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RESEARCH ARTICLE

# A link between Sas2-mediated H4 K16 acetylation, chromatin assembly in S-phase by CAF-I and Asf1, and nucleosome assembly by Spt6 during transcription

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**One sentence summary:** This study describes the dynamics of H4 K16 acetylation by the HAT Sas2 during replication and transcription.
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## ABSTRACT

The histone acetyltransferase Sas2 is part of the SAS-I complex and acetylates lysine 16 of histone H4 (H4 K16Ac) in the genome of *Saccharomyces cerevisiae*. Sas2-mediated H4 K16Ac is strongest over the coding region of genes with low expression. However, it is unclear how Sas2-mediated acetylation is incorporated into chromatin. Our previous work has shown physical interactions of SAS–I with the histone chaperones CAF-I and Asf1, suggesting a link between SAS-I-mediated acetylation and chromatin assembly. Here, we find that Sas2-dependent H4 K16Ac in bulk histones requires passage of the cells through the S-phase of the cell cycle, and the rate of increase in H4 K16Ac depends on both CAF-I and Asf1, whereas steady-state levels and genome-wide distribution of H4 K16Ac show only mild changes in their absence. Furthermore, H4 K16Ac is deposited in chromatin at genes upon repression, and this deposition requires the histone chaperone Spt6, but not CAF-I, Asf1, HIR or Rtt106. Altogether, our data indicate that Spt6 controls H4 K16Ac levels by incorporating K16-unacetylated H4 in strongly transcribed genes. Upon repression, Spt6 association is decreased, resulting in less deposition of K16-unacetylated H4 and therefore in a concomitant increase of H4 K16Ac that is recycled during transcription.

**Keywords:** histone acetylation; chromatin assembly; Sas2; Sas4; Cac1; replication

## INTRODUCTION

Post-translational modifications on histones play a critical role in controlling chromatin structure and function. One type of modification that has been extensively studied is the acetylation of lysine residues, which is found at different sites in the N-terminus or the nucleosome core region of the histones and fulfils distinct functions (reviewed in Shahbazian and Grunstein 2007). Classically, acetylation in the N-termini of histones, for instance at histone H3 lysine 9 (K9), K14, K18 and K23 as well as histone H4 K5, 8 and 12, has been associated with transcriptional activation by reinforcing the recruitment of chromatin

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remodeler complexes and the transcriptional machinery. More recently, acetylation on the lateral surface of the nucleosome (H3 K64Ac, K122Ac) has also been implicated in transcription activation (Tropberger *et al.* 2013, Di Cerbo *et al.* 2014). Furthermore, the acetylation of H3 K56, which lies in the N-terminal $\alpha$-helix of H3, is a hallmark of histones that are newly deposited during replication and transcription (Li *et al.* 2008), and this mark has been implicated in nucleosome assembly during replication and DNA repair (Masumoto *et al.* 2005; Recht *et al.* 2006) as well as in turnover of nucleosomes during transcription (Adkins, Howar and Tyler 2004; Schwabish and Struhl 2006).

In contrast to other histone acetylation sites, the acetylation of lysine 16 of histone H4 (H4 K16Ac) in yeast shows a pronounced anti-correlation with transcription in that genes with low transcription rates show high levels of H4 K16Ac over their open reading frame (ORF) and vice versa, and H4 K16Ac is low in promoter regions (Kurdistani, Tavazoie and Grunstein 2004; Liu *et al.* 2005; Heise *et al.* 2012). Also, H4 K16Ac is anti-correlated with H3 K56Ac and transcription-dependent and -independent histone exchange (Heise *et al.* 2012). So far, H4 K16Ac is the only modification known to influence higher order chromatin structure by counteracting the interaction of the H4 N-terminus with the 'acidic patch' formed by the H2A/H2B dimer surface on the neighbouring nucleosome (Shogren-Knaak *et al.* 2006). During mitosis in the yeast *Saccharomyces cerevisiae*, H4 K16Ac is removed by the histone deacetylase (HDAC) Hst2 to promote chromatin condensation (Wilkins *et al.* 2014). Approximately 60% of cellular H4 K16Ac in *S. cerevisiae* is performed by the histone acetyltransferase (HAT) complex SAS-I (Heise *et al.* 2012), which consists of the catalytic subunit Sas2 as well as the proteins Sas4 and Sas5 (Meijsing and Ehrenhofer-Murray 2001, Osada *et al.* 2001). The absence of SAS-I causes a genome-wide loss of H4 K16Ac that is most pronounced in the ORF of genes with a low transcription rate and in subtelomeric regions of the genome (Heise *et al.* 2012). In euchromatic genes, this loss of H4 K16Ac has a relatively mild effect on transcription elongation. In contrast, in subtelomeric regions, loss of H4 K16Ac causes the repression of subtelomeric genes, because the heterochromatic silent information regulator (SIR) complex, which resides at the telomeres and whose chromatin binding is inhibited by H4 K16Ac, spreads away from the telomeres into subtelomeric regions (Kimura, Umehara and Horikoshi 2002, Suka, Luo and Grunstein 2002). This results in repression/silencing of subtelomeric ORFs, but derepression at the very end of the telomere.

In contrast to other HATs, SAS-I is not recruited to chromatin via an interaction with site-specific DNA-binding factors. However, it interacts with the chromatin assembly factors/histone chaperones CAF-I and Asf1 (Meijsing and Ehrenhofer-Murray 2001, Osada *et al.* 2001). CAF-I deposits H3/H4 on newly replicated DNA (Kaufman *et al.* 1995), which it distinguishes from other cellular DNA by its interaction with PCNA (Shibahara and Stillman 1999). The histone chaperone Asf1 transfers H3/H4 to CAF-I during replication-coupled chromatin assembly (Tyler *et al.* 1999), but it also has replication-independent functions in that it is involved in the deposition of H3/H4 during transcription as well as in nucleosome disassembly at promoters during transcription activation (Adkins, Howar and Tyler 2004; Schwabish and Struhl 2006). The interaction of SAS-I with these chromatin assembly factors implies that SAS-I-mediated H4 K16 acetylation occurs in processes mediated by these factors, i.e. replication and transcription. However, bulk H4 K16Ac levels are unaffected by deletion of the gene encoding the CAF-I subunit *CAC1*, nor by *asf1*$\Delta$ (see below, data not shown). Also, a transcription-coupled relationship between Asf1 and SAS-I seems unlikely, because Asf1 is required for H3 K56 acetylation by the HAT Rtt109 (Driscoll, Hudson and Jackson 2007, Han *et al.* 2007) and deposits K56-acetylated H3 in regions of high histone turnover (Rufiange *et al.* 2007), and such regions are low for H4 K16Ac and are largely unaffected by *sas2*$\Delta$ (Heise *et al.* 2012). The functional significance of these interactions therefore remains to be determined.

Chromatin is dynamically modulated in the process of transcription (reviewed in Kwak and Lis 2013), but not all aspects of this process are entirely understood. Nucleosomes present an obstacle to the transcribing RNA polymerase, and they are displaced in front of the polymerase and reassembled after its passage through the ORF. While H2A/H2B are evicted and redeposited by FACT in this process (Belotserkovskaya *et al.* 2004), H3/H4 turnover is controlled by Asf1 (Schwabish and Struhl 2006) as well as by the histone chaperone Spt6 (Bortvin and Winston 1996). Spt6 interacts with the elongating polymerase (Yoh *et al.* 2007) and thus is predominantly recruited to transcribed genes (Ivanovska *et al.* 2011; Perales *et al.* 2013). In *S. cerevisiae* and *Schizosaccharomyces pombe*, mutation of *SPT6* causes widespread changes in gene expression and nucleosome position, most prominently a loss of histones in genes with a high histone turnover, but also an increase in the 3′ region of genes with slow turnover (DeGennaro *et al.* 2013, Perales *et al.* 2013).

