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 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 *HM* 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 ($OD_{600} = \sim 0.5$) 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, 1.5×10^9 cells were collected and used for genomic DNA isolation as described (25). Genomic DNA was digested with NcoI and subjected to 2D gel analysis as described (26,27). The conditions for the first dimension were: 0.35% agarose, $1 \times$ 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 2×10^8 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.

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

Table 1. Yeast strains

| Number	Name	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	YYZ261	W303-1A, *sas2Δ::kanMX*	This work
3	YB59	W303-1A, *orc2-1*	B. Stillman
4	YYZ263	YB59, *sas2Δ::kanMX*	This work
5	YYZ264	YB59, *sas2Δ1::TRP1*	This work
6	YB57	W303-1A, *orc5-1*	B. Stillman
7	YYZ265	YB57, *sas2Δ::kanMX*	This work
8	YYZ266	YB57, *sas2Δ1::TRP1*	This work
9	YYZ327	W303-1A, *sas4Δ::kanMX*	This work
10	YYZ328	YB59, *sas4Δ::kanMX*	This work
11	W303-1B (JRY3009)	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. Rine
12	YYZ271	W303-1B, *sas2Δ1::TRP1*	This work
13	JRY4125	W303-1B, *orc2-1*	J. Rine
14	YYZ273	JRY4125, *sas2Δ1::TRP1*	This work
15	JRY4249	W303-1B, *orc5-1*	J. Rine
16	YYZ275	JRY4249, *sas2Δ1::TRP1*	This work
17	JRY4473	W303-1B, *HMR-SSΔI*	J. Rine
18	YYZ289	JRY4473, *sas2Δ1::TRP1*	This work
19	JRY4475	JRY 4473, *orc2-1*	J. Rine
20	YYZ282	JRY4475, *sas2Δ1::TRP1*	This work
21	YZB111	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 URA3-(HML-E)-HMLα-(HML-I)*	Ref. 35
22	YYZ313	YZB111, *sas2Δ::kanMX*	This work
23	YZB111s	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 can1-100 sir3::LEU2 URA3-(HML-E)-HMLα-(HML-I)*	Ref. 35
24	YZB110	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 URA3-(HMR-E)-HMLα-(HML-I)*	Ref. 35
25	YYZ316	YZB110, *sas2Δ::kanMX*	This work
26	YZB108	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 URA3-(HML-I)-HMLα-(HML-I)*	Ref. 35
27	YYZ318	YZB108, *sas2Δ::kanMX*	This work
28	YZB102	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 (HML-E)-HMLα-(HML-I)$_{inverted}$-URA3*	Ref. 35
29	YYZ322	YZB102, *sas2Δ::kanMX*	This work
30	YZB104	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 (HML-E)-HMLα-(HMR-E)-URA3*	Ref. 35
31	YYZ323	YZB104, *sas2Δ::kanMX*	This work
32	YZB114	*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1-SUP4-SIR3 URA3-(HMR-E)$_{inverted}$-HMRa-(HMR-I)*	Ref. 35
33	YYZ320	YZB114, *sas2Δ::kanMX*	This work
34	YXB10	*MATa ura3-52 ade2-1 lys1-1 his5-1 can1-100 [cir°] LEU2-GAL10-FLP1 FRT-E-hml::β1-I-FRT*	Ref. 52
35	YYZ324	YXB10, *sas2Δ::kanMX*	This work
36	YXB810	YXB10, *sir1::URA3*	This work
37	YYZ325	YXB10, *sir1::URA3 sas2Δ::kanMX*	This work
38	YXB10s	YXB10, *sir3::URA3*	Ref. 52
39	YYZ326	YXB10, *sir3::URA3 sas2Δ::kanMX*	This work
40	YYZ301	W303-1B, *HMR::URA3*	This work
41	YYZ304	YYZ301, *sas2Δ::kanMX*	This work
42	YYZ303	JRY4249, *HMR::URA3*	This work
43	YYZ306	YYZ303, *sas2Δ::kanMX*	This work

