The histone deacetylase (HDAC) Rpd3 antagonizes heterochromatin formation at telomeres in *Saccharomyces cerevisiae*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

des Fachbereichs

Biologie und Geografie

an der

Universität Duisburg-Essen

vorgelegt von

Stefan Ehrentraut

geboren in Lauchhammer

Oktober 2008

Die der vorliegenden Arbeit zugrundeliegenden Experimente wurden am Max-Planck-Institut für Molekulare Genetik in Berlin-Dahlem sowie am Institut für Genetik des Fachbereichs Biologie und Geografie der Universität Duisburg-Essen durchgeführt.

1. Gutachter: Prof. Dr. Ann E. Ehrenhofer-Murray

2.Gutachter: Prof. Dr. Reinhard Hensel

3.Gutachter:

Vorsitzender des Prüfungsausschusses: Prof. Dr. Daniel Hoffmann

Tag der mündlichen Prüfung: 09. 02. 2009

Table of contents

Table of figures

List of tables

Abbreviations

5-FOA	5-fluoro-orotic acid
aa	amino acid
ACS	ARS consensus sequence
ARS	autonomously replicating sequence
bp	base pair
ChIP	chromatin immunoprecipitation
CoIP	Co-immunoprecipitation
HAT	Histone acetyltransferase
HDAC	histone deacetylase
HM	homothallic mating
HML / HMR	homothallic mating left / right
HMTase	histone methyltransferase
Hst	homolog of Sir two
kb(s)	kilobasepair(s)
MAT	mating type locus
NAD^+	nicotine adenine dinucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
rpm	rounds per minute

RT	room temperature
SAS	something about silencing
SDS	sodium dodecyl sulfate
Sir	silent information regulator
ts	temperature sensitive
wt	wild type
YM	yeast minimal (media)
YPD	yeast peptone dextrose (yeast full media)

Yeast genes are named according to the *Saccharomyces* genome database (SGD) gene nomenclature conventions: http://www.yeastgenome.org/help/yeastGeneNomenclature.shtml. For amino acids, the one letter code was used, for instance: K, Lysine; R, Arginine; Q, Glutamine

1 Introduction

1.1 DNA packaging in eukaryotes

The DNA of eukaryotes is divided into several linear molecules, termed chromosomes. The human genome encompasses 3.2×10^9 base pairs that would be a molecule of 1.8 meters in every single cell. Although the genome sizes between various eukaryotes differ, the basic principle of DNA organization is the same among all eukaryotes. In particular, the size of this molecule has to be adapted to the nuclear dimensions. To this end, eukaryotic DNA is packed into a nucleoprotein structure called chromatin. Furthermore, chromatin not only facilitates compaction, but also regulates the expression of genetic information.

The term chromatin describes a complex of DNA and proteins. The major proteins within chromatin are the histones. They are small, lysine and arginine rich, and thus very basic proteins. Their amino acids sequences are very conserved among various eukaryotes and only show few amino acid changes. This indicates that the function of these small molecules is very reliable during evolution, including chemical modifications of the histones that in addition to transcription factors regulate processes in DNA metabolism. The smallest subunit of chromatin is a nucleosome, consisting of 147 bp of DNA wrapped around a histone octamer consisting of two molecules of each histone type, H2A, H2B, H3 and H4 (Kornberg 1974; Luger et al. 1997). This structure is repeated approximately every 200±40 bp, thereby creating a “beads-on-a-string”-like shape with a filament diameter of 10 nm (**Figure 1**). With the aid of other proteins like the linker histone H1 in mammals (Contreras et al. 2003), this structure can further be condensed to a fibre with 30 nm in diameter. To date, different arrays of nucleosomes within this fibre are discussed that give rise to a 30 nm filament. Nearly 30 years ago, a solenoid model was suggested, where six nucleosomes are oriented in one radial turn (**Figure 1** bottom left, (McGhee et al. 1980)). Given that in this structure the helix is continued after one turn in the next turn of six nucleosomes, this structure also is defined as one-start fibre. Conversely, the crystal structure of a tetranucleosome without the linker histone H1 prompted to authors to discuss a two-start fibre (Schalch et al. 2005). In this model, the DNA zigzags back and forth and creates in complex with a second independent chromatin fibre one 30 nm filament (Schalch et al. 2005). Due to the requirement of a second fibre, this model is defined as two-start fibre. However, the structure of the 30 nm chromatin fibre remains an unsolved question.

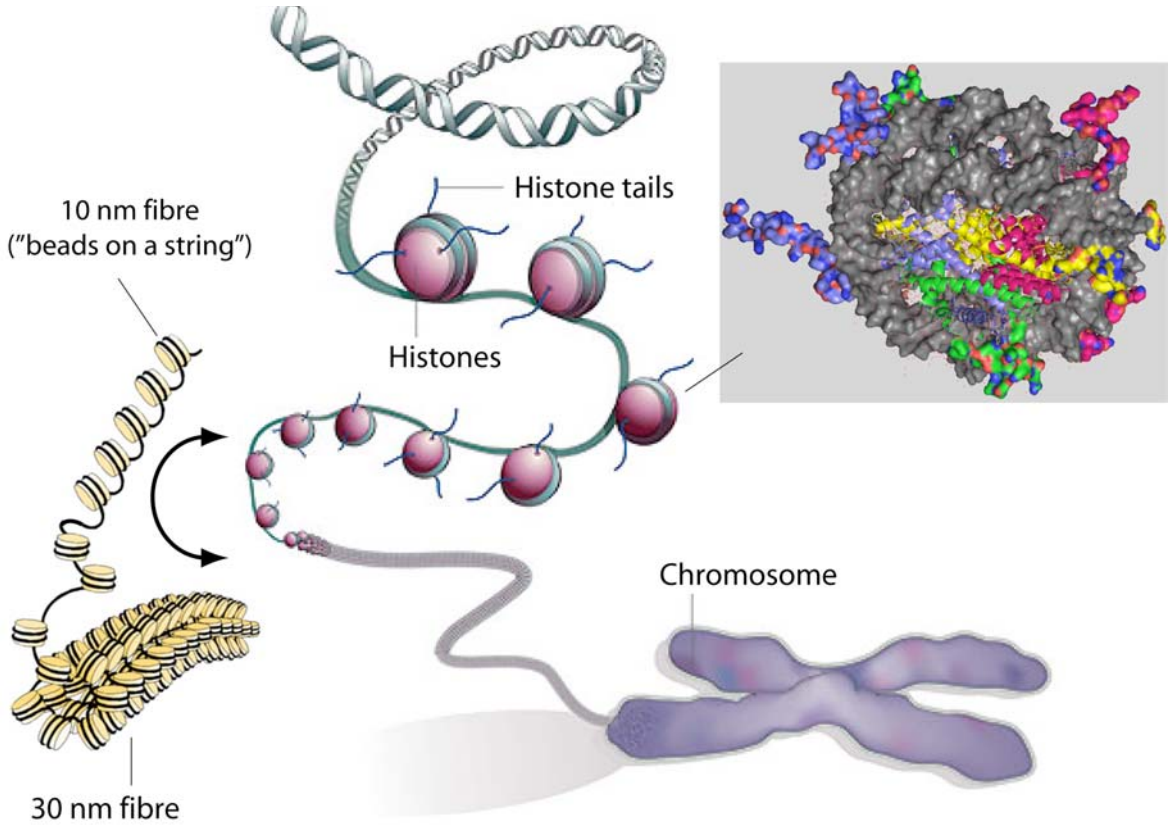

Figure 1 DNA in eukaryotic cells is packaged into nucleosomes.
The illustration (Qiu 2006) indicates the organization of eukaryotic DNA from the DNA double helix (top) up to a condensed metaphase chromosome (bottom right). The left panel indicates the structure of the "beads-on-a-string"-like 10 nm fibre, compared to the classical 30 nm fibre (McGhee et al. 1980). The crystal structure of a *Xenopus* nucleosome (Luger et al. 1997) is shown on the right. The DNA is shown in grey, the histones as follows: H2A in yellow, H2B in red, H3 in blue and H4 in green. The crystal structure was visualized with Pymol.

As mentioned above, the incorporation of DNA in a DNA-protein complex bears additional features to regulate processes like transcription, replication timing, response to DNA damage or functional distinction between chromatin domains. One important aspect of information beyond the DNA is the modification state of histones. Types of histone modifications, their functional consequences and catalysing enzymes are introduced within the next paragraphs.

1.2 Organization of chromatin

Chromatin of eukaryotic cells is organized in euchromatin and heterochromatin. Euchromatin is the open, less condensed form of chromatin that enables other factors to bind, and for instance, to initiate transcription. Heterochromatin is the condensed, transcriptionally silent form of chromatin that is characterized through the presence of silencing factors, the absence of activating marks, and therefore silenced chromatin is generally repressive (see 1.8,

reviewed in (Grewal and Moazed 2003)). Heterochromatin is often formed at repetitive elements, presumably to protect these regions from homologous recombination. Furthermore, heterochromatin is often accompanied by a highly organized nucleosomal array that leads to reduced accessibility for nucleases or DNA altering mechanisms (Wallrath and Elgin 1995). Due to the restricted access to heterochromatic DNA, these regions are generally late replicating during S-phase (Ferguson et al. 1991). A further phenomenon is the localization of heterochromatic regions to the nuclear periphery (Andrulis et al. 1998; Oki and Kamakaka 2002). However, to date it is not clear whether this localization is a cause or consequence of the heterochromatic state (also see 1.8.2).

In *S. cerevisiae*, heterochromatin forms at three independent loci (1.8). In higher organisms, heterochromatin also can form along entire chromosomes, like it is the case for mammalian female X-inactivation (Avner and Heard 2001). This mechanism is used to compensate different transcription dosage from one X-chromosome in males, compared to the otherwise twofold expression in females.

Formation of heterochromatin is generally independent of genes within the heterochromatic area. Indeed, a particular genomic region can change its expressional status through insertion into a region subjected to heterochromatin formation. This phenomenon is called position effect variegation (PEV) and was originally discovered in *Drosophila*, nearly 80 years ago (Muller 1930). In these experiments, the Drosophila gene for white eye colour (w^+) was translocated from its natural euchromatic location into a heterochromatic context, leading to repression of w^+ and a mosaic eye colour pattern.

Although heterochromatin formation is facilitated by large protein complexes like the SIR complex (see 1.8.4), one initial step in silencing is the establishment of histone modifications.