Concomitant with histone dis- and reassembly, various histone modifications show dynamic changes during transcription. The H3 K36 methyltransferase Set2 associates with the transcribing polymerase (Krogan *et al.* 2003, Li *et al.* 2003, Xiao *et al.* 2003), and H3 K36 methylation is required to recruit the HDAC complex Rpd3S, which serves to restore a hypoacetylated state after transcription and thus to prevent intragenic transcription initiation (Krogan *et al.* 2003; Carrozza *et al.* 2005). Interestingly, Spt6 is required to preserve marks of active transcription on transcribed genes in *Sc. pombe*, including H3 K36 as well as Set1-dependent H3 K4 methylation (DeGennaro *et al.* 2013, Kato *et al.* 2013). This has been interpreted to reflect a role for Spt6 in preventing transcription-coupled loss of modified histones (Kato *et al.* 2013). However, Spt6 is also required for the recruitment of the Set1/COMPASS complex as well as Set2 to transcribed genes (DeGennaro *et al.* 2013), implying that these Spt6-dependent changes in histone modifications result from reduced presence of the histone-modifying enzymes.

In this study, we sought to investigate how Sas2-dependent H4 K16Ac is deposited in the yeast genome. Using a heat-inducible degron-tagged Sas2 version, we found that the incorporation of Sas2-dependent H4 K16Ac on bulk histones required passage of the cells through the S-phase of the cell cycle, and this incorporation depended on both CAF-I and Asf1. However, this H4 K16Ac did not become immediately deposited in chromatin. Rather, chromatin deposition of H4 K16Ac was only found at genes that became repressed during the experimental regimen, and this deposition depended upon Spt6, but not the histone chaperones CAF-I, Asf1, Rtt106 or Hir1 (Eitoku *et al.* 2008). Our further analysis showed that Spt6 indirectly controlled H4 K16Ac levels by depositing H4 that is unacetylated on K16.

## MATERIALS AND METHODS

### Strains and media

*Saccharomyces cerevisiae* strains used in this study are given in Table S1 (Supporting Information). Growth and manipulation of

yeast was performed according to standard procedures (Sherman 1991). Gene knockouts and epitope tagging were performed as described (Wach *et al.* 1994), and correct integration was verified in all cases by PCR analysis and western blotting (for the introduction of tags). For the fusion of a heat-inducible degron (Dohmen and Varshavsky 2005) to *SAS2* (*SAS2-td*), a truncated *sas2* was generated and subsequently inserted into pPW66R, a plasmid containing the degron (kindly provided by Chun Liang). The truncated *sas2* was used to avoid the presence of a second functional copy of *SAS2* after integration into the yeast genome. It was generated by amplifying a *SAS2* fragment using the primers HindIII_Sas2_fwd and ClaI_Sas2_rev (see Table S2, Supporting Information), which also introduced a HindIII and a ClaI restriction site at the 5′ and 3′ end, respectively. This truncated HindIII/ClaI *sas2* fragment was then cloned into HindIII/ClaI of pPW66R to generate pAE1426. For integration into the yeast genome, pAE1426 was linearized with BclI and used to transform AEY4322 to uracil prototrophy. For detection of Sas2-td, 6 HA-tags were introduced (6HA-natNT2, primers SAS2 S2 and SAS2 S3), and expression was tested by western blotting. Inactivation of the Sas2 degron was achieved by cultivating cells overnight in galactose at 37°C. Synchronization of yeast cells in the G1-phase of the cell cycle was achieved by arresting cells first in G2/M-phase using nocodazole (10 $\mu$g/ml, 1.5–3 h) and subsequently transferring them to medium containing $\alpha$-factor (1.62 $\mu$g/ml, 1.5–3 h) at pH 4.0. For release into S-phase, cells were transferred to medium without $\alpha$-factor, but containing pronase (20 $\mu$g/ml). Cells bearing a deletion of *BAR1* were arrested in G1-phase by incubation in full medium with $\alpha$-factor at a final concentration of 25 ng/ml. Arrest in the appropriate phase of the cell cycle was monitored in all experiments by measuring the DNA content of the cells by FACS analysis.

### Chromatin immunoprecipitation (ChIP), ChIP hybridization to microarrays and quantitative PCR

ChIPs were performed as described (Heise *et al.* 2012) using the following antibodies: **α**-H4 K16Ac (Upstate, #07–329 for ChIP-chip in AEY2426, AEY2450, AEY3462; Active Motif, #39167 for all other ChIPs), **α**-H4 (Abcam #31827 for ChIP-chip in AEY2426, AEY2450, AEY3462; Abcam, #17036 for ChIP-chip in AEY4488; Millipore, #05–858 for all other ChIPs) and **α**-c-Myc (Sigma-Aldrich, #M4439). Quantitative PCR was performed as described (Heise *et al.* 2012) using SYBR Green Real MasterMix (5 PRIME) or PerfeCTa SYBR Green SuperMix (Quanta). Oligonucleotides for amplification are given in Table S2 (Supporting Information). Hybridization of ChIP samples to tiling arrays representing the complete *S. cerevisiae* genome (Affymetrix GeneChIP® *S. cerevisiae* Tiling 1.0R arrays; ChIP-chip) was done as described (Heise *et al.* 2012). ChIP-chip of H4 and H4 K16Ac in *asf1*Δ, *cac1*Δ and *cac1*Δ *asf1*Δ was performed in triplicate. For the generation of metagene profiles (Fig. S1, Supporting Information), genes with a length of over 800 bp were ranked according to their expression in wt and *sas2*Δ using previously published RNA expression data (Heise *et al.* 2012). For clustering by expression level in *asf1*Δ and *cac1*Δ, expression levels in the respective mutant were obtained by multiplying the wild-type (wt) expression level with $2^{\log2(\text{mutant}/\text{wt})}$ (Lenstra *et al.* 2011) and ranking the genes by expression level. For clustering in *asf1*Δ *cac1*Δ, the expression data of *asf1*Δ was used. For the cell-cycle experiments using *SAS2-td*, a single ChIP-chip experiment per timepoint was performed. Tiling array data analysis was performed as described (Fig. 3; Heise *et al.* 2012) or using the Bioconductor package Starr (Fig. S2, Supporting Information; Zacher, Kuan and Tresch 2010). Tiling array data is deposited in ArrayExpress (accession no. E-MTAB-2638). Data for H4 K16Ac in wt and *sas2*Δ were taken from (Heise *et al.* 2012) GEO accession no. GSE19962.

### Protein extracts and western blotting

Whole-cell yeast lysates were prepared by harvesting the equivalent of 20 $OD_{600}$ units of cells and suspending them in 200 $\mu$l of cold phosphate-buffered saline containing a protease inhibitor cocktail. Acid-washed glass beads were added, and cells were broken by vortexing six times for 30 s, with 30 s on ice in between. Around 50 $\mu$l of 4 × Lämmli buffer (Sambrook, Fritsch and Maniatis 1989) was added, and the lysate was heated to 96°C for 10 min (for histone preparations) or 5 min (for other proteins). The lysate was centrifuged for 5 min at room temperature, and the supernatant was recovered. The glass beads were washed once with 100 $\mu$l of 1 × Lämmli buffer, centrifuged again and the supernatant recovered. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Hybond ECL) according to standard procedures (Sambrook, Fritsch and Maniatis 1989). Antibodies used for western blotting were $\alpha$-HA (Covance MMS-101P), $\alpha$-H4 K16Ac (Millipore 07–329), $\alpha$-H2B (Active Motif 39237) and $\alpha$-Actin (Abcam ab8224). The specificity of the $\alpha$-H4 K16Ac antibody was verified by the absence of a signal in western blots using extracts from a strain carrying a mutation of H4 K16 (H4 K16R, not shown).

### RNA expression analysis

Cells were grown in medium with 2% galactose to mid-log phase, transferred to glucose medium (2%) and samples were taken after 0, 30 and 60 min in glucose. Heat inactivation of Spt6 using the *spt6–1004* allele was achieved by shifting cells in galactose medium to 37°C for 1.5 h prior to transferal to glucose medium (37°C) and sample collection as above. RNA was extracted using peqGOLD TriFast (peqLab), and 2.5 $\mu$g RNA was reverse transcribed with the SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen, Life Technologies) after digestion with TURBO DNase (Ambion, Life Technologies). cDNA was quantified by real-time PCR.

