Supplementary information

Materials and methods

Induction of Sir3p-HA to silence the *a1* gene

For experiments where silencing was analyzed following expression of HA-tagged Sir3p, the endogenous *SIR3* gene was deleted from the yeast strain to be analyzed (wild-type, *cac1Δ, rtt106Δ,* or *cac1Δ rtt106Δ*), and a plasmid allowing for the expression of Sir3p-HA under an inducible GAL10 promoter was integrated at the *URA3* locus as described (Cheng and Gartenberg, 2000; Katan-Khaykovich and Struhl, 2005). Cells were first arrested with 0.2 M HU for 4 hours and then released into yeast medium containing 2% galactose to induce expression of Sir3p-HA. Whole-cell extracts and total RNA were prepared at 1.5-hour intervals after cells were released from the cell cycle block and analyzed by FACS for cell cycle progression, Western blot for Sir3p-HA expression, and RT-PCR for transcription of the *a1* gene. Alternatively, following release from the HU block, yeast cells were cross-linked using formaldehyde and ChIP assays were performed using antibodies against Sir2p, Sir3p, or Sir4p.

Quantification of *a1* mRNA using RT-PCR

A standard protocol was followed to isolate RNA from yeast cells (Schmitt et al., 1990). Briefly, 5 ml of log phase cells were collected and re-suspended in 400 μl of AE buffer (50 mM sodium acetate, pH 5.3, 10 mM EDTA) and transferred into a fresh tube containing 40 μl of 10% SDS. The suspension was vortexed and an equal volume (500 μl) of fresh phenol previously equilibrated with AE buffer was added. The resulting mixture was incubated at 65ºC for 4 minutes and then chilled rapidly in a dry ice/ethanol

bath until phenol crystals appeared. The mixture was then centrifuged for 3 minutes at maximum speed to separate the aqueous and phenol phases. The RNA-containing aqueous phase was then transferred into fresh microcentrifuge tubes and extracted with 550 μl of phenol/chloroform at room temperature for 5 minutes. The RNA was then purified by ethanol precipitation, dried, and re-suspended in 40 μl of water. To quantify the expression of the *a1* gene, cDNA was first synthesized from 5 μg of total RNA using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol, and subsequently a fraction of cDNA (equivalent to 0.125 μg of total RNA) was amplified by PCR in the presence of α-^{32}P-dATP. The PCR primers used to amplify the *a1* gene were described previously (Smeal et al., 1996). Amplification of the house-keeping genes *ACT1* and *G6PDH* was used as a control. PCR products were resolved on 8% non-denaturing polyacrylamide gels in TBE buffer and quantified using a phosphorimager. Expression of the *a1* gene was normalized against expression of *ACT1* at each time point, and the ratio of *a1* mRNA to *ACT1* mRNA was set as 100% at the onset of the experiment (time 0).

Chromatin immunoprecipitation assay (ChIP) and determination of relative enrichment of immunoprecipitated DNA

Briefly, for each ChIP assay approximately 5x10^8 cells were cross-linked with 1% formaldehyde at 25°C and quenched with 65 mM glycine for 5 minutes. Yeast cells were collected, washed with cold TBS buffer twice, and lysed using mechanical disruption with glass beads. Then DNA was sheared to an average of 500 bp to 1 kb in length by sonication. After clarification, the supernatant was incubated with the appropriate

antibodies overnight, protein-G beads were added, and samples were incubated for another 1-2 hours. After extensive washing of the protein-G beads, the DNA-protein cross-linking was reversed by incubating the beads at 65ºC for 10 hours in the presence of 1% SDS, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Alternatively, cross-linking was reversed with 1% chelex 100 resins and the DNA was directly used for template as described (Nelson et al., 2006).

To quantify DNA from ChIP assays, DNA from each immunoprecipitation or whole-cell DNA was subjected to competitive PCR analysis in the presence of α-^{32}P-dATP. The primer sets have been previously described (Strahl-Bolsinger et al., 1997; Zhang et al., 2002) and are available upon request. PCR products were separated on 5% TBE-polyacrylamide gels and quantified using a phosphorimager. To determine the relative enrichment of a given PCR product following ChIP, the ratio of the PCR products over a negative control such as the *ACT1* gene or a fragment 20 kb away from the right end of chromosome VI was calculated. The resulting ratio was then divided by the ratio of PCR products obtained using whole-cell DNA as template and the same set of PCR primers.

GST pull down and immunoprecipitation

Recombinant GST-Rtt106p or GST-REGα was incubated with glutathione sepharose beads for 2 hours at 4ºC in binding buffer A (25 mM Tris, pH 8.0, 10% glycerol, 1 mM EDTA, 0.01% Nonidet P-40, 100 mM NaCl). The beads were then washed and incubated with increasing amounts of ^{35}S-labeled Sir3p or Sir4p produced by rapid TNT in vitro transcription/translation kits (Promega) for 6 hours at 4ºC. After extensive washing, the

proteins bound to GST-Rtt106p were eluted with SDS sample buffer, resolved using SDS-PAGE, and visualized by Coomassie brilliant blue staining or autoradiography. To determine whether Rtt106p interacted with Sir4p in yeast cells, wild-type or Sir4p-TAP yeast strains from which the *RTT106* gene was deleted were transformed with a CEN-containing plasmid expressing Rtt106p-Flag under the control of the endogenous *RTT106* promoter. Yeast cells were resuspended in IP buffer (20 mM Tris, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100), and frozen yeast cells were ground in dry ice. After evaporation of dry ice, 3 mM DSP (Pierce) was added to the lysate and incubated at 4ºC with shaking for 30 minutes. After quenching the cell lysate with 150 mM Tris-HCl, pH 7.5, for 5 minutes at 4ºC, the cell lysate was diluted with 150 mM KCl, 5 mM $MgCl_2$, and Triton X-100 to 1%, incubated at 4ºC for 10 minutes, clarified by centrifugation, and used for immunoprecipitation following the standard TAP purification procedure (Zhou et al., 2006). Briefly, the cell lysate was incubated with IgG beads for 2 hours at 4ºC, and the bound proteins were eluted using the TEV protease. The eluted proteins were precipitated with TCA, resuspended, and then boiled in SDS sample buffer containing 30 mM DTT to reverse cross-linking. The precipitated proteins were resolved using 10% SDS-PAGE, transferred onto nitrocellulose membrane, and detected using primary antibodies against Sir4p or the Flag epitope secondary and antibodies conjugated to horse radish peroxidase. Following addition of the ECL substrate, the membrane was exposed to film, and the film was developed.

