

Connecting the histone acetyltransferase complex SAS-I to the centromere in *S. cerevisiae*

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Abstract

The essential histone H3 variant Cse4 plays a crucial role at the centromere in *S. cerevisiae*, where it replaces histone H3 in that it assembles centromere specific (Cse4-H4)₂ tetrameres. We found in our study that the histone H3 variant was able to interact over its unique N-Terminus with two subunits of the histone acetyltransferase complex SAS-I: Sas2 and Sas4. Mutations within the acetyl-CoA binding site (HAT domain) or the zinc-finger of Sas2 disrupted the binding to Cse4, although an indirect interaction was found with co-immunoprecipitation experiments.

Additionally, the N-terminus of Cse4 interacted with Cac1, the largest subunit of the chromatin assembly factor CAF-I and Asf1 – two histone chaperones that assemble histones H3 and H4 into nucleosomes. Our findings further suggest a role of Cac1 independent of Cac2 and Cac3 as no binding to Cse4 could be detected. A role for Sas2 at the centromere was further confirmed in that a *sas2* deletion (*sas2* delta) disrupted the binding of Cse4 to Ctf19. Additionally, *sas2* delta partially rescued the temperature sensitivity of a *cse4-103* mutated strain at elevated temperatures, suggesting a role for Sas2 in improving centromere stability. An important question resulted from our studies: is Sas2 able to acetylate the histone H3 variant Cse4 ? We have circumstantial evidence that Cse4 was indeed acetylated in the cell, but whether Sas2 accounts for the acetylation remains to be determined.

Keywords: epigenetics, centromere, histone acetylation, chromatin assembly, histone code

Zusammenfassung

Die essentielle Histon H3 Variante Cse4 ersetzt am Centromer das Standard Histon H3 und bildet zusammen mit Histon H4 funktionelle Cse4-H4 Tetramere aus. In dieser Studie konnte gezeigt werden, dass Cse4 über seinen einzigartigen N-Terminus mit zwei Komponenten des Histon-Acetyltransferase-Komplexes SAS-I interagiert: der enzymatischen Untereinheit Sas2 und Sas4. Mutationen innerhalb des atypischen C2HC Zink-Fingers oder der HAT-Aktivierungsdomäne von Sas2 verhindern eine Bindung an Cse4, obwohl mit Hilfe von Co-Immunoprecipitationsexperimenten eine indirekte Interaktion nachgewiesen werden konnte.

Weiterhin wurde gezeigt, dass Cse4 mit Cac1, der größten Untereinheit des Chromatin-Assemblierungsfaktors CAF-I und Asf1 interagiert – zwei Histon Chaperonen, die Histon H3 und H4 in Chromatin assemblieren. Unsere Ergebnisse lassen weiterhin auf eine separate Rolle von Cac1, unabhängig von den beiden anderen Untereinheiten schließen. Die Interaktion von Cse4 und Ctf19 wird durch eine Deletion von Sas2 verhindert. Ebenfalls kann die Temperatur-Sensitivität eines cse4-103 mutierten Hefestamms durch eine Sas2-Deletion partiell supprimiert. Somit kann man darauf schließen, dass Sas2 eine Funktion bei der Stabilisierung des Centromers aufweist.

Die bisherigen Ergebnisse lassen die Frage aufkommen, ob Cse4 in der Zelle acetyliert ist und ob es möglicherweise als Histon H3 Variante ebenfalls ein Substrat von SAS-I darstellt. Wir konnten zeigen, dass Cse4 tatsächlich in einem acetylierten Status vorliegt, ob SAS-I jedoch für die Acetylierung verantwortlich ist bleibt nachzuweisen.

Schlagwörter: Epigenetik, Centromer, Histon Acetylierung, Chromatin Assemblierung, Histon Code

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1. Introduction

1.1. Organisation of chromatin

In eukaryotic cells, genomic DNA is packaged into chromatin in the nucleus. Chromatin is able to undergo dynamic changes during replication, recombination, transcription and DNA repair, but also to control temporal gene expression (Wolffe and Kurumizaka, 1998). The fundamental core structure of chromatin are nucleosomes, which are repetitive units of approximately 147 bp DNA wrapped around a histone core in a left-handed superhelix (Fig. 1) (Luger, et al., 1997). The histone proteins H2A, H2B, H3 and H4 are evolutionarily conserved. They form a tripartite protein helix with a H3-H4 tetramere in the middle flanked by an H2A-H2B dimer within the nucleosome.

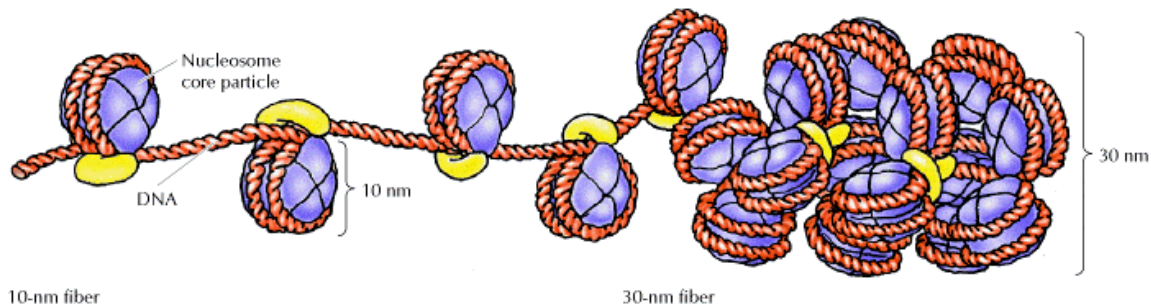


Fig. 1: Molecular assembly of nucleosomes (picture taken from http://148.216.10.83/CELULA/4,2_chromosomes_and_chromatin.htm). The DNA (red) is wrapped around the histone octamer (blue) and both form the nucleosome core particle. This structure is locked in mammals by the linker histone H1 (yellow). The chromatin fiber is further folded into a thicker fiber, the so-called solenoid that is 30 nm in diameter.

In mammals, the linker histone H1 binds between the single nucleosome core particles. Although an H1-homologue (*HHO1*) has been found in *S. cerevisiae*, it is still unknown whether it has the same function in stabilizing the nucleosome (Freidkin and Katcoff, 2001; Landsman, 1996). Six to eight nucleosomes form a solenoid structure by further coiling with a diameter of 30 nm.

Chromatin can be divided into heterochromatin and euchromatin. Heterochromatin is more compact than euchromatin and contains genes that are not actively transcribed. One of the best known examples for heterochromatin is the X-chromosome in female mammals, which is inactivated in a process called dosage compensation and forms the so-called Barr body. In general, heterochromatin replicates late in the S-phase of the cell cycle and can be found in regions containing no or only few genes, such as the telomeres and the centromere. In contrast, euchromatin is “active” chromatin, which contains DNA sequences that are transcribed into RNA.

1.1.1. Chromatin assembly

After DNA replication, recombination or repair, DNA is re-assembled into nucleosomes. This mechanism involves several protein complexes, which function as chaperones and help to integrate histones and DNA into a highly organized chromatin structure. During S-phase of the cell cycle, the parental nucleosomes become temporarily separated. After they pass the replication fork, nucleosomes are newly assembled onto the two DNA daughter strands by integrating pre-existing histones as well as newly synthesized histones.

Chromatin assembly factor I (CAF-I)

In *S. cerevisiae*, CAF-I is a chromatin assembly factor that delivers histone H3 and H4 to DNA during DNA replication or DNA repair (Gaillard, et al., 1996; Kamakaka, et al., 1996; Kaufman, et al., 1997). In order to bind to the replication fork, CAF-I interacts with the proliferating cell nuclear antigen (PCNA) (Verreault, et al., 1996).

CAF-I is an evolutionary conserved heterotrimeric complex with the subunits Cac1, Cac2 and Cac3. Deletion of any of the three *CAC* genes leads to an increase

in ultraviolet (UV) radiation sensitivity, implying a defect in nucleotide excision repair (Game and Kaufman, 1999). Additionally, *CAC* deletions reduce position dependent gene silencing at the telomeres (Enomoto, et al., 1997; Kaufman, et al., 1997), the rDNA locus (Smith, et al., 1999) and the mating-type loci (Enomoto and Berman, 1998; Kaufman, et al., 1998), suggesting a role for CAF-I in heterochromatin formation. As a deletion of the three CAF-I subunits does not result in a G2 arrest and is not lethal for the cell (Kaufman, et al., 1997), it is likely that one or more independent pathways for chromatin assembly exist.

Anti-silencing factor 1 (Asf1)

Asf1 is thought to act in concert with CAF-I as a chromatin assembly factor. It also promotes assembly of nucleosomes *in vitro* (Tyler, et al., 1999) and the *D. melanogaster* homologue of Asf1 was shown to interact with histones H3 and H4 that carry the acetylation pattern of newly synthesized histones (Tyler, et al., 2001).

ASF1 was originally identified in a screen for high-dosage disrupters of silencing at the mating-type loci in *S. cerevisiae* (Le, et al., 1997). When overexpressed, it also leads to reduced silencing at the telomeres (Le, et al., 1997; Singer, et al., 1998) and at the rDNA locus (Singer, et al., 1998). Asf1 is not essential for the cell, but a deletion causes defects in heterochromatic gene silencing, slow growth due to a lengthened S-phase, sensitivity to DNA damaging and replication blocking agents (Le, et al., 1997; Tyler, et al., 1999) and an increase in chromosome loss (Le, et al., 1997).

Additionally, Asf1 interacts with the CAF-I subunit Cac2 and increases CAF-I activity in nucleosome assembly in *Drosophila* as well as in yeast (Mello, et al., 2002; Tyler, et al., 2001). Inactivation of both CAF-I and Asf1 leads to a synergistic reduction in heterochromatic gene silencing, since double mutants display more severe phenotypes than strains with either single mutant (Tyler, et al., 1999). These results imply that CAF-I and Asf1 are both chromatin assembly

factors that integrate histone H3 and H4 into chromatin in partially overlapping pathways. Nevertheless, CAF-I and Asf1 also have distinct roles in chromatin assembly, as they show different interactions with proteins involved in cell cycle checkpoint, non-homologous end joining (NHEJ) or H2A phosphorylation and because mutations in either *CAC* or *ASF1* result in different gross chromosomal rearrangement (GCR) rates (Myung, et al., 2003).

Histone regulation genes (Hir)

Gene products from the *HIR* (histone regulatory) genes *HIR1* and *HIR2* have been shown to interact with Asf1 *in vitro* and *in vivo* (Sharp, et al., 2001; Sutton, et al., 2001), implying that they function together in a silencing pathway that is also PCNA dependent and partially overlaps with the CAF-I silencing pathway (Krawitz, et al., 2002).

The *HIR* genes *HIR1*, *HIR2*, *HIR3* and *HPC2* encode proteins that tightly regulate histone gene transcription (Osley and Lycan, 1987; Xu, et al., 1992). They code for repressors proteins that bind to the histone promoters in early G1-, late S- and in G2/M-phase and therefore prevent the histone genes from being transcribed (Osley, et al., 1986). Additionally, Hir proteins contribute to Asf1-mediated nucleosome assembly (Sharp, et al., 2001). The *HIR*-genes are exclusively expressed during G1/S transition in yeast, whereas in other phases of the cell cycle they become repressed by a mechanism that is thought to involve a specialized chromatin structure (Dimova, et al., 1999).

Mutations in *HIR* genes have only minor effects on silencing at the telomeres and the *HM* loci (Kaufman, et al., 1998), but when combined with mutations in CAF-I subunits, yeast cells display synergistic reduction of position-dependent gene silencing both at the *HM* loci and the telomeres, increased sensitivity to DNA damaging agents and slow growth (Kaufman, et al., 1998; Qian, et al., 1998; Sharp, et al., 2001). A new role for histone interacting and –deposition proteins has been described at the centromere, where CAF-I and Hir proteins function in

maintaining the centromeric chromatin structure (Sharp, et al., 2002). *cac1Δ* *hir1Δ* cells have increased chromosome missegregation, genetic synergies with mutations in kinetochore protein genes and an alteration in centromeric chromatin structure. CAF-I and Hir proteins are not absolutely required for deposition of the histone H3 variant Cse4 at the centromere, but their absence leads to Cse4 deposition outside of centromeric chromatin.

Nucleosome assembly protein 1 (Nap1)

After deposition of histone H3 and H4, histone H2A and H2B are integrated into chromatin to form the core nucleosome. The nucleocytoplasmatic shuttle protein Nap1 (*nucleosome assembly protein 1*) binds to histone H2A and H2B and helps to assemble chromatin from newly synthesized DNA (Ito, et al., 1996). Since Nap1 has been localized in several cell compartments, the current model posits that Nap1 functions as a histone chaperone that binds H2A and H2B already in the cytoplasm. Due to interaction with Kar114p, the primary karyopherin/importin responsible for the nuclear import of H2A and H2B, it helps to import them into the nucleus, where it incorporates the histones into newly replicated DNA (Mosammaparast, et al., 2002).

1.1.2. Post-translational modification of histones

Histones consist of a highly conserved globular histone-fold domain and an N-terminal domain that remains outside of the nucleosome (Luger, et al., 1997). The N-termini as well as the core region are targets for posttranslational modifications, e.g. acetylation, phosphorylation, methylation and ubiquitination, which result in changes of gene activity (Spencer and Davie, 1999).

Methylation or acetylation occurs on the ϵ -amino group of positively charged lysines (Fig. 2). In the case of acetylation, the charge of the amino group is then

changed to neutral, whereas methylation does not change the positive charge. Additionally, histones can be methylated on arginine groups.

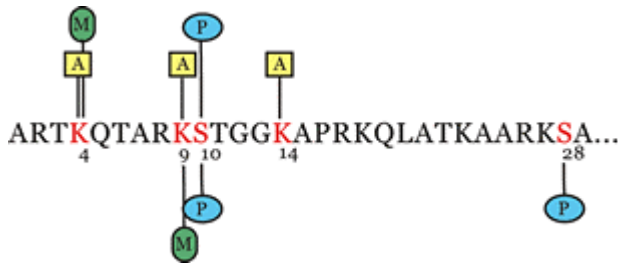


Fig. 2: Possible modifications of amino acids at the example of the N-terminus of histone H3 from *S. cerevisiae*. A=acetylation, M=methylation, P=phosphorylation

Several histone methyltransferases (HMTs) have been characterized. One example are the SUV39 family proteins that exist in *D. melanogaster*, humans and *S. pombe*, and selectively methylate histone H3 lysine 9 (Rea, et al., 2000). This methylation has been shown to be a prerequisite for binding of the heterochromatin binding protein 1 (HP1), which connects H3 K9 methylation to silencing and heterochromatin formation (Bannister, et al., 2001; Lachner, et al., 2001). Additionally, methylation on H3 K9 prevents phosphorylation of H3 S10, which is known as a modification involved in gene activation (Lo, et al., 2001; Lo, et al., 2000). Methylation can also lead to transcriptional activation, e.g. methylation of H3 K4 in *S. cerevisiae* (Noma, et al., 2001). It is becoming clear that histone modifications are able to influence each other and work together in the form of a “histone code”, thereby creating a specific chromatin structure that is connected with gene activity.