## RESULTS

### Deposition of Sas2-mediated H4 K16Ac is dependent upon passage through S-phase

Previous work has shown that the SAS-I complex performs genome-wide acetylation of H4 K16, but it is not targeted to specific genomic regions via interaction with a DNA-binding protein or association with RNA polymerases (Heise *et al.* 2012). Since SAS-I interacts with the chromatin assembly factors CAF-I and Asf1 that cooperate in DNA replication-coupled chromatin assembly (Meijsing and Ehrenhofer-Murray 2001, Osada *et al.* 2001), this suggested that SAS-I-dependent H4 K16 acetylation may require passage through the S-phase of the cell cycle.

In order to investigate this, a repressible variant of the catalytic subunit of SAS-I, Sas2, was constructed by introducing a heat-inducible degron at the N-terminus of Sas2 (Sas2-td). For this purpose, *SAS2* was fused to a temperature-sensitive N-terminal fragment of mouse dihydrofolate reductase, which contains a cryptic N-degron that is only activated at the restrictive temperature of 37°C and leads to degradation of the protein (Dohmen and Varshavsky 2005). Full repression of *SAS2* could only be achieved by simultaneous overexpression of the E3

ubiquitin ligase Ubr1, which was under control of a galactose-inducible promoter (*GALpr-UBR1*) (Makise *et al.* 2008, data not shown). Functionality of Sas2-td was tested by determining whether it supported *HML* silencing in the absence of Sir1, since *sir1*Δ *sas2*Δ causes strong derepression of *HML* (Reifsnyder *et al.* 1996; Ehrenhofer-Murray, Rivier and Rine 1997). Sas2-td was fully functional, because a *sir1*Δ *SAS2-td* strain showed strong repression of *HML* under permissive conditions (30°C, glucose). Importantly, *SAS2-td* could be completely inactivated, because *sir1*Δ *SAS2-td* strains lost *HML* silencing under *SAS2-td*-inactivating conditions (37°C, galactose) (Fig. 1A). Furthermore, in asynchronous cultures, H4 K16Ac was reduced under restrictive and increased under permissive growth conditions for the *SAS2-td* strain (Fig. 1B). Of note, H4 K16Ac was not completely absent upon Sas2 repression, because Sas2 is responsible for approximately 60%, but not 100%, of cellular H4 K16Ac.

We next were interested to determine whether Sas2-mediated H4 K16Ac depended on a particular phase of the cell cycle. For this purpose, cells were arrested in G1 using α-factor mating pheromone, and Sas2-td was inactivated by culturing cells at the restrictive temperature in galactose. Subsequently, Sas2-td was activated either in cells maintained in G1 or cells were simultaneously released into S-phase. Samples were taken at regular intervals, and H4 K16Ac on bulk histones was determined by western blotting. Significantly, there was a pronounced increase of H4 K16Ac in the course of Sas2 induction, but only in cells that passaged through S-phase, and not in the cells that were maintained in G1-phase (Fig. 1C; see Fig. S1, Supporting Information, for quantitation of four independent experiments). A trivial explanation for this result is that *SAS2-td* is not effectively activated in G1. However, we observed no appreciable difference in the appearance of Sas2-td protein, regardless of whether cells were maintained in G1 or released into S-phase (Fig. 1C). Therefore, these results showed that the Sas2-mediated acetylation of H4 K16 on bulk histones required passage of the cells through the S-phase of the cell cycle.

## CAF-I and Asf1 are required for efficient Sas2-dependent H4 K16Ac during S-phase

CAF-I and Asf1 cooperate to assemble H3 and H4 on newly replicated DNA (reviewed in Eitoku *et al.* 2008). SAS-I interacts with both these factors (Meijsing and Ehrenhofer-Murray 2001), suggesting that this interaction is required for the incorporation of Sas2-mediated acetylation during S-phase. However, CAF-I and Asf1 are not required for steady-state H4 K16Ac in asynchronous cultures, because H4 K16Ac levels are unaffected by *cac1*Δ or *asf1*Δ (data not shown). Furthermore, the genome-wide analysis of H4 K16Ac in *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ cells as measured by ChIP hybridization to high-resolution tiling arrays (ChIP-chip) showed that the levels were similar to those of wt over the ORF of genes (Fig. S2A, Supporting Information, top row). If anything, there was a slight increase, not a decrease, in H4 K16Ac, but the magnitude of the effect was very mild compared to the decrease of H4 K16Ac observed in *sas2*Δ cells (Fig. S2A, bottom row; Fig. S2B and C, Supporting Information).

We therefore tested whether CAF-I or Asf1 influenced the rate of increase in H4 K16Ac, rather than steady-state levels, upon Sas2 induction in or outside of S-phase. Importantly, in *cac1*Δ cells, there was an increase of H4 K16Ac in cells released into S-phase upon Sas2 induction (Fig. 1C). However, the increase was delayed as compared to wt cells, and even after 120 min of release in *cac1*Δ cells, the increase was not as pronounced as in wt cells after 90 min of release (Fig. 1C). Notably, this was not due to a delay in progression of *cac1*Δ cells through S-phase, since the cell-cycle profile of these cells was indistinguishable from that of wt (data not shown). Furthermore, as before for wt cells, there was no increase of H4 K16Ac in cells that were maintained in G1 (Fig. 1C). Interestingly, *asf1*Δ cells showed only a marginal increase of H4 K16Ac in S-phase upon Sas2 induction and only at the latest timepoint (120 min, Fig. 1C). Even though S-phase in *asf1*Δ cells was approximately 15 min slower than wt, the cells had completed S-phase at the last timepoints, such that the delay in S-phase cannot explain the lack of increase in H4 K16Ac. Of note, the absence of H4 K16Ac was not due to a reduced ability of *cac1*Δ or *asf1*Δ cells to induce Sas2-td (not shown). Taken together, these results showed that Cac1 as well as Asf1 were required for the dynamics of Sas2-dependent H4 K16Ac incorporation during the S-phase of the cell cycle.

## Sas2-dependent H4 K16Ac is deposited in genes upon repression

We next sought to determine where in the genome H4 K16Ac is deposited into chromatin upon Sas2 induction. Since an increase of H4 K16Ac was observed in S-phase, but not in G1-arrested cells, the expectation was that H4 K16Ac incorporation in S-phase would be found over broad genomic regions, and that there would be no incorporation in G1-arrested cells. To investigate this, genome-wide H4 K16Ac (relative to H4) was measured by ChIP-chip upon Sas2-td activation in cells maintained in G1 for 1 h or after 1 h of release into S-phase and were compared to relative H4 K16Ac at timepoint 'zero', when Sas2 was 'off' (Fig. 2A). For this purpose, ChIP was performed from the samples with an antibody against H4 K16Ac and, as a control for changes in H4 distribution, against unmodified H4 and hybridized to tiling arrays, and all data show H4 K16Ac values relative to H4.

Surprisingly, there was only limited deposition of relative H4 K16Ac across the genome after Sas2-td activation compared to before activation ('G1 arrest/time 0', 'S release/time 0', Fig. 2A), and a more pronounced deposition was observed only at few restricted sites. Strikingly, the place of strongest enrichment corresponded to the *GAL* genes (Fig. 2A, enlargement) and the heat shock genes (e.g. *SSA1*, Fig. S3, Supporting Information). Of note, these genes become repressed during the course of Sas2-td activation, when cells are shifted from 37°C and galactose (degron activated) to 30°C and glucose medium. Furthermore, there was no appreciable difference in H4 K16Ac deposition between the G1-arrested cells and those released into S-phase (Fig. 2A and Fig. S2, Supporting Information). We also verified by conventional ChIP that there was no net increase of relative H4 K16Ac in S-phase released cells versus those kept in G1 (Fig. S2C, Supporting Information). Altogether, these results suggested that the major place of H4 K16Ac incorporation in the genome upon Sas2 induction was in genes that became repressed in the course of the experiment. For G1-arrested cells, this increase of H4 K16Ac e.g. at the *GAL10* gene was surprising, because bulk H4 K16Ac was not increased (Fig. 1C). However, it was in agreement with the observation that H4 K16Ac in euchromatic regions is highest in genes with a low expression rate (Heise *et al.* 2012).