incubated for 2.5 h to induce the expression of P*GAL10-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
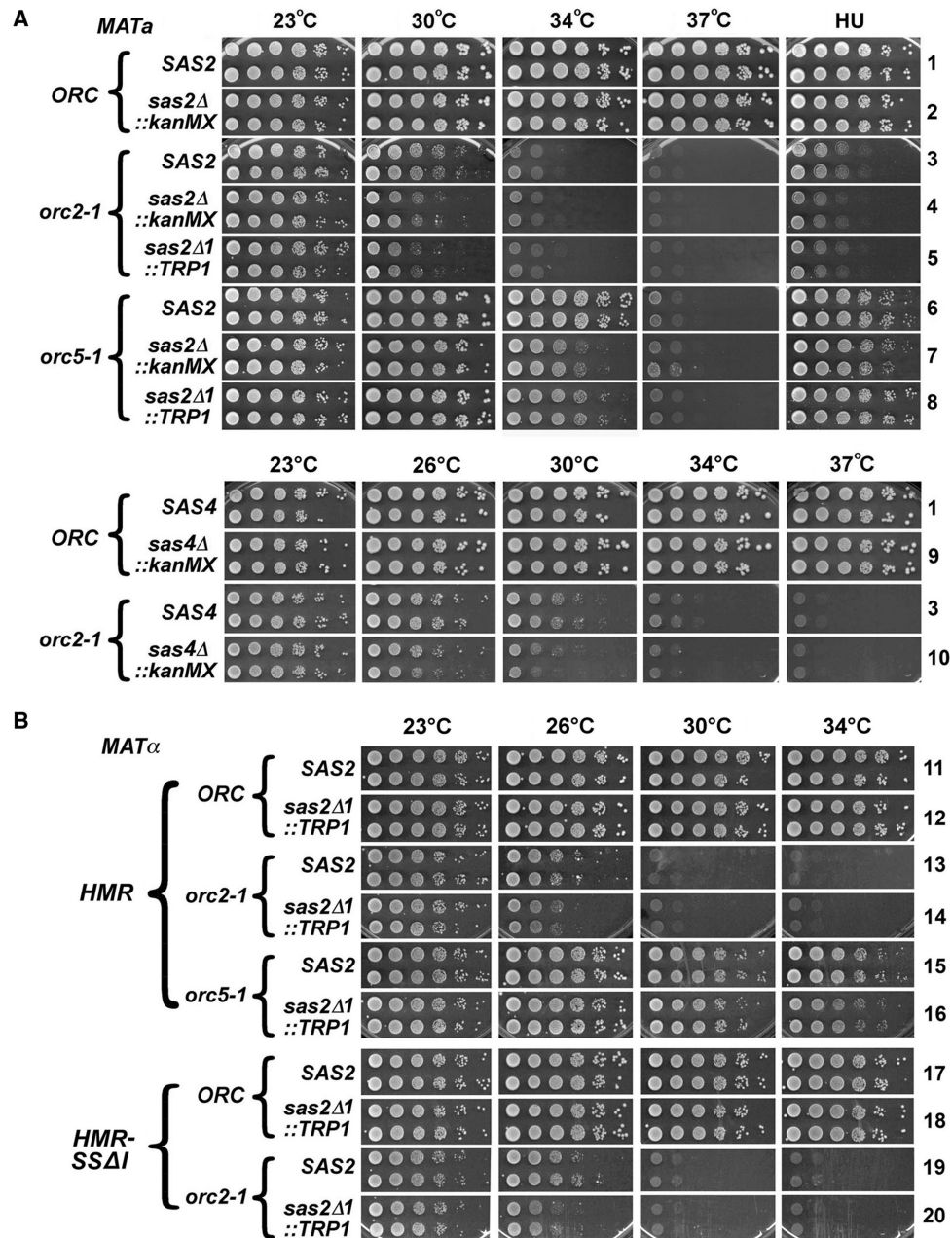
Figure 1. Synthetic interactions between *sas2Δ* and *orc* mutations. (**A**) Synthetic interactions between *sas2Δ* and *orc* mutations in the *MATa* background. Cells of strains 1–10 were grown to log phase in synthetic complete (SC) liquid medium. Serial 10-fold dilutions were spotted on four SC plates and incubated for 3 days at 23, 30, 34 and 37°C, respectively. Strains 1, 3, 9 and 10 were also grown at 26°C. Cells were also spotted on SC plates supplemented with 50 mM HU and incubated at 23°C for 3 days. Two independent clones of each strain were examined. (**B**) Synthetic interactions between *sas2Δ* and *orc* mutations in the *MATα* background. Cells of strains 11–20 were grown to log phase and serial 10-fold dilutions were spotted on SC plates and incubated for 3 days at 23, 26, 30 and 34°C, respectively. Two independent clones of each strain were examined.