Histone acetylation is carried out by several known histone acetyltransferase (HATs), and as acetylation is a reversible process, there also exist histone deacetylases (HDACs) as antagonists in the cell. In general, histones tend to be hyperacetylated in actively transcribed regions, whereas histones are hypoacetylated in transcriptionally repressed regions. The simplest model of gene

repression involves a histone deacetylase that removes acetyl groups from the N-termini of histones in a defined area, thereby building up a repressive chromatin structure. As a consequence, transcriptional activators or other transcription factors are unable to bind to their promotor elements, which leads to transcriptional inhibition.

Histone deacetylases (HDACs)

Histone deacetylation has been linked to the establishment of transcriptional inactive heterochromatin. At least ten HDACs exist in *S. cerevisiae*: Hda1, Hos1-3, Rpd3, Hst1-4 and Sir2. Hos 1/Hos3 and Hos 2 mainly affect ribosomal DNA and ribosomal protein genes, whereas Rpd3 and Hda1, which preferentially deacetylate distinct promoters, function in histone deacetylase complexes and their absence results in hyperacetylation of histone H3 and H4 (Kuo and Allis, 1998; Robyr, et al., 2002).

The first hint that Sir2 functions as a histone deacetylase was the observation that *SIR2* overexpression leads to hypoacetylation of histones in yeast (Braunstein, et al., 1993). Sir2 has now been characterized as a phylogenetically conserved NAD⁺-dependent HDAC that efficiently deacetylates histone H4 lysine 16 and histone H3 lysine 9 (Imai, et al., 2000a; Landry, et al., 2000; Smith, et al., 2000). One role of Sir2 is the establishment of silencing at the rDNA locus (Bryk, et al., 1997; Smith and Boeke, 1997), as well as at silent mating-type loci *HML* and *HMR* and at the telomeres in a complex with other Sir proteins (Sir3 and Sir4, at the *HM* loci additionally Sir1) (Moretti, et al., 1994; Rine and Herskowitz, 1987). Recently, a role in ageing has been described for Sir2 in *C. elegans* and in other organisms, including yeast (Guarente, 2000; Guarente, 2001; Imai, et al., 2000b). Overexpression of Sir2 leads to an increase in life span (Kaeberlein, et al., 1999), which is determined in yeast by how many times a mother yeast cell is able to divide, whereas *SIR2* deletion or NAD⁺-deprivation leads to a increased recombination rate and prevents longevity (Lin, et al., 2000).

Histone acetyltransferases (HATs)

In contrast to HDACs, histone acetyltransferases have the opposite effect on silencing, since the acetylation of lysine residues generally leads to the formation of euchromatin and transcriptional activation. The various existing HATs are grouped into five families based on sequence similarities. These families also include the MYST family with its representatives in yeast (*Sas2*, *Sas3*, *Esa1*), *D. melanogaster* (*MOF*, *Chameau*) and human (*MOZ*, *MORF*, *Tip60*, *HBO1*). Members of the evolutionary conserved MYST family share the MYST homology domain with a binding site for acetyl-CoA (HAT domain) and an atypical C2HC zinc-finger motif.

SAS2 was originally identified in a screen for suppressors of silencing defects at *HMR* (Ehrenhofer-Murray, et al., 1997). In contrast to its role at the *HMR* locus, a deletion of *SAS2* (*sas2Δ*) decreases silencing at *HML* in *sir1Δ* strains and derepresses silencing at the telomeres (Ehrenhofer-Murray, et al., 1997; Reifsnnyder, et al., 1996). The conserved acetyl-CoA binding domain as well as the HAT domain is essential for *HML* and telomeric silencing, because mutations in these domains lead to the same silencing phenotypes as a *SAS2* deletion (Meijsing and Ehrenhofer-Murray, 2001; Osada, et al., 2001). Additionally, (Meijsing and Ehrenhofer-Murray, 2001) have shown that a mutation in histone H4 lysine 16 to arginine phenocopies the effect of a *sas2Δ*, implying that H4 K16 is one target for *Sas2* acetylation.

Sas2 exists in a complex called SAS-I together with two other proteins involved in silencing, *Sas4* and *Sas5* (Meijsing and Ehrenhofer-Murray, 2001; Osada, et al., 2001) and acetylates next to histone H4 lysine 16 also H3 lysine 14 *in vitro* (Sutton, et al., 2003). The association with *Sas4* is essential for *Sas2*'s acetyltransferase activity, whereas binding of *Sas5* is required for improving the activity of the complex (Sutton, et al., 2003). Furthermore, *Sas2* has been shown to interact with the chromatin assembly factors CAF-I and *Asf1* (Meijsing and

Ehrenhofer-Murray, 2001; Osada, et al., 2001), proposing a model where Sas2 is recruited to the replication fork via Cac1 and/or Asf1, where it can acetylate the incorporated histones. Thereby, Sas2 might link DNA replication and chromatin assembly to histone modification. It is unknown whether Sas2 also acetylates histone variants that exist in the cell.

1.1.3. Silencing in *S. cerevisiae*

Three silenced regions are known in the yeast *S. cerevisiae*: the silent mating-type loci *HML* and *HMR* on chromosome III, the rDNA locus on chromosome XII and the telomeres. Both silencing at the *HM* loci and at the telomeres requires a common set of *trans*-acting factors, which include the Sir (silent information regulator)-proteins, the N-termini of histone H3 and H4, Abf1 and the repressor-activator protein Rap1. The structure of the different silenced regions and their common and distinct features are discussed below.

Silencing at the mating-type loci HML and HMR

Haploid yeast cells can display either an a- or a α -mating-type. The cell type is defined by the allele present in the *MAT* locus on chromosome III. Whereas cells with a *MAT*_a allele are of a-mating-type, and cells with the *MAT* _{α} allele are of α -mating-type. Under certain circumstances, a cell can switch its mating-type by cleaving the mating-type locus with HO endonuclease and replacing the *MAT* with information from *HML* or *HMR*.

Additionally to *MAT*, the cell contains unexpressed copies of the mating-type information genes near the telomeres on the same chromosome: *HMR* generally codes for a-information and *HML* for α -information. The repression of the *HM* loci is maintained by flanking silencing elements (*E* and *I* silencer) as well as by silencer binding proteins. Whereas the *E*-silencer is absolutely essential for

silencing, a deletion of the *I*-silencer does not influence the repression (Brand, et al., 1985). Since the *I*-silencer is a protosilencer, it becomes important in compromised situations, e.g. when the *E* silencer is not fully functional. The flanking silencers from the *HM* loci cause repression of a- or α -information transcription in addition to *MAT* gene expression.

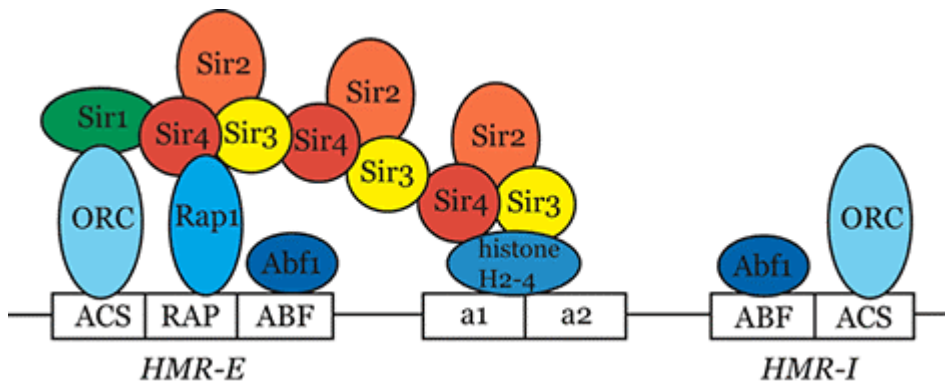


Fig. 3: Structure of the silent mating-type locus HMR in *S. cerevisiae* with silencer binding proteins and proteins involved in the establishment and maintenance of silencing.

To exemplify silencing at the *HM* loci, silencing at the well-studied *HMR* silencer is explained subsequently and in Fig. 3. The a-information at *HMR* is flanked by the *HMR-E* and the *HMR-I* silencers. The ~140 bp *HMR-E* contains binding sites for the origin recognition complex ORC, Rap1 and Abf1, whereas the *HMR-I* silencer lacks the Rap1 binding site. Rap1 and Abf1 are regulatory proteins that function as transcriptional activators in other positions in the genome beside their role in silencing (Shore, 1997).

ORC, Rap1 and Abf1 serve as a platform for binding of the Sir-proteins to *HMR-E*. Deletion of Sir1 only causes a weak silencing defect, whereas deletion of *SIR2-4* leads to complete derepression. Through the interaction with ORC, Sir1 facilitates the binding of Sir2, Sir3 and Sir4 to the silencer. Hereby, Sir3 and Sir4 interact directly with Rap 1 and the N-termini of histone H3 and H4 (Moretti, et al., 1994; Triolo and Sternglanz, 1996). Sir2 further improves efficient spreading

of the Sir-proteins throughout *HMR* via its NAD⁺-dependent deacetylation activity (Rusche, et al., 2002).

Silencing at the rDNA locus

Another silencing locus in *S. cerevisiae* is the highly repetitive rDNA locus on chromosome XII. The rDNA locus encodes the ribosomal RNAs, which are not translated into proteins, but establish the ribosome together with ribosomal proteins. One rDNA unit consists of ~9.1 kb, which is tandemly repeated in 100-200 copies per cell. Silencing at this locus is thought to regulate the access of RNA polymerase I, but prevents RNA polymerase II from transcription (Shou, et al., 2001).

At the rDNA locus, Sir2 assembles together with Net1, Cdc14, Nan1 and PolI into the so-called RENT (regulator of nucleolar silencing and telophase exit) complex. In *sir2* Δ cells, the rDNA silencing is decreased and the chromatin structure is less compact (Smith and Boeke, 1997), whereas the recombination rate is increased (Gottlieb and Esposito, 1989). Thus, Sir2 improves rDNA silencing via its deacetylation activity.

The structural core component of the RENT complex is Net1, which recruits the other RENT-proteins to the rDNA locus. Net1 binds within the RENT complex to PolI, the RNA polymerase I, and stimulates its enzymatic activity (Shou, et al., 2001). Whether Net1 directly binds to DNA is still unknown, but it is possible that other still unknown proteins contribute to rDNA silencing. Interestingly, when *NET1* is overexpressed, it is also associated with the *HMR-E* silencer, whereas *net1-1* acts indirectly in *HMR* silencing by releasing Sir2 from the nucleolus (Kasulke, et al., 2002).

Silencing at the telomeres

Telomeres can be found at the ends of all 16 chromosomes in yeast. They consist of a 300-350 bp region with irregular TG₁₋₃ repeats, which are disrupted by X- and Y-elements of variable size (Palladino and Gasser, 1994). The Rap1 binds on average every 18 bp in the telomeric repeats and is a structural core component of the telomeres. The Rap1 binding region is also referred to as the telosome and is characterized by its nuclease resistance.

Rap1 in turn recruits Sir2, Sir3 and Sir4 to the telomeres, where Sir3 and Sir4 bind to the N-termini of histone H3 and H4. The Sir proteins spread in a histone-dependent manner up to 2.8 kb into the core telomeric heterochromatin. (Strahl-Bolsinger, et al., 1997) have proposed a model in which telosomal Rap1 folds back onto subtelomeric regions. This allows a condensation at telomeric heterochromatin due to the interaction between Rap1 and the Sir's, as well as among the Sir proteins themselves.

Silencing at the telomeres and subsequent condensation prevents the chromosome ends from being degraded and improves their replication (Palladino, et al., 1993). Additionally, the positioning of the telomeres to the nuclear periphery is maintained.

1.2. The centromere-kinetochore complex

Centromeres are the site of kinetochore formation and microtubuli attachment on the chromosome. They vary in size and form in eukaryotes, but all have a key role in chromosome inheritance and are therefore essential for the transfer of genetic information from one cell to its daughter cell. Most eukaryotes contain monocentric centromeres, where the centromere-kinetochore complex is formed at a single point on the chromosome. One exception are holocentric organisms like the nematode *Caenorhabditis elegans*, which have holocentric kinetochores formed along the entire chromosome (Albertson and Thomson, 1982).

Centromeres consist of centromeric DNA (*CEN* DNA) and an assembled specialized nucleoprotein complex termed kinetochore (Clarke, 1998). After chromosomes have replicated during S-phase, sister chromatids are still attached to each other at the kinetochore. During mitosis, several spindle microtubuli bind to the kinetochore and mediate chromosome movement to the nuclei of the daughter cells. In order to assure accurate segregation, microtubuli attachment is closely monitored, so that the cell cycle can be arrested in case of incomplete or inappropriate attachment (Nicklas, et al., 1995; Rudner and Murray, 1996).

Although the centromere was first identified by Walther Flemming more than 120 years ago, the complex structure and function is still not completely elucidated. In multicellular organisms, the centromere is embedded within constitutive centric heterochromatin (Fig. 4). The outer kinetochore is responsible for attachment of spindle microtubuli, the transition from metaphase to anaphase and poleward movement. Additionally, it has a key role in binding proteins that keep the attachment of microtubuli to the kinetochore under a surveillance mechanism called the spindle assembly checkpoint (SAC) or the mitotic checkpoint (Shah and Cleveland, 2000). The inner kinetochore/centromere serves as an attachment site for kinetochore proteins and is important for centromere identity.

Several studies have revealed that a universal centromere sequence conserved among eukaryotes does not exist. Whereas the centromere in *H. sapiens* covers 500-5000 kb, essential centromere sequences could be narrowed down to 420 bp in *D. melanogaster* (Murphy and Karpen, 1995). Next to complex base sequences, transposons and series of simple satellite sequences can also be found within this fragment.

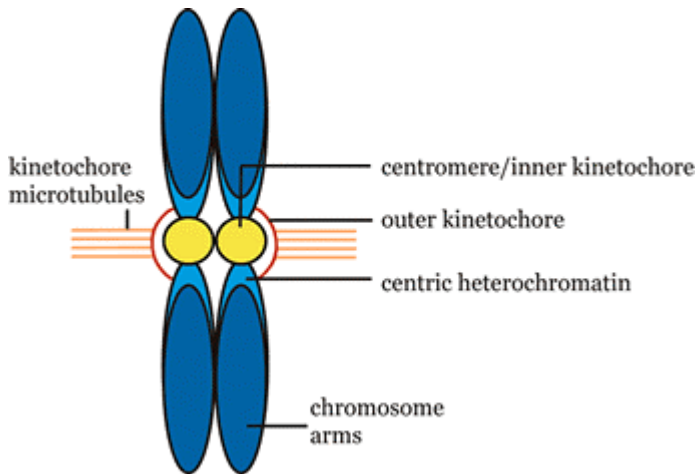


Fig. 4: Structural and functional regions of the centromere. The model shows the two sister chromatids with their chromosome arms, the centric heterochromatin and the centromere. Attached to the centromeric regions are proteins that assemble to form the outer kinetochore, which in turn serves as the binding site for microtubules.

The mammalian centromere is composed of highly repetitive α -satellite DNA that consists of 171 bp monomer sequences (Willard, 1991). The proteins binding to the inner kinetochore can be divided into constitutive proteins, e.g. CENP-A, -B, -C and -G, and facultative proteins like tubulin, CENP-E and CENP-F (Van Hooser, et al., 1999). Whereas CENP-B binds specifically to a 17 bp α -satellite motif, CENP-A, -C and -G are located near/at the inner kinetochore plate. CENP-A is one of the best-studied centromeric proteins. It was originally identified as an essential 17 kD antigen recognized by anti-centromere autoantibodies from calcinosis/Raynaud's phenomenon/esophageal dysmotility/ sclerodactyly/telangiectasia (CREST) serum (Earnshaw and Rothfield, 1985). CENP-A is 62%

identical to the carboxy-terminal domain of histone H3, and is therefore a centromeric histone H3 variant widely conserved from yeast to human (Sullivan, et al., 1994; Yoda, et al., 2000).