We next asked whether the observed deposition of H4 K16Ac in genes upon repression was also seen in wild-type cells, without the complication of switching Sas2 off and on using Sas2-td. In order to measure replication-independent H4 K16Ac deposition, cells were maintained in G1-phase using α-factor,

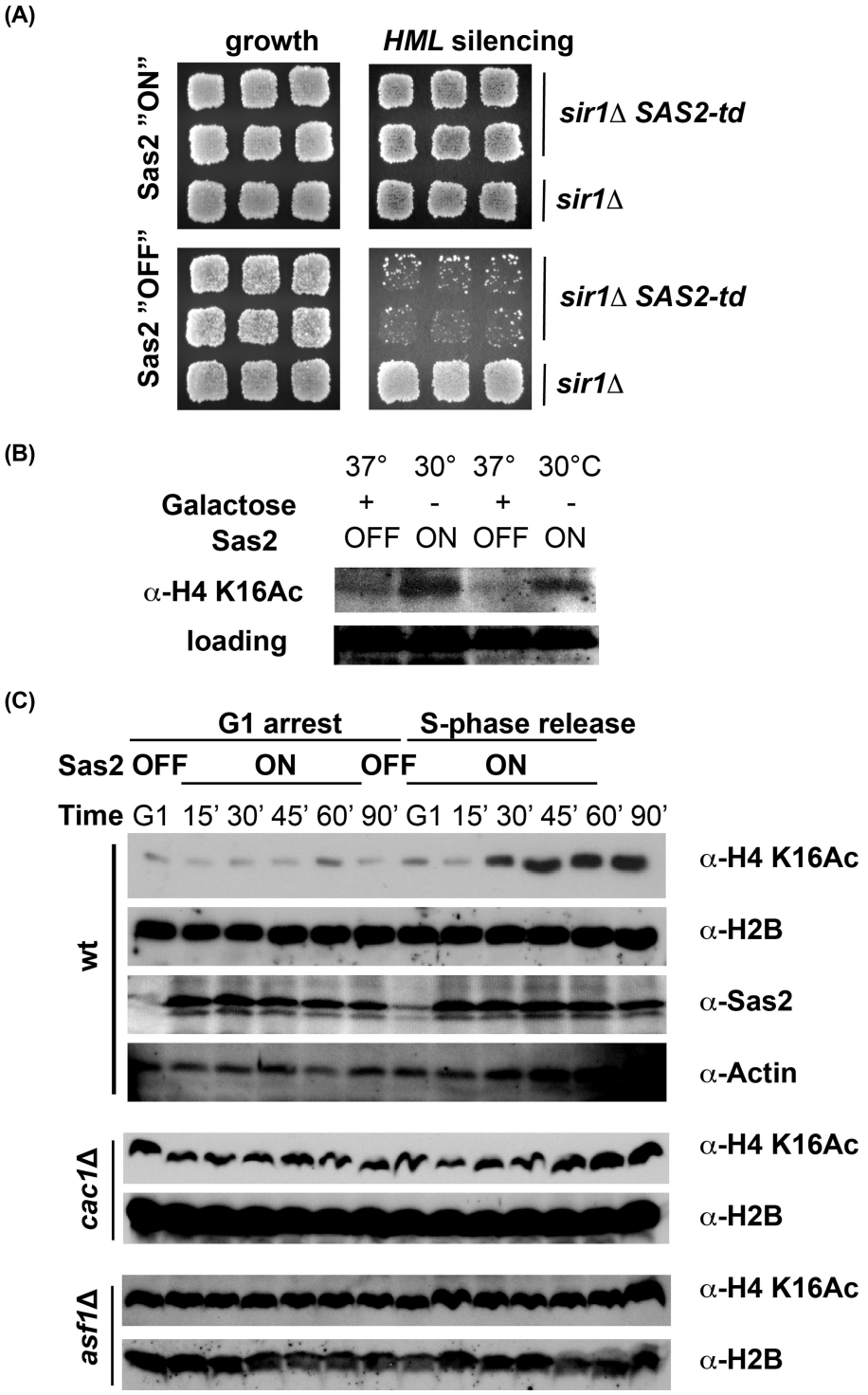


**Figure 1.** Sas2-dependent H4 K16Ac on bulk histones required passage through the S-phase of the cell cycle. (**A**) A degron version of *SAS2* (*SAS2-td*, *GALpr-UBR1*) was fully functional at low temperature (30°C) and on glucose medium (Sas2 'ON') and was efficiently inactivated upon activation of the degron at high temperature (37°C) and on galactose medium (Sas2 'OFF'). Silencing of *HML* in the *sir1*Δ background was determined by measuring the ability of a *MAT***a** *sir1*Δ *SAS2-td* (AEY4490) and a *MAT***a** *sir1*Δ strain (AEY345) to mate with a *MATα* tester strain. Loss of Sas2 activity causes *HML* derepression in *sir1*Δ (44, 45). (**B**) H4 K16Ac was reduced upon inactivation of Sas2 using Sas2-td. Whole-cell extracts from cells grown under the indicated conditions (AEY4488) were separated by SDS-PAGE and probed by western blotting for H4 K16Ac. An unspecific band of the α-H4 K16Ac antibody served as a loading control. (**C**) Upon activation of Sas2-td, H4 K16Ac only increased in cells released from G1 into S-phase, but not in cells maintained in G1, and the S-phase-dependent increase required the histone chaperones CAF-I and Asf1. Top, the *SAS2-td* strain (wt, AEY4488) was grown at 37°C in galactose and arrested in G1 using α-factor. Cells were then either maintained in G1 or they were released into S-phase and simultaneously shifted to glucose at 30°C. Samples were taken at the indicated timepoints after activation of Sas2-td. Whole-cell extracts were analysed for H4 K16Ac as in B and, as a control, for H2B, actin and Sas2-td (in AEY5495). Middle, S-phase-dependent H4 K16Ac upon Sas2 activation was strongly diminished in the absence of Cac1, the large subunit of CAF-I. Samples were generated as above, but using the *SAS2-td cac1*Δ strain (AEY4810). Bottom, the increase of H4 K16Ac upon Sas2-td induction was abrogated in *asf1*Δ. The experiment was performed as above, but using the *SAS2-td asf1*Δ strain (AEY4812).