Western blot for analysis of Sir3p-HA expression

Approximately $5x10^7$ yeast cells were pelleted, washed with H_2O, and cell lysates were prepared by mechanically disrupting cells with glass beads at 4°C. Proteins were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with primary antibodies against the HA epitope (12CA5) and secondary antibodies conjugated to horse radish peroxidase. Following addition of the ECL substrate, the membrane was exposed to film, and the film was developed.

References

Cheng, T. H., and Gartenberg, M. R. (2000). Yeast heterochromatin is a dynamic structure that requires silencers continuously. Genes Dev *14*, 452-463.

Katan-Khaykovich, Y., and Struhl, K. (2005). Heterochromatin formation involves changes in histone modifications over multiple cell generations. Embo J *24*, 2138-2149.

Kimura, A., Umehara, T., and Horikoshi, M. (2002). Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. Nat Genet *32*, 370-377.

Nelson, J. D., Denisenko, O., Sova, P., and Bomsztyk, K. (2006). Fast chromatin immunoprecipitation assay. Nucleic Acids Res *34*, e2.

Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res *18*, 3091-3092.

Smeal, T., Claus, J., Kennedy, B., Cole, F., and Guarente, L. (1996). Loss of transcriptional silencing causes sterility in old mother cells of S. cerevisiae. Cell *84*, 633-642.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev *11*, 83-93.

Suka, N., Luo, K., and Grunstein, M. (2002). Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat Genet *32*, 378-383.

Zhang, Z., Hayashi, M. K., Merkel, O., Stillman, B., and Xu, R. M. (2002). Structure and function of the BAH-containing domain of Orc1p in epigenetic silencing. EMBO J *21*, 4600-4611.

Zhou, H., Madden, B. J., Muddiman, D. C., and Zhang, Z. (2006). Chromatin assembly factor 1 interacts with histone h3 methylated at lysine 79 in the processes of epigenetic silencing and DNA repair. Biochemistry *45*, 2852-2861.

Supplementary Figure legends

Figure S1. Combined deletion of the histone chaperones *CAC1* and *RTT106*, *CAC1* and *ASF1*, or *CAC1* and *HIR1* reduces the association of Sir proteins with telomeric heterochromatin of chromosome VI-R. A – D, *cac1Δ rtt106Δ*, *cac1Δ asf1Δ*, and *cac1Δ hir1Δ* double mutant cells exhibit reduced binding of Sir2p (A and B) and Sir3p (C and D) to telomeric heterochromatin as compared to wild-type cells. As controls, ChIP assays were also performed using *sir3Δ* or *sas2Δ* mutant cells. ChIP assays were performed using antibodies against Sir2p (A and B) and Sir3p (C and D), and the immunoprecipitated DNA was analyzed using primers that annealed to the sub-telomeric region of the right arm of chromosome VI, as described for Figure 1A of the main text. PCR products were resolved on native polyacrylamide gels (A and C) and analyzed using a phosphorimager (B and D). We did not observe spreading of Sir2p and Sir3p into euchromatin in *sas2Δ* mutant cells, as observed by others (Kimura et al., 2002; Suka et al., 2002). This could be due to the fact that the relative enrichment of DNA using antibodies against Sir2p and Sir3p is low compared to that enriched by antibodies against Sir4p. Consequently, the spreading of Sir2p and Sir3p into euchromatin in *sas2Δ* mutant cells could be too low to be detected in our experiments.

Figure S2. Sir4p binding to telomeric heterochromatin of chromosome VI-R is reduced in *cac1Δ asf1Δ* and *cac1Δ hir1Δ* double mutant cells. The experiments were performed essentially as described for Figure 2 of the main text except that data for two more mutant strains, *cac1Δ asf1Δ* and *cac1Δ hir1Δ*, are shown. The *cac1Δ asf1Δ, cac1Δ hir1Δ*, and

cac1Δ rtt106Δ double mutants all exhibit a reduced binding of Sir4p to telomeric heterochromatin.

Figure S3. Acetylation of H4-K16 at the telomere of chromosome VI-R is affected in *cac1Δ asf1Δ* and *cac1Δ hir1Δ* double mutant cells in a manner distinct from that of *sas2Δ* mutant cells. The experiments were performed essentially as described for Figure 3 of the main text except that data for two more mutant strains, *cac1Δ asf1Δ* and *cac1Δ hir1Δ*, are shown. No differences in the effects on H4-K16 acetylation were detected among the *cac1Δ asf1Δ, cac1Δ hir1Δ*, and *cac1Δ rtt106Δ* double mutant cells; however, the H4-K16 acetylation pattern detected in these mutants did differ from that detected in *sas2Δ* mutant cells.

Figure S4. Formation of silent chromatin at the *HMR* locus is delayed in unsynchronized *cac1Δ rtt106Δ* double mutant cells. A – B, silencing of the *a1* gene, a distal site of the *HMR* locus, is delayed in unsynchronized *cac1Δ rtt106Δ* double mutant cells as compared to wild-type cells. In wild-type cells, amplification of *a1* transcripts is barely detectable 3 hours after induction of Sir3p-HA, whereas in *cac1Δ rtt106Δ* double mutant cells amplification of *a1* transcripts is still detectable 7.5 hours after induction of Sir3p-HA. The endogenous *SIR3* gene was deleted from wild-type and *cac1Δ rtt106Δ* strains and a plasmid allowing for the induction of Sir3p-HA expression under control of the GAL10 promoter was integrated at the *URA3* locus. Sir3p-HA expression was induced using 2% galactose in each of the two strains, cells were collected at different time points, as indicated, and total RNA was isolated. The expression of the *a1* gene was

monitored using RT-PCR in the presence of ^{32}P-dATP. PCR products were resolved on native polyacrylamide gels (A) and quantified using a phosphorimager (B). Amplification of the pyruvate dehydrogenase alpha (PDA1) gene was used as a control. The relative amount of mRNA amplified immediately after induction of Sir3p-HA (time 0) was set at 100% for both strains.