1.2.1. The centromere in *S. cerevisiae*

The organization of the centromere of *S. cerevisiae* is relatively simple compared to higher eukaryotes. It consists of 125 bp centromeric DNA sequences, which can be subdivided into three conserved regions named CDEI, CDEII and CDEIII (Hyman and Sorger, 1995). CDEI (8 bp) and CDEIII (25bp) are palindromic sequences flanking the A/T-rich 78-86 bp CDEII. Whereas CDEIII is absolutely necessary for centromeric function, mutations in CDEI and CDEII can be tolerated.

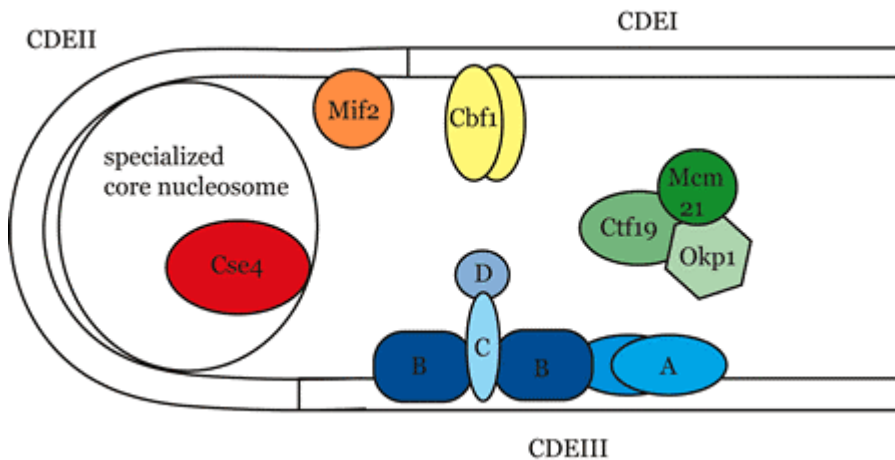


Fig. 5: The current model of the yeast centromere. The three centromeric regions CDEI, CDEII and CDEIII serve as binding sites for different centromere binding proteins, e.g. Cse4. Cse4 replaces histone H3 and therefore forms a specialized core nucleosome with histone H2A, H2B and H4. The connection between CDEI, CDEII and the essential CDEIII-region is made by a three protein containing complex (Ctf19-Okp1-Mcm21).

CDEIII contains the binding site for the essential four-protein complex CBF3 (Cbf2-Cep3-Ctf13-Skp1) (Connelly and Hieter, 1996); to CDEI binds a Cbf1 homodimer that increases the accuracy of chromosome segregation to the factor ten (Hegemann and Fleig, 1993). Mif2, a CENP-C homologue from mammals (Meluh and Koshland, 1995), and the histone H3 homologue Cse4 are associated to CDEIII. An essential END domain (amino acids 28-60) lies within the N-terminus of Cse4 that mediates binding to Ctf19 (Chen, et al., 2000). Ctf19 can be found in a complex together with Okp1 and Mcm21. This complex mediates the connection between CDEI, CDEII and CDEIII by binding to Cse4 as well as to Mif2 and CBF3, and therefore forms a stabilized centromere structure and a functional kinetochore (Ortiz, et al., 1999).

Recently, (Sharp, et al., 2003) discovered, that the silencing protein Sir1 is a functional component of centromeric chromatin. This was the first time that a protein that functions in heterochromatic gene silencing at the *HM* loci was found to play a role at the centromere. Here, Sir1 functions in a partially overlapping pathway with CAF-I and Hir1 to maintain chromosome stability. It interacts with Cac1, the largest subunit of CAF-I and helps to maintain the chromatin assembly factor at the centromere.

Figure 4 illustrates the current model of the yeast centromere. Many protein-protein and DNA-protein interactions have been demonstrated by two-hybrid analysis, co-immunoprecipitation and chromatin-immunoprecipitation (ChIP) experiments, but it is expected that other still unknown factors might also play a role at the centromere.

1.2.2. The histone H3 variant Cse4

Histone H3 variants are a common element found at functional centromeres in eukaryotes. Centromere specific histone H3 variants have first been described with CENP-A in mammals (Palmer, et al., 1987; Sullivan, et al., 1994), but CENP-

A homologues have also been found in *D. melanogaster* (Cid) (Henikoff, et al., 2000), *S. pombe* (Cnp1) (Takahashi, et al., 2000), *C. albicans* (CaCse4) (Sanyal and Carbon, 2002), *C. elegans* (HCP-3) (Buchwitz, et al., 1999) and in *S. cerevisiae* (Cse4) (Stoler, et al., 1995). They all share highly conserved C-terminal histone H3-like sequences, but carry unique and widely different N-termini (Malik and Henikoff, 2001). Inactivation of CENP-A family proteins leads to severe chromosome missegregation events or even to cell death.

The histone H3 variant *CSE4* from *S. cerevisiae* was first identified in a screen for mutants with defects for **chromosome *se*gregation**. Here, the mutant *cse4-1* increases the frequency of chromosome non-disjunction and leads to a G2-M cell cycle arrest at elevated temperatures (Stoler, et al., 1995).

CSE4 encodes an essential 27 kD protein with a unique 135 aa N-terminus containing two putative nuclear localization signals (NLS), and a C-terminus with >64% homology to the histone-fold-domain of histone H3. The N-terminus is localized outside the centromeric nucleosome core and has at least one essential function, because deletion of the first 50 amino acids leads to cell death (Keith, et al., 1999). This region was further characterized by (Chen, et al., 2000), who delineated a 33 amino acid region (aa 28-60) termed END domain (essential N-terminal domain) by deletion mutagenesis, which is essential and sufficient for wildtype Cse4 function.

| | |
|-----|--|
| 1 | MSSKQQWVSSAIQSDSSGRSLSNVRLAGDQQSINDRALS |
| 41 | LLQRTRATKNLFPREERRRYESSKSDLDIETDYEDQAGN |
| 81 | EIETENEEEAEMETEVPAVVRTHSYALDRYVRQKRREKQ |
| 121 | RKQSLKRVEKKYTPSELALYEIRKYQRSTDLLISKIPFAR |
| 161 | LVKEVTDEFTTKDQDLRWQSMAIMALQEASEAYLVGLLEH |
| 201 | TNLLALHAKRITIMKKDMQLARRIRGQFI |

Fig. 6: Amino acid sequence of the histone H3 variant Cse4. Putative acetylation sites are lysine residues (K) and marked in red.

One function of the END domain is the interaction with the kinetochore protein Ctf19 (Chen, et al., 2000; Ortiz, et al., 1999), which exists in a complex together with Okp1 and Mcm21. *MCM21* is a suppressor of END domain mutations, whereas simultaneous mutations within the Ctf19-Mcm21-Okp1 complex and the END domain are synthetically lethal. Additionally, putative modifications of amino acid residues within the END domain were investigated. The N-terminal tail of standard core histones are posttranslationally modified, bearing a critical role in chromatin structure and transcriptional regulation, but neither acetylation of K49 nor phosphorylation of S33, S40, T45 or T48 could be found within the END domain of Cse4 (Chen, et al., 2000).

The C-terminus of Cse4 is highly homologous to the histone-fold-domain of histone H3. The histone-fold-domain consists of three helices (helix I-III) that are separated by two β -loop stands. In front of helix I an N-loop can be found and in case of histone H3 an additional N-terminal helix. Substitutions of helix II or helix III of Cse4 with analogous histone H3 amino acids are critical and often lead to increased chromosome loss and lethality. Replacing the histone-fold-domain of Cse4 with that of CENP-A is unable to rescue the *cse4* null phenotype, so leading to the conclusion that specific domains within the histone-fold-domain are essential for the function of the yeast centromere (Keith, et al., 1999).

Both histone-histone interactions as well as histone-DNA interactions occur via the globular histone-fold domain, which was demonstrated in a genetic screen for Cse4 histone-fold-mutants. The current model suggests that Cse4 replaces both copies of histone H3 at the centromere and forms together with histone H4 centromere specific (Cse4-H4)₂ tetrameres (Glowczewski, et al., 2000). Supporting this model, overexpression of Cse4 can suppress a temperature sensitive phenotype in a mutant histone H4-allele (*hhf1-20*), which leads to a G2-M cell cycle arrest and increased chromosome missegregation (Meluh, et al., 1998; Smith, et al., 1996). Reciprocally, overexpressing histone H4 can repress centromere defects in *cse4* mutants.

Histone H2A has also been described to have a function at the centromere (Pinto and Winston, 2000). *hta1*-mutants lead to a cell cycle delay in G2-M, display increased chromosome loss and show genetic interaction with mutations in genes coding for kinetochore proteins. Further analysis of histone H2A and H2B function at the centromere will be required to determine their precise role.

Cse4 competes against histone H3 for binding to histone H4. Normal core histones are transcribed exclusively during S-phase of the cell cycle from histone genes that have a special 3' untranscribed region instead of poly(A) tails (Hereford, et al., 1981; Osley, 1991). In contrast, Cse4 mRNA is polyadenylated and transcribed at low constant levels during the whole cell cycle. How the competition between Cse4 and H3 is regulated is still unknown, but overexpression of histone H3 in *cse4* mutant cells is lethal (Glowczewski, et al., 2000).

So far no modifications have been found at the histone H3 variant Cse4, although acetylation and phosphorylation have been investigated within the END domain of Cse4 (Chen, et al., 2000). We found with two-hybrid analysis that Cse4 interacted with the histone acetyltransferase Sas2 and further analyzed if Cse4 is able to interact with other components of the SAS-I complex. Additionally, we investigated the role of Sas2 at the centromere and if the acetyltransferase is able to acetylate Cse4.

2. Materials and Methods

2.1. Material

2.1.1. Bacterial strains

| | |
|----------------|--|
| DH5 α | F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYAargF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_k⁻</i> , <i>m_k</i>) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1 tonA</i> (Chemically competent: Gibco BRL) |
| TOP10 | F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str^R) <i>endA1 nupG</i> (Chemically competent: Invitrogen)</i> |
| BL21(DE3) Star | F ⁻ <i>ompT hsdS_B</i> (<i>r_B⁻ m_B⁻</i>) <i>gal dcm mel31</i> (DE3) (Chemically competent: Invitrogen) |

2.1.2. Yeast strains

| Strain | Genotype | Source |
|---------|--|----------------|
| AEY1 | <i>MATα ade2-101 his3-11, 15 trp1-1 leu2-3, 112 ura3-1</i> | J. Rine |
| AEY2 | <i>MATα ade2-101 his3-11, 15 trp1-1 leu2-3, 112 ura3-1</i> | J. Rine |
| AEY2661 | AEY1 <i>cse4</i> Δ :: <i>kanMX ADE2-1 lys2</i> + pRS313-3xHA-CSE4 | |
| AEY2666 | AEY1 <i>cse4</i> Δ :: <i>kanMX sas2</i> Δ :: <i>TRP1 ADE2 lys2</i> + pRS313-3xHA-CSE4 | |
| AEY2781 | AEY2661 but pRS426-3xHA-CSE4 | |
| AEY2782 | AEY2666 but pRS426-3xHA-CSE4 | |
| AEY1162 | <i>MATα cse4-103 his3</i> Δ 200 <i>ura3-52 leu2, 112 trp1</i> Δ 1 pPY13 (<i>CEN/ARS/TRP1/cse4</i> :: <i>LEU2</i>) | L. Glowczewski |
| AEY1781 | AEY1162 <i>sas2</i> Δ :: <i>HIS3</i> | |
| AEY2373 | AEY1162 <i>cac1</i> Δ :: <i>kanMX</i> | |

| | | |
|---------|--|----------------|
| AEY2374 | AEY1162 <i>cac1Δ::kanMX sas2Δ::HIS3</i> | |
| AEY1194 | <i>MATα cse4Δ::LEU2 his3Δ200 ura3-52 leu2, 112 trp1Δ1 + pPY13 (CEN/ARS/TRP1/CSE4)</i> | L. Glowczewski |
| AEY1558 | <i>MATa leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112</i> | E.W. Jones |
| AEY1559 | AEY1558 <i>sas2Δ::TRP1</i> | |
| AEY1808 | AEY1558 <i>cac1Δ::kanMX</i> | |
| AEY2461 | AEY1558 <i>sas4Δ::kanMX</i> | |
| AEY2463 | AEY1558 <i>CAC3-9myc::TRP1</i> | |
| AEY2493 | AEY1558 <i>ASF1-4HA::TRP1</i> | |
| L40c | <i>MATa his 3Δ200 trp1-901 leu2-3. 112 ade2 lys2-801 am LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4</i> | E. Wanker |
| AEY1695 | L40c <i>sas2Δ::HIS3</i> | |

Strains between horizontal lines are isogenic

2.1.3. Plasmids

| Strain | Genotype | Source |
|--------|------------------------------------|--------|
| pAE90 | <i>URA3, 2μ, GPD-SAS2-PGK</i> | |
| pAE249 | <i>pRS315-SAS2-HAT^r</i> | |
| pAE388 | <i>pRS316-SAS2-Zn^r</i> | |
| pAE431 | <i>pRS315-sas2Δ::HIS3</i> | |
| pAE436 | <i>pBTM117c- SAS2</i> | |
| pAE439 | <i>pACT2-SAS2</i> | |
| pAE451 | <i>pBTM117c-SAS4</i> | |
| pAE454 | <i>pBTM117c-CAC1</i> | |
| pAE465 | <i>pACT2-CSE4 (aa 28-229)</i> | |
| pAE493 | <i>pBTM117c-CSE4 (aa 93-229)</i> | |
| pAE535 | <i>pACT2-ASF1</i> | |
| pAE613 | <i>pRS424-6x-myc-SAS4</i> | |
| pAE614 | <i>pRS426-6x-myc-CAC1</i> | |
| pAE625 | <i>pRS426-HA-SAS5</i> | |

| | | |
|---------------------------------|--|---------------|
| pAE686 | pACT2-N-term.- <i>CSE4</i> (aa 11-139) | |
| pAE687 | pACT2-C-term.- <i>CSE4</i> (aa 137-229) | |
| pAE688 | pBTM117c-N-term.- <i>CSE4</i> (aa 11-139) | |
| pAE689 | pBTM117c-C-term.- <i>CSE4</i> (aa 137-229) | |
| pAE690 | pBTM117c- <i>SAS5</i> | |
| pAE794 | pBTM117c- <i>CSE4</i> (full length) | |
| pAE817 | pBTM117c- <i>SAS2-HAT</i> | |
| pAE818 | pBTM117c- <i>SAS2-Zn</i> | |
| pAE820 | pRS424-6x <i>myc-CAC2</i> | |
| pAE872 | pACT2- <i>SAS4</i> | |
| pAE901 | pRS426-6x <i>myc-CSE4</i> | |
| pAE956 | pBTM117c- <i>CTF19</i> | |
| pAE974 | pRS423-3x <i>HA-CSE4</i> | |
| pAE975 | pRS424-6x <i>myc-CSE4</i> | |
| pAE976 | pRS425-3x <i>HA-CSE4</i> | |
| pAE977 | pRS426-3x <i>HA-CSE4</i> | |
| pAE994 | pET15b- <i>CSE4</i> | |
| pET15b | <i>lacI</i> , MCS, <i>HIS</i> -tag | Novagen |
| pUN60 | CEN/ARS, <i>URA3</i> , <i>SUP11</i> | ATCC |
| pCR [®] -Blunt TOPO | II- MCS, TOPO-cloning site | Invitrogen |
| pACT2 | <i>LEU2</i> , MCS, <i>GAL4</i> -AD | Elledge, 1988 |
| pBTM117c | <i>TRP1</i> , MCS, LexA | E. Wanker |

