2 sas2Δ::TRP1]                                      | D. Rivier       |
| YJW265   | a SAS2-13Myc::His3MX6                                                    |                 |
| YJW269   | a SAS2-13Myc::His3MX6 sas4Δ::kan                                         |                 |
| YJW270   | a SAS2-13Myc::kanMX6 sas5Δ::HIS3                                         |                 |
| YJW271   | a SAS2-13Myc::TRP1 sasΔ::kanMX4 sas5Δ::HIS3                               |                 |
| YJW275   | LPY1382 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]                        | L. Pillus       |
| YJW276   | YJW228 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]                         |                 |
| YJW278   | YJW278 [pESC/URA/PGAL10-6xHIS-SAS2-M1-FLAG (pS117)]                      |                 |
| YJW279   | YJW279 [pESC/URA/PGAL10-6xHIS-SAS2-M2-FLAG (pS118)]                      |                 |
| YJW280   | YJW280 [pESC/URA/PGAL10-6xHIS-SAS2-M3-FLAG (pS119)]                      |                 |
| YJW281   | YJW252 [pESC/URA (pS99)]                                                 |                 |
| YJW282   | YJW258 [pESC/URA (pS99)]                                                 |                 |
| YJW283   | YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]                         |                 |
| YJW284   | YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M1-FLAG (pS117)]                      |                 |
| YJW285   | YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M2-FLAG (pS118)]                      |                 |
| YJW286   | YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M3-FLAG (pS119)]                      |                 |
| YJW288   | YJW252 [pRS416/CEN/URA (pS15)]                                           |                 |
| YJW289   | YJW258 [pRS426/2μ/URA (pS23)]                                            |                 |
| YJW290   | YJW258 [pRS416/CEN/URA (pS15)]                                           |                 |
| YJW291   | YJW258 [pRS426/2μ/URA (pS23)]                                            |                 |
| YJW315   | YJW258 [pRS416/CEN/URA/PSAS2-SAS2 (pS126)]                               |                 |
| YJW352   | YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M1 [pS136]                             |                 |
| YJW352   | YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M2 [pS137]                             |                 |
| YJW355   | YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M3 [pS138]                             |                 |
| YJW356   | YJW258 [pRS426/2μ/URA/PSAS2-SAS2 [pS127]]                               |                 |
| YJW357   | YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M1 [pS142]]                             |                 |
| YJW358   | YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M2 [pS143]]                             |                 |
| YJW359   | YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M3 [pS144]]                             |                 |
| YJW414   | YJW265 [pRS426/2μ/URA3/ASF1 (pAS89)]                                     |                 |
| YJW415   | YJW265 [pRS426/2μ/URA3/ASF1-3HA (pAS90)]                                 |                 |
| YJW416   | YJW228 [pRS426/2μ/URA3/ASF1 (pAS89)]                                     |                 |
| YJW417   | YJW228 [pRS426/2μ/URA3/ASF1-3HA (pAS90)]                                 |                 |
| YJW418   | YJW229 [pRS426/2μ/URA3/ASF1 (pAS89)]                                     |                 |
| YJW419   | YJW229 [pRS426/2μ/URA3/ASF1-3HA (pAS90)]                                 |                 |
| YJW433   | aasf1Δ::HIS3                                                             |                 |
| YJW436   | aasf1Δ::HIS3 sir1Δ::LEU2                                                 |                 |
| YJW437   | aasf1Δ::HIS3 sas2Δ::TRP1 sir1Δ::LEU2                                      |                 |
| YJW457   | W303-1a [pRS424/2μ/TRP1/PASF1-ASF1-9Myc [pAS92]]                         | R. Rothstein    |
| YJW454   | YJW213 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc [pAS92]]                          |                 |
| YJW459   | YJW219 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc [pAS92]]                          |                 |
| YJW460   | YJW220 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc [pAS92]]                          |                 |
| W303-1b  | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100                  | R. Rothstein    |
| YGM75    | hmrΔΔΔB                                                                  | G. Micklem      |
| SY560    | hmrΔΔΔB asf1::His5                                                       |                 |
Table 1. (Continued)