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 compare 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

semi-permissive temperatures (Figure 1A, compare 10 to 3 at 26 and 30°C). These results suggest that the SAS complex performs an overlapping role with ORC in cell growth.

As we considered the cause of the discrepancy between our results and previously published results regarding genetic interactions between *orc* mutants and *sas2Δ*, we noted that the genetic background of our strains was different from that of the previous strains. Our *sas2Δ* 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 *sas2Δ1::TRP1* (13,14). We analyzed the sequence of *sas2Δ1::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 *sas2Δ1::TRP1* differed from *sas2Δ::kanMX* in its interaction with *orc2-1* or *orc5-1*, we made *orc2-1 sas2Δ1::TRP1* and *orc5-1 sas2Δ1::TRP1* double mutants in the W303-1A background. The growth phenotypes of these double mutants at various temperatures were similar to *orc2-1 sas2Δ:: kanMX* and *orc5-1 sas2Δ::kanMX* strains, respectively (Figure 1A, compare 5 to 4 and 3, and 8 to 7 and 6). Therefore, the *sas2Δ::kanMX* and *sas2Δ1::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 (*MATα*) instead of W303-1A (*MATa*). We therefore examined if the cell type affected genetic interactions between *orc* mutants and *sas2Δ*. We remade *orc2-1 sas2Δ1::TRP1* and *orc5-1 sas2Δ1::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 *MATα* background than in the *MATa* background as the *MATα 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-SSΔI*) (13), whereas all our strains were wild-type for *HMR*. We remade the double mutant *orc2-1 sas2Δ1::TRP1* in the *MATα HMR-SSΔI* 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 *sas2Δ* 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 (Figuer 1A, compare 3 to 1 on HU-containing medium; ref. 31). We found that *sas2Δ* 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.
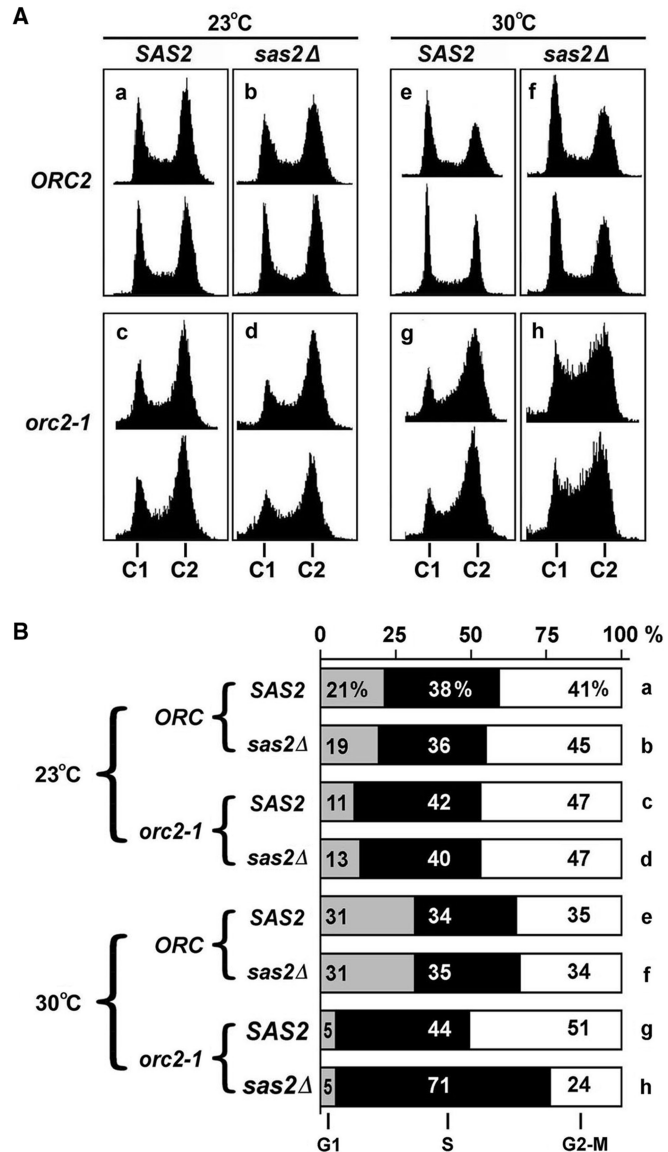
Synthetic effect of *sas2Δ* and *orc2-1* on cell cycle progression through S-phase