2.1.4. Media

| | |
|-----|--|
| LB | 10 g/l Caseinpepton, 5 g/l Yeast extract, 5 g/l NaCl, pH 7,2 (Lennox, 1955) |
| TY | 16 g/l Trypton, 10 g/l Yeast extract, 5 g/l NaCl |
| SOC | 2 g/l Trypton, 500 mg/l Yeast extract, 10 mM NaCl, 2-5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , 20 mM Glucose |

| | |
|--------------------|--|
| YM | 6,7 g/l Yeast Nitrogen Base w/o amino acids, 2 g/l Glucose |
| YPD | 10 g/l Yeast extract, 20 g/l Peptone, 2 g/l Glucose |
| FOA | 14 g/L Yeast Nitrogen Base w/o amino acids, 4 g/l Glucose, 2 g/l FOA, 40 mg/l Uracil |
| Sporulation medium | 19 g/l KAc, 0,675 mM ZnAc |

2.1.5. Buffers and Solutions

| | |
|-----------------------|--|
| TAE | 4.84 g/l Tris/HCl, 0.744 g/l EDTA, 1.142 mg/l Acetic acid |
| PI-Mix (1000x) | 1 M PMSF, 2 mg/ml Benzamidin, 1.4 mg/ml Pepstatin, 1 mg/ml Leupeptin, 100 mg/ml Bacitracin, 1 ml DMSO |
| TBST | 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.0005 % Tween-20 |
| PBS | 8 g/l NaCl, 0.2 g/l KCl, 1.14 g/l Na ₃ HPO ₄ , 0.2 g/l KH ₂ PO ₄ |
| TE | 10 mM Tris/HCl, pH 8, 1 mM EDTA |
| Z-Buffer | 60 mM Na ₂ HPO ₄ , 40 mM Na ₃ HPO ₄ , 10 mM KCl, 1 mM MgSO ₄ , pH 7 |
| 2x L-Buffer | 250 mM Tris/HCl, pH 7.5, 20 % Glycerol, 200 mM NaCl, 2 mM EDTA, 20 mM MgOAc, 2 mM DTT, 1xPI-Mix |
| Bead Buffer | 50 mM Tris/Hcl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM DTT, 1x PI-Mix |
| Dilution Buffer | 60 mM Tris-HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100, 1 mM DTT, 1x PI-Mix |
| Urea-Wash | 50 mM Tris/Hcl, pH 7.4, 2 M Urea, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0.2 % SDS |
| IP Buffer | 50 mM Tris/HCl, pH 7,4, 150 mM NaCl, 5 mM EDTA , 1 % Triton X-100, 0.2 % SDS |
| Low Salt | 150 mM NaCl |
| 4x Laemmli | 12.5 % SDS, 2.5 % Glycerin, 5 % β-Mercaptoethanol, 125 mM Tris/HCl, pH 6.8, Bromphenolblue |
| 5x DNA loading buffer | 40% Ficoll 400, 0.1 M EDTA, 1% SDS, Bromphenolblue |

| | |
|--------------------|--|
| Zymolyase solution | 1 M Sorbitol, 0.1 M Na-Citrat, 60 mM EDTA pH 8.5, 5 mg/ml Zymolyase (Seikagaku) |
| 5x HAT-Buffer | 250 mM Tris/HCl, pH 8.5, 50% Glycerin, 0.5 mM EDTA, 5 mM DTT |
| Lysis Buffer | 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole, 1% Triton X-100, 1 mM PMSF, 1xPI-Mix |
| Wash Buffer | 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 20 mM imidazole, 1% Triton X-100 |
| Elution Buffer | 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.25-1 M imidazole |

| | |
|--------------------------------------|------------|
| BenchMark™ Prestained Protein Ladder | Invitrogen |
| ReadyLoad™ 1 kb DNA ladder | Invitrogen |
| ECL Reagents | Amersham |

2.1.6. Antibodies

| | | |
|--------------------------|----------|------------------------------|
| α -HA | 1:1000 | Covance |
| α -acetyl-lysine | 1:1000 | Upstate |
| α -Sas2 | 1:40.000 | Meijsing + Ehrenhofer-Murray |
| α -myc | 1:5000 | Invitrogen |
| α -His | 1:1000 | Sigma |
| α -mouse-HRP | 1:1000 | Sigma |
| α -rabbit-HRP | 1:3000 | Sigma |
| α -guinea pig-HRP | 1:1000 | Sigma |

2.1.7. Peptides

The Cse4 peptide used in this study was synthesized by Sigma Genosys. It consists of the N-terminal amino acids 112-134 with the following sequence (putative acetylation sites are marked in red):

YVRQKRREKQRKQSLKRVEKKYTPS

1 mg/ml peptide was solubilized in dH₂O and stored at -80°C.

2.1.8. Primer

The primers used in this study were synthesized by Metabion and applied in a dilution of 10 pmol/μl. Sequences derived from the Saccharomyces Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>) were used to design optimal primers with the assistance from the Oligonucleotide Properties Calculator (<http://www.basic.nwu.edu/biotools/oligocalc.html>).

2.2. Methods

2.2.1. Molecular methods

2.2.1.1. Cell cultivation

The cultivation of bacteria with plasmids was carried out in LB-media or on LB-plates at 37°C unless indicated otherwise. For the bacterial expression of 3xHA-Cse4, nutrient rich TY-medium was used. For the maintenance of plasmids the appropriate antibiotics were added to the media (ampicillin, kanamycin, chloramphenicol).

Yeast strains were grown on YPD full media or on YM minimal media at 30°C or 23°C (temperature sensitive mutants). Minimal media were supplemented as appropriate.

2.2.1.2. Transformation of *E. coli* and *S. cerevisiae*

Chemically competent bacterial strains were obtained from Gibco BRL (DH5 α) and Invitrogen (TOP10, BL21 (DE3) Star). The transformation was carried out as suggested by the manufacturer.

For the preparation of competent yeast cells and the transformation of DNA into yeast we used a method described from (Klebe, et al., 1983). Competent yeast cells were used immediately or were stored after the addition of DMSO to 5.5% at -80°C.

2.2.1.3. DNA isolation

The isolation of plasmids from bacteria was performed with the Mini- or Midi-Kit from QIAGEN according to the manufacturers instruction.

Genomic yeast DNA was isolated as described from (Hoffman and Winston, 1987). For PCR (*polymerase chain reaction*) mediated analysis of gene knock-outs in yeast, a single yeast colony was heated for one minute in a microwave. The PCR mix was added subsequently and the DNA amplified in a thermocycler.

2.2.1.4. Plasmid constructions

The epitope tagged *HA-CSE4* plasmid was constructed by inserting three *HA*-tags into the *XbaI*-site of *CSE4* analogous to (Stoler, et al., 1995). For the insertion of the N- and C-terminal *CSE4* fragments into the pACT2 and pBTM117c vector the amino acids 11-139 and the amino acids 137-229 were PCR- amplified with *CSE4*-primers containing specific restriction sites and subcloned into pCR®BluntII-TOPO (Invitrogen). For insertion into pACT2, the N-terminus was excised with *PstI/SpeI* and the C-terminus with *SacI/PstI*. The overlapping ends were filled in with T4 DNA-polymerase and the fragments were cloned into *SmaI*-linearized pACT2. To obtain the N-/C-terminal *CSE4* in the LexA-vector, the fragments were excised with *AccI/NotI* and inserted into *AccI/NotI*-linearized pBTM117c.

2.2.1.5. *S. cerevisiae* strain construction

To generate yeast strains with a specific genotype, the appropriate strains were crossed, sporulated and the resulting tetrads were dissected. For this purpose, small amounts of the yeast strains to be crossed were mixed in dH₂O and were allowed to mate for ≥ 8 h. To select for diploid cells, the cells were then transferred onto supplemented YM and grown at 30°C. After two days, diploids were restreaked onto YPD before they were transferred to sporulation plates and incubated at 30°C for 3-4 days. The cell wall of the sporulated strain was digested with zymolyase solution for 10 minutes at room temperature; the reaction was stopped by the addition of 160 μ l dH₂O. The spores from the asci were separated under the microscope (Zeiss Axioskop FS) with a micromanipulator (Narishige) and analyzed for their genotype.

For specific gene knock-outs, we took advantage of homologous recombination. In most cases the *kanMX*-system was used, which lead after successful homologous recombination to geneticin (G418) resistance in *S. cerevisiae*.

The basic principle of the system is to amplify the *kanMX*-gene from pF6a-*kanMX4* (pAE478) with homologous sequences at the ends from the gene to be knocked out via PCR. The PCR product is then transformed into competent yeast cells, which are plated onto G418 plates (YPD + 200 mg/l G418). After 2-3 days at 23°C/30°C, the grown colonies are analyzed with PCR.

2.2.1.6. Polymerase chain reaction

Standard conditions:

| | | |
|-----------------|------------|---|
| 1. Denaturation | 7 min | 93°C |
| 2. Denaturation | 1 min | 93°C |
| 3. Annealing | 30 sec | depending on primers, in general with formula $72.4 + (0,41 * \% GC) - 650/\text{length}$ |
| 4. Extention | 1 min/ 1kb | 72°C |
| 5. Extension | 10 min | 72°C |
| 6. Cool down | ∞ | 4°C |

PCR for subsequent cloning: repeat step 2-4 for 20-25 cycles

Control PCR: repeat step 2-4 for 25-30 cycles

2.2.1.7. DNA sequencing

Sequencing of DNA templates was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit. For this purpose, 0.5-1 µl DNA was mixed with 4 µl Terminator Ready Reaction Mix, 1 µl Primer and dH₂O and amplified in the thermocycler.

PCR conditions for 25 cycles were:

| | | |
|---------------|--------|---------|
| Denaturation: | 10 sec | 96°C |
| Annealing: | 5 sec | 50-60°C |
| Extension: | 4 min | 60°C |
| Cool down | ∞ | 4°C |

After amplification, the DNA was precipitated with Na-acetate/EtOH, dried and analyzed in the sequencing department of the MPIMG.

2.2.1.8. Two-hybrid system

The Two-hybrid system can be used to detect protein-protein interactions in yeast. The method is based on the *GAL4* gene from *Saccharomyces cerevisiae*, whose gene product activates several genes from the galactose synthesis pathway. For their activation, Gal4p binds with its DNA-binding domain (DNA-BD) to sequences upstream of the genes (UAS).

Additionally, Gal4p consists of an activation domain (AD), which binds via proteins to the DNA-binding domain and leads to transcription of the reporter genes (*lacZ*, *HIS3*). If the two domains remain separated from each other, the transcription from the reporter genes is blocked.

For our experiments we used the pACT2-vector with the activation domain and pBTM117c with the DNA-BD. pBTM117c contains instead of the Gal4 DNA-BD LexA, a bacterial protein that normally binds to *lexA* promoters. The advantage of LexA based two-hybrid is that less false positive interactions occur and that weak interactions are effectively amplified due to multiple LexA operators. The proteins to be tested were cloned into the vectors and both plasmids were cotransformed into the two-hybrid strain L40c. Protein-protein interactions were monitored with the β -galactosidase filter assay and the expression of *HIS3*.

2.2.1.8.1. β -galactosidase filter assay

Upon activation of the reporter gene *lacZ*, the enzyme β -galactosidase is expressed in the cells. In this assay, the enzyme uses the colourless X-Gal (5-Brom-4-chlor-3-indolyl- β -D-galactopyranoside) as a substrate instead of galactose, and cleaves it into the dark blue 5-brom-4-chlor-indigo. For this purpose the yeast cells were transferred onto a nitrocellulose filter and broken in liquid nitrogen. The filter was incubated with 2,5 ml buffer Z and 50 μ l X-gal for up to 2 h at 30°C. During this time course, a positive interaction between the proteins should lead to a blue coloration.

2.2.1.8.2. *HIS3* reporter assay

The reporter gene expression was also tested with the *HIS3* reporter assay. The two-hybrid strain with the appropriate plasmids was streaked onto media lacking histidine. L40c itself is auxotroph for histidine, but due to the interaction of the proteins to be tested, *HIS3* gene expression is activated, and the strain can grow in the absence of histidine.

2.2.1.9. FACS – fluorescent activating cell sorting

To perform FACS analysis, yeast cells were cultured in an appropriate volume (~3 ml) until they reached $OD_{600}=0.05-0.1$. After brief centrifugation and washing, cells were fixed in 70% EtOH at 4°C over night. Cells were then incubated for 4 h in 20x TE + 1 μ g/ml RNase A, washed twice in PBS and subsequently stained over night in PBS + 100 μ g/ml propidium-iodide (Sigma). After the suspension was 10x diluted, cell were separated from each other by sonication (3x 5 sec, 60%) and maintained in the dark. FACS analysis was performed with a flow cytometer (FACSCalibur) at the Deutsche Rheumaforschungszentrum in Berlin.

2.2.2. Biochemical methods

2.2.2.1. Protein extract preparation

Protein extract preparation from bacteria

After reaching the desired optical density (OD), the bacterial culture was centrifuged for 10 minutes at 5000 rpm and the pellet resuspended in column buffer. The cells were broken with ultrasound (4 x 1 minute, 60 %) or with the french press. To obtain the protein extract, the broken cells were centrifuged for 30 minutes at 20.000 rpm and the supernatant was frozen at -80°C.

Protein extract preparation from yeast

Yeast cell cultures were grown to an $OD_{600} = 0,8-1$ and harvested by centrifugation for 20 minutes at 5000 rpm at 4°C. After washing the cells with dH₂O, the cell pellet was resuspended in the appropriate buffer.

Native whole cell extracts from yeast cultures <200 ml were prepared in 2x buffer L by glass bead lysis as described by (Moazed and Johnson, 1996), except that the concentration of NaCl was adjusted to 200 mM. For denatured protein extracts, the cells were lysed in Bead Buffer, boiled for 10 minutes at 95°C and diluted in IP dilution buffer for subsequent immunoprecipitation.

Larger cell cultures were resuspended in 2x buffer L or bead buffer, respectively, and the cells were broken using a french press. After 1 h centrifugation at 40.000 rpm, protein extracts were analyzed or frozen at -80°C for further use.

2.2.2.2. SDS-PAGE and immunoblotting

Proteins were analyzed according to their molecular weight with 8/10/12% SDS-PAGE gels. The transfer from the gel to a nitrocellulose membrane was performed either at 0,8 A/cm² for 1 h with a Semi-Dry Blot from BIO-RAD, or at 110 V for 45 minutes with a wet blot from BIO-RAD. The efficiency of the transfer was visualized with Ponceau S dye. Subsequently, the membrane was blocked for 1 h with 5% fat free milk/TBST and incubated with the primary antibody in 5% fat free milk/TBST at 4°C overnight. After washing the membrane four times with TBST, the secondary HRP-conjugated antibody was added for 30 minutes. The membrane was washed with TBST for six times and proteins were then detected with ECL-solution from Amersham.