| Strain     | Genotype                                                      | Source   |
|------------|---------------------------------------------------------------|----------|
| YAB53      | hmrΔΔΔΔE                                                      | A. Brand |
| SY557      | hmrΔΔΔΔE asf1::His5*                                          | A. Brandt|
| YAB197     | hmrΔB sir1::LEU2                                              | A. Brand |
| SY558      | hmrΔB sir1::LEU2 asf1::His5*                                   | A. Brand |
| YJW251c    | JRY2728 MATa his4                                             | D. Rivier|
| AY1281c    | MATa his1                                                     | K. Arndt |
| AY1283c    | MATa his1                                                     | K. Arndt |
| YJW287d    | LPY917/UC1001 [a TEL(VII) adh4::URA3 ade2-101<sup>c</sup> his3Δ200 leu2-Δ1 lys2-801<sup>c</sup> trp1-Δ1 ura3-52] | L. Pillus|
| YJW288     | LPY1285 a sir1Δ::HIS3                                         | L. Pillus|
| YJW289     | LPY2058 a sas2Δ::TRP1                                         | L. Pillus|
| YJW290     | LPY2062 a sas2Δ::TRP1 sir1Δ::HIS3                              | L. Pillus|
| YJW324     | YJW287 [pRS425/2µ/LEU (pS20)]                                 |         |
| YJW326     | YJW288 [pRS425/2µ/LEU (pS20)]                                 |         |
| YJW328     | YJW289 [pRS425/2µ/LEU (pS20)]                                 |         |
| YJW330     | YJW289 [pRS425/2µ/LEU (pS123)]                                |         |
| YJW332     | YJW290 [pRS425/2µ/LEU (pS20)]                                 |         |
| YJW334     | YJW290 [pRS425/2µ/LEU (pS123)]                                |         |
| YJW369     | YJW289 [pRS425/2µ/LEU (pS139)]                                |         |
| YJW370     | YJW289 [pRS425/2µ/LEU (pS140)]                                |         |
| YJW371     | YJW289 [pRS425/2µ/LEU (pS141)]                                |         |
| YJW375     | YJW290 [pRS425/2µ/LEU (pS139)]                                |         |
| YJW376     | YJW290 [pRS425/2µ/LEU (pS140)]                                |         |
| YJW377     | YJW290 [pRS425/2µ/LEU (pS141)]                                |         |

<sup>a</sup>Strains below are isogenic with W303-1a.
<sup>b</sup>Strains below are isogenic with W303-1b.
<sup>c</sup>YJW251, AY1281, and AY1283 are lawn tester strains for mating assays.
<sup>d</sup>Strains below are isogenic with YJW287.

to the ALL-1 gene in leukemias with translocations (Tkachuk et al. 1992; Negrini et al. 1993). Further analysis of the function of Sas5p in Sas2p-mediated silencing might help us understand how human t2f proteins such as ENL, AF-9, and GAS41 contribute to tumorigenesis in humans.

**ASF1 and SAS2 enhance the silencing defect at HML in a sir1 mutant**

We showed that chromatin assembly factor Asf1p interacts with components of the SAS complex in vivo. Individually, asf1 mutants do not show significant derepression of silencing at mating-type loci (Le et al. 1997; Singer et al. 1998; Tyler et al. 1999), but the double asf1 sir1 deletion strain displayed a much more severe silencing defect at HML than did deletion of either gene alone. This phenotype of asf1 is similar to that which is observed for sas genes on silencing at HML (Xu et al. 1999b). Furthermore, asf1 mutations, like sas mutations, restored silencing at mutated HMR silencers. These genetic results suggest that ASF1 and SAS2/4/5 function in the same pathway to regulate silencing at HML and HMR. Although asf1, sas2, sas4, and sas5 mutants have very similar silencing phenotypes at HML and HMR, they have drastically different properties with respect to telomeric silencing. Although asf1 mutants show little or no telomeric silencing defect (Singer et al. 1998), sas2, sas4, and sas5 mutants are totally defective in telomeric silencing (Reifsnnyder et al. 1996).

Nucleosome assembly may be important to generate the silent state, because deletion of CAF-1 subunits leads to a significant decrease in silencing at telomeres and a subtle weakening of silencing at HML and HMR (Enomoto and Berman 1998). CAC1 encodes the largest subunit of CAF-1 that assembles newly synthesized histones onto replicated DNA. A cac1 mutation enhanced the mating defect of a sir1 deletion strain. CAF-1 contributes to the maintenance, but not the re-establishment, of silencing at the mating loci (Enomoto and Berman 1998). Mutations in the genes for either chromatin assembly factor, CAF-1 or Asf1p, enhance the silencing defect of sir1 mutants. ASF1 function also might be involved in the maintenance of silent mating type genes. Because ASF1 and SAS2/4/5 genetically function in the same pathway to repress the HML locus, the interaction between the SAS complex and Asf1p might have a role for maintenance.