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 *sas2Δ* 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 *sas2Δ* (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 *sas2Δ* 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 *sas2Δ* 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 sas2Δ* cells were in S-phase at 30°C than at 23°C (Figure 3, compare h to g), indicating that *sas2Δ* delayed or arrested progression of *orc2-1* cells through S-phase.

SAS2 plays 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 *sas2Δ* 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 *sas2Δ* 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,

Figure 2. Synthetic effect of *sas2Δ* and *orc2-1* on cell cycle progression. (**A**) FACS analysis. Cells were grown to log phase and subjected to FACS analysis. Two independent clones of each strain grown at 23 and 30°C, respectively, were examined. The *x*-axis represents DNA content and the *y*-axis the number of cells in the culture. Data for strains 1–4 at 23°C are shown in panels a–d, respectively, and data for strains 1–4 at 30°C are shown in panels e–h, respectively. (**B**) Quantification of the proportions of G1, S and G2-M cells. The percentages of G1, S and G2-M cells for each strain are the averages of data for the two independent clones examined by FACS in (A).

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 4 60 989–4 64 386) (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 36 563–41 669). 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 micrococcol nuclease (MNase) digestion followed by indirect end labeling. The *ARS1* region is associated
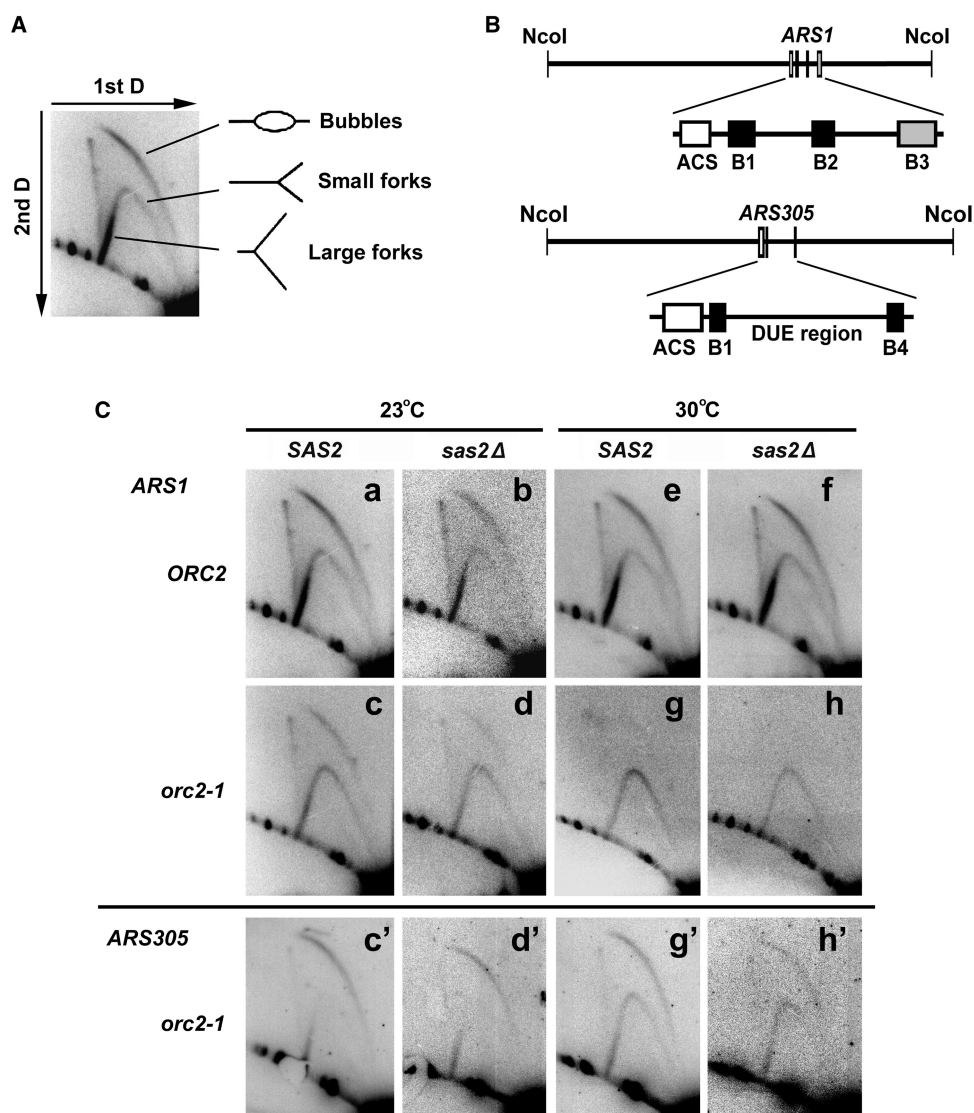
Figure 3. Synthetic effect of *sas2Δ* and *orc2-1* on the initiation of DNA replication. (**A**) Scheme of replication intermediates fractionated by 2D gel electrophoresis. The bubble arc, small- and large-fork arcs are indicated. (**B**) Schematics of the genomic NcoI fragment of chromosome IV containing *ARS1* and the NcoI fragment of chromosome III containing *ARS305*. The ACS (ARS consensus sequence), B1–B4 and DUE sequence in the two ARSs are shown. Note that ACS is the recognition site of ORC, and B3 is an Abf1p-binding site. (**C**) 2D gel analysis of replication intermediates. Data for *ARS1* in strains 1–4 at 23°C are shown in panels a–d, respectively, and data for *ARS1* in strains 1–4 at 30°C are shown in panels e–h, respectively. Data for *ARS305* in strains 3 and 4 at 23°C are shown in panels c' and d', respectively. Data for *ARS305* in strains 3 and 4 at 30°C are shown in g' and h', respectively. See text for descriptions.