2.2.2.3. Detection methods for proteins

To detect proteins directly in SDS-PAGE gels, the gel was stained for 1 h with Coomassie Brilliant Blue R250. Subsequently, the gel was destained with 25% methanol/10% acetic acid, such that the staining of the proteins was maintained.

If small protein concentrations (~0,1 ng/mm²) were to be detected, we used the Silver Stain Kit from Bio-Rad. The staining of the proteins in an SDS-PAGE gel was performed according to the manufacturer's instructions. Protein concentrations in solutions were determined with the Bradford Assay (Bio-Rad Protein Assay Kit).

2.2.2.4. Concentration of protein solutions

To concentrate proteins in a smaller volume, Centricon-columns (amicon) with an exclusion matrix of 10 kD were used. After one hour centrifugation at 5000 g, protein solutions were concentrated from 2 ml to approximately 50-200 µl. If

necessary, an additional buffer change was performed with three washing steps in 2 ml buffer.

2.2.2.5. Solo- and Co-immunoprecipitation

Immunoprecipitation experiments were performed to isolate proteins and protein complexes. If the immunoprecipitation was carried out under denaturing conditions, the protein extract was boiled before use for 10 minutes at 95°C. The appropriate antibody was added to the whole cell protein extract and incubated for one hour with shaking at 4°C. Subsequently, protein G sepharose beads (Pharmacia) were added and the lysate-antibody-protein G mix was incubated over night.

Immunoprecipitates from native extracts were washed four times with 1x buffer L and resuspended in SDS sample buffer; precipitates from denatured extracts were washed with Urea wash buffer, IP buffer and detergent free wash buffer before resuspension in SDS sample buffer. After boiling for 10 min at 70°C and centrifugation, the immunoprecipitates were analyzed by immunoblotting.

2.2.2.6. Bacterial expression of Cse4

For bacterial expression of large amounts of Cse4, N-terminal His-tagged Cse4 was generated by inserting *CSE4* into the *XhoI/BamHI*-site of pET15b and transforming the resulting plasmid (pAE994) into BL21(DE3) Star cells. The expression of His-Cse4 in 2 l LB-culture was induced by adding 1 mM IPTG to the medium at $OD_{600}=0.8$ and subsequent growth for additional 2h at 37°C. The cells were harvested and proteins extracted in Lysis Buffer by sonication (3x 20 sec, 40-50%). After centrifugation, the supernatant was added to 2 ml 50% Ni-NTA matrix and incubated with rotation for 1 h at 4°C. Proteins that did not bind the to matrix were washed off with 15 ml Wash Buffer.

The bound His-Cse4 was eluted by adding 8x 500 µl Elution Buffer with 250 mM imidazole, 1x with 350 mM imidazole and 1x with 1 M imidazole to the matrix. The samples were dialyzed against water, concentrated and further analyzed for His-Cse4 by immunoblotting.

2.2.2.7. Acetylation assay

To investigate the acetylation of proteins, we took advantage of an *in vitro* acetylation assay. In a total volume of 25 µl we mixed 2 µg recombinant histone H4 and/or 2 µg His-Cse4 together with the enzyme (200 µg rSAS-I or 500 µg recombinant PCAF), 5xHAT-Buffer and 0,5 µl [¹⁴C] acetyl-CoA (50-62 mCi/mmol Amersham). After one hour incubation at 30°C, the mix was run on a 15 % SDS-PAGE gel. The gel was dried in a gel dryer (BioRad) for 1 hour at 80°C, and the acetylated proteins were detected after over night exposure with a phosphoimager.

3. Results

3.1. Interactions between Cse4, SAS-I and chromatin assembly factors

Sas2 belongs to the MYST family of HATs and acetylates lysine 16 on histone H4 (Meijsing and Ehrenhofer-Murray, 2001; Suka, et al., 2002) and lysine 14 on histone H3 (Sutton, et al., 2003). To identify proteins that interact with Sas2, we performed a two-hybrid screen and identified a fragment of the histone H3 variant Cse4 (Table 1).

| GAD- LexA- | Cse4 | Sas2 | Sas4 | Cac1 | Asf1 | - |
|-----------------------|------|------|------|------|------|---|
| Cse4 | + | + | + | + | + | - |
| Cse4 (aa11-139) | n.d. | + | + | + | n.d. | - |
| Cse4 (aa137-229) | n.d. | - | - | - | n.d. | - |
| Sas2 | + | + | + | + | + | - |
| Sas2-HAT ⁻ | - | n.d. | - | - | n.d. | - |
| Sas2-Zn ⁻ | - | n.d. | - | - | n.d. | - |
| Sas4 | + | + | + | + | + | - |
| Sas5 | - | - | + | + | + | - |
| - | - | - | - | - | - | - |

Table 1: Two-hybrid interactions between the histone H3 variant Cse4, the SAS-I complex and chromatin assembly factors. The reporter strain L40c was cotransformed with a plasmid expressing Cse4 and a plasmid containing components of the SAS-I complex or chromatin assembly factors. The genes were fused either to a Gal4 activation domain (GAD) or to a DNA binding domain (LexA). Interactions between proteins were measured as expression of lacZ and HIS3. Positive interactions (+) became blue in a β -galactosidase filter assay within 2 h and were able to grow on minimal media lacking histidine, negative interactions (-) did not become blue and were unable to grow on media lacking histidine; n.d. = not determined.

The interaction between Cse4 and Sas2 was further narrowed down to the N-terminus (amino acids 11-139), whereas the C-terminal region (amino acids 137-229) showed no interaction with Sas2. Since the C-terminal region is highly similar to the globular domain of H3, this suggested that Sas2 interacted with a portion of Cse4 that lies outside of the centromeric nucleosome core.

In order to test whether Cse4 and Sas2 also interact *in vivo*, we performed co-immunoprecipitation experiments (Fig. 7A). We constructed a 3x-HA-tagged version of *CSE4* that was fully functional in that it rescued the lethality of a *CSE4* deletion (data not shown). In an immunoprecipitation with a Sas2-antibody, we were able to detect HA-Cse4, whereas the antibody did not precipitate HA-Cse4 in the absence of Sas2.

Since Sas2 exists in the SAS-I complex together with Sas4 and Sas5 (Meijsing and Ehrenhofer-Murray, 2001), we asked whether Cse4 was able to interact with Sas4 and Sas5. Whereas Cse4 interacted via its N-terminus also with Sas4, we were unable to find two-hybrid interactions between Cse4 and Sas5. The binding of Cse4 to Sas4 *in vivo* was confirmed by co-immunoprecipitation. Interestingly, Sas5 could be precipitated in this assay (Fig. 7A). Thus, we suggest that Cse4 and Sas5 did not interact directly with each other, but that they can be found in the same complex *in vivo*. Possible mediators between these two proteins are Sas2 or Sas4.

As a member of the MYST family of HATs, Sas2 contains an acetyl-CoA-binding site (HAT) and an atypical zinc-finger (C2HC motif), which is necessary for substrate recognition in the Sas2 homologue MOF in *D. melanogaster* (Clarke, et al., 1999). We tested the relevance of these motifs for the interaction with Cse4 (Table 1, Figure 7B). Both a mutation in the atypical zinc-finger (C106L) and in the acetyl-CoA-binding site (P213A/P214V) resulted in a loss of the Cse4-Sas2 two-hybrid interaction, showing that both motifs were essential for Sas2 binding to Cse4.

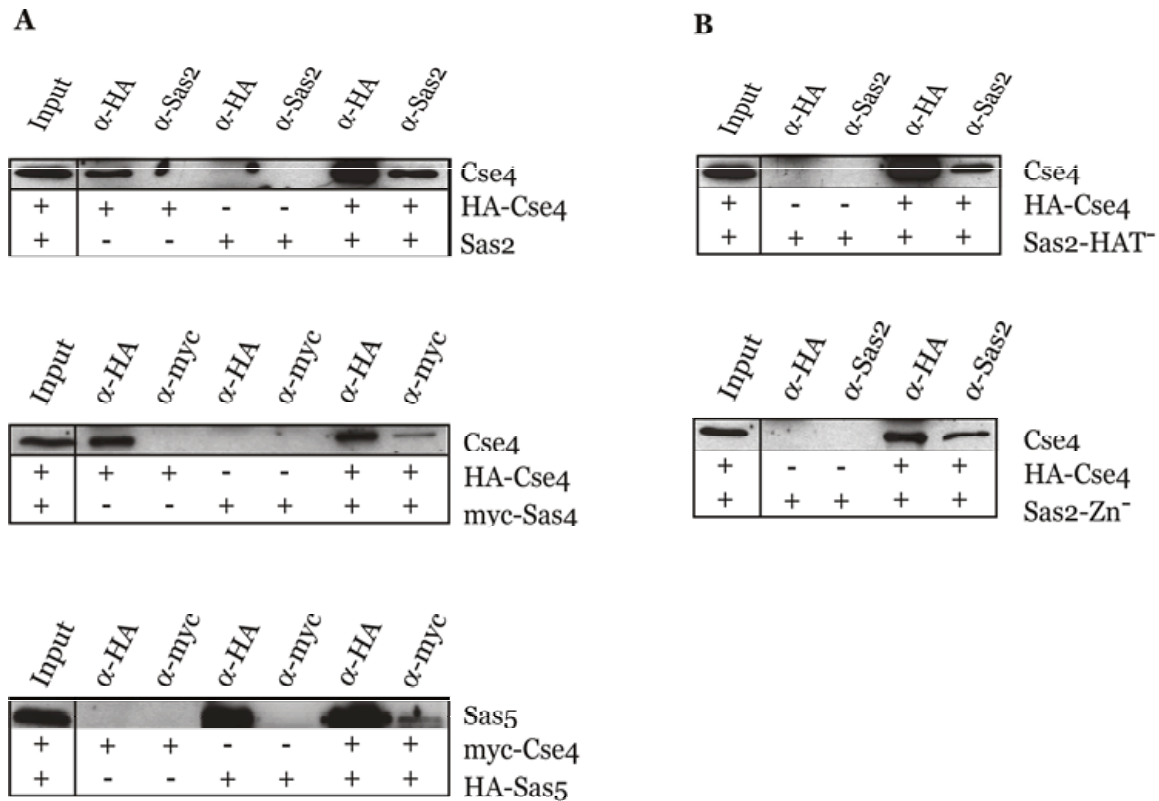


Fig. 7: Interactions between Cse4 and the SAS-I complex. The antibodies for precipitation are indicated above the panel. (A) Cse4 immunoprecipitated with Sas2, myc-Sas4 and myc-Sas5. (Top) The protease deficient yeast strain AEY1558 was transformed with plasmids containing 3x-HA-CSE4 (pAE977) and SAS2 (pAE90). Precipitates were detected with α -HA-antibody. (Middle) For immunoprecipitation between 3x-HA-Cse4 (pAE977) and 6x-myc-Sas4 (pAE613) AEY2461 was used. Precipitates were analyzed with α -HA-antibody. (Bottom) Sas5 immunoprecipitated with Cse4. AEY1558 was cotransformed with plasmids containing myc-CSE4 (pAE975) and HA-SAS5 (pAE625). Detection of proteins was performed with α -HA-antibody. (B) Cse4 immunoprecipitated with Sas2 variants, carrying single point mutations in the acetyl-CoA-binding site (HAT⁻, P213A/P214V) or in the zink-finger (Zn⁻, C106L). For this purpose, 3x-HA-Cse4 (pAE976/pAE977) and Sas2-Zn⁻ (pAE388) or Sas2-HAT⁻ (pAE249), respectively, were introduced into AEY1559. For detection in precipitates α -HA-antibody was used.

However, the Sas2 mutants still co-immunoprecipitated with Cse4 (Fig. 7B), suggesting that the Cse4-Sas2 interaction was stabilized *in vivo* by other proteins. Alternatively, the folding of Sas2 in the two-hybrid may be partially compromised.

The SAS-I complex interacts with Cac1, the largest subunit of CAF-I, as well as with the nucleosome assembly factor Asf1 (Meijsing and Ehrenhofer-Murray, 2001). Since SAS-I interacted with Cse4, we next sought to determine whether Cac1 and Asf1 interacted with Cse4. With both two-hybrid and co-immunoprecipitation, we could demonstrate that Cse4 interacted with Cac1 (Table 1, Fig. 8A), where the interacting region of Cse4 could further be narrowed down to the N-terminus, and Asf1 (Table 1, Fig. 8B).

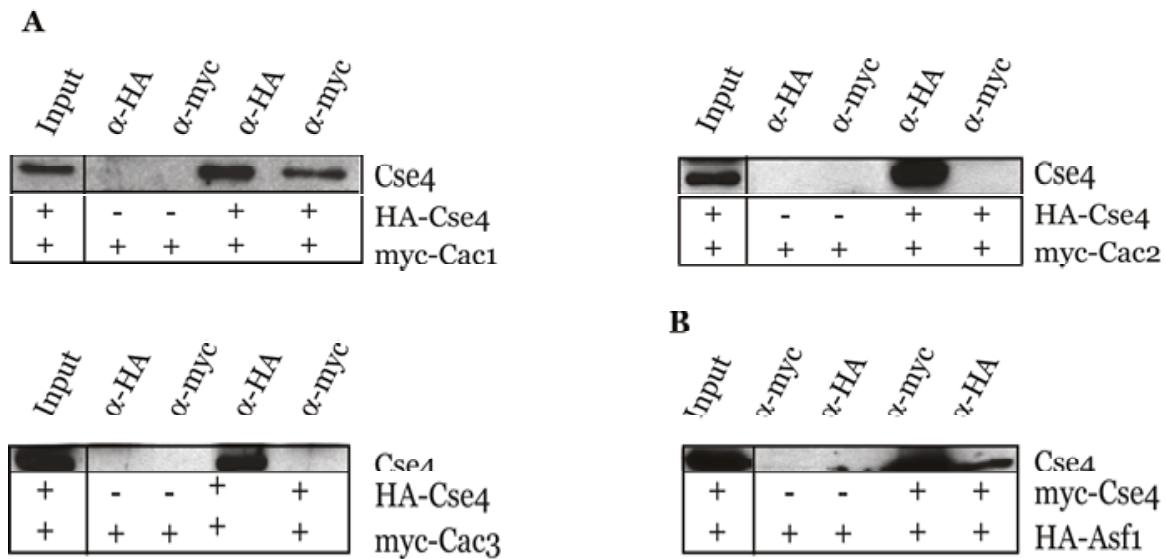


Fig. 8: Interactions between Cse4 and chromatin assembly factors. The antibodies for precipitation are indicated above the panel. (A) Cse4 immunoprecipitated with Cac1, but not with the other two subunits of the CAF-I complex, Cac2 and Cac3. (Top Left). AEY1808 was cotransformed with plasmids containing 3x-HA-CSE4 (pAE976) and 6x-myc-CAC1 (pAE614). Precipitation was carried out with α -HA-antibody. (Top Right) No interaction was detected between 3x-HA-Cse4 (pAE977) and 6x-myc-Cac2 in AEY1558. Detection of precipitates was performed with α -HA-antibody. (Bottom Left) 3x-HA-Cse4 (pAE977) did not interact with 6x-myc-Cac3 in AEY1558 *cac1* Δ . Precipitates were analyzed with α -HA-antibody. (B) Cse4 interacted with Asf1 in vivo. Therefore AEY2493 with genomic 3xHA-tagged Asf1 was transformed with 6x-myc-CSE4 (pAE901). For detection of precipitated Cse4 α -myc-antibody was used.