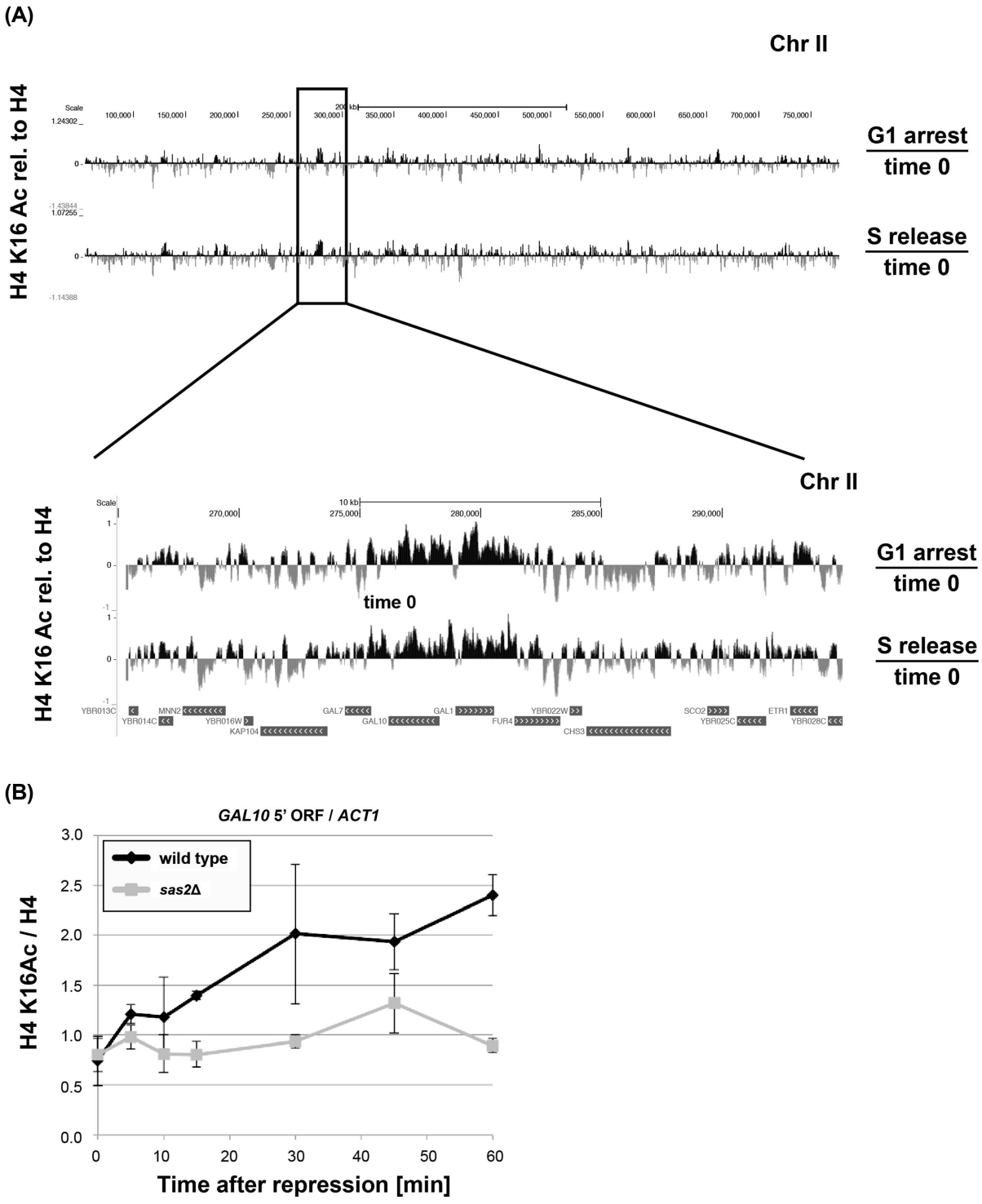


**Figure 2.** Deposition of Sas2-dependent H4 K16Ac in the 26 genome was most prominent in genes that became repressed during activation of Sas2-td. (**A**) *SAS2-td* cells (AEY4488) were treated using the experimental setup as in Fig. 1C, and samples were taken at timepoint 0 (before Sas2 induction) and after 1 h either upon S-phase release or in cells maintained in G1. H4 K16Ac levels were determined relative to H4 levels by hybridization of ChIP samples to high-resolution tiling microarrays (ChIP-chip). H4 K16Ac levels relative to H4 were averaged over the whole yeast genome for each sample individually, and the change of relative H4 K16Ac upon Sas2 induction in G1-arrested cells (top) or S-phase released cells (bottom) versus Sas2-uninduced cells (time 0) on chromosome II is shown. The average change over the genome was set to zero (baseline); more-than-average H4 K16Ac results in positive values (black, upward bars), whereas less-than-average H4 K16Ac results in negative values (grey, downward bars). Below, zoom into the region highlighted above, which contains the genes *GAL7*, *GAL10* and *GAL1* that are repressed in the course of the experiment. (**B**) The S-phase-independent increase of H4 K16Ac at *GAL10* upon repression in glucose required Sas2. Wt (AEY5258) or *sas2*Δ (AEY5260) cells were arrested in G1-phase using $\alpha$-factor in galactose medium and subsequently shifted to glucose medium containing $\alpha$-factor. Samples were taken at the indicated timepoints, and H4 K16Ac levels were determined relative to H4 at *GAL10* and *ACT1* (as a control). Error bars represent the standard deviation from three independent biological replicates.

were transferred from galactose to glucose in order to repress galactose-inducible genes and the level of H4 K16Ac relative to H4 was determined using ChIP. In line with the above results, there was a pronounced increase of H4 K16Ac at *GAL10* in wt cells (Fig. 2B). However, no increase was seen in *sas2*Δ cells, showing that the incorporated H4 K16Ac depended on Sas2 and not another cellular HAT. Of note, this increase of H4 K16Ac was not simply a result of more H4 being deposited on the *GAL10* gene upon repression, because we report here H4 K16Ac levels that were determined relative to H4 at *GAL10*.

The absence of an increase in chromatin-bound H4 K16Ac as cells passage through S-phase was unexpected, because it indicates that bulk H4 becomes acetylated upon Sas2 induction, but the acetylated H4 does not immediately become incorporated into chromatin.

## Deposition of H4 K16Ac upon gene repression requires the histone chaperone Spt6

The incorporation of H4 K16Ac in genes upon repression begged the following question: If cells do not passage through S-phase, how is new H4 K16Ac incorporated into chromatin? One simple possibility is that SAS-I is recruited to genes that become repressed and acetylates H4 K16. However, we and others have been unable to find chromatin association of Sas2 by ChIP (data not shown; Dang *et al.* 2009), suggesting that another mechanism is at play. An alternative explanation is that Sas2 acetylates all H4 before incorporation, but that there is less turnover of nucleosomes as a gene becomes repressed, such that Sas2-dependent H4 K16Ac remains in chromatin. During strong transcription, the chromatin is partially disassembled as the transcription machinery passes through the body of the gene and is reassembled after its passage by histone chaperones, for instance Asf1 and Spt6 (reviewed in Kwak and Lis 2013). We therefore asked whether any of the known chromatin assembly factors and histone chaperones were required for S-phase-independent H4 K16Ac deposition upon repression of *GAL10*.

Importantly, inactivation of Spt6 using the *spt6–1004* allele completely abrogated H4 K16Ac deposition upon repression of *GAL10* (Fig. 3A). This was also observed at two other sites in the middle and at the 3′ end of *GAL10*, as well as at another galactose-repressed gene, *GAL3* (data not shown), indicating that this was a general effect of Spt6 during glucose-mediated gene repression. This showed that Spt6 was required for transcriptional repression-dependent H4 K16Ac deposition.

We furthermore tested the effect of CAF-I and Asf1. While CAF-I function is restricted to replication-coupled chromatin assembly, Asf1 also has roles both in assembly and disassembly of H3/H4 during transcription (Adkins, Howar and Tyler 2004; Schwabish and Struhl 2006). *cac1*Δ did not decrease H4 K16Ac deposition (Fig. 3B), which may not be surprising, since the cells in this experiment were maintained in G1-phase. *asf1*Δ showed a slight tendency towards less H4 K16Ac incorporation, but the effect was not significant over three biological replicates (Fig. 3B). Furthermore, the absence of Hir1, which in some instances cooperates with Asf1 as a histone chaperone (Eitoku *et al.* 2008), also did not reduce H4 K16Ac incorporation (Fig. 3C), and the absence of Rtt106 (Huang *et al.* 2005), as for Asf1, caused a mild decrease that was not statistically significant. We conclude that Spt6 has a major impact on the deposition of Sas2-dependent H4 K16Ac upon gene repression. Asf1 and Rtt106 may have minor effects, and H4 K16Ac incorporation is unaffected by CAF-I and Hir1.