with three major MNase cleavage sites (designated as α, β and γ) 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 *sas2Δ* single mutations or the *orc2-1 sas2Δ* 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 (*HMR*a-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-SSΔI*) (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
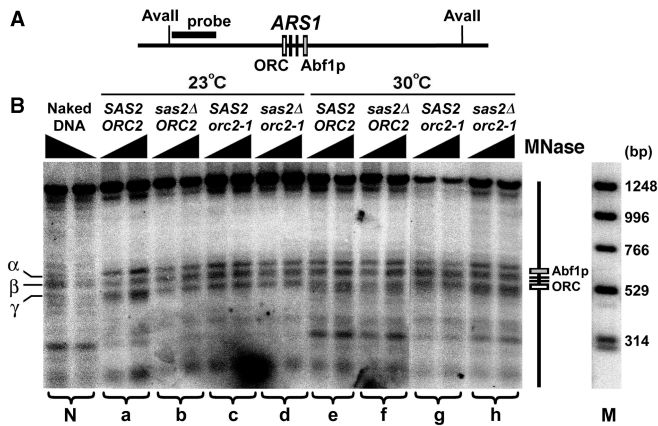
Figure 4. The *sas2Δ* does not affect chromatin structure at *ARS1*. (**A**) Schematic representation of the *ARS1* locus. Two AvaII sites flanking *ARS1* and the position of the probe used in indirect end labeling are shown. (**B**) Results of chromatin mapping around *ARS1* in strains 1, 2, 3 and 4 at 23°C (lanes a, b, c and d) or 30°C (e, f, g and h). Chromatin was treated with MNase at 120 or 160 U/ml, and DNA was isolated and digested with AvaII. Naked DNA sample (N) from strain 1 was also treated with MNase and digested with AvaII. DNA samples were subjected to gel electrophoresis and Southern blotting. Three major MNase cleavage sites are marked as α–γ. The positions of Abf1p and ORC sites in *ARS1* are indicated on the right.