1.3 Histone modifications

Histones contain many lysine and arginine residues. Common to these two amino acids is not only the basic character, but they also can be chemically modified. Several amino acids in the histones have been found to be a target of posttranslational modification. For instance, lysine (K) residues can be acetylated, methylated or ubiquitinated, arginines can be methylated, and serines or threonines can be phosphorylated (**Figure 2**). A recent study further revealed that histones can be sumoylated, with the consequence of transcriptional repression (Nathan et al. 2006). One general model for consequences of these posttranslational modifications is that for

instance the acetylation at a lysine residue neutralizes the positive charge and therefore weakens the interaction with the negatively charged DNA. This in consequence led to the assumption that histone acetylation is a mark for weaker histone-DNA contacts, and thus characterizes euchromatin, whereas the stronger histone-DNA contact upon deacetylation is seen as a repressive, typical heterochromatic mark. Indeed, hypoacetylation is a mark for heterochromatic regions (1.8, (Braunstein et al. 1996)). Generally, the strength of histone-DNA contact can be seen as a determinant for the overall chromatin structure, in that the stronger the contact is, the more the chromatin is condensed.

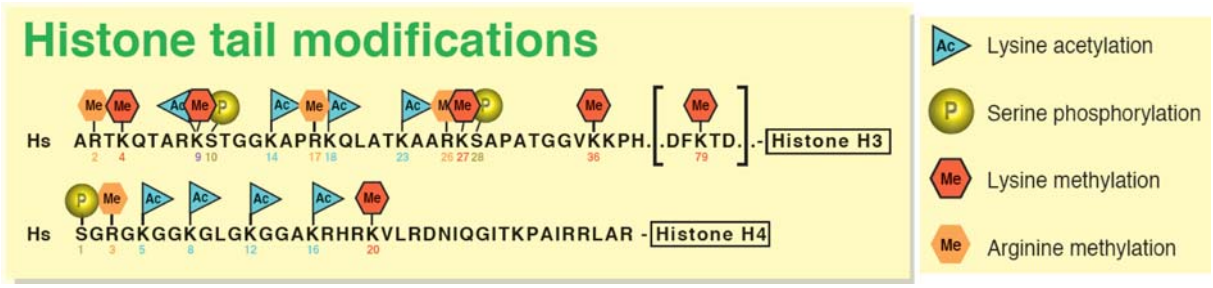

Figure 2 Posttranslational modifications within the histone tails.
Representation of the human histone H3 and H4 N-terminal tails and possible modifications within them. The picture is adapted from (Lachner et al. 2003).

In addition to the direct influence of histone-DNA interaction via acetylation, posttranslational modifications further were shown to create or alter binding sites for proteins that influence the chromatin structure. Due to the diversity of modifications, this "histone code" hypothesis heavily increases the amount of information beyond DNA sequence (Jenuwein and Allis 2001). Such a "code" might recruit several different factors. The most prominent example is the deacetylated state of H4 K16, established by the Sir2 histone deacetylase (HDAC), which creates high affinity binding site for Sir3 and Sir4 to bind to the deacetylated histone tail (Hecht et al. 1995). Conversely, acetylated H4 K16 can be bound by the bromodomain protein Bdf1, leading to protection against deacetylation through the SIR complex (1.9, (Ladurner et al. 2003)). In agreement with this crucial role of H4 K16 in determining the chromatin state, mutations of H4 K16 lead to derepression of heterochromatin, irrespective of the introduced amino acid (3.1.7, (Meijsing and Ehrenhofer-Murray 2001)).

Several proteins have been defined to bind different "histone codes". In higher eukaryotes, methylation of H3 K9 acts as recruiting factor for the heterochromatic protein 1 (HP1) (Eissenberg et al. 1990; Lachner et al. 2001). Binding of HP1 to methylated H3 K9 is

facilitated through a chromodomain (Lachner et al. 2001). Similarly, a domain that binds to acetyl-lysines is called bromodomain (reviewed in (Jenuwein and Allis 2001)). Several chromatin remodelling complexes, the SAGA complex with the HAT Gcn5 (Jeanmougin et al. 1997; Ornaghi et al. 1999) and the transcription factor TFIID (Jacobson et al. 2000) were shown to contain bromodomain subunits that bind acetyl-lysines. Therefore the general assumption of a correlation between histone acetylation and transcription might be based on the activity of bromodomain proteins.

An additional recruiting factor for chromatin modifying activities is DNA methylation that also is involved in the inheritance of epigenetic states. Generally, DNA methylation is linked to repression (Bird 2002). One of the first discoveries reported that the onset of DNA methylation in plants is controlled by histone H3 K9 methylation (Tamaru and Selker 2001). Conversely, studies reported that mammalian H3 K9 methylation is facilitated through recruitment of the histone methyltransferase by the methyl-CpG-binding protein MeCP2, which additionally interacts with an HDAC complex to induce silencing (Fuks et al. 2003). Thus, DNA methylation creates an additional epigenetic mark, and several diseases, for instance Prader-Willi-syndrome, are caused by abnormalities in the DNA methylation imprint (Driscoll et al. 1992).

Another phenomenon that is linked to silencing in the fission yeast *Schizosaccharomyces pombe* is the involvement of the RNAi machinery in the initiation of heterochromatin formation. Disruption of RNAi components like the argonaute homolog *ago1*Δ leads to a significant loss of silencing (Hall et al. 2002; Volpe et al. 2002). Another example for RNA facilitated repression is the involvement of a non-coding RNA (ncRNA) in mammalian rDNA silencing. In this case, the small ncRNA is complementary to an rDNA promoter sequence and thus facilitates binding of the repressive nucleolar remodelling complex NoRC (Mayer et al. 2006). Furthermore, whole chromosomes can be inactivated through mechanisms depending on RNA molecules, like X-inactivation in mammals through Xist RNA (Avner and Heard 2001).

Up to now, heterochromatic processes coupled to DNA methylation or RNAi are just starting to be uncovered. However, due to the lack of DNA methylation and RNAi mechanisms, these processes cannot be studied in the small model eukaryote *Saccharomyces cerevisiae*.

1.4 Histone acetyltransferases (HATs)

As mentioned above, histones are target to posttranslational modifications. Perhaps best studied among these modifications is histone acetylation. Acetylation refers to the transfer of an acetyl moiety from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of the lysine residue. Through this esterification, the positive charge of the ε-amino group is neutralized, which has several functional consequences (see above).

In general, HATs are classified in five families based on the homology between them (Roth et al. 2001). This classification distinguishes between (I) the GNAT (Gcn5-related), (II) the MYST, (III) the p300, (IV) the general transcription factor related HATs and (V) the nuclear receptor related family of HATs (Roth et al. 2001). Among them, the most prominent families are the GNAT and the MYST family.

The GNAT family encompasses Gcn5 and homologs from higher eukaryotes like PCAF, a general transcriptional activator, and Elp3, which is part of the elongator complex (Wittschieben et al. 1999), but also the cytoplasmatic HAT-B Hat1 that acetylates free histones prior to incorporation into chromatin (Ruiz-Garcia et al. 1998). As the name implies, these HATs all show structural similarity to yeast Gcn5.

The MYST family of HATs was named for the human and yeast homologs MOZ, Ybf2/Sas3, Sas2 and Tip60. This family includes Sas2, Sas3 and Esa1, as well as the human and *Drosophila* homologs Tip60, MOF and HBO1. Sas3, a component of the NuA3 complex, preferentially acetylates histone H3 (John et al. 2000), whereas Esa1, a component of the NuA4 complex, primarily acetylates histone H4 (Smith et al. 1998a; Clarke et al. 1999). Common to these HATs is a MYST domain, consisting of an acetyl-CoA binding motif and a C_2HC zinc finger, both of which are important for the function of these HATs (Meijsing and Ehrenhofer-Murray 2001).

Most of the HATs work in large multiprotein complexes, like Gcn5 in the SAGA complex, Esa1 in NuA4, Sas3 in NuA3 and Sas2 in the SAS-I complex (reviewed in (Carrozza et al. 2003)). This multiprotein composition facilitates several functions of these complexes like recruitment to promoters to acetylate and open the chromatin and therefore to prepare the chromatin for transcription initiation (Carrozza et al. 2003). Esa1, the only essential HAT in yeast (Smith et al. 1998a; Clarke et al. 1999), was further implicated to be required for DNA double strand break repair (Bird et al. 2002).

While histone acetylation covalently adds the acetyl group to lysines, this state can be reversed by histone deacetylases (HDACs, see 1.6).

1.5 The HAT complex SAS-I

When the SAS-I complex and its catalytic subunit, the HAT Sas2 were discovered, the authors linked the proteins phenomenologically to gene silencing and called them something about silencing (SAS) (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). In particular, the SAS-I complex bears histone acetyltransferase activity through its catalytically active subunit Sas2. Sas2 belongs to the MYST family of HATs, including the MYST domain (Meijsing and Ehrenhofer-Murray 2001). Sas2 works in the SAS-I complex, together with Sas4 and Sas5, that additionally are required for the acetylation function of the SAS-I complex (Sutton et al. 2003). Although Sas2 *in vitro* acetylates histone H4 K16 and H3 K14, Sas2 shows weak activity in acetylating free histones, and the HAT activity required the other complex components, Sas4 and Sas5 (Sutton et al. 2003).

The absence of Sas2 is not lethal to yeast cells, but causes specific defects in *HM*, telomeric and rDNA silencing (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). On the other hand, disruption of Sas2 suppresses the silencing defects of a nonfunctional silencer element (Ehrenhofer-Murray et al. 1997). This indicates that the acetylation through Sas2 is able to antagonize heterochromatin spreading.

SAS-I has been implicated in several aspects of chromatin. For instance, SAS-I works together with chromatin assembly factors to re-establish H4 K16 acetylation on newly synthesized histones after replication (1.10, (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001)) because SAS-I directly interacts with CAF-I and Asf1. Therefore, SAS-I works globally, but also can act at heterochromatin boundaries. At the junctions between euchromatin and heterochromatin, SAS-I establishes a boundary against heterochromatin spreading by reversing the deacetylated state that is generated by deacetylation via Sir2 (1.9, (Kimura et al. 2002; Suka et al. 2002). Thus, SAS-I is needed to restrict telomeric heterochromatin (Kimura et al. 2002; Suka et al. 2002), as well as heterochromatin at the *HM* loci (Ehrenhofer-Murray et al. 1997). Furthermore, SAS-I dependent H4 K16 acetylation was shown to be a prerequisite for the incorporation of the histone variant H2A.Z at subtelomeric boundaries (1.9, (Shia et al. 2006)). The function of Sas2 to establish boundaries against heterochromatin invasion is also conserved in other organisms, in that the *Drosophila* homolog Chameau was found to be a suppressor of position effect variegation (Grienenberger et al. 2002).