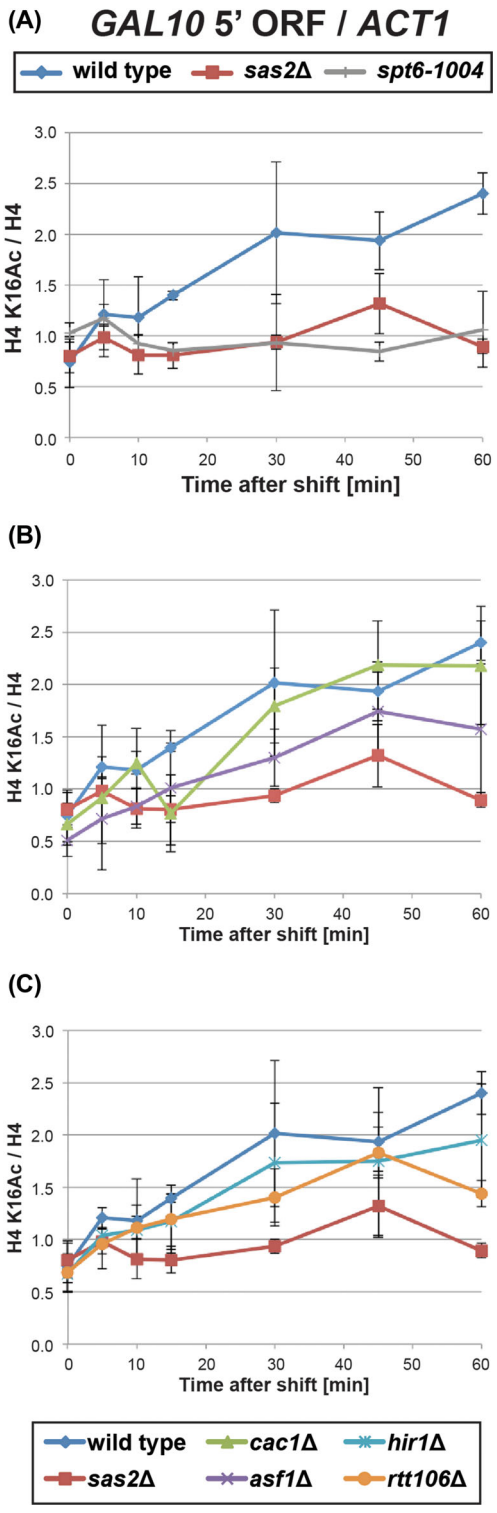


**Figure 3.** The deposition of H4 K16Ac upon gene repression required the histone chaperone Spt6, but not CAF-I, Asf1, Hir1 and Rtt106. (**A**) The increase of H4 K16Ac at *GAL10* in cells arrested in G1 phase was measured in wt (AEY5258), *sas2*Δ (AEY5260) and *spt6–1004* cells (AEY5412) as in Fig. 2C. Values are given as H4 K16Ac relative to H4 at *GAL10* and were normalized to H4 51 K16Ac/ H4 at *ACT1*. Values are the average +/– standard deviation from three independent biological replicates. (**B**) Cac1 and Asf1 were not required for H4 K16Ac deposition upon repression of *GAL10*. Experiment was performed as in A with *cac1*Δ (AEY5262) and *asf1*Δ (AEY5281) strains. (**C**) Hir1 and Rtt106 were not required for increased deposition of H4 K16Ac upon repression of *GAL10*. Experiment as in A using *hir1*Δ (AEY5370) and *rtt106*Δ (AEY5380) strains.

### Spt6 controls the level of H4 K16Ac via transcription-coupled deposition of H4 that is not acetylated on lysine 16

How does Spt6 affect H4 K16Ac incorporation upon gene repression? Spt6 has been shown to interact with several factors during transcription elongation (Yoh *et al.* 2007), including with the elongating form of RNA PolII (Yoh *et al.* 2007), and it is required to redeposit histones on chromatin after passage of the RNA polymerase (Ivanovska *et al.* 2011; Perales *et al.* 2013). At face value, the observation that H4 K16Ac levels do not increase upon gene repression in the *spt6–1004* mutant suggests that Spt6 deposits H4 K16Ac in repressed genes. However, this is at odds with the previous observation that Spt6 shows higher association with more strongly expressed genes (Ivanovska *et al.* 2011; DeGennaro *et al.* 2013, Perales *et al.* 2013), and we also observed higher association of Spt6 at the *GAL10* 5′ region when *GAL10* was expressed (data not shown). This implies that Spt6 is required for the deposition of H4 unacetylated at K16 at highly transcribed genes, such that a reduced activity/presence of Spt6 leads to less incorporation of 'K16-unacetylated' H4 and therefore 'tips the balance' towards a higher level of recycled H4 K16Ac.

This hypothesis makes the prediction that the inactivation of Spt6 leads to less incorporation of unacetylated H4 and thus higher H4 K16Ac levels at any gene whose ORF normally is bound by Spt6, and the effect should be more pronounced at strongly expressed genes that have more Spt6 associated. In agreement with this, we observed higher H4 K16Ac levels (relative to H4) at *GAL10* (under inducing conditions), the house-keeping gene *ACT1* and the poorly expressed *CSF1* gene in *spt6–1004* compared to wt cells (Fig. 4A), further supporting the notion that Spt6 deposits 'K16-unacetylated' H4 on ORFs. Of note, Spt6 inactivation has previously been shown to have a tendency to reduce nucleosome occupancy at strongly transcribed genes and to increase it at weakly transcribed genes, though not all genes follow this rule (Perales *et al.* 2013). Accordingly, we observed reduced H4 occupancy at *ACT1* and *CSF1*, and more H4 at the 3′ end, but less H4 in the middle of *GAL10* in *spt6–1004* cells compared to wt (Fig. 4B), indicating that Spt6 was required for proper H4 deposition at these genes. Again, the H4 K16Ac levels reported here are measured relative to H4 and thus are unaffected by changes in total H4 on genes in the *spt6* mutant.

Altered H4 occupancy and increased H4 K16Ac levels in the *spt6–1004* mutant raised the question whether this affected the ability of the cells to repress *GAL10*, also since mutation of *SPT6* is known to cause widespread changes in gene expression (Cheung *et al.* 2008; Ivanovska *et al.* 2011). While *GAL10* was effectively repressed upon a shift from galactose to glucose in wt, *GAL10* repression in *spt6–1004* was less pronounced than in wt (a drop to approximately 50%, Fig. 4C). Therefore, as has been observed earlier for other genes (Ivanovska *et al.* 2011), Spt6 was required for efficient repression of *GAL10*. Most likely, this slight defect in gene repression is caused by the *spt6*-mediated change in histone occupancy, rather than by changes in relative H4 K16Ac levels.

In summary, the results above showed that Spt6 was required to maintain high levels of 'K16-unacetylated' H4 at strongly transcribed genes, and that the loss of Spt6 activity resulted in higher levels of relative H4 K16Ac. Given its function as a nucleosome assembly factor (Bortvin and Winston 1996), this indicated that Spt6 deposits 'K16-unacetylated' H4 in the wake of transcription and thus indirectly controls H4 K16Ac levels over the body of the gene.

## DISCUSSION

Histone acetylation is subject to dynamic changes through the action of HATs and HDACs, but also through nucleosome disassembly and reassembly during regulatory processes on chromatin, for instance transcription activation, elongation and during replication-coupled chromatin assembly. Here, we have investigated how H4 K16Ac that is mediated by the HAT complex SAS-I is incorporated into histones and chromatin. Significantly, Sas2-mediated H4 K16Ac in bulk histones required passage of the cells through the S-phase of the cell cycle. Furthermore, the appearance of H4 K16Ac in bulk histones required the presence of the chromatin assembly complex CAF-I as well as the histone chaperone Asf1. However, H4 K16Ac did not become immediately deposited in the genome, but rather, its deposition was restricted to genes that became repressed during the experiment (Fig. 5A). We observed a cell-cycle independent increase of H4 K16Ac in genes upon their repression, and this increase required the histone chaperone Spt6. The effect of Spt6 on H4 K16Ac levels was likely to be indirect in that Spt6 associates with strongly transcribed genes and deposits H4 that is unacetylated at K16. Thus, our model is that reduced Spt6 activity leads to reduced H4 turnover/deposition. This indirectly leads to increased H4 K16Ac levels, because more K16-acetylated H4 is recycled during the process of transcription (Fig. 5B).