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 *SAS2* 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 *HMR*a-e** or *HMR-SS*), reporters (e.g. *HMRa* 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
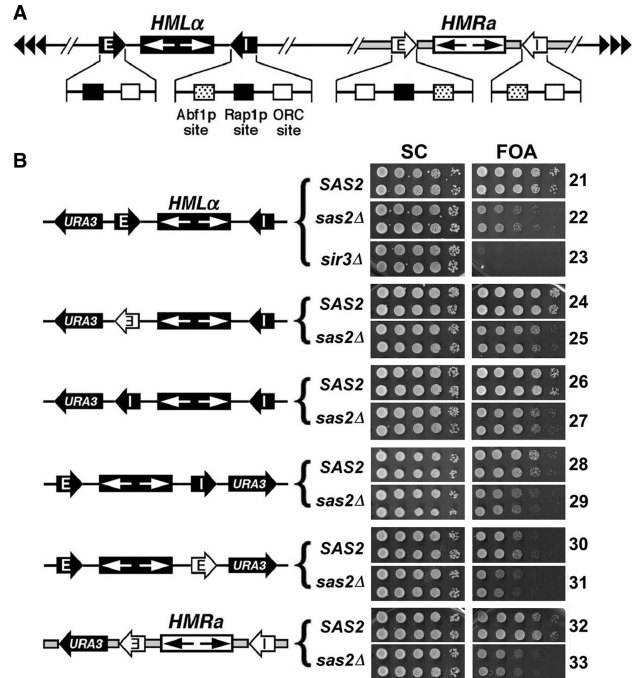
Figure 5. A positive role of *SAS2* in silencer function. (**A**) Schematics of the *HML* and *HMR* loci on chromosome III in *S. cerevisiae* (not drawn to scale). *HML-E* and *-I* silencers are shown as filled arrows with white letters, and *HMR-E* and *-I* are shown as open arrows with black letters. The directions of silencers are drawn pointing toward the inside of *HML* or *HMR*. The sites for Abf1p, Rap1p and ORC binding in silencers are indicated by stippled, filled and open rectangles, respectively. *HML*α genes at *HML* and *HMR*a genes at *HMR* are also indicated. Tandem arrowheads indicate telomeric repeats. (**B**) Growth phenotypes of strains 21–33. Left, modified *HML* and *HMR* loci in strains 21–33. Cells of each strain were grown to log phase, and serial 10-fold dilutions were spotted on test plates and allowed to grow for 3 days. Two independent clones of each strain were examined. FOA, SC plus 1 mg/ml of 5-fluoroorotic acid (FOA).

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 with *HMR-E* 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 P_{GAL}–*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 *sir*⁻ 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
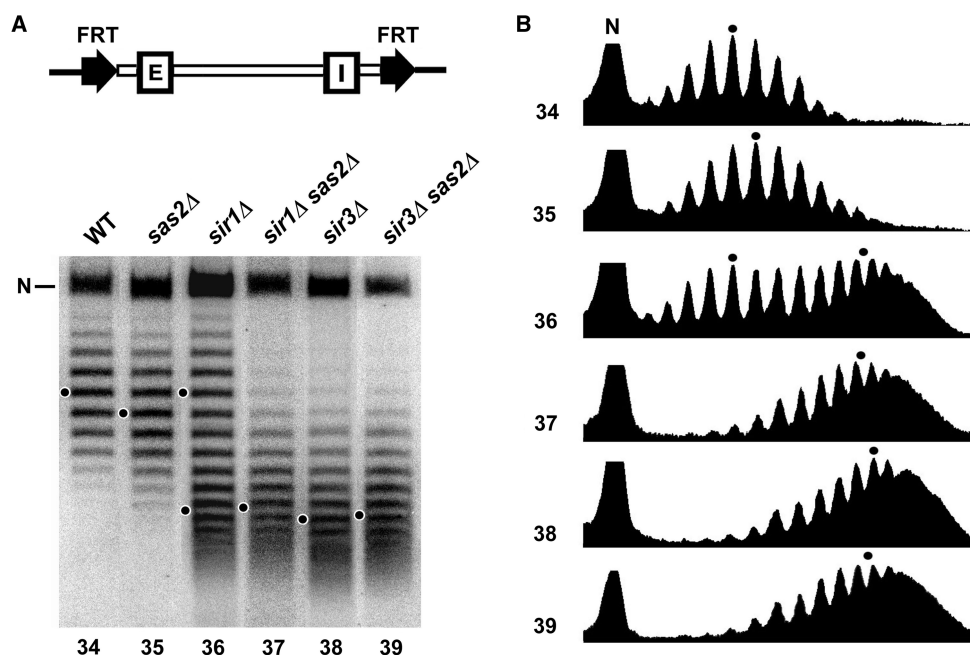
Figure 6. *sas2Δ* reduces the negative supercoiling of silent *HML* DNA. (**A**) Top, diagram of the modified *HML* locus in strains 34–39. The *HML-E* and *-I* silencers and a pair of FRTs were shown. Each strain bears a P_{GAL}–*FLP1* gene in the genome. Bottom, the topology of *HML* DNA in strains 34–39. DNA isolated from each strain was subjected to agarose gel electrophoresis in the presence of 13 μg/ml chloroquine. Under this condition, more negatively supercoiled circles migrate more slowly. After Southern blotting, topoisomers of the *HML* circle were detected by an *HML*-specific probe. The center of distribution of topoisomers in each lane was marked by a dot. The nicked/relaxed form of the *HML* circle was indicated as N. (**B**) Profiles of topoisomers in lanes 34–39 shown in (A) as determined by using NIH Image.