1.6 Histone deacetylases (HDACs)

The enzymes that reverse the acetylated state of a lysine are called histone deacetylases (HDACs). Deacetylation restores the positive charge of the ε-amino group with the functional consequence of stronger histone-DNA contacts (see above).

HDACs are classified in three classes (reviewed in (Ekwall 2005)). The class (I) HDACs contain Rpd3, Hos1 and Hos2, and the class (II) HDACs contain Hda1 and Hos3. Funding member of the class (III) HDACs, also called Sirtuins, is Sir2 and its homologs (Hst1, Hst2, Hst3 and Hst4 – homolog of Sir two). Another possibility to classify HDACs is the need for NAD^+ as a cofactor, in that class (I) and (II) are NAD^+-independent, whereas the sirtuins are NAD^+-dependent HDACs (Imai et al. 2000). A prominent member of the class (III), NAD^+-dependent HDACs is Sir2. For instance, Sir2 is required for all forms of silencing in yeast (see1.8.4), and is further discussed in lifespan extension as well as in the establishment of cancer (reviewed in (Blander and Guarente 2004)). The mechanism of its deacetylation reaction may be relevant for silencing. During the NAD^+-dependent deacetylation reaction, the acetyl group is transferred from the acetylated lysine to NAD^+ that thereby is hydrolysed to nicotinamide and the ADP-Ribose derivative 2'-O-Acetyl-ADP-ribose (OAADPR) (**Figure 3**). Within the cells, this reaction product is quickly isomerised to 3'-OAADPR and builds a mixture of both isoforms (Gasser and Cockell 2001). The potential functional relevance of this derivative is described in (1.8.4). The mechanism of NAD^+-dependent deacetylation requires binding of this cofactor to a particular binding pocket within Sir2. Indeed, mutations within this binding pocket (N345A) abrogated the deacetylation function of Sir2 (Imai et al. 2000).

In contrast to the sirtuins, the class (I) and (II) HDACs are NAD^+-independent. Thus, during deacetylation through HDACs of these classes, no cofactor is needed, and no metabolite is generated. The different enzymatic mechanisms also bear other differences, for instance concerning the mechanisms of inhibition. Most notably, Rpd3 shows high sensitivity to the HDAC inhibitor trichostatin A (TSA), whereas the sirtuins are insensitive to TSA (Yoshida et al. 1990; Bernstein et al. 2000).

Genome-wide analysis of HDAC functions has suggested a "division of labour" among different HDACs (Ekwall 2005). For instance, Rpd3 controls the acetylation of meiotic genes or genes involved in carbohydrate biosynthesis, whereas Hda1 preferentially deacetylates in the HAST regions, contiguous chromatin domains adjacent to the subtelomeres (Robyr et al. 2002). Furthermore, Hos1, Hos2 and Hos3 were described to deacetylate rDNA genes (Robyr

et al. 2002). Not only genomic regions are target to different deacetylases, but particular HDACs might also be specific for a special set of residues. Whereas Rpd3 at gene promoters can deacetylate all H4, H3, H2B or H2A sites (Suka et al. 2001), Hda1 mostly deacetylates H3 and H2B (Wu et al. 2001). Furthermore, Sir2 also deacetylates several residues *in vivo* (Suka et al. 2001), whereas its homologs like Hst3 and Hst4 are specific to H3 K56 (Maas et al. 2006). Therefore, beyond the prominent Sir2, Rpd3 is another HDAC that acts very broadly within the cells.

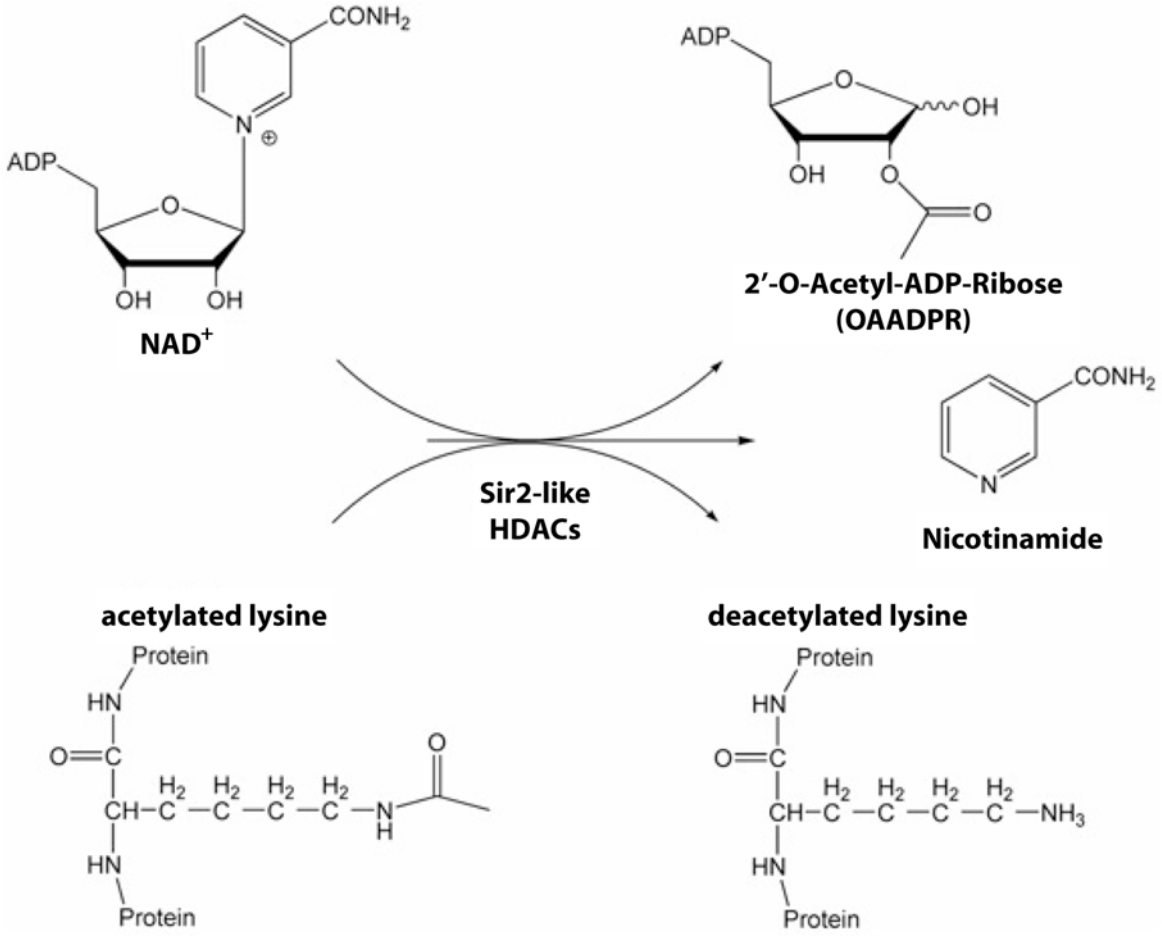

Figure 3 The NAD^+-dependent acetyl-lysine deacetylation reaction.
Reaction scheme of NAD^+-dependent deacetylation. The acetyl group is transferred to the second ribose moiety of NAD^+, thereby hydrolysing NAD^+ to 2' or 3'-O-Acetyl-ADP-Ribose (OAADPR) and nicotinamide. The picture originates from (Yang and Sauve 2006).

1.7 The histone deacetylase (HDAC) Rpd3

Rpd3 belongs to the class (I) HDACs. Homologs among all eukaryotic species, like human HDAC1, are well conserved (reviewed in (Ekwall 2005)). Originally, Rpd3 was discovered as

a suppressor of mutations in the potassium transporter gene *trk1*, and therefore was termed reduced potassium dependency (Rpd3) (Vidal et al. 1990).

Rpd3 has several functions in the cells, ranging from genome wide control of gene expression to the control of replication initiation timing (Vogelauer et al. 2002; Aparicio et al. 2004). In line with the global function of Rpd3, the overall level of histone H4 K5 and K12 acetylation is increased in *rpd3*Δ cells (Vogelauer et al. 2000). Additionally, late replicating origins initiate replication significantly earlier in *rpd3*Δ cells, implicating Rpd3 as a factor that is required for the control of replication timing (Vogelauer et al. 2002; Aparicio et al. 2004). Rpd3 has further functions in gene repression. Rpd3 can be targeted to promoters of specific genes via the corepressor Ume6 (Rundlett et al. 1998), and deacetylates all lysine residues on all histones (Rundlett et al. 1998; Suka et al. 2001).

Rpd3 works together with Sin3 (Kadosh and Struhl 1997), and is present in two functionally and structurally distinct complexes, Rpd3 (L) and Rpd3 (S) (Carrozza et al. 2005; Keogh et al. 2005). The Rpd3 (L) complex can be targeted to promoters of specific genes and provides promoter deacetylation (Rundlett et al. 1998). Furthermore, the Rpd3 (L) complex has a global role in controlling genome wide acetylation, whereas Rpd3 (S) is specifically recruited to ORFs to deacetylate within the body of genes to prevent intragenic transcription starting from cryptic promoters (Carrozza et al. 2005; Keogh et al. 2005). In this case, Rpd3 (S) is recruited to ORF through Set2-mediated H3 K36 methylation, a modification specifically bound by the chromodomain subunit of Rpd3 (S), Eaf3 (Li et al. 2007).

In contrast to the general description of a correlation between HDACs and gene repression, Rpd3 and another class (I) HDAC, Hos2, were found to have an additional role in gene activation (Wang et al. 2002; De Nadal et al. 2004). In this case, targeting of Rpd3 to osmo-sensitive genes upon stress was necessary to activate the transcription of these genes (De Nadal et al. 2004), though the mechanism for this activation remains unclear.

Another observation, more than ten years ago, was that the absence of Rpd3 enhanced position effect variegation in *Drosophila* (De Rubertis et al. 1996), and also in yeast, subtelomeric reporters become more silenced in *rpd3*Δ cells (Sun and Hampsey 1999). This was counterintuitive, because histone deacetylation is generally thought to be beneficial for silencing. One would then assume that *rpd3*Δ decreases silencing. With the description of Rpd3 as boundary element against heterochromatin spreading in this study, we now can shed further light on this observation.