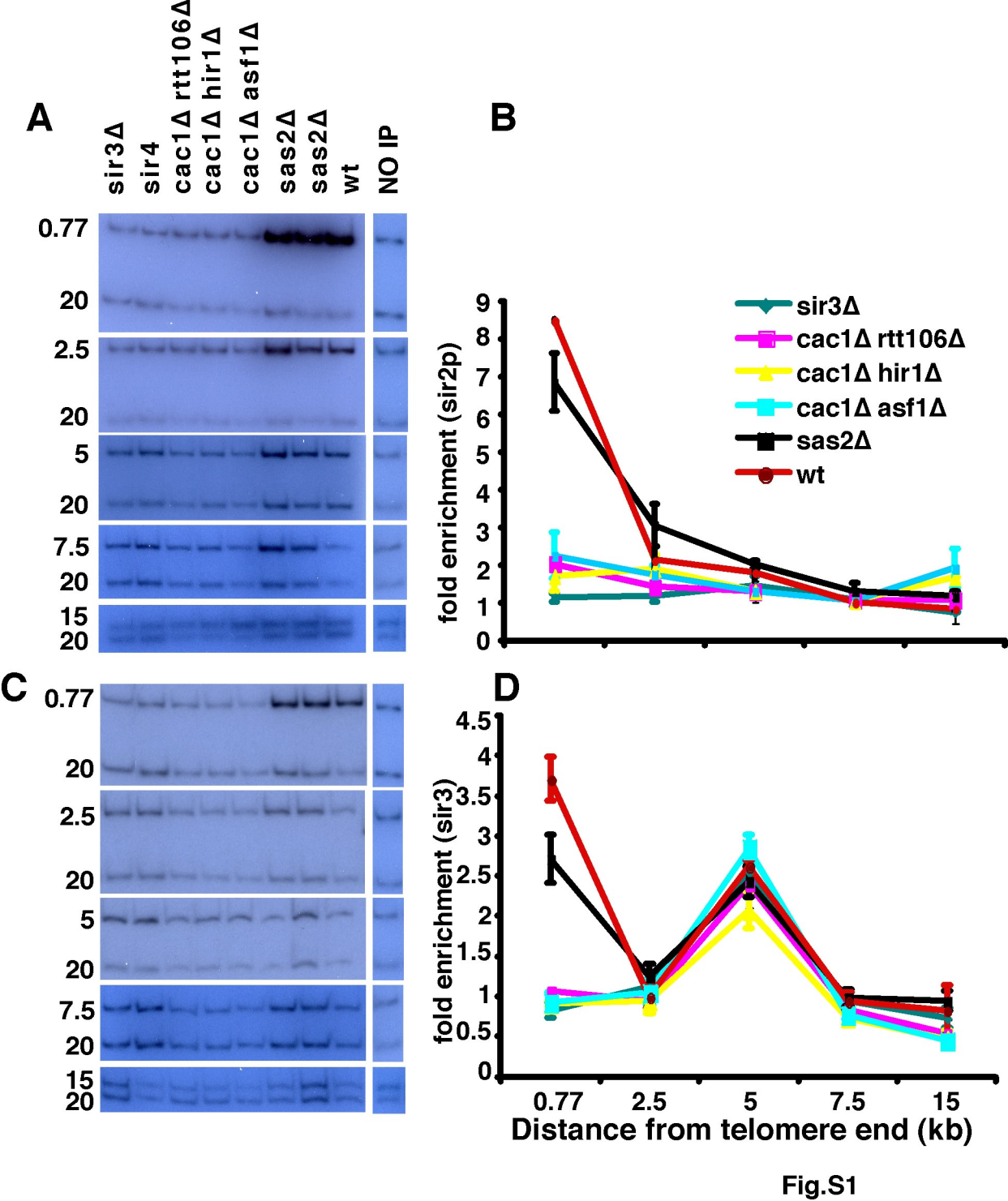

A
sir3Δ
sir4
cac1Δ rtt106Δ
cac1Δ hir1Δ
cac1Δ asf1Δ
sas2Δ
sas2Δ
wt
NO IP
0.77
20
2.5
20
5
20
7.5
20
15
20
B
fold enrichment (sir2p)
sir3Δ
cac1Δ rtt106Δ
cac1Δ hir1Δ
cac1Δ asf1Δ
sas2Δ
wt
C
D
fold enrichment (sir3)
Distance from telomere end (kb)
0.77
2.5
5
7.5
15

