Positive roles of SAS2 in DNA replication and transcriptional silencing in yeast

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ABSTRACT
Sas2p is a histone acetyltransferase implicated in the regulation of transcriptional silencing, and ORC (ORC) is the six-subunit origin recognition complex involved in the initiation of DNA replication and the establishment of transcriptionally silent chromatin by silencers in yeast. We show here that SAS2 deletion (sas2Δ) exacerbates the temperature sensitivity of the ORC mutants orc2-1 and orc5-1. Moreover, sas2Δ and orc2-1 have a synthetic effect on cell cycle progression through S phase and initiation of DNA replication. These results suggest that SAS2 plays a positive role in DNA replication and cell cycle progression. We also show that sas2Δ and orc5-1 have a synthetic effect on transcriptional silencing at the HMR locus. Moreover, we demonstrate that sas2Δ reduces the silencing activities of silencers regardless of their locations and contexts, indicating that SAS2 plays a positive role in silencer function. In addition, we show that SAS2 is required for maintaining the structure of transcriptionally silent chromatin.

INTRODUCTION
Eukaryotic DNA is packed into chromatin through the formation of nucleosomes. Chromatin can be roughly divided into condensed heterochromatin and decondensed euchromatin based on its cytological and molecular properties. Chromatin plays a pivotal role in the regulation of DNA-dependent processes including gene expression, DNA replication and DNA repair. Chromatin is subject to various modifications that differentially affect its compaction and accessibility to DNA/chromatin-interacting factors (1). Acetylation of lysine residues of histones may alter nucleosomal conformation and/or chromatin compaction. Histone acetylation is usually associated with euchromatin, whereas histone hypoacetylation is a hallmark of heterochromatin. Histone acetylation is carried out by histone acetyltransferases (HATs) that fall into distinct families with specific substrate preferences (2,3). For example, Saccharomyces cerevisiae Sas2p belongs to the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) HAT family that preferentially acetylates histones H4 and H2A. A native HAT usually functions as part of a multi-subunit complex that helps direct the HAT to proper targets (2). Sas2p is the catalytic subunit of the SAS complex consisting of Sas2p, Sas4p and Sas5p that specifically acetylates histone H4-K16 (3).

In S. cerevisiae, DNA replication is initiated at defined origins named autonomous replicating sequences (ARSs) across the genome (4). The origin recognition complex (ORC) plays a key role in replication initiation. ORC is composed of six subunits (Orc1p–Orc6p), all of which are required for cell survival. ORC associates with replication origins throughout the cell cycle, but its main function is to nucleate an initiation competent pre-replication complex (pre-RC) at origins in the G1 phase (4). Defects in pre-RC formation caused by ORC mutations may result in incomplete DNA replication and increased chances of replication fork stalling/breakage that triggers DNA damage and spindle assembly checkpoint pathways leading to mitotic arrest (5). There is also evidence that ORC participates in sister-chromatid cohesion, which is distinct from its role in DNA replication (6).

The timing and efficiency of the firing of replication origins appear to depend on their genomic locations and contexts. There is evidence that chromatin structure affects origin activity (7,8). Histone acetylation surrounding an origin directly influences its time of firing (9). Removal of the histone deacetylase (HDAC) Rpd3p causes DNA replication to occur early in S-phase, and targeting the HAT Gcn5p to a late firing origin causes it to fire earlier (9,10). There is also evidence that the HDAC Sir2p involved in transcriptional silencing negatively regulates initiation of DNA replication (11). The HAT Hat1p was recently shown to physically interact with ORC, and deletion of HAT1 exacerbated the temperature sensitivity of orc mutants (12). These studies suggest that HATs play positive roles, and HDACs play negative roles in the initiation of DNA replication. However, interestingly, there is also evidence suggesting that the HAT Sas2p negatively regulate ORC function (13,14).

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ORC also plays a role in the establishment of yeast silent chromatin that is akin to metazoan heterochromatin (15). Formation of silent chromatin at the HML and HMR loci is promoted by cis-acting E and I silencers flanking these loci (15). Each silencer consists of recognition sites for ORC, Rap1p and/or Abf1p. The silencer-binding factors recruit the Sir complex composed of Sir2p, Sir3p and Sir4p through a direct interaction between ORC and Sir1p, which binds to Sir4p, and the binding of Rap1p to Sir3p or Sir4p. Sir2p is a HDAC that is responsible for characteristic hypoacetylation of histones in silent chromatin (15,16). The Sir complex recruited to a silencer is believed to deacetylate histones in adjacent nucleosomes. The newly deacetylated nucleosomes then bind additional Sir complexes. This is because the Sir complex self-interacts and preferentially binds hypoacetylated histones. Through repeated cycles of histone deacetylation and Sir complex recruitment, Sir complexes propagate along an array of nucleosomes.