1.8 Heterochromatin in *S. cerevisiae*

Heterochromatin was originally defined cytologically as regions of the genome that remain condensed throughout the cell cycle (Schultz 1936). Heterochromatin in *S. cerevisiae* shares its main characteristics with other eukaryotes, like inaccessibility of DNA for transcription factors and hypoacetylation of histones (Loo and Rine 1995; Braunstein et al. 1996; Lustig 1998). Thus, heterochromatin forms a condensed structure that inhibits transcription independently of genes and their promoters within the heterochromatic region.

Three classes of gene silencing are known in *S. cerevisiae*: (I) repression of the silent mating type loci *HML* and *HMR*, (II) telomeric repression and (III) rDNA silencing (reviewed in (Stone and Pillus 1998)). One important structural component of heterochromatin is the SIR (Silent Information Regulator) complex. The SIR complex consists of the Sir2, Sir3 and Sir4 subunits, and at the HM loci SIR additionally contains Sir1. Sir2 is an HDAC that deacetylates the histones H3 and H4, thereby creating binding sites for Sir3 and Sir4 to bind to the deacetylated histones (reviewed in (Rusche et al. 2003)). Since only the Sir2 HDAC is common to all three silenced regions, the mechanisms of silencing differentiate the regions and are described in the following paragraph.

1.8.1 Silencing at the *HM* loci

Haploid *S. cerevisiae* cells show one of two possible mating types, **a** or α. The mating type of a yeast cell is determined by the mating type locus, *MAT*. The *MAT* locus encodes for proteins that regulate expression of mating specific genes (Mat **a**1/**a**2 in *MAT***a** cells, MAT α1/α2 in *MAT*α cells) (Herskowitz I. 1977). In addition to *MAT*, the two *HM* loci contain a second copy of mating type genes. The *MAT* locus and both *HM* loci are located on chromosome III, with *HML* (homothallic mating left) on the left arm and *HMR* on the right, respectively. The *HML* locus contains α information and *HMR* contains **a** information. During mating, cells of opposing mating types fuse and form diploid cells (**a** and α) that undergo meiosis during sporulation and form haploid progeny. Since mating requires opposing mating types, derepression of the *HM* loci leads to pseudodiploid yeast cells that express both types of cell-type information (**a** and α), like diploid cells, and therefore show lower mating efficiency. To prevent this mating inability, the *HM* loci are kept silent by incorporation into heterochromatin.

Formation of heterochromatin is facilitated by *cis*-acting elements called silencers and *trans*-acting proteins. The *HM* loci are flanked by the E and I silencers (**Figure 4**). These

silencers are *cis*-acting regulatory DNA elements that create binding sites for *trans*-acting DNA binding proteins like Rap1 (repressor activator protein), Abf1 and the origin recognition complex, ORC, that binds to ars consensus sequences (ACS) (**Figure 4**). Although the occurrence of Rap1 or Abf1 (ars binding factor) binding sites is variable, all silencers contain an ACS. ORC normally functions in the initiation of replication (Foss et al. 1993), and indeed, the ACS sequences of the silencers are able to initiate replication when inserted into plasmids (Sharma et al. 2001). Although establishment of silencing at *HM* requires passage of the cells through S-phase, it does not require replication initiation or replication fork passage (Kirchmaier and Rine 2001). Significantly, all of the *cis*-acting proteins have distinct roles elsewhere in the cell (transcriptional regulation for Rap1, Abf1 (Planta et al. 1995), replication initiation for ORC), but at the silencers, they act as recruiting factor for Sir proteins (reviewed in (Lustig 1998)). In line with this, it was speculated that it is the combination and the close proximity of these factors that converts these DNA elements to silencers that effectively recruit the SIR complex (Lustig 1998), like it is the case for several Rap1 binding sites at telomeres ((Cockell et al. 1995),1.8.2). In fact, Rap1 interacts with Sir3 or Sir4 in order to subsequently forming heterochromatin (Liu and Lustig 1996). In analogy to this, Rap1 and Abf1 at the *HM* loci associate with Sir3 to nucleate silencing (Lustig 1998). Furthermore ORC also has the ability to recruit the SIR complex, in that Orc1 interacts with Sir1, and Sir1 interacts with Sir4 (Triolo and Sternglanz 1996). Thus, Sir1 acts as a mediator between ORC and SIR at the *HM* silencers (Rusche et al. 2002). Furthermore, this involvement of ORC and Sir1 creates a situation that is exclusive to the *HM* loci (see 1.8.2).

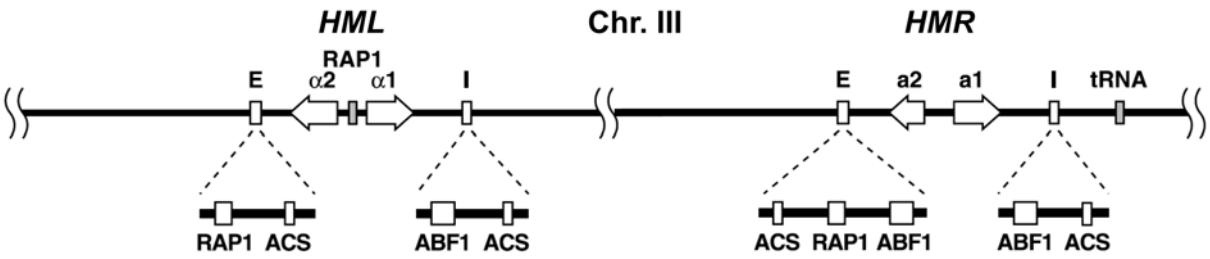

Figure 4 The *HM* loci of *Saccharomyces cerevisiae*.
The illustration indicates the *HML* and *HMR* loci with the E silencers (E), the I silencers (I); binding sites for ORC (ACS), Rap1 and Abf1 and the open reading frames for the **a**1, **a**2, α1 and α2 mating type genes. The picture is taken from (Rusche et al. 2003).

The four silencers not only vary in their composition, but they are also of different importance for the silencing state of the region. For instance, at *HML*, each of the silencers alone is sufficient for silencing (Mahoney and Broach 1989), whereas at *HMR*, the I silencer is

dispensable, but the *HMR*-E silencer is essential to keep *HMR* silent (Rivier et al. 1999). Furthermore, *HMR*-E is special in that at least two of the three binding sites (or the corresponding binding proteins) have to be disrupted to cause loss of silencing.

After recruitment of SIR through Rap1, Abf1 and ORC, the process of stepwise polymerization of the SIR complex starts, including binding of Sir3 and Sir4 to Rap1. Sir2 is brought to the locus as Sir2/Sir4 heterodimer. Next, histones become deacetylated via Sir2 (Imai et al. 2000), and association of the deacetylated histone tails with Sir3 and Sir4 continues the polymerization of the SIR complex (Rusche et al. 2002). This cycle is repeated, resulting in multimerization and spreading of Sir proteins along the chromosome (also see 1.8.4). The SIR complexes spread multidirectionally in both directions along the chromosome and are stopped by so called boundary elements that prevent regions adjacent to heterochromatin from repression through SIR complexes (see 1.9).

1.8.2 Telomeric silencing

The telomeres are the very ends of the chromosome and consist of a short single stranded DNA overhang and a nucleosome free region of approximately 300 bp long C_{1-3}/TG_{1-3} repeats. If left unprotected, these regions would be subject to end-to-end fusions and homologues recombination. Furthermore, telomeres need to be protected from DNA degradation that would lead to telomere shortening and in consequence to a shorter replicative lifespan and cellular senescence. To prevent this, telomeres are incorporated into heterochromatin.

The 300 bp long C_{1-3}/TG_{1-3} repeat sequence contains several Rap1 binding sites. In contrast to *HM*, here a multitude of Rap1 proteins serve to recruit the Sir2/Sir4 heterodimer and Sir3. Sir2/Sir4 and Sir3 then form a complex in a similar way to the *HM* loci, and this complex spreads away from the telomeric end (**Figure 5**). When getting into contact with nucleosomes adjacent to the nucleosome free region, Sir2 deacetylates histones, and the SIR proteins further spread into subtelomeric regions (Luo et al. 2002).

In principle, the multitude of Rap1 binding sites is also able to induce silencing when integrated elsewhere in the genome (Stavenhagen and Zakian 1994), and therefore is sufficient for silencing. However, natural telomeres additionally contain a so-called CoreX element, which contains an ACS and in many cases an Abf1 binding site. Silencing at natural telomeres is discontinuous and is enhanced around the CoreX element (Fourel et al. 1999; Pryde and Louis 1999). Given that ORC binding at the ACS of the CoreX element is able to

recruit Sir1 and to stabilize silencing like at the *HM* loci, some controversies are discussed about the dependence of Sir1 for telomeric silencing. Whereas some studies show that Sir1 is not required for telomeric silencing (Aparicio et al. 1991), others report a partial loss of telomeric silencing upon disruption of Sir1 (Pryde and Louis 1999). One possible explanation is that screens for telomeric silencing use a reporter system that was generated through insertion of reporter genes in a way that artificially truncated the CoreX element (Gottschling et al. 1990). The current working model for this discrepancy is that telomeric silencing *per se* is stable but can further be stabilized by forming a loop back to the CoreX element and thus further stabilizes silencing (**Figure 5**, (Strahl-Bolsinger et al. 1997; Pryde and Louis 1999)).