**Materials and methods**

**Yeast strains and manipulations**

Strains used in this study are described in Table 1. All of the chromosomal integrated tagged-strains were generated by the one-step PCR-mediated technique as described elsewhere (Longtine et al. 1998). The template plasmids were kindly provided by Dr. Peter Philippsen (Institut für Angewandte Mikrobiologie, Biozentrum, Universität Basel, Switzerland). A DNA fragment used to replace the ASF1 open reading frame (ORF) with the S. pombe his5<sup>c</sup> gene was synthesized by PCR using...
Osada et al.

synthetic oligonucleotides and the plasmid pME3 as described (Wach et al. 1997). Standard yeast manipulations were performed as described [Guthrie and Fink 1991].

**Plasmid construction**

The C-terminal Flag-tagged Sas2p, Sas4p, and Sas5p expression vectors, pS100, pS120, and pS104, were constructed by fusing the SAS2, SAS4, and SAS5 ORFs, which were amplified by PCR, and the products were subcloned into the SpeI site of the Flag-tagged protein expression vector pESC/URA [pS99; Stratagene]. For the N-terminal 6xHis-tagged and the C-terminal Flag-tagged Sas2p, Sas4p, and Sas5p expression vectors pS116, pS128, and pS129, the double-stranded oligonucleotide containing 6xHis-tagged sequence was ligated into the NotI site of pS100, pS120, and pS104. Plasmids expressing Sas2p regulated by the endogenous promoter were generated by PCR amplification of the SAS2-coding sequences along with 1 kb upstream region sequence from yeast genomic DNA [pS123, pS126, pS127]. The amplified product was cloned into pRS416 [pS15], pRS425 [pS20], and pRS426 [pS23]. All fragments generated by PCR were verified by sequencing. Mutations introduced using the QuickChange site-directed mutagenesis kit (Stratagene) following manufacturer’s protocols, and mutations were confirmed by sequencing. ASB90 [ASFI–[HA]3 in pRS426] was created as follows. First, PCR mutagenesis was used to replace the stop codon at the 3’ end of the ASFI ORF in pLS67 [ASFI in pUC18] with a NotI site with a NotI site to create pASB31. A fragment encoding a triple repeat of the HA epitope flanked by NotI restriction sites was inserted into the NotI site of pASB31 to create pASB35. An Xbal–EcoRI fragment encoding the C terminus of Asf1p in plasmid pLS27 (full length ASFI in Ycp50) was replaced with an Xbal–EcoRI fragment from pASB35 to create pASB41 [ASFI–[HA]3 in Ycp50]. A SalI–EcoRI fragment from pASB41 containing ASFI–[HA]3 was cloned into pRS426 to create ASB90. ASB92 [ASFI–[Myc]3] was created as follows. A NotI fragment containing 13 tandem copies of the Myc epitope was inserted into the NotI site of ASB31 so that the Myc tag was in-frame with the C terminus of Asf1p. A NdeI fragment that encodes the Myc-tagged C terminus of Asf1p was then used to replace the sequences for the untagged C terminus of ASFI in pRS424. ASB99 [ASFI in pRS426] was created by cloning a BamHI–HindIII fragment containing ASFI from pLS27 into pRS426. ASB92 ASFI–[Myc]3 was created by cloning the GST-Asf1p fusion protein expression vector [pS173], the ORF of ASFI gene was subcloned into the BamHI and EcoRI sites of the glutathione S-transferase (GST) expression vector pGEX-2T (Amersham-Pharmacia).

**Purification of the SAS complex**

Whole cell extracts were prepared essentially as a previously published procedure [Eberhart et al. 1998]. Approximately 4 mg and 0.4 mg of whole cell extracts were loaded onto a 24-mL Superose 6 HR 10/30 and a 2.4-mL Superdex 200 PC 3.2/30 column [Amersham-Pharmacia], respectively, equilibrated in buffer C (40 mM HEPEs at pH 7.8, 500 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.5 µg/mL leupeptin, and 0.5 µg/mL pepstatin A). For Figure 1, whole cell extracts from two liters of SAS2–HA strain [YJW214] were mixed with 1.6 mL of Ni2+-NTA agarose (Qiagen) overnight. The unbound fraction was loaded onto a 25-column-volume gradient from 0.1 M to 0.5 M NaCl in buffer B [50 mM Tris at pH 8.0, 10% glycerol, 0.1% Tween 20, and protease inhibitors described above]. Fractions containing Sas2––HA were concentrated over a 1-mL MonoQ HR 5/5 column [Amersham-Pharmacia] using the same gradient as described above. The Sas2–HA fractions were subjected to gel filtration by a Superose 6. The fractions were precipitated with TCA before gel electrophoresis. All gel filtration columns were calibrated using a high-molecular-weight filtration calibration kit (Amersham-Pharmacia).