Silent chromatin is regulated by various factors including Sas2p. SAS2 deletion (sas2Δ) was originally found to suppress the defect in HMR silencing caused by mutations in the HMR-E silencer, but decrease HML silencing in the sir1Δ background (13,17). However, it was also found that sas2Δ decreased silencing of ectopic reporter genes inserted at an otherwise intact HMR locus (18,19). Therefore, SAS2 seems to have the ability to positively or negatively regulate HML silencing. What determines the mode of Sas2p action has not been resolved. On the other hand, sas2Δ has been consistently found to abolish telomeric silencing (17), and it is proposed that Sas2p contributes to telomeric silencing by helping retain Sir complexes in telomeric silent chromatin (20,21). This is based on the finding that acetylation of H4-K16 in subtelomeric euchromatic regions by Sas2p hinders ectopic spreading of Sir complex from telomeric silent chromatin (20,21).

In this report, we examined functional relationships between Sas2p and ORC in DNA replication and transcriptional silencing in different genetic backgrounds. We found that sas2Δ exacerbated the temperature sensitivity of orc2-1 and orc5-1 mutants. We further showed that sas2Δ retarded cell cycle progression through S-phase and reduced the origin activity of ARS1 in the orc2-1 mutant. These results suggest that Sas2p plays a positive role in DNA replication. We also obtained evidence suggesting that Sas2p positively regulates silencer function regardless of whether it is at HML or HMR. Moreover, we showed that Sas2p played a role in maintaining transcriptionally silent chromatin structure.

**MATERIALS AND METHODS**

**Yeast strains**

Yeast strains are listed in Table 1. Strains carrying the sas2Δ::kanMX allele were made by transforming their corresponding parents to geneticin-resistant with a PCR-generated fragment composed of kanMX bracketed by 5' and 3' flanking sequences of the SAS2 open reading frame (ORF). Strains carrying the sas2Δ1:::TRP1 allele were made by transforming their parents with plasmid pJR1642 (13) digested with XhoI and BamHI. Plasmid pUC-HMR was made by inserting the genomic HindIII-HMR-Hind III sequence (289 227–294 210 of chromosome III) into pUC12. An XbaI-URA3-XbaI fragment was used to replace an XbaI fragment of pUC-HMR, making p206. Strains 36 and 37 were made by transforming strains 34 and 35, respectively, to Ura+ with plasmid pES17 (22) digested with BamHI and Hind III. Strains 38 and 39 were made by transforming strains 34 and 35, respectively, to Ura+ with EcoRI-digested plasmid pDM42 (23). Strains 40 and 42 were made by transforming strains 11 and 15, respectively, to Ura+ with HindIII-digested plasmid p206. The relevant genotypes of all strains were confirmed by Southern blotting.

**Fluorescence activated cell sorting**

Cells for fluorescence activated cell sorting (FACS) analysis were grown at 23°C to log phase (OD600 = 0.5) and divided into two aliquots that were grown for another 90 min at 23 and 30°C, respectively. Samples were prepared as described (24) and analyzed on a FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Data acquisition was performed with the CellQuest software. Quantification of the percentages of cells in G1 phase (with C1 DNA content), S-phase and G2-M (with C2 DNA content) were performed using the MODFIT software program (Verity Software House).

**Two-dimensional gel electrophoresis**

Cells used for isolating replication intermediates (RIs) were grown at 23°C to log phase and divided into two aliquots that were grown for another 90 min at 23 and 30°C, respectively. For each sample, 1.5 × 10⁸ cells were collected and used for genomic DNA isolation as described (25). Genomic DNA was digested with Neol and subjected to 2D gel analysis as described (26,27). The conditions for the first dimension were: 0.35% agarose, 1 × TBE buffer, 0.6 V/cm for 45 h, room temperature; and for the second dimension: 0.95% agarose, 0.3 µg/ml ethidium bromide, 2.8 V/cm for 20 h, 4°C. The Southern blot was first hybridized with an ARS1 probe to reveal RIs associated with ARS1. The blot was then stripped of the ARS1 probe and hybridized with an ARS305 probe to reveal RIs associated with ARS305.

**Chromatin mapping**

Chromatin mapping was carried out as described (28). About 2 × 10⁹ permeabilized spheroplasts were treated with MNase at 120 or 160 U/ml at 37°C for 5 min. Purified genomic (naked) DNA from untreated cells was digested with MNase at 7.5 U/ml. The conditions for the first dimension were: 0.35% agarose, 1 × TBE buffer, 0.6 V/cm for 45 h, room temperature; and for the second dimension: 0.95% agarose, 0.3 µg/ml ethidium bromide, 2.8 V/cm for 20 h, 4°C. The Southern blot was first hybridized with an ARS1 probe to reveal RIs associated with ARS1. The blot was then stripped of the ARS1 probe and hybridized with an ARS305 probe to reveal RIs associated with ARS305.

**Analysis of DNA topology**

Cells were grown in YPR medium (1% yeast extract, 2% bacto-peptone and 2% raffinose) to log phase. Galactose (2%) was added to the culture, which was further
incubated for 2.5 h to induce the expression of PGAL10-FLP1. Nucleic acids were isolated using the glass bead method and fractionated on an agarose gel supplemented with 13 μg/ml chloroquine. DNA circles were detected by Southern blotting.