A

HMR::URA3 ▭ E ▷ *URA3* ▭ ◀——▶ HMRa ▭ I ▷

B
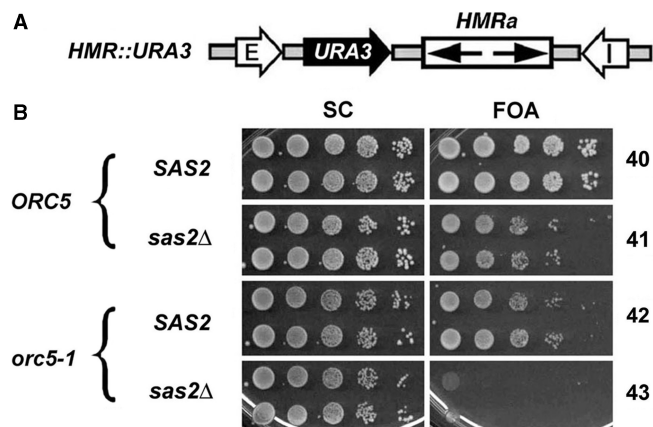
Figure 7. Synthetic effect of *sas2Δ* and *orc5-1* on transcriptional silencing. (**A**) Schematic representation of the *HMR* locus in strains 40 through 43 (not drawn to scale). The *HMR-E* and *-I* silencers, the *URA3* and *HMRa* genes are indicated. (**B**) Growth phenotypes of strains 40–43 on SC and FOA media at 30°C.

those from *sir⁻* cells where *HML* is derepressed (Figure 6, compare 36 to 34 and 38). However, in a *sas2Δ sir1Δ* double mutant, there was only one population of *HML* circles, which is similar to that in *sir⁻* cells (Figure 6, compare 37 and 38). Therefore, *sas2Δ* and *sir1Δ* have a synthetic effect on the topology of *HML* DNA, which is consistent with the finding that *sas2Δ* abolished *HML* silencing in a *sir1Δ* background (13,17,19).

Synthetic effect of *sas2Δ* and *orc5-1* on transcriptional silencing

Given the aforementioned functional interactions between *sas2Δ* and *orc* mutants regarding cell growth and DNA replication, we asked whether they also interact to affect transcriptional silencing. We showed that either *orc5-1* or *sas2Δ* alone reduced silencing of a *URA3* reporter integrated at *HMR* in W303-1B (Figure 7, compare 41 and 42 to 40 on FOA), and *sas2Δ orc5-1* double mutation abolished *URA3* silencing (Figure 7, compare 43 to 40). Therefore, *SAS2* is required for *HMR* silencing in an *orc5-1* mutant.

DISCUSSION

A positive role of *SAS2* in DNA replication

The initiation of DNA replication in eukaryotes depends on the assembly of the pre-RC via the sequential binding to the origin of ORC, Cdc6p, Cdt1p and the MCM complex (4). We have presented evidence suggesting that the HAT Sas2p plays a positive role in replication initiation in yeast. This is in line with the notion that chromatin structure regulates origin function. It has been shown that increasing histone acetylation around a replication origin in yeast enables it to fire earlier in S phase (9). Recently, it was found that the yeast HAT Hat1p physically interacts with ORC and *HAT1* deletion exacerbates the temperature-sensitivity of *orc* mutants (12). Hat1p acetylates histone H4-K5, K12 and, to a lesser extent, histone H2A (40), and it may contribute to replication initiation by

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 *sir3Δ* (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 wells 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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