For mass spectrometry analyses, whole cell extracts were prepared from 12 L of yeast strain YJW276. The extracts were bound to 10 mL of Ni2+-NTA agarose resin overnight. Then resin was washed with the extraction buffer (40 mM HEPEs at pH 7.5, 350 mM NaCl, 10% glycerol, 0.1% Tween 20, and protease inhibitors) and 20 mM imidazole solution containing 100 mM NaCl, 10% glycerol, 0.1% Tween 20, and protease inhibitors described above. Bound proteins were eluted with 30 mL of 300 mM imidazole solution. The imidazole eluate was loaded onto a 1-mL MonoQ column. Retained proteins were eluted in a 10-column-volume gradient from 0.1 M to 0.8 M NaCl in buffer B. Dithiothreitol [DTT] and anti-Flag M2 affinity resin was added to the fraction containing Sas4p–Myc. Beads were washed extensively with buffer B containing 0.5 M NaCl and 1 mM DTT, and bound proteins were eluted with buffer B containing 0.5 M NaCl, 1 mM DTT and 0.7 mg/mL Flag peptide (Sigma). The elute was subjected to a 2.4-mL Superdex 200 PC 3.2/30 gel filtration column equilibrated in the buffer C.

**Western blotting and antibodies**

Ten microliters of each fraction were electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose, and detected by ECL Western blotting analysis detection system [Amersham-Pharmacia]. Anti-Myc [clone 9E10, Boehringer], horseradish peroxidase-conjugated anti-HA [clone 3F10, Boehringer], and anti-Flag M2 [Sigma] antibodies were used.

**Immunoprecipitations and GST pull-down assays**

One microliter of the anti-HA antibody (clone 16B12, Covance) was mixed with 400 µg of whole cell extracts or 72 µL of Superose 6 fractions that were diluted to appropriate salt concentration of 150 mM NaCl with buffer and incubated on ice for 8 h. Twenty microliters of protein G–Sepharose beads were added to the sample and incubated at 4°C for 8 h. In some experiments, ethidium bromide [EtBr] was added [50 µg/mL], and the extracts were incubated on ice for 30 min. Precipitates were removed by centrifugation at 4°C for 10 min, and the supernatant was used for immunoprecipitation experiments. For micrococcal nuclease [MNase] treatment, extracts were treated with 0.4 units of MNase, 0.5 mM CaCl2, and 0.5 mM MgCl2 at 27°C for 10 min. Beads were washed with extraction buffer or Buffer C containing 150 mM NaCl before gel electrophoresis. GST fusion proteins were expressed in *Escherichia coli* as described by the manufacturer (Amersham-Pharmacia) and cross-linked to glutathione sepharose 4B with dimethylpimelimidate [Harlow and Lane 1988]. The N-terminal 6xHis-tagged and the C-terminal Flag-tagged Sas2, Sas4, and Sas5 proteins were individually expressed from pS116, pS128, and pS129 with T3 polymerase using TNT-coupled reticulocyte lysate system [Promega]. Five microliters of the reticulocyte lysate reaction containing [35S]methionine-labeled Sas proteins were incubated in buffer B containing 350 mM NaCl at 4°C overnight with GST-fusion proteins cross-linked to glutathione Sepharose 4B. Beads were washed with buffer B containing 350 mM NaCl before gel electrophoresis.
Mass spectrometry

The Sas2 complex fraction was electrophoresed through a 4% to 15% SDS–polyacrylamide gradient gel. Silver-stained protein bands were excised and digested in gel trypsin. Proteins were identified by microcolumn high-performance liquid chromatography electrospray ionization tandem mass spectrometry and database searching (Grant et al. 1998).

Mating and telomeric silencing assays

Mating assays were performed as described previously (Sprague 1991). The patches from the strains containing pRS416- and pRS426-based plasmids were replica plated to a lawn of α-cells (AY1283) grown on dextrose plates. Galactose plates were used for mating assays with the strains carrying the pESC plasmids. Quantitative mating assay was performed as described previously (Ehrenhofer-Murray et al. 1997). Serial dilutions of test strains were mixed with 1.0 × 10⁷ cells of a MATα lawn (AY1283 or YW251) or a MATα lawn (AY1281) and plated onto selective medium and rich medium to determine the number of viable cells. For the experiments in Figure 5, mating efficiency was expressed as a percentage of diploid cells formed per viable cells. For the experiments in Figure 7, mating efficiency was expressed as the number of diploids formed per viable cells and was normalized to the efficiency of an isogenic wild-type strain. Mating efficiencies are the average of three independent experiments. For the telomeric silencing assay, three microliters of mating mixture containing plates containing 5-FOA containing plates.

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