Histone H3 and H4 acetylation levels during transcription are determined by histone turnover and histone chaperones, but also by the cotranscriptional recruitment of HDACs to the ORF, for instance Rpd3-containing complexes (Carrozza *et al.* 2005, Keogh *et al.* 2005; Li *et al.* 2007), Hda1 and Hos2 (Govind *et al.* 2010). It is therefore possible that the dissociation of these HDACs from the ORFs upon gene repression also contribute to the increase in H4 K16Ac that we observe. However, if this were the predominant mechanism of H4 K16Ac increase upon repression, we would also expect such an increase in *sas2*Δ cells. However, the effect observed here was strictly Sas2-dependent (Fig. 3C), arguing that the H4 K16Ac increase was not solely due to a reduced activity of HDACs at the repressed ORF.

One interpretation for the S-phase dependence of H4 K16Ac is that the genes encoding histone H4 (*HHF1* and *HHF2*) are predominantly expressed in S-phase (Eriksson *et al.* 2012), and that Sas2 has specificity for newly synthesized histones. Notably, although histone gene expression is strong in S-phase, it is not completely absent in G1 (Verzijlbergen *et al.* 2010), but, it is possible that this level of new H4 expression is too low for the resulting H4 K16Ac to be detected by western blotting. However, even in *asf1*Δ cells, which show a deregulation of histone gene expression (Sutton *et al.* 2001), we did not observe an increase of H4 K16Ac upon Sas2 activation in G1-arrested cells, arguing for an S-phase-specific event that is required to activate SAS-I. For instance, the SAS-I complex may require activation by a post-translational modification, like an S-phase-specific phosphorylation by a cyclin-dependent kinase. Furthermore, the fact that the S-phase increase of H4 K16Ac was diminished in *cac1*Δ and *asf1*Δ cells suggests that the respective histone chaperones bind and present histone H4 to the SAS-I complex for acetylation. This is consistent with earlier work showing that some CAF-I-associated histone H4 is acetylated on H4 K16 (Zhou *et al.* 2006). Interestingly, however, Asf1 inhibits acetylation by SAS-I *in vitro* (Sutton *et al.* 2003). Perhaps Asf1 passes unacetylated H4 to CAF-I, which then presents it for acetylation to SAS-I. Of note, CAF-I and Asf1 apparently contribute to the rate/efficiency of H4 K16 acetylation, but not to the steady-state level, and in their absence, H4 K16Ac (as does H4) eventually becomes incorporated

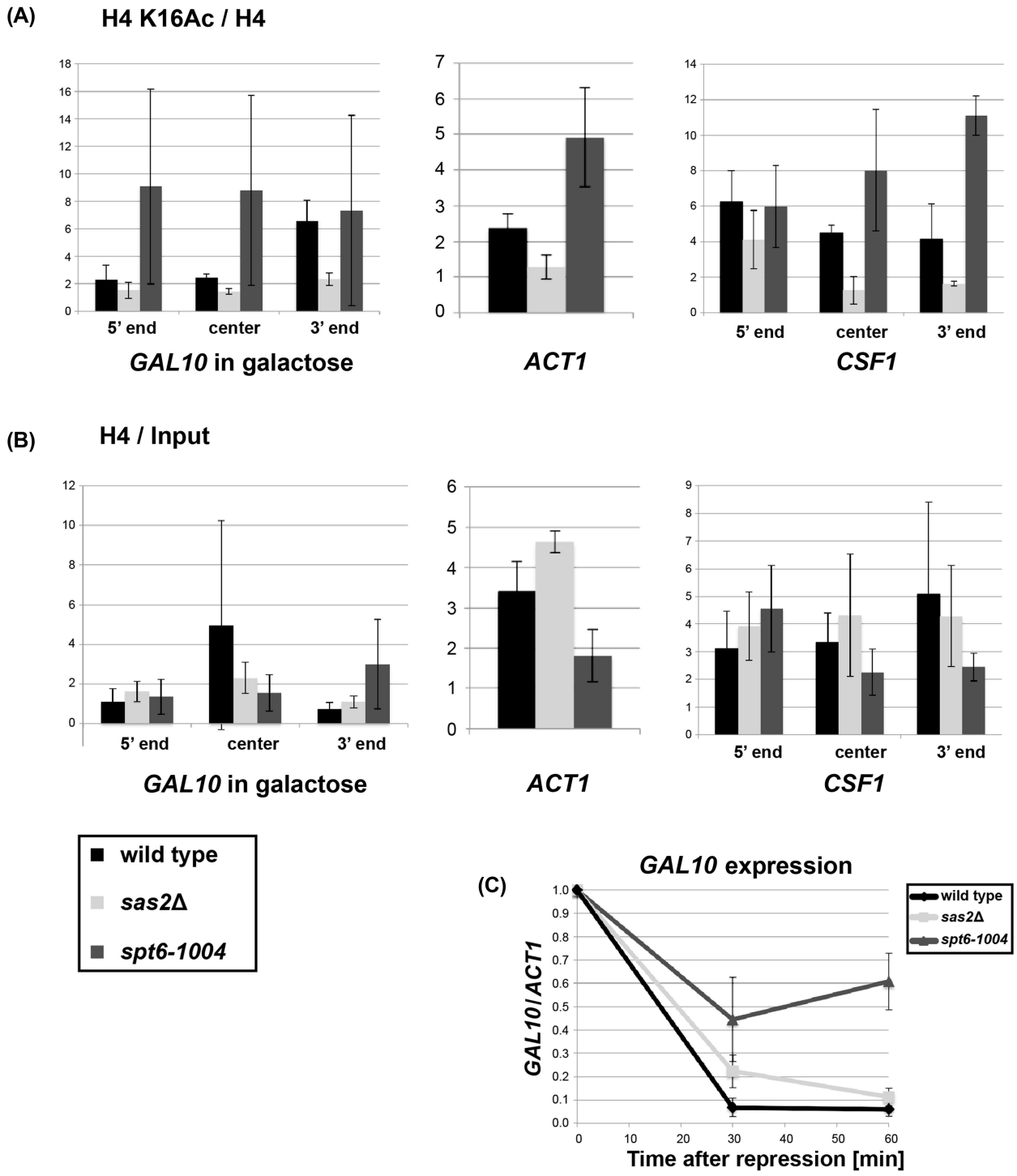


**Figure 4.** (**A**) Inactivation of Spt6 in *spt6–1004* caused an increase of relative H4 K16Ac at *GAL10*, *ACT1* and *CSF1*. The *spt6–1004* strain (AEY5412) was grown at 37°C, the wt and *sas2*Δ strains (AEY5258 and AEY5260, respectively) at 30°C. Values are given as the average +/– standard deviation of three independent biological replicates. (**B**) Changes in total H4 level by *spt6–1004* at *GAL10*, *ACT1* and *CSF1*. Values were determined as in A. (**C**) Quantification of *GAL10* glucose repression (relative to *ACT1*) in wt, *spt6–1004* and *sas2*Δ cells.

into the genome, since genome-wide H4 K16Ac levels show only mild changes in *cac1*Δ and *asf1*Δ cells.