Fig.S1

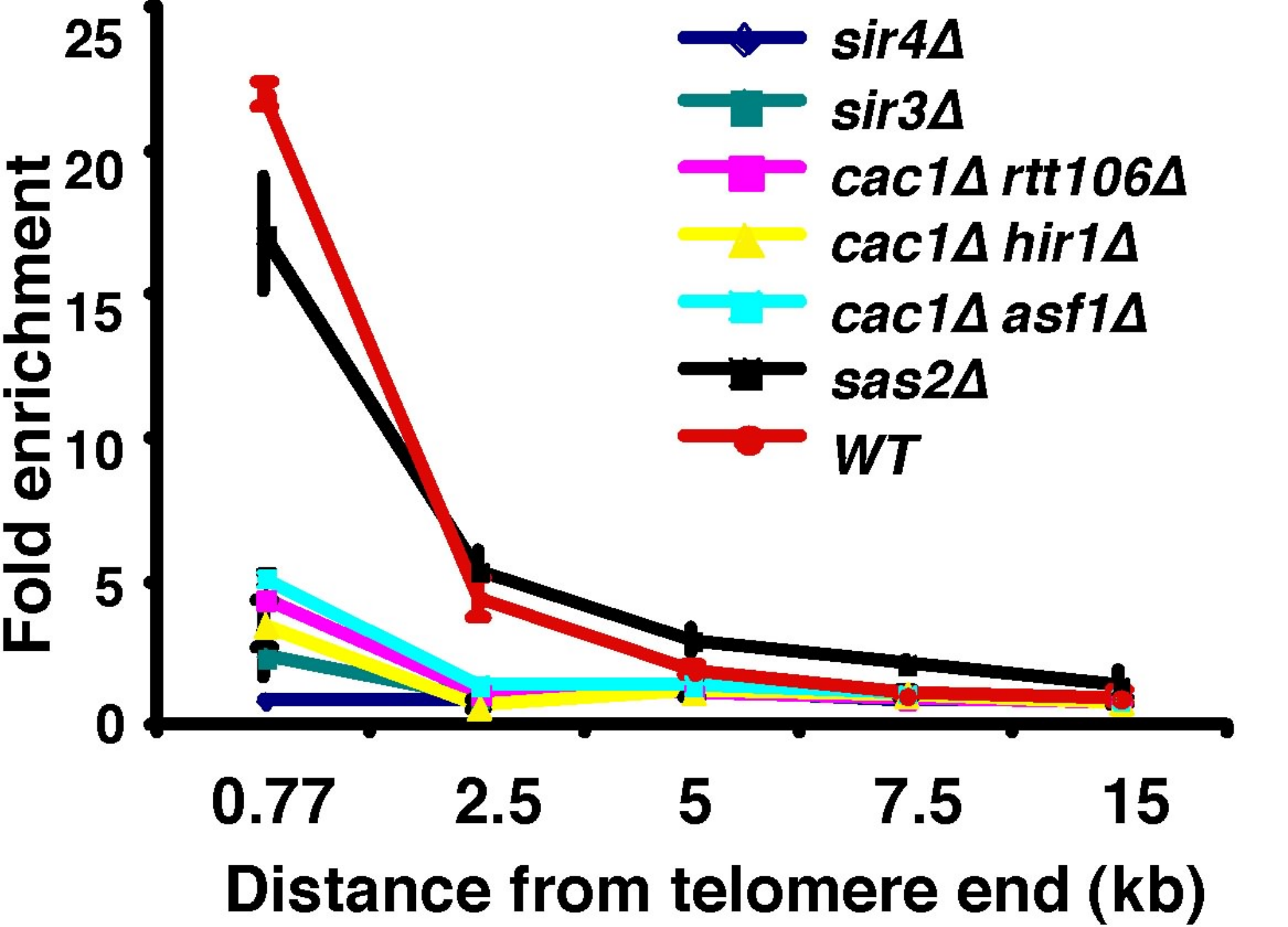

25
20
15
10
5
0
Fold enrichment
sir4Δ
sir3Δ
cac1Δ rtt106Δ
cac1Δ hir1Δ
cac1Δ asf1Δ
sas2Δ
WT
0.77
2.5
5
7.5
15
Distance from telomere end (kb)

Fig.S2

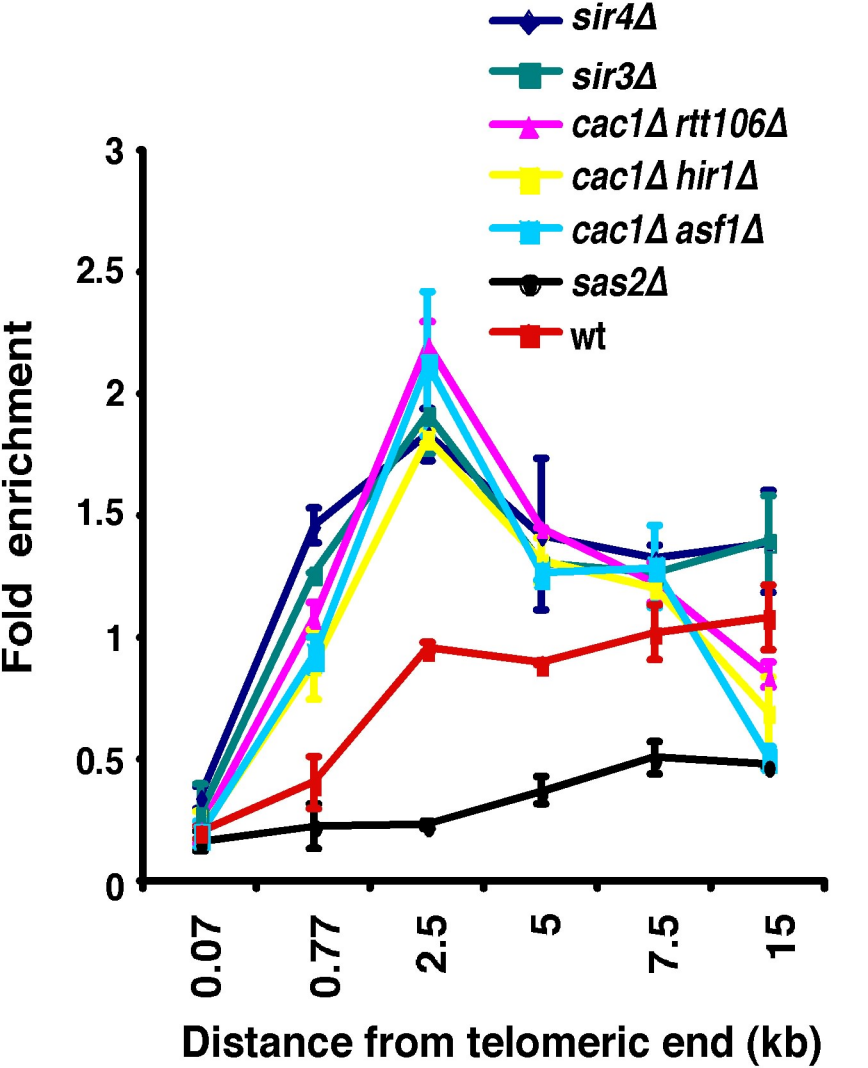
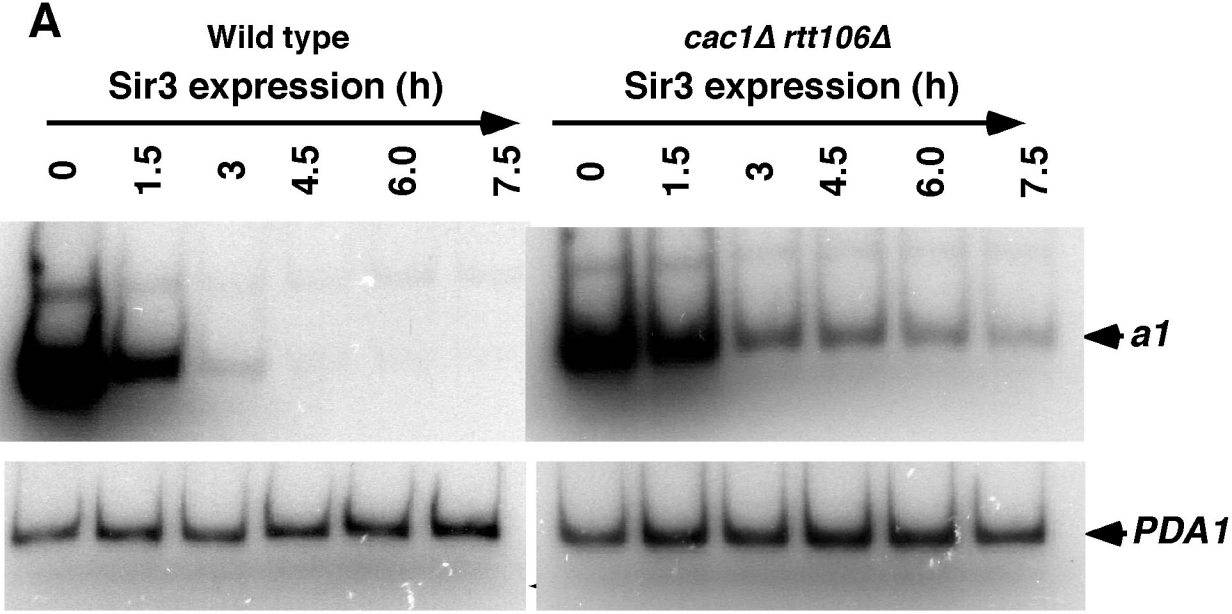