RESULTS

Synthetic genetic interactions between SAS2 and ORC genes

All six subunits of ORC, Orc1p-Orc6p, are essential for cell viability, but temperature sensitive mutations of the

Table 1. Yeast strains

| Number | Yeast strains | Relevant genotype | Reference/Source |
|--------|---------------|-------------------|------------------|
| 1      | W303-1A       | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | B. Stillman |
| 2      | YYY261        | W303-1A, sas2A::kanMX | This work |
| 3      | YYY263        | YB59, sas2A::kanMX | B. Stillman |
| 4      | YYY266        | YB59, sas2A::TRP1 | This work |
| 5      | YYY267        | YB57, sas2A::kanMX | This work |
| 6      | YYY268        | YB57, sas2A::TRP1 | This work |
| 7      | YYY269        | W303-1A, sas4A::kanMX | This work |
| 8      | YYY270        | W303-1A, sas4A::kanMX | This work |
| 9      | YYY271        | W303-1A, sas4A::kanMX | This work |
| 10     | YYY272        | W303-1A, sas4A::kanMX | This work |
| 11     | YYY273        | W303-1A, sas4A::kanMX | This work |
| 12     | YYY274        | W303-1A, sas4A::kanMX | This work |
| 13     | YYY275        | W303-1A, sas4A::kanMX | This work |
| 14     | YYY276        | W303-1A, sas4A::kanMX | This work |
| 15     | YYY277        | W303-1A, sas4A::kanMX | This work |
| 16     | YYY278        | W303-1A, sas4A::kanMX | This work |
| 17     | YYY279        | W303-1A, sas4A::kanMX | This work |
| 18     | YYY280        | W303-1A, sas4A::kanMX | This work |
| 19     | YYY281        | W303-1A, sas4A::kanMX | This work |
| 20     | YYY282        | W303-1A, sas4A::kanMX | This work |
| 21     | YYY283        | W303-1A, sas4A::kanMX | This work |
| 22     | YYY284        | W303-1A, sas4A::kanMX | This work |
| 23     | YYY285        | W303-1A, sas4A::kanMX | This work |
| 24     | YYY286        | W303-1A, sas4A::kanMX | This work |
| 25     | YYY287        | W303-1A, sas4A::kanMX | This work |
| 26     | YYY288        | W303-1A, sas4A::kanMX | This work |
| 27     | YYY289        | W303-1A, sas4A::kanMX | This work |
| 28     | YYY290        | W303-1A, sas4A::kanMX | This work |
| 29     | YYY291        | W303-1A, sas4A::kanMX | This work |
| 30     | YYY292        | W303-1A, sas4A::kanMX | This work |
| 31     | YYY293        | W303-1A, sas4A::kanMX | This work |
| 32     | YYY294        | W303-1A, sas4A::kanMX | This work |
| 33     | YYY295        | W303-1A, sas4A::kanMX | This work |
| 34     | YYY296        | W303-1A, sas4A::kanMX | This work |
| 35     | YYY297        | W303-1A, sas4A::kanMX | This work |
| 36     | YYY298        | W303-1A, sas4A::kanMX | This work |
| 37     | YYY299        | W303-1A, sas4A::kanMX | This work |
| 38     | YYY300        | W303-1A, sas4A::kanMX | This work |
| 39     | YYY301        | W303-1A, sas4A::kanMX | This work |
| 40     | YYY302        | W303-1A, sas4A::kanMX | This work |
| 41     | YYY303        | W303-1A, sas4A::kanMX | This work |
| 42     | YYY304        | W303-1A, sas4A::kanMX | This work |
| 43     | YYY305        | W303-1A, sas4A::kanMX | This work |
ORC genes such as orc2-1 and orc5-1 have been described (4,29,30). It was reported previously that sas2Δ partially suppressed the temperature-sensitivity of the orc2-1 mutant (13). In an attempt to further examine functional interactions between the SAS2 and ORC genes, we made orc2-1 sas2Δ::kanMX and orc5-1 sas2Δ::kanMX double mutants in the W303-1A (MATa) background. We were surprised to find that our orc2-1 sas2Δ double mutant grew less robustly than the orc2-1 and sas2Δ single mutants at 30°C, a semi-permissive temperature for orc2-1 (Figure 1A, compare 4 to 3). In line with this finding, our orc5-1 sas2Δ double mutant also showed reduced growth compared to the orc5-1 single mutant at the semi-permissive temperature of 34°C (Figure 1A, compare 7 to 6). These results suggest that sas2Δ has a synthetic effect on cell growth with orc2-1 or orc5-1. Since Sas2p is a subunit of the SAS complex that also contains Sas4p and Sas5p, we asked whether SAS4 also genetically interacts with the ORC genes. We found that similar to sas2Δ, sas4Δ exacerbated the growth defect of orc2-1 at