As Cac1 exists in the CAF-I complex together with Cac2 and Cac3 (Kaufman, et al., 1997), we asked whether Cse4 interacted with the whole CAF-I complex or with Cac1 alone. By co-immunoprecipitation, we could exclude the possibility that Cse4 existed in a complex with Cac2 or Cac3 as no precipitation could be detected. Recently, (Sharp, et al., 2002) reported a function for CAF-I and Hir1 outside of S-phase at the centromere. Additionally, they found a genetic interaction between Cse4 and Cac1, as *cac1Δ hir1Δ cse4-107* cells were unable to grow at semipermissive temperatures. Besides, Cac1 and Cac2 colocalized at the centromere, which would support our finding that the centromeric H3 variant Cse4 and Cac1 directly interact with each other.

We found that the chromatin assembly factor Asf1 was also able to interact with Cse4. This interaction might be important at earlier steps in the assembly pathway, e.g. by delivering Cse4 to CAF-I, which in turn incorporates Cse4 at the centromere. As Cse4 is able to interact with subunits of the Asf1 and CAF-I histone deposition factors, there may exist different steps or mechanisms in assembling Cse4 into chromatin. In contrast to *cacΔ hirΔ* cells, *cacΔ asf1Δ* cells showed a G2/M delay independent of spindle assembly checkpoint and segregated a reporter minichromosome at wildtype frequencies. This result indicates, that the centromere needs Hir1, but not Asf1 in the absence of CAF-I function, so that the *HIR/ASF1* chromatin assembly pathway might be bifurcated at some point.

Since Cse4 interacted with Cac1 as well as with SAS-I, we next sought to determine the dependence of these interactions on SAS-I components and Cac1, respectively. As can be seen in Figure 9A, a deletion of *SAS2* did not disturb the interaction between Cse4 and Sas4 or Cac1, respectively. This suggested that the interaction between Cse4 and Sas4 or Cac1 was stable and that the presence of Sas2 was not necessary for maintaining the association between Cse4, Sas4 and Cac1.

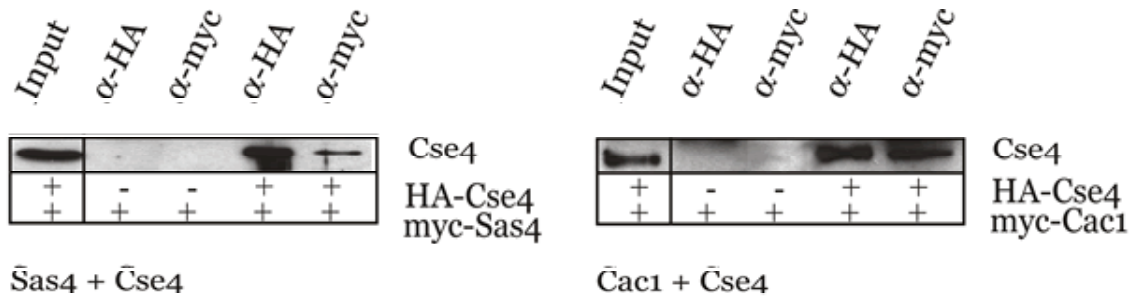
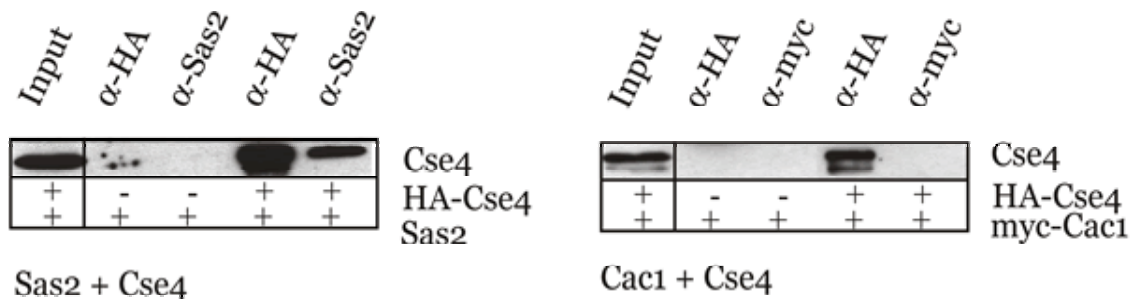
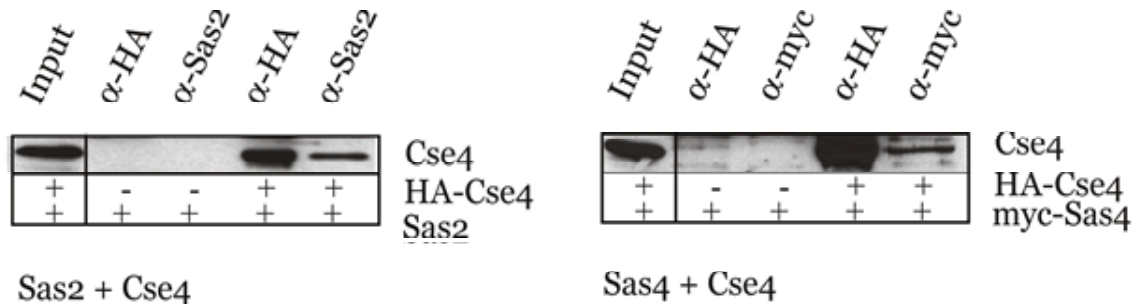
A *sas2* Δ **B** *sas4* Δ **C** *cac1* Δ 

Fig. 9: Consequences of different mutations on the interaction with Cse4. The antibodies for precipitation are indicated above the panel. (A) The interaction between HA-Cse4 (pAE977) and myc-Sas4 (pAE613) and myc-Cac1 (pAE614), respectively, in a *sas2* Δ strain (AEY1559). Cse4 was precipitated with α -myc-antibody and precipitates were immunoblotted using α -HA-antibody. (B) HA-Cse4 (pAE977) and Sas2 (pAE90) were still able to interact with each other in a *sas4* Δ strain (AEY2461), whereas the interaction between HA-Cse4 and myc-Cac1 (pAE614) was disrupted. The detection was carried out with α -HA-antibody. (C) The chromatin assembly factor is not necessary for the interaction between HA-Cse4 and Sas2 (pAE90) or myc-Sas4 (pAE613), respectively. The interaction was tested in a *cac1* Δ strain (AEY1808). HA-Cse4 was precipitated with α -Sas2 or α -myc, immunoblotting was performed with α -HA-antibody.

In contrast to a *sas2* Δ , a deletion of *SAS4* had consequences on the integrity of the complex (Fig. 9B). Whereas Cse4 and Sas2 were still able to interact with each other in a *sas4* Δ strain, the association of Cse4 with Cac1 was disrupted. A deletion of *SAS4* leads to a disruption of the SAS-I complex, because Sas2 and Sas5 are unable to interact with each other (Meijsing and Ehrenhofer-Murray, 2001). Thus, we hypothesize that either Sas4 or Sas5 were essential for maintaining the interaction between Cse4 and Cac1.

In contrast, the chromatin assembly factor Cac1 was not essential to maintain the interaction between Cse4 and the SAS-I complex (Fig. 9C). Sas2 as well as Sas4 were still associated with Cse4 in a *cac1* Δ strain, showing that the interaction between Cse4 and SAS-I was not affected by the presence or absence of Cac1.

3.2. Effect of mutations in SAS-I, CAF-I and Asf1 on centromere function

The results from the two-hybrid assay and the co-immunoprecipitation experiments indicated, that Sas2 might indeed have a role at the centromere. As Sas2 is a histone acetyltransferase that acetylates histone H3 K14 and histone H4 K16, we hypothesized that the histone acetylation might be important for the centromere as well. One possibility is that Sas2 interacts with the histone H3 variant Cse4 because it is an additional acetylation target. We next asked, if a *sas2* deletion, and therefore a missing acetylation, had any effect in combination with *cse4* mutations at the centromere. As a *CSE4* deletion is lethal, we used a temperature sensitive mutant for the subsequent experiment.

The *cse4-103* strain, which has two amino acid exchanges in the histone-fold-domain (I156V, L193Q), was generated by random mutagenesis. This temperature sensitivity is due to the weakened interaction between *cse4-103* and histone H4, because the L193Q substitution is predicted to disrupt the Cse4-H4 interface (Glowczewski, et al., 2000). We inserted both *cac1* and *sas2* single deletions as well as *cac1 sas2* double deletions into the *cse4-103*-strain and investigated the temperature sensitivity at 23°C, 34°C and 37°C. At the permissive temperature all strains grew equally well, whereas at 37°C all *cse4-103* mutants were unable to survive. At 34°C, the *cse4-103* mutant strain grew poorly compared to wildtype (Fig. 10).

When *SAS2* was deleted, the *cse4-103* strain grew better at 34°C as compared to the single *cse4-103* mutation. Perhaps a missing acetylation on histones H3, H4 or Cse4 by Sas2 stabilizes the structure and function of the centromere. As Cse4 together with histone H4 forms stable (Cse4-H4)₂ tetrameres, one possible explanation is that the target of acetylation was histone H4, because the acetylation of free histone H4 at lysine 16 has previously been described (Sutton, et al., 2003). One hypothesis is that the missing acetylation on histone H4 lysine

16 in *sas2* Δ cells forms a more compact chromatin structure at the centromere, comparable to heterochromatin at silenced regions.

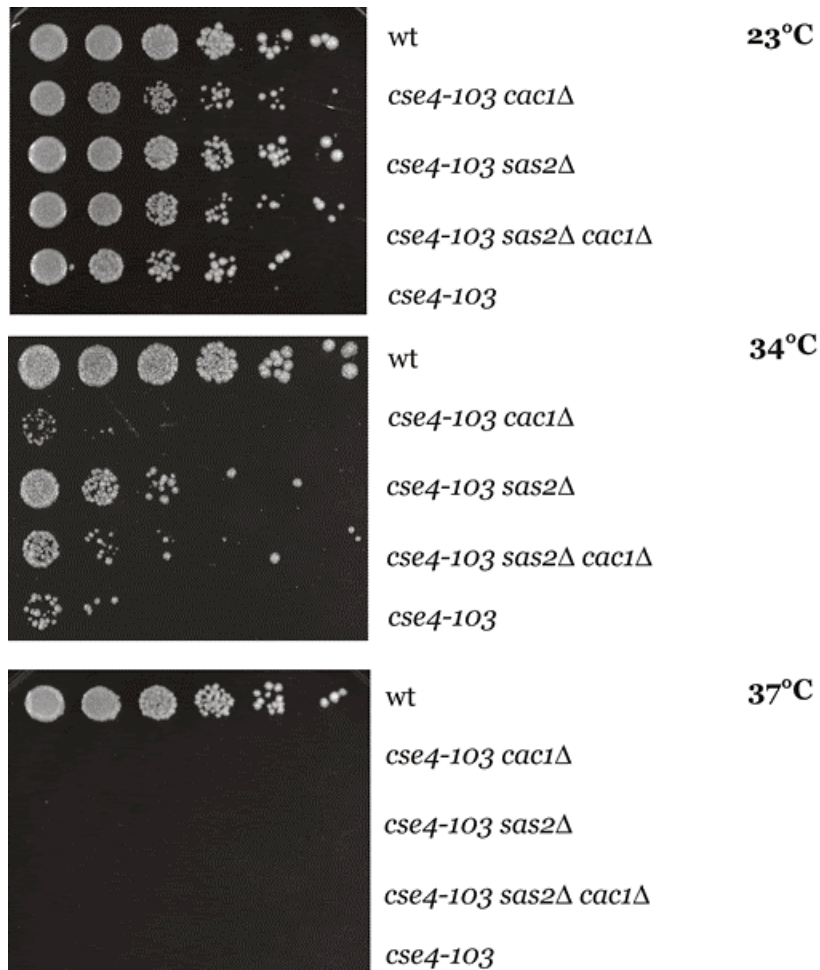


Fig. 10: Partial suppression of the *cse4-103* temperature sensitivity by *sas2* Δ . The strains were spotted in serial dilutions onto selective media and grown for 2 days at 34°C. Strains used in this assay were AEY1194 (wildtype, wt), AEY1781 (*cse4-103 sas2* Δ), AEY1162 (*cse4-103*), AEY2373 (*cse4-103 cac1* Δ) and AEY2374 (*cse4-103 sas2* Δ *cac1* Δ).

Thus, the *cse4-103 sas2* Δ strain is still temperature sensitive, but not to the same extent as the single *cse4-103* mutation. Another possibility with a similar result would be if Cse4 also was a target for acetylation by Sas2.

A deletion of *CAC1* in *cse4-103* or *cse4-103 sas2Δ* strains did not lead to a change in temperature sensitivity. Interestingly, in *cse4-103 sas2Δ cac1Δ* strains, growth at 34°C was not improved as in *cse4-103 sas2Δ* strains, but was comparable to the single *cse4-103* mutation. (Sharp, et al., 2002) described a function for the chromatin assembly factor Cac1 and Hir1 at the centromere. In *cac1Δ* cells, the distribution of Cse4 was disturbed: additionally to the centromere, extra-centromeric localization of Cse4 could be observed. This could also be the reason for our observation that the temperature-sensitivity was not improved when *CAC1* as well as *SAS2* were deleted in the *cse4-103* strain. Taken together, Cac1 might be important for the integrity and stability of the centromere in *cse4* mutated strains.

Drugs like benomyl or nocodazole affect the correct formation of microtubuli. When a yeast strain has an additional mutation in a gene that is important for the correct formation of the centromere-kinetochore complex, it becomes sensitive and is unable to grow on plates with benomyl or nocodazole. We investigated if a deletion of *ASF1* or components of the SAS-I and CAF-I complex were benomyl or nocodazole sensitive, which might point to a role during centromere-kinetochore formation. None of the investigated deletions resulted in an increased or decreased benomyl sensitivity. We deduced from our data that all examined genes were dispensable for the correct formation of the centromere-kinetochore complex.

(Sharp, et al., 2003) found that a deletion of *SIR1* lead to a greater resistance to the microtubule-depolymerizing drug benomyl in a point mutated *cse4-107* background. *cse4-107* contains a single point mutation at L175F, and is predicted to be in close association with the histone fold domain of histone H4 (Glowczewski, et al., 2000). We were now interested whether a *SAS2* deletion also had an effect on benomyl sensitivity in combination with a *cse4-103* mutation, which is also thought to disrupt binding to histone H4. However, benomyl sensitivity was neither increased nor decreased. In light of our results

we concluded that the deletion of *SAS2* did not cause such a dramatic effect on centromere-kinetochore assembly such that it could be monitored with microtubule-depolymerizing drugs.

Next to components of the SAS-I complex, *Cse4* additionally interacted in co-immunoprecipitation experiments with chromatin assembly factors. Chromatin assembly factors like CAF-I and Asf1, but also associated proteins like Hir1 have distinct roles in the cell cycle. Apart from their functions in heterochromatic gene silencing, they also display various cell cycle dependent phenotypes.

A single deletion of *cac1* or *hir1* does not have any effects on cell cycle progression as can be seen in their FACS profiles or in assays monitoring chromosome loss (Sharp, et al., 2002). However, if yeast cells have an *asf1* deletion or a *cac1 hir1* double deletion, respectively, are delayed in G2-M phase in the cell cycle (Sharp, et al., 2002; Tyler, et al., 1999) and have an increased rate of chromosome loss compared to wildtype cells (Le, et al., 1997; Sharp, et al., 2002).