sir4Δ
sir3Δ
cac1Δ rtt106Δ
cac1Δ hir1Δ
cac1Δ asf1Δ
sas2Δ
wt
Fold enrichment
3
2.5
2
1.5
1
0.5
0
0.07
0.77
2.5
5
7.5
15
Distance from telomeric end (kb)

Fig.S3


A
Wild type
Sir3 expression (h)
0
1.5
3
4.5
6.0
7.5
cac1Δ rtt106Δ
Sir3 expression (h)
0
1.5
3
4.5
6.0
7.5
a1
PDA1

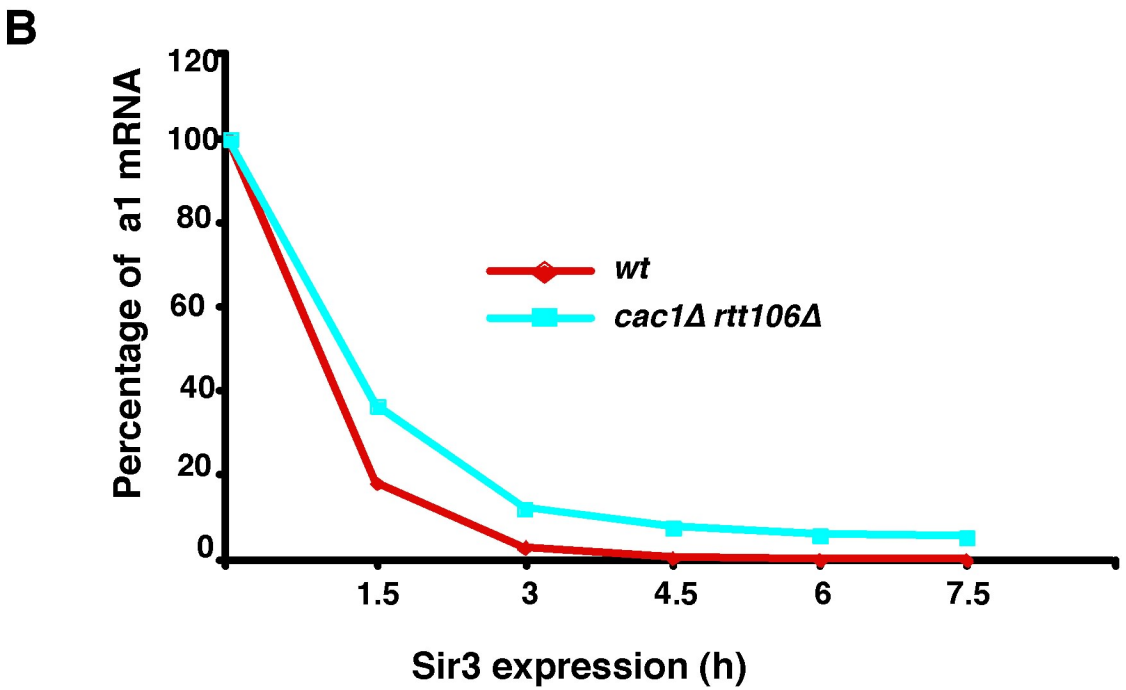

B
Percentage of a1 mRNA
120
100
80
60
40
20
0
wt
cac1Δ rtt106Δ
1.5
3
4.5
6
7.5
Sir3 expression (h)

Fig.S4

Please note that images of the gels represented in Figures 1, 2, 3, and 5 were processed to simplify presentation. However, in no case were the images cropped or manipulated in such a way as to alter the data. To support this point, we have detailed below how each image was processed and attached the original images following the text.