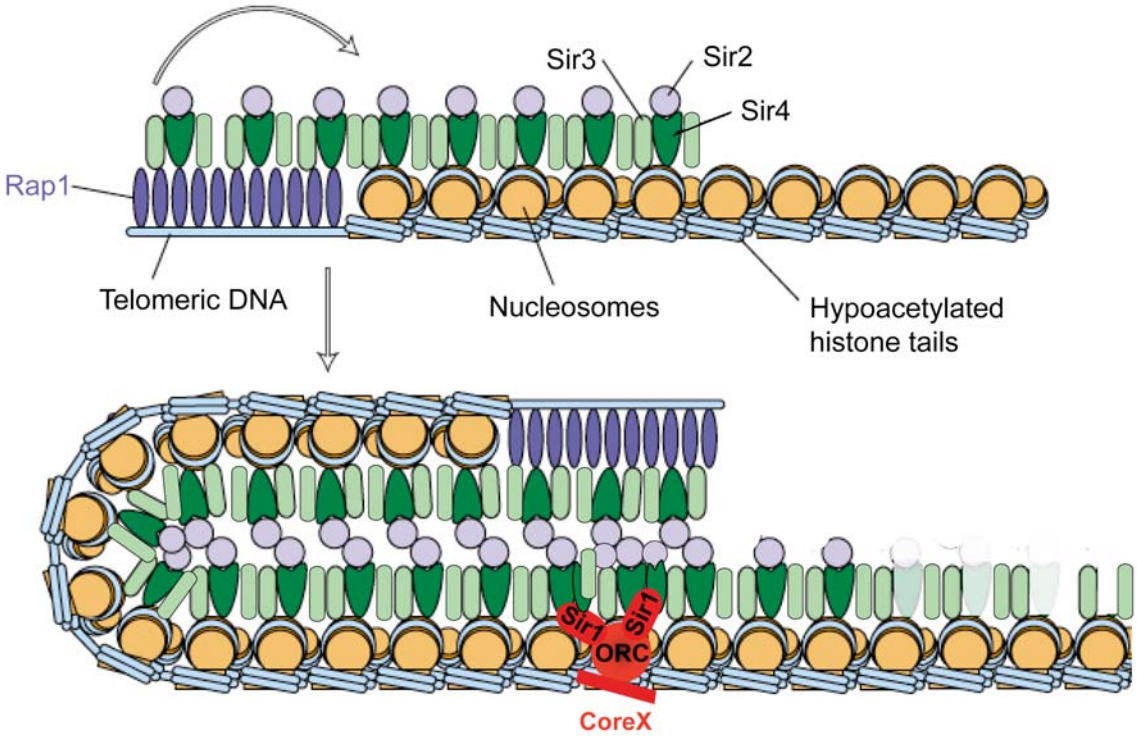

Figure 5 Telomeres in *Saccharomyces cerevisiae*.
The upper panel indicates the 300 bp long $C_{1\text{-}3}/TG_{1\text{-}3}$ repeat sequence (telomeric DNA) with several Rap1 binding sites and the SIR proteins recruited through Rap1. The lower panel includes the CoreX element and the loop structure suggested by (Strahl-Bolsinger et al. 1997; Pryde and Louis 1999). This slightly modified picture originates from the website of Bruce Stillman:
http://departments.oxy.edu/biology/Stillman/bi221/110300/rna_polymerases.htm.

Furthermore, Sir3 is limiting for SIR complex propagation along the chromosome (Renauld et al. 1993). Overexpression of Sir3 extends the heterochromatic domain from approximately 3 kb up to 16 kb away from the telomere (Hecht et al. 1995), coinciding with the spread of Sir3, whereas Sir2 and Sir4 levels decrease with increasing distance from the telomere (Strahl-Bolsinger et al. 1997).

Another process that is thought to help in silencing is the placement of the telomeres at the nuclear periphery (Gotta et al. 1996). It is thought that the telomeres are brought there through interaction with the nuclear pore complex (Galy et al. 2000). However, to date it is unclear whether silencing is the cause or a consequence of localization to the nuclear periphery. This localization of specific genomic regions to the nuclear envelope is another way of controlling gene expression by insulating the region at nuclear substructures and therefore inactivating them for transcription (see 1.9).

1.8.3 rDNA silencing

The rDNA locus contains the highly repetitive ribosomal DNA that is protected from recombination or formation of extrachromosomal rDNA rings through incorporation into heterochromatin (Christman et al. 1988). The *S. cerevisiae* rDNA locus consists of a 9.1 kb sequence that is repeated 100 to 200 times (Petes and Botstein 1977). The rDNA contains 35S rDNA as precursor for the 25S, 18S and 5.8S ribosomal rRNA (**Figure 6**). Only a fraction of the rDNA genes are transcribed at a given time, and the majority remains silenced by the action of Sir2 (Smith and Boeke 1997). Although *NTS2* contains an ACS site, silencing at rDNA is different from that of telomeres and the *HM* loci in that it is independent of Sir1 and Sir3 (Smith and Boeke 1997). Sir2 in this context does not act via Sir4, but is part of the nucleolar RENT (regulator of nucleolar silencing and telophase) complex (Straight et al. 1999). However, the mechanistic aspects of rDNA silencing are still unclear.

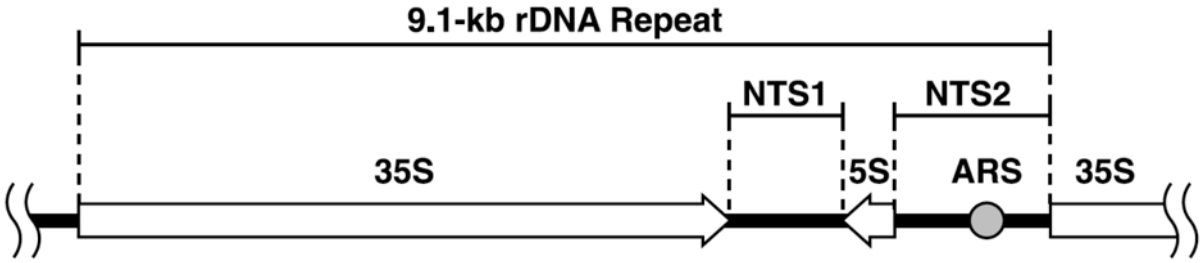

Figure 6 The rDNA locus of *Saccharomyces cerevisiae*.
Ribosomal DNA (rDNA) is present in a tandem array of 100-200 copies of a 9.1 kb repeat. Each repeat encodes a 5S RNA, transcribed by RNA PolIII, and a 35S precursor RNA, transcribed by RNA Pol I and subsequently processed to 18S, 5.8S, and 25S RNA. The 35S coding regions are separated by nontranscribed spacers, NTS1 and NTS2 The picture was adopted from (Rusche et al. 2003).

Altogether all silenced loci may be linked by competition through limiting amounts of Sir proteins. In line with this, rDNA silencing is negatively regulated by titrating Sir proteins out of the nucleolus towards other silenced loci (Smith et al. 1998b).

1.8.4 The Silent Information Regulator (SIR) complex as a key component of heterochromatin

One important structural component of silenced chromatin is the SIR complex. Sir proteins are essential for silencing, but not essential for growth (Rine and Herskowitz 1987). At least four different proteins belong to the SIRs, with Sir2, Sir3 and Sir4 as essential structural components of yeast heterochromatin. In contrast to the other SIR proteins, Sir1 contributes to, but is not essential for silencing (Pillus and Rine 1989). The role of Sir1 rather is to facilitate binding of the other Sir proteins to the ACS within silencers (see 1.8.1-1.8.2).

The four *SIR* genes were originally identified in a screen for factors that are required for *HM* silencing (Rine and Herskowitz 1987). Within the cell, Sir2 and Sir4 form a soluble complex that does not contain Sir3 (Moazed et al. 1997). Conversely, after recruitment of the Sir proteins to a silencer, several interactions form the SIR complex that consists of Sir2, Sir3 and Sir4 and is able to spread along the chromatin fibre (Rusche et al. 2002). Sir2 is a NAD^+-dependent HDAC that deacetylates histone H3 and H4 (Imai et al. 2000). This hypoacetylation of histones creates high affinity binding sites for Sir3 and Sir4 (Hecht et al. 1995). In contrast to Sir2, Sir3 and Sir4 have no measurable enzymatic activities, although Sir3 was shown to contain a domain with homology to the class of AAA+ ATPase-like proteins and therefore is thought to play a critical role in binding the reaction product of NAD^+-dependent deacetylation (see below, (Gasser and Cockell 2001)). SIR spreading involves ordered events of recruiting Sir3 or Sir4 through Rap1, Abf1 or Sir1-ORC, deacetylation via Sir2 and binding of Sir3 and Sir4 to the deacetylated histone tails (Rusche et al. 2002). Through recruitment of further Sir2 proteins, nucleosomes adjacent to the not yet deacetylated ones also can be deacetylated. This creates further binding sites for Sir3 and Sir4, thus allowing spreading of the SIR complex over several kbs along the chromatin fibre.

The NAD^+-dependent deacetylase activity of Sir2 is required for SIR spreading (3.1.7, (Rusche et al. 2002)). Furthermore, NAD^+-dependent deacetylation *per se* plays a vital role in SIR complex assembly (Gasser and Cockell 2001). Significantly, Sir2 and its homologs (Hst1-4) couple the deacetylation reaction to the hydrolysis of NAD^+ (**Figure 3**, (Imai et al. 2000)). During this reaction, the acetyl group is transferred from the acetyl-lysine to NAD^+, generating OAADPR and nicotinamide (Tanner et al. 2000). One of the outstanding questions is, whether OAADPR has a further role after the deacetylation reaction has taken place. As mentioned above, Sir3 is classified to belong to the family of AAA+ ATPases (Neuwald et al. 1999). Members of the AAA+ superfamily were described to couple the hydrolysis of ATP to conformational changes that drive the assembly or disassembly of large protein complexes.

However, Sir3 lacks the critical amino acids that are needed for ATP hydrolysis, but nevertheless it was speculated that Sir3 has the potential to bind OAADPR and thus might induce a conformational change within the SIR complex (Gasser and Cockell 2001). Indeed, recombinant Sir proteins together with oligonucleosomes change their conformation after addition of OAADPR and form chromatin filaments visible under an electron microscope (Onishi et al. 2007). However, the exact role of OAADPR in SIR complex assembly still remains elusive.

Some controversies are discussed regarding the substrate specificity of Sir2. Although Sir2 *in vitro* preferentially deacetylates histone H3 K9 and K14 and H4 K16 (Imai et al. 2000), *in vivo* all lysines of H3 and H4 were deacetylated in heterochromatic regions (Suka et al. 2001). This suggests that Sir2 has a relatively broad substrate range. For instance, Sir2 also deacetylates lysines within the core region of histones, like H3 K56 (Xu et al. 2007).

In addition, Sir2 homologs from other organisms deacetylate also substrates other than histones. For instance, the human p53 protein is deacetylated by the human Sir2 homolog, SIRT1 (Luo et al. 2001; Vaziri et al. 2001). Interestingly, the acetylated form of p53 activates the apoptosis pathway, whereas the deacetylated form remains bound to SIRT1 after deacetylation and therefore cannot activate apoptosis (Luo et al. 2001; Vaziri et al. 2001). This implicates SIRT1 as a possible cancer related-factor.