![Figure 1](image-url)
As we considered the cause of the discrepancy between our results and previously published results regarding genetic interactions between *orc* mutants and *sas2A*, we noted that the genetic background of our strains was different from that of the previous strains. Our *sas2A* strains differed from published ones in the way of *SAS2* disruption. In our strains, the *kanMX* cassette was used to precisely replace the entire ORF of *SAS2*, whereas in the previous strains *TRP1* was used to replace most of the *SAS2* ORF resulting in *sas2A1::TRP1* (13, 14). We analyzed the sequence of *sas2A1::TRP1* and found it contained a new ORF encoding a short peptide composed of the N-terminal four amino acids of Sas2p followed by 20 other residues. To test whether *sas2A1::TRP1* differed from *sas2A::kanMX* in its interaction with *orc2-1* or *orc5-1*, we made *orc2-1 sas2A1::TRP1* and *orc5-1 sas2A1::TRP1* double mutants in the W303-1A background. The growth phenotypes of these double mutants at various temperatures were similar to *orc2-1 sas2A::kanMX* and *orc5-1 sas2A::kanMX* strains, respectively (Figure 1A, compare 5 to 4 and 3, and 8 to 7 and 6). Therefore, the *sas2A::kanMX* and *sas2A1::TRP1* alleles interact similarly with *orc2-1* or *cor5-1*, ruling out the method of *SAS2* disruption as the cause of the discrepancy between our data and previously published results.

The *orc* mutants used in previous studies were derivatives of W303-1B (*MATa*) instead of W303-1A (*MATa*). We therefore examined if the cell type affected genetic interactions between *orc* mutants and *sas2A*. We remade *orc2-1 sas2A1::TRP1* and *orc5-1 sas2A1::TRP1* double mutants in the W303-1B background and compared their growth phenotypes with *orc* single mutants. The double mutants exhibited more severe temperature sensitivity than their corresponding *orc* single mutants (Figure 1B, compare 14 to 13, and 16 to 15), which was similar to our results concerning W303-1A derivatives (Figure 1A). We noticed that the temperature sensitivity of *orc* mutants was more severe in the *MATa* background than in the *MATa* background as the *MATa orc2-1* strain was not able to grow at 30°C, whereas the *MATa orc2-1* strain was (albeit poorly) (Figure 1, compare 13 to 3). Possible causes of this phenomenon are noted later in the Discussion section.

Another difference between our strains and previous strains was that the latter contained a modified *HMR* locus with a synthetic *HMR-E* silencer and deletion of the *HMR-I* silencer (*HMR-SSAI*) (13), whereas all our strains were wild-type for *HMR*. We remade the double mutant *orc2-1 sas2A1::TRP1* in the *MATa HMR-SSAI* background and found that it still exhibited growth defects compared to the *orc2-1* single mutant (Figure 1B, compare 20 to 19).

The above data demonstrate that *sas2A* exacerbates the temperature sensitivity of *orc* mutants. In addition to its temperature sensitivity, the *orc2-1* mutant is sensitive to hydroxyurea (HU), which induces replicative DNA damage at permissive temperature (Figure 1A, compare 3 to 1 on HU-containing medium; ref. 31). We found that *sas2A* rendered *orc2-1* cells moderately more sensitive to HU (Figure 1A, compare 4 and 5 to 3 on HU-containing medium). Taken together, our results suggest that *SAS2* plays a positive role in *ORC*-mediated functions.

**Synthetic effect of *sas2A* and *orc2-1* on cell cycle progression through S-phase**

*ORC* is involved in the initiation of DNA replication, and defects in *ORC* function interrupt cell cycle progression (30, 32, 33). To further examine the role of Sas2p in *ORC*-mediated functions, we tested whether *sas2A* affected cell cycle progression of *ORC2* and *orc2-1* cells using FACS (fluorescence-activated cell sorting) analysis. Exponentially growing asynchronous cultures of two independent clones of each strain were subjected to FACS analysis and the results are shown in Figure 2A. The percentages of cells in G1 phase (with C1 DNA content), S-phase, and G2-M (with C2 DNA content) were measured for each culture and shown in Figure 2B.

At 23°C, the wild-type culture has about 21% G1, 38% S and 41% G2-M cells (Figure 2A), which was not significantly affected by *sas2A* (Figure 2, compare b to a). The *orc2-1* reduced the proportion of cells in G1 (Figure 2, compare c to a), which is consistent with previous results showing that temperature-sensitive mutations in *ORC1, ORC2* and *ORC5* all reduce the proportion of G1 cells (33). The *sas2A* had no effect on the proportions of G1, S and G2-M cells in the *orc2-1* culture (Figure 2, compare d to c). After being incubated at the semi-permissive temperature of 30°C for 90 min, *SAS2* and *sas2A* cells were roughly equally divided among the G1, S and G2-M phases (Figure 2B, E and F). As for the *orc2-1* mutant, the proportion of G1 cells decreased, whereas that of S or G2-M cells increased at 30°C (Figure 2, compare g to c), which suggests that compromising Orc2p function induces a delay of progression through S and/or G2-M phases. Compared to *orc2-1* cells, a strikingly larger percentage of *orc2-1 sas2A* cells were in S-phase at 30°C than at 23°C (Figure 3, compare h to g), indicating that *sas2A* delayed or arrested progression of *orc2-1* cells through S-phase.