Figure 1A. Three lanes were removed from the original image of the gel. (1) PCR products using whole-cell DNA as template. This was used for calculations of relative enrichment and will not yield any information if presented. (2) An empty lane to separate the products generated from immunoprecipitated DNA with those generated from whole-cell DNA. (3) The Sir4p ChIP result showing that Sir4p binding to telomeric ends is reduced in *cac1Δ rtt106Δ* double mutant cells. As described in the text, this reduction is likely due to the reduced binding of Sir4p at telomeric heterochromatin in these double mutant cells, rather than their effects on telomeric ends. We took out this lane to avoid potential confusion to the readers and/or reviewers.

Figure 1B. The lanes showing the effects of *cac1Δ* and *rtt106Δ* single mutants and *cac1Δ rtt106Δ* double mutants on the binding of Sir4p to the *HMR-E* silencer were removed to simplify the figure.

Figure 2B. The lanes showing the effect of the *sir4* single-site mutant on the binding of Sir4p to telomeric heterochromatin were removed, because this *sir4* single-site mutant affects Sir4p binding differently than the *sir4Δ* deletion mutant. At present, the reasoning behind this is unclear, and we would like to further investigate the silencing defect of this

sir4 single-site mutant. We removed lanes showing the effects of *cac1Δ hir1Δ* and *cac1Δ asf1Δ* double mutants on the binding of Sir4p to telomeric heterochromatin to simplify the figure. Three lanes of PCR products using different whole-cell DNA as template were also removed to simplify the figure.

Figure 3A. We show analysis of whole-cell DNA from only one sample, out of seven total, and have removed the others to simplify the figure. Moreover, significant differences were not detected among the samples upon analysis of the whole-cell DNA. The lanes showing the effects of *cac1Δ hir1Δ* and *cac1Δ asf1Δ* double mutants on H4-K16 acetylation were also removed to simplify the figure.

Figure 5B. Lanes containing samples of standardized protein markers were removed.

Figure 5C. Empty lanes separating samples at different time points were removed. All of these samples were analyzed on one large gel, which was cut to fit into our cassette for exposure to a single film. Thus, it appears that there are separations between time points. However, importantly, all four strains (WT, *cac1Δ, rtt106Δ*, and *cac1Δ rtt106Δ*) are on the same piece of gel.

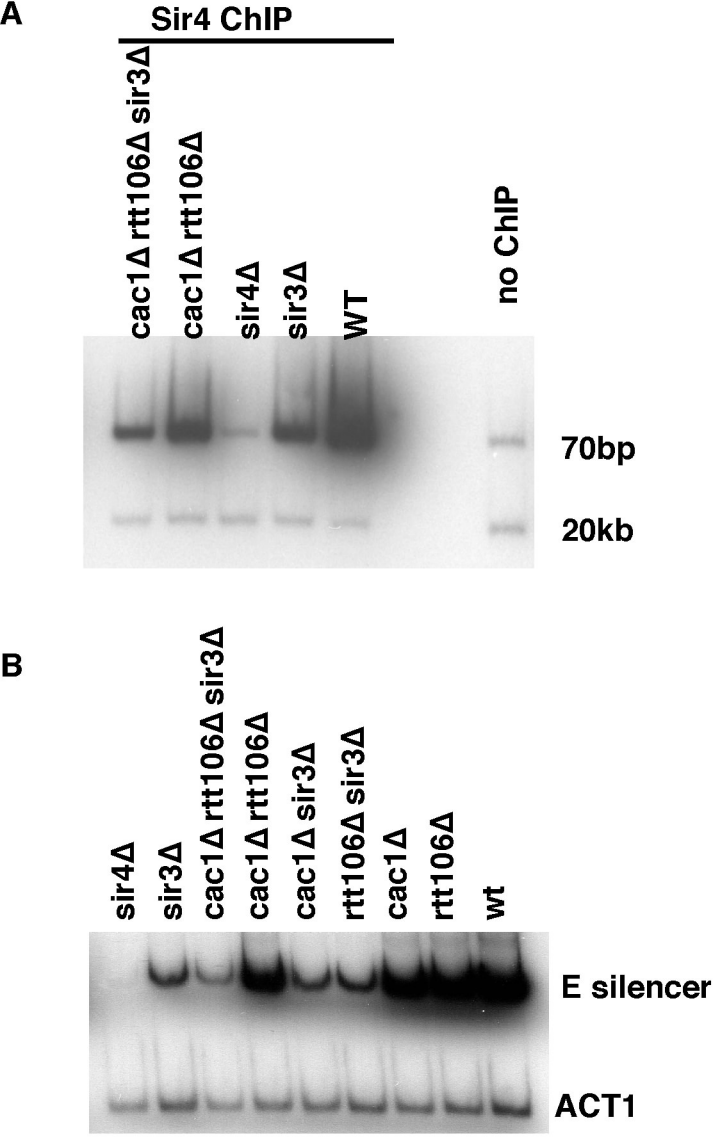


Fig. 1 (original)