We were interested in examining the effect of a *sas2* Δ on cell cycle progression. In order to investigate if a deletion causes a delay in the cell cycle, we performed FACS analysis with wildtype, *cac1* Δ , *hir1* Δ , *sas2* Δ , *asf1* Δ , *cac1* Δ *hir1* Δ , *hir1* Δ *sas2* Δ , and *asf1* Δ *sas2* Δ cells. As expected, *cac1* Δ *hir1* Δ as well as *asf1* Δ cells displayed a G2-M arrest in the cell cycle. In contrast, neither a single *sas2* deletion nor a *sas2 hir1* double deletion caused any effect. When Sas2 and Asf1 were both missing, the cells displayed the same phenotypes as an *asf1* deletion alone (data not shown).

Another method to investigate defects within the cell cycle is to monitor loss of plasmids or chromosomes. (Stoler, et al., 1995) constructed a strain where chromosome loss could be monitored. This strain is disomic for chromosome III and contains several markers on the additional *cen 130-3* mutated chromosome. Loss of the original or the additional chromosome III could be followed by

mating assay (*MATa/MAT α*), FOA resistance (*URA3/ura3-52*) and the colour of cells (*SUP11/ade2-101*). We inserted several deletions in SB230 in order to analyze if single (*sas2 Δ* , *cac1 Δ* , *hir1 Δ* , *asf1 Δ*) or double deletions (*sas2 Δ hir1 Δ* , *sas2 Δ asf1 Δ* , *sas2 Δ cac1 Δ*) of genes involved in histone acetylation and chromatin assembly lead to elevated chromosome missegregation and chromosome loss. None of the analyzed mutated strains resulted in elevated chromosome loss compared to wildtype, except for *asf1 Δ* and *asf1 Δ sas2 Δ* where the loss rate of the additional chromosome III was comparable.

Another method to investigate correct segregation is to monitor plasmid loss. In order to do so, we transformed *sas2 Δ* , *hir1 Δ* , *cac1 Δ* single, double and triple mutated strains with a plasmid carrying *SUP11* as a marker (pUN90). We were able to verify the elevated chromosomal instability in *cac1 Δ hir1 Δ* cells that has already been reported by (Sharp, et al., 2002). A single *SAS2* deletion or *sas2 Δ* in combination with *cac1 Δ* and *hir1 Δ* did not cause a further increase in plasmid loss. In summary we concluded that a single or an additional *SAS2* deletion had no detectable influence on cell cycle progression or chromosome and plasmid stability under the conditions tested here.

3.3. A *SAS2*-deletion abrogated the interaction between Cse4 and Ctf19

Ctf19 is a Cse4-interacting protein at the centromere that mediates the connection between CDEI and CDEIII in a complex with Okp1 and Mcm21 (Ortiz, et al., 1999). Ctf19 is nonessential, and mutations result in chromosome missegregation, increased benomyl sensitivity and accumulation in the G2/M phase of the cell cycle (Grienenberger, et al., 2002). Since Ctf19 and Cse4 interact by two-hybrid analysis, we asked whether a *SAS2*-deletion had an effect on their association.

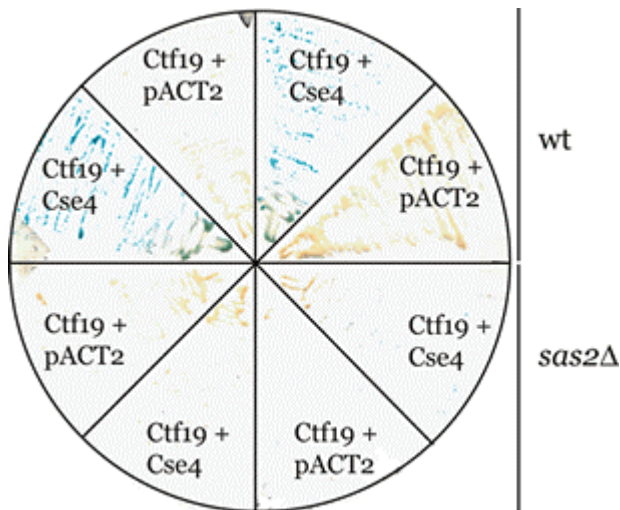


Fig. 11: Two-Hybrid interaction between Cse4 and Ctf19 in wt (L40c) and *sas2Δ* (AEY1695) cells. Yeast strains were plated on YM media and grown at 30°C for 1-2 days. The interaction between Cse4 and Ctf19 was tested with the β -galactosidase filter assay, where positive interactions became blue after incubation at 30°C over night. Picture courtesy of Uta Marchfelder.

In contrast to the wildtype situation, where Ctf19 and Cse4 interacted with each other, no association was found when *SAS2* was deleted (Fig.11). This suggested that Sas2 had a role at the centromere, e.g. by stabilizing the cohesion between the two centromeric proteins Ctf19 and Cse4 via putative acetylation of Cse4.

3.4. Does Sas2 acetylate the histone H3 variant Cse4 ?

As Sas2 is a histone acetyltransferase for histone H4 K16 and histone H3 K14 (Meijsing and Ehrenhofer-Murray, 2001; Osada, et al., 2001; Sutton, et al., 2003) and additionally interacts with the histone H3 variant Cse4, we asked whether Sas2 was also able to acetylate Cse4 *in vivo*. To determine the putative acetylation, we analyzed denatured protein extracts from wildtype and *sas2Δ* strains with α -acetyl-lysine-antibodies. If Sas2 acetylated Cse4, we expected to be able to precipitate Cse4 in the wildtype protein extract but less or none in the *sas2Δ* protein extract. It was important to denature the proteins, because otherwise Cse4 might be precipitated in a complex together with histone H4, which would lead to a signal due to histone H4's acetylation pattern.

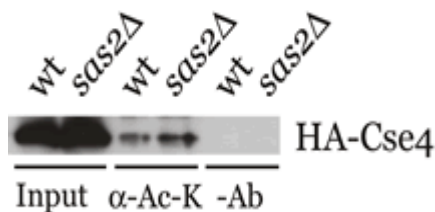


Fig. 12: Immunoprecipitation of HA-Cse4 with α -acetyl-lysine-antibody. HA-Cse4 was isolated with α -HA-antibody from whole cell protein extracts from wildtype (AEY2661) and *sas2Δ* (AEY2666) cells. The concentrated HA-Cse4 was then incubated with α -acetyl-lysine-antibody and detected via immunoblotting with α -HA-antibody.

Furthermore, to reduce the complexity of the α -acetyl-lysine precipitation, we isolated the protein extracts and performed a first immunoprecipitation with α -HA-antibody to isolate Cse4 from the extract. This additional immunoprecipitation step was necessary to partially purify Cse4 for the second immunoprecipitation with α -acetyl-lysine-antibody. After washing off the other proteins, we removed the bound Cse4 with Bead Buffer containing 1 % SDS that was then diluted to 0.1 % SDS for the subsequent second immunoprecipitation

step with α -acetyl-lysine-antibody (Upstate). The precipitated Cse4 was detected in an immunoblot with α -HA-antibody.

As shown in Figure 12, the amount of HA-Cse4 applied was comparable in both wildtype and *sas2* Δ protein extracts (Input). After immunoprecipitation with α -acetyl-lysine-antibody, HA-Cse4 could be detected in wildtype and *sas2* Δ extracts, whereas the control without antibody (-Ab) did not show a HA-Cse4 specific signal. We concluded that an acetylated Cse4 form existed in the cell, although differences in the intensity of the signal from wildtype and *sas2* Δ extracts could not be detected. As the signal varied in independent experiments, we cannot rule out the possibility that Cse4 might only be temporarily acetylated during the cell cycle, so that the acetyl group is removed at some point. Furthermore, an associated histone deacetylase might be co-purified in the assay. In this case, the acetyl-group from acetylated Cse4 could be removed during the purification, so that in consequence we were unable to detect Cse4. Additionally, it might still be possible that after removing the bound HA-Cse4 with 1% SDS from the α -HA-antibody, protein complexes containing Cse4 and histone H4 are restored upon dilution of SDS to 0,1%. In this scenario, the precipitation of HA-Cse4 with the α -acetyl-lysine-antibody may be via acetylated histone H4.

Because it was difficult to determine if Cse4 is acetylated in the cell with immunoprecipitation experiments, we also took advantage of another independent method to determine a putative Cse4 acetylation via Sas2: an *in vitro* acetylation assay. For this purpose, we used the bacterially expressed and purified SAS-I-complex (pAS134, plasmid and purification as described by (Sutton, et al., 2003)) and Cse4. The advantage of this method was that large quantities of purified proteins could be purified from bacteria. On the other hand, co-factors or other modifications that are important for the reaction might be missing.

For the *in vitro* acetylation assay we used the whole purified recombinant SAS-I complex, because (Sutton, et al., 2003) reported that Sas4 and Sas5 are essential and important for Sas2's acetylation activity. The same experiments were performed with the TAP-purified SAS-I complex from yeast (data not shown). As a positive control we applied histone H4 alone or in the same reaction with His-Cse4, so that the functionality of the assay could be monitored.

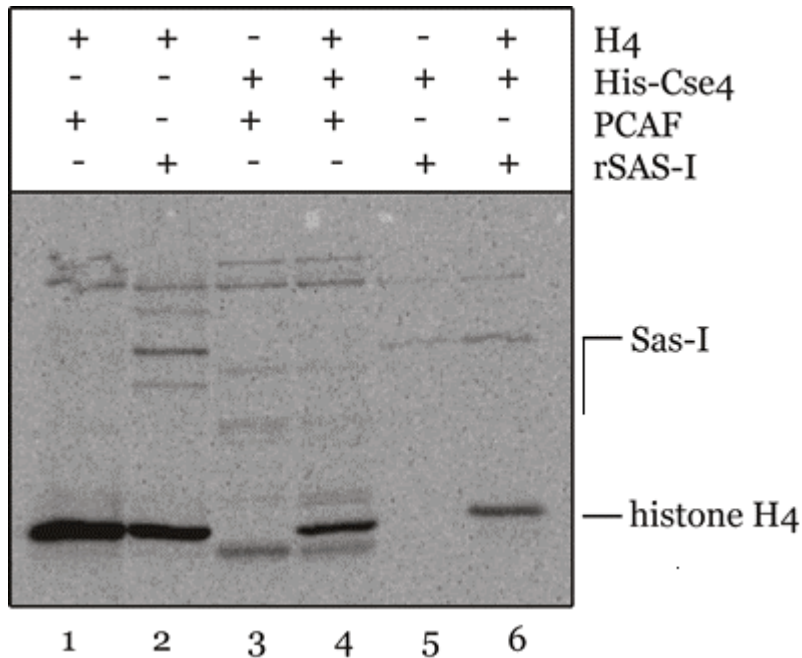


Fig. 13: Acetylation assay with the purified and recombinant His-Cse4 and the SAS-I complex. SAS-I and His-Cse4 were purified from bacteria, whereas histone H4 and PCAF were commercially available. In this assay 2 μ g substrate (histone H4, Cse4) was added to 200 ng SAS-I and 500 ng PCAF, respectively, and mixed together with 0,5 μ g [14 C] acetyl-CoA. After 1 h incubation the samples were loaded onto a 15 % SDS-gel, which was analyzed after the run in a phosphoimager. Figure courtesy of Jacqueline Franke.

As can be seen in Figure 13, PCAF as well as the SAS-I complex were functional and able to acetylate recombinant histone H4 (lane 1+2). Additionally, self-acetylation of components from the SAS-I complex could be detected in lane 2. When His-Cse4 was added to PCAF in the assay (lane3), no Cse4-specific acetylation signal could be detected. The signals obtained in lanes 3 and 4 could not be assigned to Cse4, because they didn't match His-Cse4 in Coomassie blue

stained SDS-PAGE gels and immunoblots using an α -His antibody, respectively. To be sure that the enzymes in this assay were still functional in the presence of His-Cse4, we added His-Cse4 as well as histone H4 in the same reaction and could indeed find acetylated histone H4, although the acetylation signal was weaker than in lane 2 (lane 4). Next to the identified signals from histone H4, additional unidentified signals were obtained (lane 3+4). These signals may result from co-purified proteins that bound to His-Cse4, and were also an acetylation target of PCAF.

The main question of this assay was, whether the SAS-I complex was able to acetylate the histone H3 variant Cse4. Thus, we incubated SAS-I together with purified His-Cse4, but we were unable to detect acetylated His-Cse4 with the applied concentrations, even when self-acetylation of the SAS-I complex could be seen (lane5). The addition of histone H4 to the sample lead to its acetylation, but acetylated His-Cse4 was still not detectable (lane 6).

Additionally to the purified His-Cse4 from bacteria, we used a Cse4 peptide for our *in vitro* acetylation assays. As Cse4 contains several putative acetylation sites (9 lysines in the N-terminus, 7 lysines in the C-terminal histone fold domain), we chose a lysine rich region (aa 112-134 with K115, 119, 122, 126, 130, 131) within the N-terminus for this purpose. As Cse4 interacted via its N-terminus with the acetyltransferase Sas2, we hypothesized to find a putative acetylation site in this region, but we were unable to find a Cse4 specific acetylation signal with SAS-I in the *in vitro* assay (data not shown).

4. Discussion

Cse4 is an essential, evolutionarily conserved core component of the centromere. Homologues have been found in *S. pombe*, *C. elegans*, *D. melanogaster* and mammals. In all organisms, Cse4 and its homologues replace the histone H3 at the centromere by building specific (Cse4-H4)₂ tetrameres.

In this work we were able to demonstrate that the histone H3 variant Cse4 was able to interact with subunits of the SAS-I complex as well as with chromatin assembly factors. All interactions could be shown by two-hybrid analysis and co-immunoprecipitation experiments and occurred mainly over the unique 135 aa N-terminus of Cse4. The absence of single proteins did not result in destabilizing the complex, except for the Cac1-Cse4 interaction in a *sas4Δ*-strain. The biochemical interaction between Cse4 and Sas2 could further be confirmed with genetic interactions. We observed that the temperature sensitivity of the *cse4-103* mutant at 34°C was partially suppressed by an additional *sas2* deletion. Sas2 was also shown to have a direct role at the centromere in that its deletion disrupted the interaction between Cse4 and Ctf19. Additionally, we were able to show that Cse4 existed in an acetylated state in the cell, but if Cse4 was an acetylation target for the acetyltransferase Sas2 could not be verified.

4.1. Cse4 interacts with the SAS-I complex and the chromatin assembly factors Cac1 and Asf1

In this study we were able to show with two-hybrid experiments that the histone H3 homologue Cse4 interacted with the acetyltransferase Sas2. We further narrowed the Sas2-interacting region of Cse4 down a region within the N-terminus (amino acids 11-139). Therefore, we propose that Sas2 interacted with a

portion of Cse4 that lies outside of the centromeric nucleosome core, whereas the essential C-terminal histone-fold domain was dispensable for the interaction.

As Sas2 is a member of the MYST-family of HATs, it contains an acetyl-CoA-binding site (HAT) and an atypical zinc finger (C2HC motif). Both motifs are essential for the acetylation activity of Sas2. We tested if the interaction between Cse4 and Sas2 was still intact when the HAT-domain or the atypical zinc finger, respectively, was mutated. Clearly, the interaction between Cse4 and the point-mutated Sas2 was disturbed, so that we concluded that both motifs were important for the binding of Sas2 to Cse4. As the zinc-finger is absolutely required for substrate recognition in the *Drosophila* Sas2-homologue MOF (Akhtar and Becker, 2001), one way of explanation could be that Cse4 was an acetylation target for Sas2.