*ORC1* has a positive role in ORC-dependent initiation of DNA replication

The above results suggest that *SAS2* is important for the passage of the *orc2-1* mutant through S-phase. Sas2p may regulate replication initiation that is ORC-dependent, and/or replication elongation that is ORC-independent. We directly examined whether *sas2A* affected initiation of DNA replication. A neutral–neutral 2D gel electrophoresis technique was used to examine the abundance of RIs associated with *ARS1*, an early-firing origin of replication, in wild-type as well as *orc2-1* and *sas2A* single and double mutants. This type of 2D gel separates replication bubbles, forks and linear DNA based on the size and shape of the DNA fragment containing a replication origin (26). A fragment containing an active replication origin in the middle would yield a bubble arc,
whereas a fragment being replicated by passing replication forks would yield a fork arc (Figure 3A).

ARS1 activity in wild-type, sas2Δ, orc2-1, and sas2Δ orc2-1 strains was examined. For each strain, cells were grown to log phase at 23°C and divided into two halves that were grown for another 90 min, one at 23°C and the other at 30°C, before being harvested. Genomic DNA was isolated from each sample and digested with NcoI, and subjected to 2D gel analysis followed by Southern blotting. ARS1 is located in a 4.7 kb NcoI fragment of chromosome IV (coordinates 460989–464386) (Figure 3B).

Because the position of ARS1 is asymmetric, replication initiated from ARS1 will initially yield bubbles and will then produce large forks when one of the two replication forks reaches one end of the fragment before the other (Figure 3A).

In the wild-type strain at either 23 or 30°C, the majority of the RIs were bubbles and large forks, which is consistent with the notion that ARS1 is an active origin (Figure 3C, a and e). The presence of some small forks indicated that the ARS1 did not fire in a small fraction of the cells in the culture (Figure 3C, a and e; ref. 34). The sas2Δ alone had little or no effect on ARS1's origin activity at either 23 or 30°C (Figure 3C, compare b to a, and f to e). The orc2-1 mutation reduced ARS1 activity at 23°C as evidenced by a decrease in the relative intensity of the bubble arc compared to the fork arc (Figure 3C, compare c to a). The sas2Δ further reduced the relative intensity of the bubble arc in the orc2-1 mutant (Figure 3C, compare d to c), suggesting that SAS2 is required for efficient ARS1 function in an orc2-1 mutant. At 30°C, the bubble arc in the orc2-1 mutant was barely detectable, which is consistent with the temperature sensitivity of orc2-1 (Figure 3C, compare g to c). We were not able to detect a bubble arc in the orc2-1 sas2Δ double mutant at 30°C (Figure 3C, h). These results suggest that Sas2p plays a positive role in the initiation of DNA replication at ARS1 in an orc2-1 mutant.

We next tested whether sas2Δ also affected the activities of other origins in an orc2-1 mutant. We examined ARS305, which is also an early firing origin but differs from ARS1 in sequence/structure (Figure 3B). ARS305 is asymmetrically located in a 5.1 kb NcoI fragment of chromosome III (coordinates 36563–41669). In the orc2-1 mutant, bubble and large-fork arcs were readily detectable, but little or no small-fork arc was observed at 23°C (Figure 3C, c'), suggesting that ARS305 was a very active origin. Raising the temperature from 23°C to 30°C increased the intensity of small fork arc (Figure 3C, compare g' to c'). However, the bubble arc was still readily detectable (Figure 3C, g'), indicating that ARS305 was less sensitive to orc2-1 mutation than ARS1 (Figure 3C, compare g' and g). The sas2Δ does not significantly affect ARS305 activity in the orc2-1 background at either 23 or 30°C (Figure 3C, compare d' to c', and h' to g'). Therefore, compared to ARS1, replication initiation at ARS305 seems less sensitive to orc2-1 and sas2Δ.

SAS2 does not affect chromatin structure at replication origins

ORC bound to a replication origin serves as a platform for the formation of the pre-RC. Meanwhile, it also directs the establishment of proper chromatin structure around the origin that is important for its function (8). Because Sas2p is a HAT that can acetylate histone H4 in chromatin, and plays a positive role in replication initiation at ARS1, we wondered whether it contributes to chromatin structure at ARS1. We examined chromatin around ARS1 in sas2Δ and orc2-1 single and double mutants using micrococcal nuclease (MNase) digestion followed by indirect end labeling. The ARS1 region is associated
with three major MNase cleavage sites (designated as $\alpha$, $\beta$ and $\gamma$) corresponding to a nucleosome-free region defined by ORC and Abf1p bound to the ACS and B3 elements of ARS1, respectively (Figure 4; ref. 8). The pattern of MNase sensitive sites around ARS1 was not significantly altered by orc2-1 and sas2D single mutations or the orc2-1 sas2D double mutation at 23 or 30°C (Figure 4, compare b, c and d to a, and f, g, h to e). Therefore, Sas2p does not seem to contribute to chromatin structure at ARS1.