The Sir protein is highly conserved through organisms ranging from archea to humans (Brachmann et al. 1995). Sir2 family members contribute to cell cycle progression, radiation resistance and genetic stability. For instance the yeast Sir2 homologs Hst3 and Hst4 were shown to control cell cycle progression through deacetylation of H3 K56 (Maas et al. 2006). Furthermore Sir2 and Hst1 have been implicated in the control of replication initiation (Pappas et al. 2004; Irlbacher et al. 2005). This suggests that Sir2 and its homologs are involved in several processes beyond gene silencing and are interesting subjects for studies of their working mechanisms, as well as for the identification of inhibitors or activators for this family of enzymes.

1.9 Restriction of heterochromatin spreading through boundary elements

Once heterochromatin is formed, this structure is restrictive to various enzymes (Gottschling 1992; Loo and Rine 1994). Therefore cells need to ensure that heterochromatin spreading is prevented prior to invasion of heterochromatin into normally transcriptionally active

euchromatin. To maintain the transcriptional status of a region, junctions, so called boundaries exist that separate active from inactive regions.

These boundaries, so far, have been associated with chromatin opening activities, for instance histone acetylation, methylation, the incorporation of histone variants or even histone eviction at a boundary (reviewed in (Oki and Kamakaka 2002)). In particular histone acetylation by several HATs (Donze and Kamakaka 2001; Jacobson and Pillus 2004; Oki et al. 2004; Oki and Kamakaka 2005), histone H3 K79 methylation by Dot1 (van Leeuwen et al. 2002) and H3 K4 methylation through Set1 (Santos-Rosa et al. 2004) have been implicated to create boundaries. In addition, chromatin alterations like the complete loss of nucleosomes (Oki et al. 2004; Oki and Kamakaka 2005) or the incorporation of the histone variant H2A.Z (Meneghini et al. 2003) may contribute to the formation of a barrier against heterochromatin. Some modifications further may also recruit chromatin remodelling factors to initiate boundary formation (Oki and Kamakaka 2005).

While heterochromatin formation starts at silencer elements (see 1.8), some boundaries form adjacent to the silencing nucleation sites. One example is the right barrier to *HMR* that is build by the strongly expressed t-RNA^{THR} gene, adjacent to the *HMR*-I silencer (Oki and Kamakaka 2005). Deletion of this t-RNA gene results in the further spread of SIR proteins (Donze and Kamakaka 2001). In this case the barrier is a fixed, nucleosome free element determined by the position of the t-RNA gene (Oki and Kamakaka 2005).

In contrast to this example for a fixed border, boundaries further can be flexible. These boundaries are characterized by a balance of opposing enzymatic activities, and therefore were termed “negotiable” boundary (Kimura and Horikoshi 2004). For instance, at the telomeres the histone H4 K16 deacetylation activity of Sir2 is antagonized by the parallel onset of H4 K16 acetylation through Sas2 (Kimura et al. 2002; Suka et al. 2002). In this case the establishment of heterochromatin starts at the telomeric Rap1 sites and spreads towards the centromere (1.8.2, reviewed in (Rusche et al. 2003)). Since Sas2 globally acetylates H4 K16 (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001), the counteracting mechanisms to SIR spreading at telomeres are not solely specified by DNA elements like promoters. Thus, the boundary between telomeric heterochromatin and subtelomeric euchromatin is a result of the competition between the opposing enzymatic activities of Sir2 and Sas2 (Kimura et al. 2002; Suka et al. 2002; Kimura and Horikoshi 2004).

Target of the negotiation between Sir2 and Sas2 is the acetylation state of H4 K16. Indeed, this is a critical residue in that the deacetylated state is preferentially bound by Sir3 and Sir4, followed by SIR spreading (1.8.4, (Rusche et al. 2002)). Conversely, the

bromodomain proteins Bdf1 and Bdf2 (Ladurner et al. 2003) can bind the acetylated state of H4 K16. This is thought to prevent from the deacetylation through Sir2, and therefore aids in boundary formation (Ladurner et al. 2003).

The terms fixed or negotiable boundary described above designates the position on the DNA. But also the "fixation" of a genomic region within certain subcompartments of the cell can influence the epigenetic fate of this region. For instance, mutated HM loci can regain silencing when targeted to the nuclear periphery (Andrulis et al. 1998). Furthermore, also the telomeres are located at the nuclear periphery (Gotta et al. 1996), although it is not clear if the heterochromatic state is the cause or the consequence of this location.

As the initiation of heterochromatin formation works through silencers, also the formation of boundaries can work through DNA elements. These elements, termed insulators recruit activating factors that work against SIR spreading (reviewed in (Oki and Kamakaka 2002)). For instance, the telomeres are flanked by subtelomeric repetitive sequences and the X and Y' elements (Louis and Haber 1992). These elements were named subtelomeric anti-silencing regions (STARs), and contain Reb1 (RNA polymerase I enhancer binding protein) and Tbf1 (TTAGGG repeat binding factor) binding sites. When these binding sites are integrated into heterochromatic regions, they are able to protect from heterochromatin spreading (Fourel et al. 1999).

All in all, the described mechanisms that work against heterochromatin formation are generally viewed as being activating mechanisms, i.e. histone acetylation or the recruitment of the transcription machinery. In this study we show that also the opposite, histone deacetylation is capable to protect from heterochromatin invasion.

Another important question is how the epigenetic information is inherited from generation to generation to ensure the re-establishment of epigenetic states. Indeed, this inheritance uses the mentioned *cis*-acting DNA elements and *trans*-acting proteins, and therefore works through the described principles of initiation, establishment and restriction of chromatin states (reviewed in (Ehrenhofer-Murray 2004)).

1.10 Replication of chromatin

The Duplication of genetic information in eukaryotic cells requires not only the replication of the DNA sequence, but also that the epigenetic information carried on the histones is inherited to the daughter cells. The correct duplication of epigenetic information during replication is important to restore the expressional characteristics of a genomic region.

Replication of chromatin in a first step requires removal of nucleosomes prior to DNA replication. Therefore, in front of a replication fork, histones become disassembled to H3-H4 tetramers and H2A-H2B dimers (Gruss et al. 1993). After DNA replication, chromatin is reassembled by deposition of H3-H4 in a first step, followed by H2A-H2B incorporation. Deposition of histones onto the DNA is facilitated by the chromatin assembly factors CAF-I and Asf1 (Adams and Kamakaka 1999; Tyler et al. 1999). Histone deposition is coupled to the DNA replication machinery through the proliferating cell nuclear antigen (PCNA) protein (Shibahara et al. 2000) that remains topologically linked to the freshly replicated DNA, and thus recruits chromatin assembly factors (e.g. CAF-I) that are necessary to restore the chromatin fibre.

Doubling of DNA requires also doubling of histones, and therefore synthesis of new histones. The incorporation of newly synthesized or parental histones is random, and therefore old and freshly synthesized histones become mixed on both DNA strands (Sogo et al. 1986). *In vitro,* CAF-I incorporates histones regardless of their modification pattern (Shibahara et al. 2000). *In vivo,* newly synthesized histones carry a histone modification pattern that was established by the cytoplasmatic HAT-B complex HAT1, and this pattern is characterized by acetylation of histone H4 K5 and K12 (Ruiz-Garcia et al. 1998). These evolutionary conserved histone deposition related marks (Sobel et al. 1995) in higher eukaryotes colocalize with CAF-I (Taddei et al. 1999), suggesting that CAF-I incorporates these newly synthesized histone H4 molecules into chromatin. However, these modifications are removed after chromatin assembly, and this removal is sensitive to the HDAC inhibitor TSA (Yoshida et al. 1990; Taddei et al. 1999). This implies that an HDAC is involved in the contemporary removal of these modifications, supposedly coupled to replication (Ehrenhofer-Murray 2004). This further implies that chromatin assembly factors may act as a platform for the reestablishment of epigenetic marks after replication. Indeed, this was found to be the case, like for the establishment of H4 K16 acetylation (Meijsing and Ehrenhofer-Murray 2001). To establish this modification, which is absent on newly synthesized histones, the HAT complex SAS-I is recruited to the replication fork through interaction with the chromatin assembly factors CAF-I and Asf1 (Meijsing and Ehrenhofer-Murray 2001). However, the question whether the removal of cytoplasmatic acetylation marks through an HDAC also works through chromatin assembly factors to recruit histone modifying enzymes has not yet been solved and will be addressed in this study.

1.11 Outline of this thesis

The functional distinction between euchromatic and heterochromatic domains is necessary in order to maintain the transcriptional status of a genomic region (Oki and Kamakaka 2002). Heterochromatic chromatin condensation prevents inappropriate gene expression, DNA degradation or recombination, whereas heterochromatin spreading leads to silencing of otherwise transcriptionally active genes. To maintain the transcriptional state of a region, boundaries ensure that the respective chromatin states do not spill over into the other one. One factor that was known previously to restrict the spreading of telomeric heterochromatin into euchromatin is the SAS-I complex (see 1.5 and 1.9, (Kimura et al. 2002; Suka et al. 2002)). Sas2 counteracts the deacetylated state that is generated by the heterochromatic SIR complex (Kimura et al. 2002; Suka et al. 2002). Several other factors have been implicated to have boundary activity (1.9, (Oki and Kamakaka 2002)). The aim of this study was to gain further insights into the biology of junctions between active and repressive chromatin states. Therefore, we performed a synthetic lethal screen for factors that become essential in the absence of the HAT Sas2.

Interestingly, we found that deletion of the gene encoding the HDAC Rpd3 was lethal in *sas2*Δ cells. This was counterintuitive, since these factors have opposing enzymatic activities. Given that a synthetic lethal screen is commonly used to identify factors that share similar biological functions, we hypothesized that both factors act in the same pathway despite their contrary enzymology. Since Sas2 has boundary activity, this means that Rpd3 also is required to restrict heterochromatin to the telomeres, and that the parallel loss of both factors might lead to SIR spreading to a degree that is lethal to the cells. Indeed, the absence of Rpd3 led to increased Sir spreading into subtelomeric regions, and disrupting the SIR complex abrogated the synthetic lethality between *rpd3*Δ and *sas2*Δ, indicating that excessive spreading of heterochromatic SIR complexes was responsible for the lethality of *rpd3*Δ *sas2*Δ cells. Notably, Rpd3 was necessary to restrict the SIR proteins to the telomeres, and targeting of Rpd3 to normally silent chromatin created a barrier to the spreading of SIR-dependent repression.