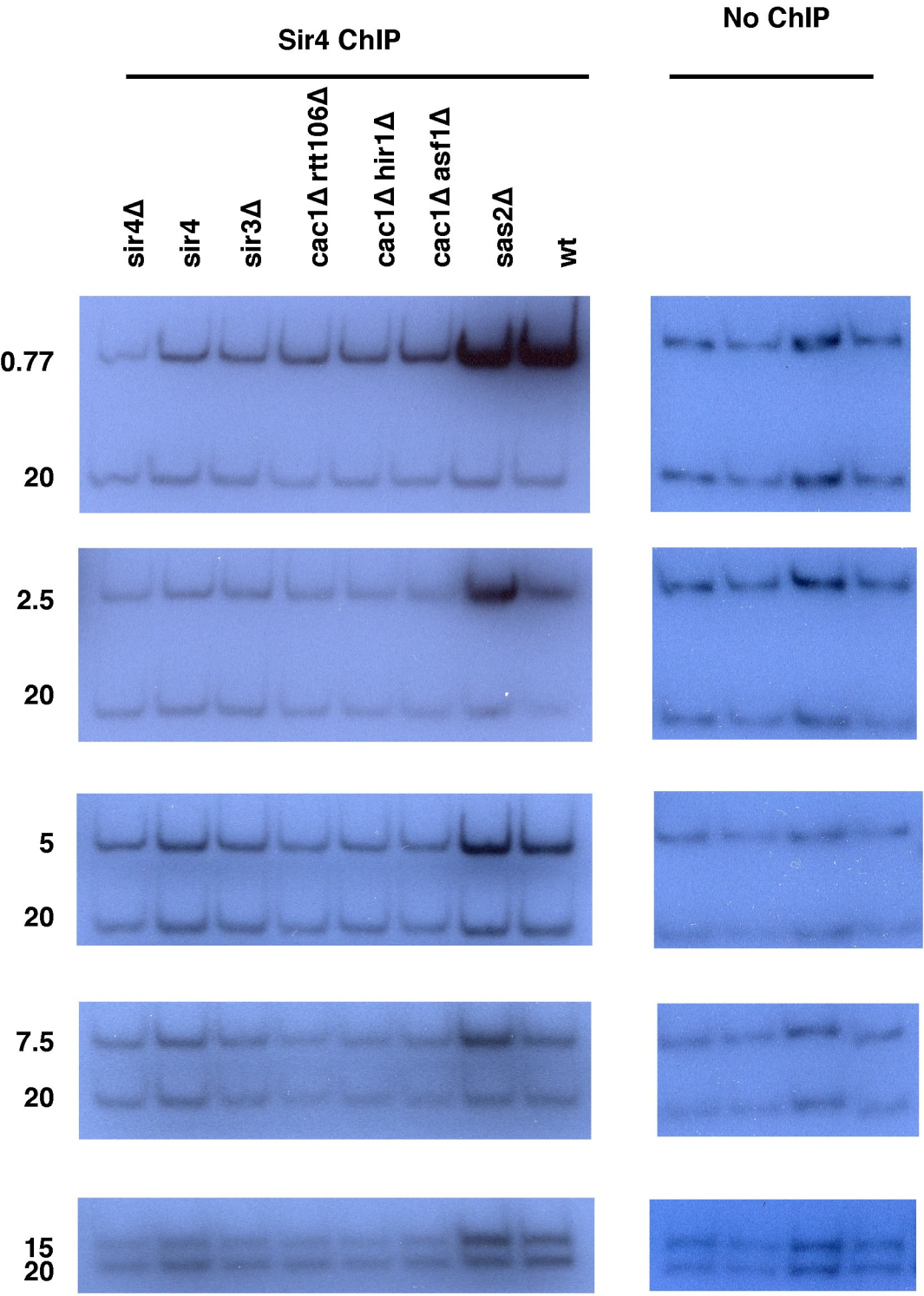
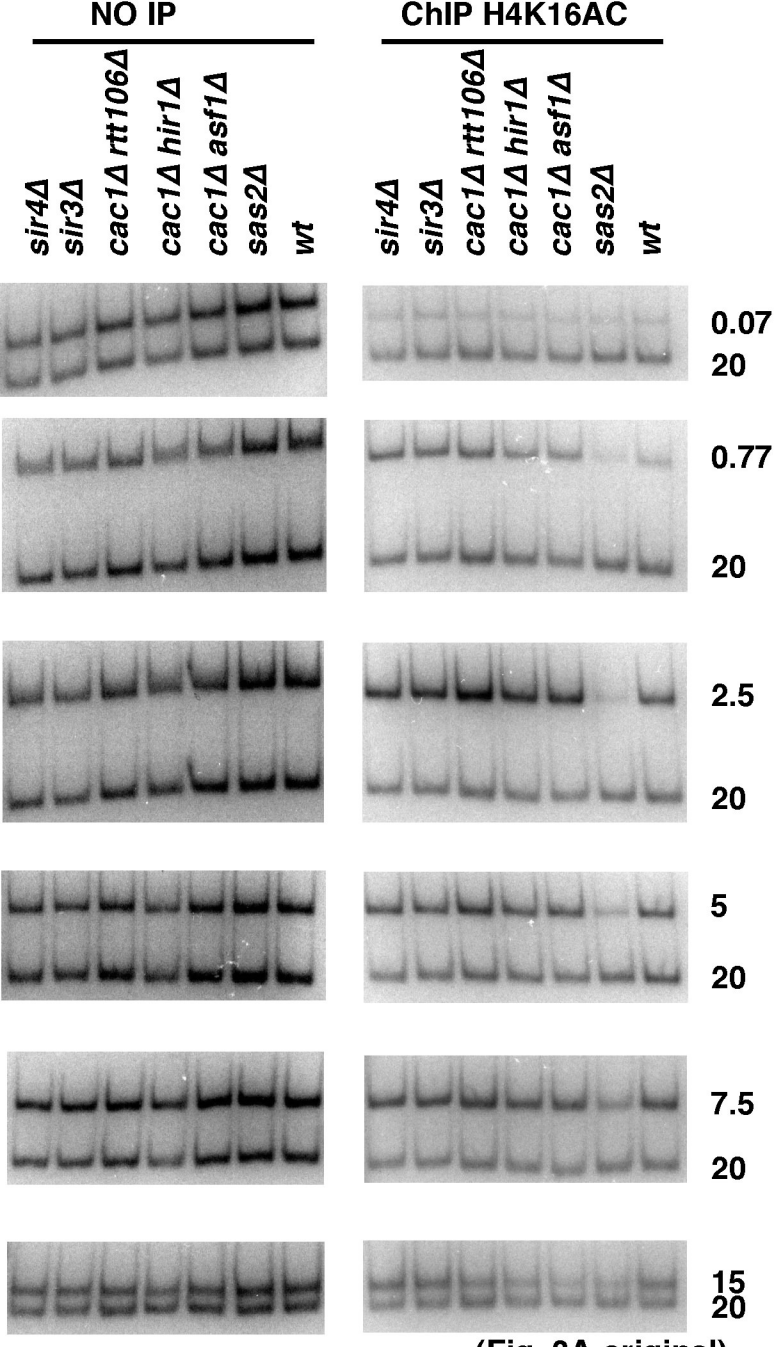