A positive role of SAS2 in silencer function

SAS2 was originally identified as a gene involved in transcriptional silencing (13,17). However, conflicting effects of sas2Δ on silencing have been reported. The sas2Δ was found to eliminate HML silencing in a sir1Δ mutant, and abolish telomeric silencing (13,17). These results indicate that SAS2 plays a positive role in silencing at HML and telomeres. On the other hand, it was shown that sas2Δ suppressed the silencing defect of a HMR-E silencer with mutated Rap1p- and Abf1p-binding sites (HMRa-e**) (13,17). It also suppressed the silencing defect of a synthetic HMR-E silencer at the HMR locus deleted for the HMR-I silencer (HMR-SSA1) (13). These results suggest that SAS2 plays a negative role in silencing at HMR. Other investigations into SAS2 function in silencing have only added more conflicting evidence to this apparent paradox. For instance, it was shown that sas2Δ decreased silencing of an ADE2 reporter inserted at HMR (18). A recent study found that sas2Δ reduced
silencing at both HML and HMR, and sas2Δ and sir1Δ had a synthetic effect on both HML and HMR silencing (19). In this work, the reporters for HML and HMR silencing were yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) genes respectively, under the control of the URA3 promoter, and silencing in individual cells was examined (19).

Reconciling the conflicting results concerning the effect of SAD2 on silencing has been complicated by the fact that they were obtained from various silencing assays that involved different loci (e.g. HML versus HMR), silencers (e.g. intact HMR-E versus HMRα-e** or HMR-SS), reporters (e.g. HMRα versus ADE2) and/or genetic backgrounds (SIR1 versus sir1Δ). Since distinct silencers have different properties in silencing, and the function of a silencer is affected by its genomic context (15, 35), we wondered whether the mode of Sas2p action on a silencer is also dependent on the intrinsic features and/or the context of the silencer. To avoid potential complications that might result from comparing data from different silencing assays, we examined the effect of sas2Δ on the silencing of the URA3 reporter by the HML-E, HMR-E and HML-I silencers in the same context of the HML-E silencer (Figure 5B, strains 21–27). Cells expressing URA3 are sensitive to the drug 5-fluoroorotic acid (FOA), so cell growth on medium containing FOA is a measure of URA3 silencing (36). 

URA3 inserted to the left (telomere-proximal) side of HML was strongly silenced by HML-E (Figure 5B, robust growth of strain 21 on FOA). HML-I and HMR-E placed in the context of HML-E also efficiently silenced URA3 (Figure 5B, 24 and 26 on FOA). The sas2Δ decreased the growth of strain 22 on FOA medium (Figure 5B, compare 22 to 21). Since sas2Δ does not affect the function of the URA3 promoter per se (19), decreased growth of strain 22 on FOA medium demonstrates that URA3 silencing by HML-E is reduced by sas2Δ. It is noteworthy that in the sas2Δ mutant, silencing was not completely abolished as it was in the sir3Δ strain (Figure 5B, compare 22 to 23). The sizes of colonies of the sas2Δ strain 22 on FOA were uniformly smaller than those of the SAS2 strain 21 (Figure 5B), suggesting that URA3 silencing was reduced to an intermediate level by sas2Δ. The sas2Δ also decreased URA3 silencing by HMR-E and HML-I (Figure 5B, compare 25 to 24, and 27 to 26). Taken together, these results demonstrate that SAS2 plays a positive role in silencing by all three silencers in the context of HML-E.

We next examined whether the location of a silencer affects its response to sas2Δ. We deleted SAS2 from strains in which URA3 inserted to the right of HML is subject to silencing by inverted HML-I or HMR-E (Figure 5B, strains 28 and 30). Growth of these sas2Δ strains on FOA was reduced compared to their SAS2 counterparts (Figure 5B, compare 29 to 28, and 31 to 30). Therefore, the activities of HML-I and HMR-E in the context of HML-I were also reduced by sas2Δ. We next deleted SAS2 from strains 32 in which URA3...
was inserted to the left of an inverted HMR-E silencer at HMR (Figure 5B). We found again that sas2Δ reduced URA3 silencing by HMR-E (Figure 5B, compare 33 to 32). Moreover, sas2Δ also decreased the silencing of URA3 inserted at the endogenous HMR locus in its natural orientation (compare 41 to 40 in Figure 7). We conclude from the above results that SAS2 is required for efficient silencing by all tested silencers in the three contexts of HML-E, HML-I and HMR-E.

A role of SAS2 in maintaining transcriptionally silent chromatin structure

Transcriptional silencing in yeast is mediated by a special silent chromatin (15). One of the characteristics of silent chromatin is a distinct topology of its DNA. DNA at HML and HMR is more negatively supercoiled when these loci are silenced than when they are derepressed (37,38). As the topology of eukaryotic DNA reflects the density and conformation of nucleosomes, the degree of negative supercoiling of HM DNA can be used as a measure of the state of silent chromatin (37,38).