The surprising observation that histone deacetylation through Rpd3 was prohibitive, rather than conducive to silencing raised the question for a mechanism how Rpd3 might be able to do so. By analysing the different deacetylation mechanisms between Rpd3 and Sir2, we found that the NAD^+-independent deacetylation through Rpd3 precluded deacetylation through Sir2. Thus, the production of the metabolite OAADPR during NAD^+-dependent

deacetylation through Sir2 was abrogated through the prior removal of acetyl groups through Rpd3. The lack of OAADPR in essence prevented the propagation of the SIR complex.

OAADPR was shown to influence the assembly of the SIR complex (Gasser and Cockell 2001; Liou et al. 2005; Onishi et al. 2007). The exact role of OAADPR in the assembly of the SIR complex yet is unknown. Here we provide genetic evidence that OAADPR binds to Sir3 and that mutation within the binding pocket for OAADPR abolished the function of the SIR complex.

Another question was to address how Rpd3 is recruited to the regions adjacent to heterochromatin to perform its boundary function. Surprisingly, a genome-wide binding map for Rpd3 showed a significant underrepresentation of Rpd3 in subtelomeric regions (Kurdistani et al. 2002). This led us to the hypothesis that Rpd3 deacetylates these regions through a transient contact to chromatin. Significantly we found that Rpd3 *in vivo* showed interaction with the large subunit of the chromatin assembly complex CAF-I, Cac1. Through this interaction, Rpd3 was able to deacetylate cytoplasmatic acetylation marks coupled to chromatin assembly and therefore likely performed its boundary function through this transient replication-coupled contact to chromatin.

In summary, our data indicate that histone deacetylation through Rpd3 effectively halts SIR propagations through removal of Sir2 substrates. Thus, although traditionally associated with repression, histone deacetylation by Rpd3 was also capable of the opposite, namely of creating a block to silent chromatin in the context of telomeres. This finding provides novel insights into the formation of euchromatin-heterochromatin boundaries and the role of histone modifications in this process.

2 Material & Methods

2.1 *E. coli* strains

TOP10 F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139Δ(*ara−leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen).

DH5α F⁻ φ80d*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻ (Invitrogen).

BL21 (DE3) F⁻ *omp*T *hsd*S($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* λ(DE3) endA Hte [*argU ileY leuW* Cam^r]

2.2 Media and growth conditions

E. coli strains used for plasmid amplification were cultured according to standard procedures (Sambrook 1989) at 37°C in Luria-Bertani (LB) medium supplemented with either 100 µg/ml ampicillin or 50 µg/ml kanamycin. Media for the growth of *S. cerevisiae* were as described previously (Sherman, 1991). YM (yeast minimal: 6,7 g/L yeast nitrogen base w/o amino acids) medium was supplemented with 2% Glucose and as required with 20 µg/ml for adenine, uracil, tryptophan, methionine and histidine or 30 µg/ml leucine and lysine. YM + 5-FOA (5-fluoro-orotic acid; US Biological) medium contained 5-FOA at 1 mg/ml, 20 µg/ml uracil and 2% glucose. Strains were grown at 30°C unless indicated otherwise.

2.3 *Saccharomyces cerevisiae* strains

Yeast strains used in this study are listed in **Table 1**. Yeast was grown and manipulated according to standard procedures (Sherman 1991). Unless indicated otherwise, yeast were grown on full media (YPD: 10 g/L yeast extract, 20 g/L peptone, 2 g/L glucose). Marker selection was performed on selective minimal plates (YM), and plates containing 5-fluoro-orotic acid were used to select against *URA3*. The growth of yeast strains in the presence of different concentrations of TSA was tested by placing filters containing different amounts of a 7.5 mM TSA solution (in DMSO) on lawns of 2×10^5 cells on YPD plates.

2.4 Genetic manipulation of *S. cerevisiae* strains

Unless indicated otherwise, yeast strains listed in **Table 1** were generated during this study by direct deletion, chromosomal integration, transformation with plasmids or by crossing of yeast cells or originated from the laboratory strains collection.

2.4.1 Crossing, sporulation and dissection of asci

For crosses, the two parental yeast strains of different mating types were streaked together in a drop of YPD media and incubated for eight hours at 30°C on a YPD plate. The mixture was subsequently streaked on a selective YM plate to isolate diploids.

To induce sporulation, diploids were plated on sporulation medium (19 g/L KAc, 0.675 mM ZnAc, 20 g/L agar) and incubated for at least three days at 30°C. For dissection of asci, a loop of cell material was suspended in zymolyase buffer (1 M Sorbitol, 0.1 M NaCitrate, 60 mM EDTA pH 8.0, 5 mg/ml zymolyase) and incubated for 6 min at room temperature. The reaction was stopped by adding 100 µl H_2O. Subsequently, the digested ascospores were dissected using a micromanipulator (Narishige) connected to a Zeiss Axioscope FS microscope. The plates were incubated for two to three days at 30°C. To follow the segregation of markers, plates were replica plated on selective medium.

2.4.2 DNA techniques in *S. cerevisiae*

Gene knock-outs with *kanMX* were performed as described (Wach et al. 1994). *HisMX or HIS3* knockouts were performed using the PCR-mediated knockout technique, thereby replacing the complete open reading frame by the *HisMX* or *HIS3* sequence. Correct integration in all cases was verified by PCR analysis. The deletion of *SAS2* by *TRP1* was as described (Ehrenhofer-Murray et al. 1997). Double mutant *S. cerevisiae* strains were generated by isogenic crosses, followed by tetrad dissection and analysis of marker segregation. The construction of strains carrying telomeric *ADE2* was achieved by integrating TEL VII-L::*ADE2* (*Sal*I/*Not*I linearized pVII-L *ADE2-URA3*-TEL (Gottschling et al. 1990)) into wt and *rpd3*Δ strains. Strains carrying *SAS2* alleles with mutations in the acetyl-CoA binding site (P213A/P214V, HAT$^-$) or the zinc finger (C106L, Zn$^-$) were generated by integrating linearized pAE227 (*SAS2* wt), pAE230 (*sas2* HAT $^-$) or pAE389 (*sas2* Zn$^-$) into AEY3923. The *myc*-epitope tagged versions of *SIR2* in strains deleted for *SAS2* or *RPD3* were constructed as described (Zachariae et al. 1998).

Table 1 *S. cerevisiae* strains used in this study

Strain[a]	Genotype	Source[b]
AEY1	*MATα ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1 can1-100* (=W303-1B)	
AEY2	*MAT***a** *ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1 can1-100* (= W303-1A)	
AEY15	AEY1 *sir2*Δ*::HIS3*	
AEY264	*MAT***a** *his4*	
AEY265	*MATα his4*	
AEY266	AEY2 *sas2*Δ*::TRP1*	
AEY269	AEY1 *sas2*Δ*::TRP1*	
AEY468	AEY2, but *ADE2 lys2*Δ, *hat1*Δ*::HIS3*	
AEY795	AEY2 *rad6*Δ*::URA3*	P. Kaufman
AEY921	AEY1 *HMR***a-e**** *sas3*Δ*::HIS3*	
AEY1012	AEY1 *hmr*Δ*::URA3 hho1*Δ*::HIS3*	
AEY1280*	*MAT***a** *his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::14lexAop-HIS3 URA3::8lexAop-lacZ-GAL4*	
AEY1403	AEY2 *cac1*Δ*::LEU2*	
AEY1492	AEY2 *sas4*Δ*::KanMX, but ADE2*	D. Rivier
AEY1493	AEY2 *sas5*Δ*::HIS3, but ADE2*	D. Rivier
AEY1499	AEY2 *hst1*Δ*::KanMX*	
AEY1501	AEY2 *hst2*Δ*::KanMX*	
AEY1503	AEY2 *hst3*Δ*::KanMX*	
AEY1505	AEY2 *hst4*Δ*::KanMX*	
AEY1507	AEY2 *hdf1*Δ*::KanMX*	
AEY1558*	MAT**a** *leu2-3, 112 trp1-1 ura3-52 prc1-407 pep4-3 prb1-112*	
AEY2247	AEY1 *lys2*Δ *sas2*Δ*::TRP1 sir2*Δ*::URA3* + pRS305-*SIR2*	
AEY2493*	AEY1558 *ASF1-3×HA::TRP1*	
AEY2554*	AEY1558 *SIR2-myc::TRP1*	
AEY2652	AEY1, but *lys2*Δ *ADE2, sas2*Δ*::HIS3*	
AEY2724*	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 ppr1*Δ*::HIS3 hda1*Δ*::KanMX*	
AEY2856*	AEY2554 *sas2*Δ*::KanMX*	
AEY2895	AEY2 *asf1*Δ*::KanMX*	