Fig. 2B(original)



(Fig. 3A original)

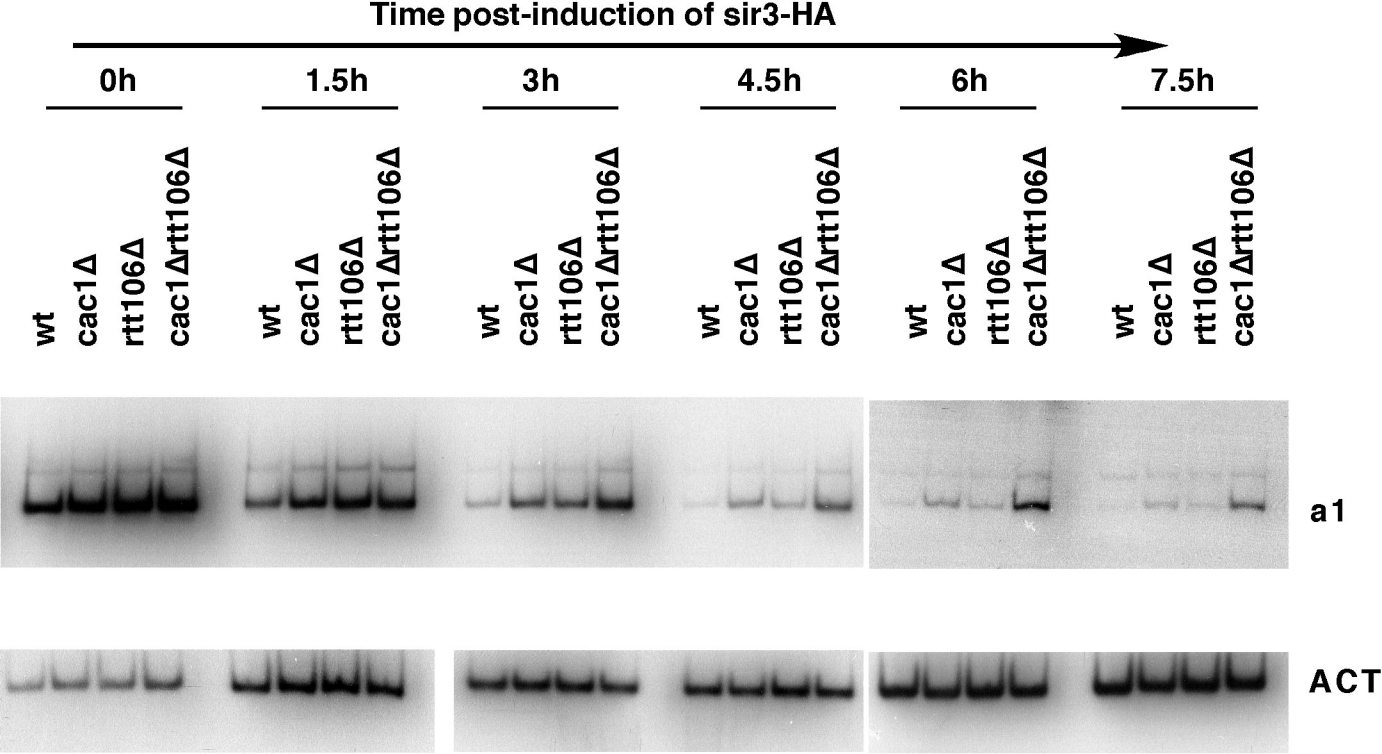


Fig. 5B (original)

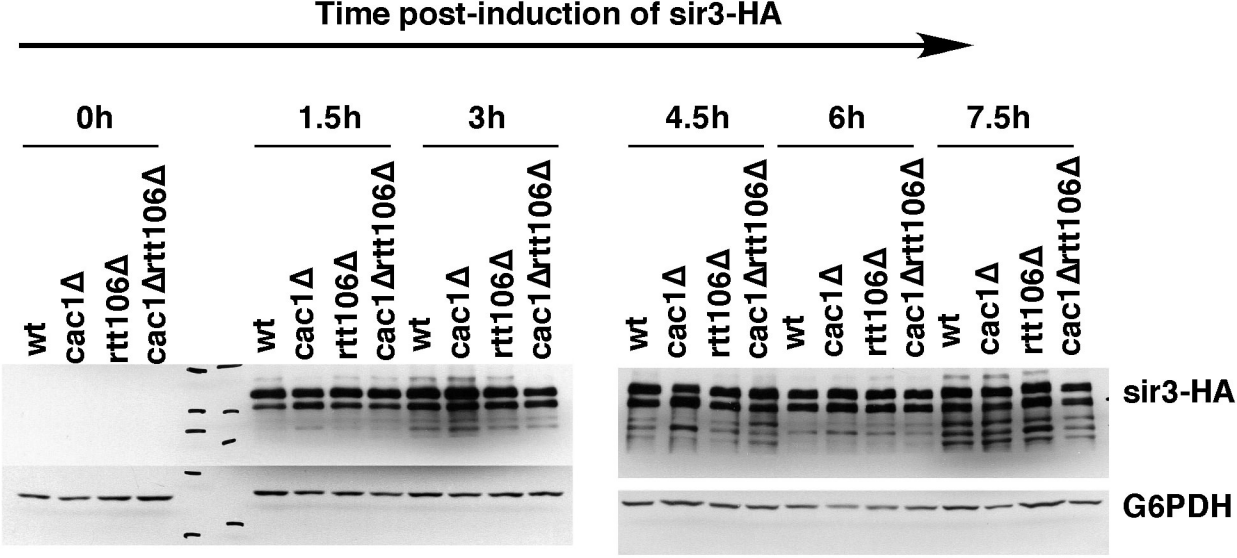


Figure 5C (original)