To investigate whether sas2Δ affects the structure of silent chromatin, we compared the supercoiling of HML DNA in wild-type and sas2Δ strains. In strain 34, the modified HML locus was bracketed by two copies of FRT (Flp1p recombination target), the recognition site for the site-specific recombinase Flp1p (Figure 6A, top). Induction by galactose of a PGAL–FLP1 fusion gene resident elsewhere in the genome would lead to the expression of Flp1p and recombination between the FRT sites resulting in the excision of HML as a closed minichromosome circle. After being deproteinized, the supercoiling of the circle can be examined by gel electrophoresis in the presence of the DNA intercalator chloroquine (Figure 6A, strain 34). Deletion of SIR3 reduced the negative supercoiling of HML DNA by a linking number change (ΔLk) of 7 (Figure 6, compare the centers of topoisomer distributions in strains 38 and 34; note more negatively supercoiled circles migrate more slowly under the condition used). The sas2Δ induced a reduction in negative supercoiling of HML DNA of a ΔLk of 1 (Figure 6, compare 35 and 34). This effect was specific to silent HML, as sas2Δ did not reduce the negative supercoiling of derepressed HML in a sirΔ background (Figure 6, compare 39 and 38). Therefore, the supercoiling of HML (hence the configuration of silent chromatin) in a sas2Δ strain is in an intermediate state between fully silenced and derepressed states (Figure 6, compare 35 to 34 and 38). This is consistent with our result that sas2Δ reduced transcriptional silencing to an intermediate level (Figure 5).

As sas2Δ and sir1Δ have a synthetic effect on HML silencing (13,17,19), we tested whether they also have a synthetic effect on the topology of HML DNA. Sir1p is believed to be involved in the establishment but not the maintenance of silent chromatin at the HM loci (15). Single-cell assays revealed that a culture of SIR1 null cells consists of two populations of mitotically stable cells that differ in the state of HML silencing (19,39). HML is silenced in one population of cells, but derepressed in the other. Consistently, the topoisomers of HML circles isolated from a sir1Δ culture appear to be a mixture of topoisomers from SIR+ cells where HML is silenced and derepressed.
modifying nucleosomes near the origins. There is also evidence suggesting that Hat1p contributes to chromatin assembly during replication elongation (12,40). In vertebrates, Hbo1p, a member of the MYST family of HATs, has been shown to interact with ORC and MCM and positively regulate pre-RC formation (41,42). Depletion of Hbo1p does not affect chromatin binding of ORC and Cdc6p but prevents the recruitment of the MCM complex. It is proposed that Hbo1p facilitates MCM loading by acetylating chromatin at the origin and/or components of the pre-RC complex such as Orc2p (42).

We showed that SAS2 deletion (sas2Δ) does not affect DNA replication in the presence of an intact ORC, but reduces origin firing when ORC function is compromised by orc2-1 mutation (Figure 3). Sas2p’s role in DNA replication may overlap with that of other factors involved in ORC function such as Hat1p. It is not known whether Sas2p directly interacts with ORC like Hat1p and Hbo1p. Sas2p may help ORC function by acetylating histones near origins. We tested the possibility that Sas2p contributes to the formation of proper chromatin structure at origins such as ARS1 that is required for origin firing. However, we found that sas2Δ caused no significant change in chromatin at ARS1 in wild-type or orc2-1 cells (Figure 4). It is also possible that H4-K16 acetylation by Sas2p helps an origin fire by disrupting higher order chromatin structure. This is based on the finding that H4-K16 acetylation is inhibitory to 30 nm fiber structure formation from a nucleosome array (43). In addition, Sas2p may also contribute to chromatin assembly during DNA replication. Consistent with this notion is the finding that Sas2p physically interacts with CAF-I and Asf1p, two factors that mediate chromatin assembly during DNA synthesis (18,44).

We found, interestingly, that compared to ARS1, ARS305 firing was significantly less sensitive to orc2-1 and sas2Δ despite the fact that ARS305 is also an early-firing origin (Figure 3). ARS305 is distinct from ARS1 in sequence and structure. Whereas the ARS1 contains ACS, B1, B2 and B3 elements, ARS305 contains ACS, B1 and B4 but lacks B2 and B3 (Figure 3B; ref. 45). Moreover, ARS305 contains a DUE (DNA unwinding element) that is essential for its function (Figure 3B; ref. 46). The unique presence of this DUE sequence may be the reason why ARS305 firing is less dependent on the functions of ORC and Sas2p than ARS1.