Since Sas2 functions in a complex together with Sas4 and Sas5 as a histone acetyltransferase, we hypothesized that Cse4 also binds to Sas4 and Sas5. Indeed we could verify an interaction over the N-terminal tail of Cse4 and Sas4, whereas no binding to Sas5 was detected. From our two-hybrid data we concluded that Cse4 binds via its N-terminus to two subunits of the SAS-I complex, Sas2 and Sas4.

All two-hybrid interactions were further confirmed with co-immunoprecipitation experiments *in vivo*. Interestingly, we were also able to precipitate Sas2-HAT-, Sas2-Zn²⁺- and Sas5 with Cse4, although no direct interaction was detected with the two-hybrid assay. However, as Cse4 interacted with two subunits from the SAS-I complex, Sas2 and Sas4, the interaction with the point mutated Sas2 as well as with Sas5 could be bridged by them. In this case a positive precipitation could be found even if the proteins did not bind directly to each other.

Two other proteins could also be able to bridge these interactions – Cac1, the largest subunit of the chromatin assembly factor CAF-I, and Asf1. CAF-I and Asf1 are histone chaperones that deliver histone H3 and histone H4 to DNA after

replication or repair and function in partially overlapping pathways of nucleosome assembly. Furthermore, Asf1 and Cac2 interact with each other and increase CAF-I activity in nucleosome assembly (Mello, et al., 2002; Tyler, et al., 2001).

Both proteins, Cac1 and Asf1, have been shown to interact with the SAS-I complex (Meijsing and Ehrenhofer-Murray, 2001; Osada, et al., 2001), and we found with two-hybrid and co-immunoprecipitation experiments that they also bind to Cse4. The interaction with Cac1 could further be narrowed down to the amino acids 94-137. Although Cac1 exists in a complex, no interaction could be detected with Cac2 or Cac3, which argues for a function of Cac1 separate of CAF-I. Recently, (Sharp, et al., 2002) reported that CAF-I and Hir1 have a role in maintaining the centromeric chromatin structure. They are not absolutely required for Cse4 deposition, but their absence leads to additional deposition outside of the centromere.

Since Cac1 as well as Asf1 are both chromatin assembly factors that bind histones H3 and H4, our findings together with the results from (Sharp, et al., 2002) support a model where Cac1 and Asf1 were also responsible for delivering the histone H3 variant Cse4 to the centromere. Since CAF-I and Hir1 are not essential for recruiting Cse4 to the centromere (Sharp, et al., 2002), our results could point out an important role for the nucleosome assembly factor Asf1. In Figure 14 all interactions between Cse4, SAS-I and the chromatin assembly factors are summarized.

In our experiments we further found that neither deletion of *sas2*, *sas4* or *cac1* affected the association of Cse4 with components of the SAS-I complex or Cac1. One exception was found in a *sas4*Δ strain, where Cac1 and Cse4 were unable to bind to each other. If *sas4* is deleted, the whole SAS-I complex is disturbed, because Sas5 binds only to Sas4 and not to Sas2. In light of our results, one

interpretation could be that Sas4 and/or Sas5 are important for recruiting Cac1 to the SAS-I complex, and therefore to Cse4.

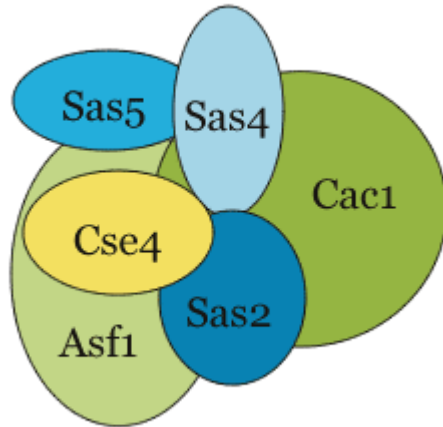


Fig. 14: A model of the histone H3 variant Cse4 and its interactions with the SAS-I complex as well as with a component of the chromatin assembly factors CAF-I and Asf1. A direct interaction is proposed with Sas2, Sas4, Cac1 and Asf1, whereas Sas5 does not bind directly to Cse4.

This model would include that Cac1 is only able to bind to the complex in the presence of the active SAS-I complex. Sas4 has been shown to be essential for histone acetyltransferase activity for Sas2 (Sutton, et al., 2003). If *SAS4* is deleted, the SAS-I complex is both incomplete and inactive. In this case, Sas2 would be unable to acetylate its target, probably the histone H3 homologue Cse4 or histone H4, which would then prevent binding of Cac1 to the complex. This hypothesis would further include that Cac1 is also unable to bind to Sas2, which remains to be tested.

(Meijsing and Ehrenhofer-Murray, 2001) have reported that Sas2, Sas4 and Sas5 coeluted in gel filtration experiments in a peak of ~220 kD, even if the calculated molecular masses of myc-Sas2, myc-Sas4 and Sas5 add up to ~140 kD. Additionally, (Osada, et al., 2001) detected by Superose 6-size exclusion chromatography and a subsequent western blot a 450 kD Sas2 containing complex. This implied that additional subunits of the complex might exist. One

possibility could be that Cse4 interacted with the SAS-I complex and could therefore be a missing component. Consequently, it would be interesting to investigate if a tagged Cse4 could also be found in the elution peak of the SAS-I complex. We tried to solve this question with a TAP-tagged SAS-I complex and mass spectrometry analysis (data not shown), but we were unable to detect Cse4. One explanation could be that the interaction with Cse4 takes place in only one phase of the cell cycle, although Cse4 and the SAS-I components are continuously expressed at low levels. Another possibility could be that the association of Cse4 to the SAS-I complex is not so strong. In consequence, Cse4 could be lost during the purification procedure so that no Cse4 could be detected although the interaction exists in the cell. Furthermore, the SAS-I bound Cse4 amounts could have been under the detection limit for mass spectrometry analysis, so that in consequence we were unable to find Cse4 in the assay.

4.2. The histone acetyltransferase Sas2 has a function at the centromere

The histone acetyltransferase Sas2 functions in the SAS-I complex together with Sas4 and Sas5 and acetylates histone H3 K14 and histone H4 K16 (Meijsing and Ehrenhofer-Murray, 2001; Sutton, et al., 2003). Additionally, Sas2 has a role in heterochromatic gene silencing at the *HM* loci and at the telomeres (Ehrenhofer-Murray, et al., 1997; Reifsnyder, et al., 1996).

Does Sas2 also have a role at the centromere ? A direct function for Sas2 at the centromere has not been described so far, but (Sharp, et al., 2003) discovered that the silencing protein Sir1 is a functional component of centromeric chromatin and helps to maintain CAF-I at the centromere. As Sas2 genetically interacts with Sir1 in silencing at *HML* (Ehrenhofer-Murray, et al., 1997), an additional function at the centromere might be possible. Furthermore, our two-hybrid and co-immunoprecipitation results that Sas2 interacts with Cse4 provide

further evidence that Sas2 has additional functions in non-heterochromatic regions of the genome.

In addition to our findings that the SAS-I complex was associated with Cse4, we found that a *SAS2* deletion leads to better growth in the temperature-sensitive *cse4-103* strain at 34°C. The *cse4-103* strain has a point mutation in two amino acids within the histone-fold-domain (I156V, L193Q). This mutation is thought to affect the interaction with the globular domain of histone H4. In consequence, the $(cse4-103/H4)_2$ tetrameres are destabilized at elevated temperatures and the cells are unable to survive.

How can a *SAS2* deletion have a positive influence on the stability of $(cse4-103/H4)_2$ tetrameres? It is known that Sas2 acetylates histone H4 at lysine 16, which is also present at the centromere in contrast to histone H3. Histone acetylation has mainly been found in the neighborhood of transcriptionally active promoters and enhancers (Kuo and Allis, 1998), where they are thought to restrict the folding of nucleosomes into the condensed 30 nm fiber (Garcia-Ramirez, et al., 1995; Tse, et al., 1998). As the yeast centromere is not packed into heterochromatin like in other organisms, one might hypothesize that the histone tails exist in an acetylated state. In *SAS2* wildtype cells, this acetylation may further destabilize the already weak interaction between *cse4-103* and histone H4, so that in consequence the cells are unable to grow at elevated temperatures. If the histone acetyltransferase Sas2 is absent, the acetylation of histone H4 K16 is missing and the nucleosomes would be able to form a more compact structure that in turn helps to tighten the $(cse4-103/H4)_2$ tetrameres. Another possibility could be that Sas2 also acetylates Cse4 next to histone H4. A missing acetylation on Cse4 would therefore have the same consequences on the stability as on histone H4 in a *sas2Δ* strain.

Here, we were able to show that Sas2 has an additional role that contributes to centromere stability. The centromeric protein Ctf19 functions in a complex

together with Okp19 and Mcm21 in centromere stability. This complex mediates the connection between CDEI, CDEII and CDEIII by binding to different centromeric proteins, e.g. via the interaction between Ctf19 and Cse4. We showed by two-hybrid experiments that a deletion of *sas2* disrupted the binding of Cse4 to Ctf19.

How can Sas2 influence the interaction between Cse4 and Ctf19 ? Post-translational modifications of histones have already been shown to be critical for protein-protein interactions. One example are the silent information regulator (SIR) proteins Sir3 and Sir4 that interact with deacetylated tails of histone H3 and histone H4 in order to build up a repressive chromatin state. An explanation for our observation could be that Sas2 acetylates one or more lysine residues on the N-terminus of Cse4. Thereby, the acetylation may provoke a conformational change in the (Cse4-H4)₂ tetrameres that are now able to interact with other proteins, e.g. Ctf19. In a *sas2*Δ cell, the N-terminus of Cse4 may remain more inflexible due to the missing acetylation so that the accessibility for other interacting proteins is decreased. In that case, the interaction between Cse4 and Ctf19 would be disrupted as can be seen in a two-hybrid assay.

Further analysis with co-immunoprecipitation experiments need to be carried out to confirm this hypothesis. Additionally, chromatin immunoprecipitation or indirect immunofluorescence experiments with Sas2 could be used to test whether it can be found at some point of the cell cycle at the centromere.

4.3. Cse4 exists in an acetylated state in the cell

We were interested in exploring the possibility that Cse4 exists in an acetylated state in the cell and if the histone acetyltransferase Sas2 is a Cse4-modifying enzyme. Indeed, we have evidence that Cse4 is acetylated, but whether Sas2 is involved in this process remains unclear.

The N-termini of the core histones H2A, H2B, H3 and H4 are post-translationally modified by methylation, acetylation and phosphorylation. These modifications are essential for modulating nucleosome structure and therefore changing gene activity. Little is known about modification of histones and histone variants at the centromere, but it has been reported that histone H3 phosphorylation in mammals precedes the phosphorylation of the Cse4-homologue CENP-A in prophase (Zeitlin, et al., 2001). This phosphorylation is necessary for directing the subcellular localization of enzymes required for completion of cytokinesis.

So far no modifications have been shown for the histone H3 variant Cse4, although they may occur because its homologue in mammals is phosphorylated. We were now able to show that Cse4 is acetylated at least at one stage in the cell cycle. This acetylation could be a specific mark for chromatin assembly factors like CAF-I and Asf1. In consequence, Cse4 is exclusively delivered to the centromere, where it replaces histone H3. One putative acetyltransferase involved in Cse4 modification could be Sas2, although no direct activity was detected. An explanation for the missing acetylation activity in our in vitro assay could be that the Cse4 concentration was too low. In that case, the radioactive signal of [¹⁴C] may be too weak to be detected. Another possibility could be that additional cofactors or pre-existing modifications at the N-terminus are needed for Cse4 acetylation. It has already been described for the histone H3 N-terminus that modifications are able to influence each other. One example is that histone H3 K4 methylation by Set7 inhibits methylation of lysine 9 by Su(var)3-9, but promotes acetylation of histone H3 by p300 (Wang, et al., 2001).

4.4. A model for chromatin-assembly at the centromere

We propose the following model from our data: during replication, newly synthesized as well as pre-existing histones need to be incorporated into nucleosomes. The histone H3 variant Cse4 replaces histone H3 at the centromere

by forming centromere specific (Cse4-H4)₂ tetrameres. But how do chromatin assembly factors distinguish between (Cse4-H4)₂ and (H3-H4)₂ tetrameres and their site of incorporation ?

First, Cse4 and histone H3 have a highly homologous C-terminal histone-fold domain, but their N-terminus is distinctly different. The 135 aa N-terminus of Cse4 shows no homology to known proteins and contains at least one essential function, since the deletion of the END domain (amino acids 28-60) is lethal to the cell (Chen, et al., 2000). The N-terminus of Cse4 may extend from the core and is able to interact with other proteins, so that in consequence chromatin assembly factors could distinguish between the standard core (H3-H4)₂ tetramere and centromere specific (Cse4-H4)₂ tetrameres. Another mechanism for helping the chromatin assembly factors to separate H3-H4 assembly from Cse4-H4 assembly is to divide their assembly in time. Whereas the standard core histones are exclusively expressed in early S-phase, Cse4 mRNA can be found at low levels throughout the whole cell cycle. Thus, the equilibrium is shifted towards Cse4-H4 after early S-phase, which may help to deposit them after H3-H4 deposition took place.

In this model, the histone acetyltransferase complex SAS-I may first bind to free Cse4 and specifically modifies one or more lysine residues on its N-terminus. The nucleosome assembly factor Asf1 is a histone chaperone that delivers histones H3 and H4 to the replication fork. Asf1 binds to the SAS-I complex via Sas4 (Meijsing and Ehrenhofer-Murray, 2001; Sutton, et al., 2001) and we were able to show that Asf1 also interacted with the histone H3 variant Cse4. Thus, Asf1 might bind to the (Cse4-H4)₂ tetrameres after identification via the N-terminus of Cse4. Here, the specific Sas2-dependent acetylation may already have an important role for recognizing the specificity for centromere deposition. (Sharp, et al., 2002) reported that Asf1 didn't play a role at the centromere, because *cacΔ asf1Δ* cells segregated a CFIII(D8B.d) reporter minichromosome at wildtype frequencies and displayed a G2-M delay independent of spindle assembly

checkpoint activation. In contrast to their observation, we found that a single *asf1* Δ already resulted in a significant increase of loss of an additional chromosome III (data not shown). Thus, the conclusion that the centromeres did not require Asf1 requires re-investigation.

In a subsequent step, the whole complex may be delivered to the centromere. Here, the chromatin assembly factor CAF-I may still be bound to the DNA after replication took place. Asf1 interacts with Cac2 (Tyler, et al., 2001) and may deliver the (Cse4-H4)₂ tetrameres to CAF-I that binds the histone H3 variant Cse4 via the Cac1 subunit, and histone H4 via Cac3 (Verreault, et al., 1996).

In this model, CAF-I releases Asf1 as well as SAS-I and now deposits Cse4 and histone H4 into centromere-specific nucleosomes and therefore replaces pre-assembled (H3-H4)₂ tetrameres. The acetylation of Cse4 may further stabilize the centromere by mobilizing the N-terminus that can now interact with other centromeric protein, e.g. Ctf19. Of course, further studies are necessary to prove this model and also to elucidate the role of the nucleosome assembly factor Asf1 as well as the dependence of the Cse4-Ctf19 interaction on the histone acetyltransferase Sas2.

5. Literature

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Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbst und nur unter Verwendung der angegebenen Quellen und Hilfsmitteln verfasst habe. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I ist mir bekannt.

Berlin, 31.01.2004

Stefanie Seitz