One surprising finding of this study was that even though bulk H4 K16Ac levels increased strongly in S-phase upon Sas2 induction (Fig. 1C), this acetylation did not appear throughout the genome during S-phase, but only at selected genes that became repressed in the course of the experiment. Notably, H4 K16Ac levels increase early in S-phase in wt cells (Wilkins *et al.* 2014). Therefore, one possibility is that H4 K16Ac has to occur prior to the start of chromatin assembly in order to be incorporated into the chromatin in the wake of DNA replication, and that the experimental regimen used here provides H4 K16Ac too late for it

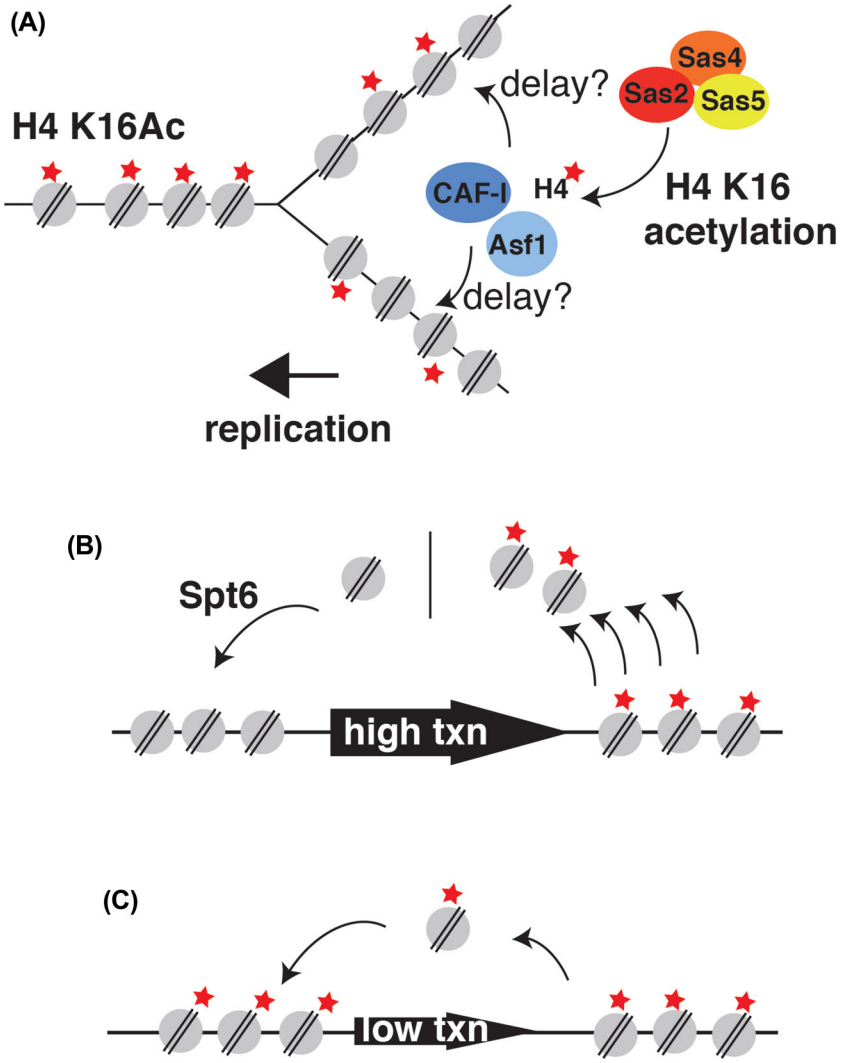


**Figure 5.** Model for dynamics of H4 K16Ac during replication and transcription. (**A**) The SAS-I complex acetylates H4 K16 during S-phase in a CAF-I and Asf1-dependent manner, but H4 K16Ac (red asterisk) incorporation into chromatin is delayed. (**B**) When transcription (txn) is high, H4 K16Ac is removed from chromatin in front of the transcription machinery (black arrow). Restoration of chromatin after transcription is performed by Spt6, which incorporates unacetylated H4 (no red asterisk). (**C**) When transcription is low, recycling of H4 K16Ac predominates, and thus, H4 K16Ac levels remain high on genes with low transcription.

to be deposited in the same S-phase. Alternatively, it is conceivable that Sas2-mediated H4 K16Ac first appears in a pool of H4 K16Ac that is bound to CAF-I, and that this pool then serves as a reservoir for H4 to be deposited in a subsequent step.

Our work furthermore sheds light on the fate of histone H4 during transcription. In agreement with earlier observations of high H4 K16Ac levels correlating with low gene expression (Kurdistani, Tavazoie and Grunstein 2004; Liu *et al.* 2005; Heise *et al.* 2012), we found that Sas2-dependent H4 K16Ac increases in genes upon their repression in an Spt6-dependent fashion, raising the question where the deposited histones come from. Our data, together with that of others (Ivanovska *et al.* 2011; Perales *et al.* 2013), suggest the following model (Fig. 5B, C). As the transcription machinery moves through the body of a gene, H4 acetylated or unacetylated at K16 (along with other histones) partially or completely dissociates from the DNA. For redeposition after passage, 'old' histones with H4 K16Ac, but also new, K16-unacetylated H4 are deposited. Spt6 is recruited to the gene body via its interaction with RNA PolII and assembles the new (i.e. K16-unacetylated) H4 behind the polymerase. As transcription diminishes upon repression, less PolII and thus less Spt6 moves along the gene, such that the reincorporation of 'old' H4 K16-acetylated histones predominates over new histones, thus leading to increased levels of H4 K16Ac over the ORF. These 'old' histones may remain associated with the DNA after FACT-mediated eviction of H2A/H2B and may be 'passed back' from in front to behind the polymerase as it navigates through this structure. Due to reduced Spt6 association, this passback is expected to be more pronounced in genes with low expression, which in fact has been observed (Radman-Livaja *et al.* 2011). Alternatively, 'old' H3/H4 is completely evicted by an unknown factor and is locally redeposited behind the polymerase.

This model also makes the prediction that a reduction in histone turnover across ORFs should lead to an increase in H4 K16Ac levels. In agreement with this notion, we observed such an increase in *asf1*Δ cells (Fig. S2, Supporting Information), which previous work has demonstrated to have reduced histone turnover (Schwabish and Struhl 2006; Rufiange *et al.* 2007). We also observed an increase of H4 K16Ac over ORFs in *cac1*Δ cells, suggesting reduced histone turnover in this mutant.

One remaining question is whether SAS-I acetylates free or chaperone-bound histones, or whether it performs the acetylation on DNA-associated H4 during transcription- or replication-coupled chromatin assembly. Notably, we have been unable to find chromatin association of Sas2 by ChIP except at the rDNA locus (Meijsing and Ehrenhofer-Murray 2001), suggesting that SAS-I is not active on chromatin-bound H4. Alternatively, the chromatin association of Sas2 may be transient, for instance during S-phase, or for other technical reasons may not be amenable to ChIP. Of note, the *in vitro* activity of SAS-I is stronger on free H4 than on nucleosomes (Sutton *et al.* 2003), which favours the interpretation that SAS-I *in vivo* is active on H4 that is either free or bound to CAF-I or Asf1, but is not nucleosomal.

What is the function of H4 K16Ac during S-phase? The most obvious effect of reduced H4 K16Ac levels in the absence of SAS-I is the spreading of the SIR complex into subtelomeric regions and concomitant gene repression (Kimura, Umehara and Horikoshi 2002, Suka, Luo and Grunstein 2002), which, if excessive (Ehrentraut *et al.* 2010), can affect cell viability. Conceivably, the counteraction of SIR spreading is most important at genes with a low histone turnover, and accordingly, such genes have high H4 K16Ac levels (Heise *et al.* 2012). During replication-coupled chromatin assembly, H4 K16Ac may be diluted through incorporation of new histones, and this needs to be counteracted by SAS-I activity specifically in S-phase in order to prevent inappropriate gene repression by SIR into telomere-adjacent regions. We propose that yeast cells have evolved a global, untargeted mechanism to achieve this feat by coupling H4 K16Ac to chromatin assembly, because such a mechanism does not necessitate conserved DNA sequence motifs at subtelomeric genes and thus leaves more evolutionary flexibility for these genes. The consequence of this is that, as a 'bystander' effect, H4 K16Ac is also incorporated in non-telomeric regions, where its absence has only minor effects on transcription. In this respect, it is surprising that global H4 K16Ac levels have been observed to drop in G2/ M-phase in order to promote chromosome condensation (Wilkins *et al.* 2014), and it remains to be determined whether this affects SIR spreading in late M- and early G1-phase. We propose that Sas2-dependent H4 K16Ac is deposited in the genome in a replication-coupled manner, and that it then is 'sculpted' by transcription-dependent and -independent nucleosome assembly, the sculpting being most pronounced in regions of high histone turnover.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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***Conflict of interest.*** None declared.

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