Our results demonstrating a synthetic genetic/functional interaction between SAS2 and ORC genes are apparently contrary to the previous finding that sas2Δ suppressed orc2-1 phenotypes (13,14). The temperature-sensitive orc2-1 and, to a lesser extent, orc5-1 mutants exhibit slow growth phenotypes even at permissive temperatures. It is possible that a spontaneous secondary mutation that suppresses the growth defect exists in one or more of the orc2-1 and orc5-1 mutants used in this and/or other studies. In fact, Shimada et al. (31) demonstrated that their batch of a widely used orc2-1 mutant JRY125 had an extragenic suppressor mutation that mitigated the temperature sensitivity of orc2-1 so that cells could grow at 30°C (but not higher temperatures). The JRY125 derivatives we made were not viable at 30°C (Figure 1B), and therefore did
not seem to contain the additional mutation. On the other hand, our MATa orc2-1 strain was able to grow, albeit slowly, at 30°C, and therefore might possess a secondary mutation that partially suppresses the growth defect of orc2-1 (Figure 1). There has been no report regarding whether orc5-1 mutants contain extragenic suppressor mutations. Nevertheless, the fact that sas2Δ showed synthetic growth defect with both orc2-1 and orc5-1 in three genetic backgrounds in this report (Figure 1) strongly supports the notion that Sas2p plays a positive role in ORC-mediated functions. As for previous results of sas2Δ suppressing orc2-1 phenotypes (13,14), it is possible that an extragenic suppressor mutation alone, or together with sas2Δ, was responsible for the suppression.

A positive role of SAS2 in transcriptional silencing

Previous studies on the role of SAS2 in transcriptional silencing yielded conflicting results, with some suggesting a positive role for SAS2 in silencing, while others concluding that Sas2p was a negative regulator (13,17–19). However, the silencing assays used in these studies were distinct from each other concerning the silencing reporter, the silent locus, and/or the genetic background, making it difficult to compare the results directly. In this work, we examined whether the intrinsic properties and/or the context of a silencer (HML versus HMR) affected its response to sas2Δ. We showed that the function of each of silencers HMR-E, HML-E and HML-I was reduced to an intermediate level when put in the same context of HML-E (Figure 5). The activity of HMR-E in its native context is also reduced to an intermediate level by sas2Δ (Figures 5 and 7). The same silencing reporter URA3 was used in all our experiments so the results can be readily compared directly. These results demonstrate that SAS2 plays a positive role in the function of any of the tested silencers regardless of their genomic contexts, which are consistent with the finding that SAS2 is required for telomeric silencing. Acetylation of histone H4-K16 by Sas2p in euchromatin was proposed to prevent ectopic spreading Sir complex from telomeric silent chromatin (20,21). This model may also apply to silent chromatin at HM loci. According to this model, sas2Δ allows a portion of Sir proteins to leave silent HML and HMR and spread into euchromatin regions, thereby reducing Sir abundance at HM loci. As a result, silent chromatin adopts an intermediate state between fully silent and fully derepressed structures.

Given the functional interactions between the SAS2 and ORC genes in DNA replication, we envision that Sas2p may also regulate silencing by affecting ORC function at the silencer. This is supported by our finding that sas2Δ and orc5-1 have a synthetic effect on HMR silencing (Figure 7). ORC bound to a silencer contributes to the establishment of silent chromatin by recruiting Sir1p, which interacts with the Sir complex (15). In addition, ORC also directs nucleosome positioning at the silencer that affects the efficiency and directionality of silencing (47). It is possible that Sas2p-mediated histone acetylation near the silencer facilitates one or both of these ORC-mediated processes.

It is noteworthy that our experiments demonstrating a positive role of SAS2 in silencing involved wild-type silencers (Figures 5–7), while previous studies reporting a negative role of SAS2 in silencing employed a mutated or synthetic HMR-E silencer that is weaker than the wild-type one (13,17). It is not clear why wild-type and mutated HMR-E silencers respond to SAS2 in opposite ways.

Role of Sas2p in maintaining a fully silenced state of chromatin

We found that sas2Δ reduced the negative supercoiling of HML DNA by a ΔLk of 1 (Figure 6). This reduction is less than the ΔLk of 7 caused by sir3A (Figure 6), but is consistent with the finding that sas2Δ decreases but does not completely abolish transcriptional silencing at the HM loci (Figures 5 and 7), and suggests that HML chromatin assumes an intermediate state that is distinct from both the fully silenced state (in SIR+ cells) and fully derepressed state (in sir− cells).

The topology of eukaryotic DNA is determined by multiple factors including the density of nucleosomes along the DNA and the conformation of individual nucleosomes. The major contribution to the negative supercoiling of DNA is its wrapping into nucleosomes with an average ΔLk of about –1 per nucleosome formed in vitro (48). Changing the conformation of a nucleosome may alter the number of supercoils constrained on it. For example, acetylation of the core histones reduces the negative supercoiling of nucleosomal DNA (49). The higher negative supercoiling of DNA in silent versus derepressed HML locus is a reflection of increased regularity and stability of nucleosomes as well as reduced histone acetylation in silent chromatin (50,51). The reduction of negative supercoiling induced by sas2Δ might be the result of a decrease in nucleosome density/stability and/or conformational changes of nucleosomes, but our preliminary chromatin mapping results suggest that the former is unlikely the case (Zou,Y. and Bi,X. unpublished results).

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