AEY3049*	AEY1558 *RPD3-9×myc::TRP1*	
AEY3051*	AEY1558 *ASF1-3×HA::URA3 RPD3-9×myc::TRP1*	
AEY3055*	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4*Δ *gal80*Δ *GAL2-ADE2 LYS2::Gal1-HIS3 met2::GAL7-lacZ*	P. James
AEY3132*	*MAT***a** *leu2-3, 112 trp1-1 esa1*Δ*::HIS3 esa1L327S::URA3*	L. Pillus
AEY3183	AEY1 *bdf1*Δ*::HIS3*	
AEY3306	AEY2 *cac1*Δ*::KanMX*	
AEY3307	AEY2 *rpd3*Δ*::HisMX*	
AEY3438	AEY2 *asf1*Δ*::KanMX rpd3*Δ*::HisMX*	
AEY3439	AEY2 *cac1*Δ*::LEU2 rpd3*Δ*::HisMX*	
AEY3440	AEY2 *asf1*Δ*::KanMX cac1*Δ*::LEU2 rpd3*Δ*::HisMX*	
AEY3458	AEY1 *rpd3*Δ*::HisMX*	
AEY3459	AEY2 *asf1*Δ*::KanMX cac1*Δ*::LEU2*	
AEY3519	AEY1 *hml*Δ*::TRP1*	
AEY3533	AEY2 *ade3*Δ *sas2*Δ*::TRP1* + p*URA3-ADE3-SAS2*	
AEY3643	*MAT***a** *ade2-1 lys2*Δ *ura3-1 his3-11,15 leu2-3, 112 trp1-1 can1-100 hht1-hhf1*Δ*::LEU2 hht2-hhf2*Δ*::HIS3 rpd3*Δ*::HisMX + pURA3-HHT1-HHF1*	
AEY3680	AEY2, but *lys2*Δ *ADE2, dot1*Δ*::KanMX*	
AEY3700	AEY1 *bdf2*Δ*::LEU2*	
AEY3703*	AEY2554 *rpd3*Δ*::KanMX*	
AEY3708	AEY2 *rpd3*Δ*::HisMX sir2*Δ*::URA3*	
AEY3712	AEY2 *sas2*Δ*::TRP1 rpd3*Δ*::HisMX sir2*Δ*::URA3*	
AEY3752	*AEY2,* but *TRP1, rco1*Δ*::KanMX*	M. Keogh
AEY3753	*AEY2,* but *TRP1, eaf3*Δ*::KanMX*	M. Keogh
AEY3754	*AEY2,* but *TRP1, rpd3*Δ*::KanMX*	M. Keogh
AEY3755	*AEY2,* but *TRP1, dep1*Δ*::KanMX*	M. Keogh
AEY3756	*AEY2,* but *TRP1, sds3*Δ*::KanMX*	M. Keogh
AEY3766	AEY1, but *lys2*Δ *ADE2, htz1*Δ*::KanMX*	
AEY3770	AEY1 *rpd3*Δ*::KanMX*	
AEY3771	AEY2, but *lys2*Δ *ADE2, rpd3*Δ*::KanMX*	
AEY3776	AEY2 *eaf3*Δ*::KanMX sas2*Δ*::HIS3*	
AEY3778	AEY2 *rco1*Δ*::KanMX sas2*Δ*::HIS3*	
AEY3780	AEY2 *dep1*Δ*::KanMX sas2*Δ*::HIS3*	
AEY3782	AEY2 *sds3*Δ*::KanMX sas2*Δ*::HIS3*	
AEY3844	AEY2, *lys2*Δ *sir3*Δ*::NatMX*	
AEY3868	*AEY1,* but *ADE2, sas2*Δ*::TRP1 hst2*Δ*::KanMX*	
AEY3908	AEY1 *adh4::URA3-*$(C_{1\text{-}3}A)_n$	L.Pillus
AEY3909	AEY1 *adh4::URA3-*UAS_{Gal}-$(C_{1\text{-}3}A)_n$	L.Pillus
AEY3923	AEY2 *lys2*Δ *sas2*Δ*::TRP1 rpd3*Δ*::KanMX* + pRS316-*SAS2*	
AEY3924	AEY3923 + pAE227	
AEY3925	AEY3923 + pAE230	
AEY3926	AEY3923 + pAE389	

AEY3927	AEY3923 + pRS313	
AEY3930	AEY1 *sas2*Δ*::TRP1 rpd3*Δ*::KanMX sir1*Δ*::LEU2* + pRS316-*SAS2*	
AEY3931	AEY2 *lys2*Δ *sas2*Δ*::TRP1 rpd3*Δ*::KanMX sir2*Δ*::HisMX* + pRS316-*SAS2*	
AEY3932	AEY1 *sas2*Δ*::TRP1 rpd3*Δ*::KanMX sir3*Δ*::LEU2* + pRS316-*SAS2*	
AEY3933	AEY1, but *lys2*Δ *ADE2, sas2*Δ*::TRP1 rpd3*Δ*::KanMX sir4*Δ*::LEU2* + pRS316-*SAS2*	
AEY3943	AEY2 *TEL VII-L::ADE2-URA3*	
AEY3944	AEY1 *rpd3*Δ*::KanMX TEL VII-L::ADE2-URA3*	
AEY3945	*MAT***a** *ade2-1 lys2*Δ *ura3-1 his3-11,15 leu2-3, 112 trp1-1 can1-100 hht1-hhf1*Δ*::LEU2 hht2-hhf2*Δ*::HIS3 rpd3*Δ*::HisMX sas2*Δ*::KanMX + pURA3-HHT1-HHF1* + pRS317-*SAS2*	
AEY4003	AEY2 *lys2*Δ *sas2*Δ*::TRP1 rpd3*Δ*::KanMX sir3*Δ*::NatMX* + pRS316-*SAS2*	
AEY4004	AEY4003 + pAE232	
AEY4005	AEY4003 + pAE1225	
AEY4021	AEY1 *sin3*Δ*::KanMX adh4::URA3-UAS*$_{Gal}$*-(C*$_{1-3}$*A)*$_n$	
AEY4144	AEY1 *rpd3*Δ*::KanMX adh4::URA3-UAS*$_{Gal}$*-(C*$_{1-3}$*A)*$_n$	
AEY4169	AEY1 *rpd3*Δ*::KanMX adh4::URA3-(C*$_{1-3}$*A)*$_n$	
AEY4179	AEY4144 + pAE1208 + pRS315	
AEY4180	AEY4144 + pAE1208 + pSB32-*RPD3*	
AEY4181	AEY4144 + pAE1208 + pRS415-*rpd3* (H150:151A)	
AEY4243	AEY2 *HML*::UAS$_{Gal}$-*ADE2*-UAS$_{Gal}$-*URA3*	R. Kamakaka
AEY4244	AEY1 *ade2*Δ*::KanMX HMR*::UAS$_{Gal}$-*ADE2*-UAS$_{Gal}$-a1prm-*URA3*	R. Kamakaka
AEY4258	AEY2 *sas2*Δ*::HIS3 rco1*Δ*::KanMX* + pRS316-*SAS2*	
AEY4259	AEY1 *sas2*Δ*::HIS3 sds3*Δ*::KanMX* + pRS316-*SAS2*	
AEY4260	AEY1 *sas2*Δ*::HIS3 rco1*Δ*::KanMX sds3*Δ*::KanMX* + pRS316-*SAS2*	
AEY4305*	*MAT***a** *leu2-3, 112 trp1-1 ura3-52 prc1-407 pep4-3 prb1-112 sir3*Δ*::KanMX* + pRS315-*SIR3-HA::TRP1*	
AEY4307*	*MAT***a** *leu2-3, 112 trp1-1 ura3-52 prc1-407 pep4-3 prb1-112 sir3*Δ*::KanMX* + pRS315-*sir3*Δ*578-585-HA::TRP1*	
AEY4316	AEY2, but *met3, sas2*Δ*::TRP1 set2*Δ*::KanMX* pRS316-*SAS2*	

[a] Strains were isogenic to W303 (AEY1, AEY2) except those marked with an asterisk.
[b] Unless indicated otherwise, strains were constructed during this study or were from the laboratory strain collection.
*set2*Δ, *set3*Δ, *set4*Δ, *set5*Δ, *set6*Δ, *set7*Δ, *hos1*Δ, *hos2*Δ, *hos3*Δ, *rtt106*Δ, *snf1*Δ, *ctk1*Δ, *cka1*Δ, *isw2*Δ and *ume6*Δ strains originate from the Research Genetics yeast deletion library.

2.5 Molecular cloning

Plasmids were generated according to standard cloning techniques (Sambrook 1989). Kits for plasmid isolation were purchased from Qiagen. Enzymes and respective buffers were used from NEB, Promega and Stratagene. Oligonucleotides used for molecular cloning, mutagenesis, gene knockouts or tagging are listed in **Table 3**. For the exchange of single amino acids or deletion of eight or nine amino acids within the *SIR3* ORF, the PCR sewing technique was used. With this technique in a first step short complementary overhangs were generated that served as polymerase start site in a second PCR reaction. Gal4 fusion constructs were generated by cloning the ORFs of the respective genes via the indicated restriction sites (**Table 3**) into the plasmid containing the Gal4 binding domain (GBD).

Table 2 Plasmids used in this study

Plasmid [a]	Description	Plasmid [a]	Description
pAE88	pRS316-*SAS2*	pAE1192	pRS314-*HHT1 hhf1-10* = H4 K5, 8, 12, 16Q (R. Morse)
pAE227	pRS303-*SAS2*		
pAE230	pRS303-*SAS2-(HAT⁻)*	pAE1197	pRS314-*HHT2 hhf2* H4 K56Q (J. Boeke[1])
pAE232	pRS315-*SIR3*	pAE1198	pRS314-*HHT2 hhf2* H4 K59Q (J. Boeke[1])
pAE389	pRS303-*SAS2-(Zn⁻)*	pAE1199	pRS314-*HHT2 hhf2* H4 K77Q (J. Boeke[1])
pAE524	pACT2	pAE1200	pRS314-*HHT2 hhf2* H4 K79Q (J. Boeke[1])
pAE525	pBTM117c	pAE1208	p*HIS3-GBD-RPD3*
pAE535	pACT2-*ASF1*	pAE1222	p*HIS3-GBD-HST2*
pAE655	pRS414-*HHT1/HHF1* K16R	pAE1223	p*HIS3-GBD-HOS1*
pAE656	pRS414-*HHT1/HHF1* K5R, K8R	pAE1224	p*HIS3-GBD-HOS2*
pAE700	pRS414-*HHT1/HHF1* K5R, K12R	pAE1225	pRS315-*sir3* Δ578-585
pAE713	pRS305-*sir2* (N345A)	pAE1227	pRS315-*sir3* Δ684-692
pAE822	pRS314-*HHF1/HHT1*	pAE1256	p*TRP1-GBD-HST1*
pAE823	pRS314-*HHF1/HHT1* K9R	pAE1257	p*TRP1-GBD-HOS3*
pAE824	pRS314-*HHF1/HHT1* K14R	pAE1258	p*TRP1-GBD-HST3*
pAE954	pBTM117c-*RPD3*	pAE1259	p*TRP1-GBD-HST4*
pAE955	pBTM117c-*HDA1*	pAE1260	pRS317-*SAS2*
pAE998	pACT2-*CAC1*	pAE1281	pRS415-*rpd3* (H150:151A)
pAE1010	pRS426-Asf1-HA	pAE1317	p*TRP1-GBD-HDA1*
pAE1012	pRS424-Rpd3-myc	pAE1352	pGAD-C2-*SIR3* (307-978)
pAE1117	p*ADE3-URA3-SAS2*	pAE1353	pGAD-C2-*sir3* (307-978) Δ578-585
pAE1186	pSB32-*RPD3*	pAE1354	pGBD-C2-*SIR3* (307-978)
pAE1191	pRS314-*HHF1 hht1-3* = H3 K4, 9, 14, 18, 23, 27Q (R. Morse)	pAE1355	pGBD-C2-*SIR4* (839-1358)

[a] Plasmids were generated according to standard cloning techniques; [1] (Hyland et al. 2005)
Unless indicated otherwise, plasmids were constructed during this study or originated from the laboratory plasmid collection.

Table 3 Oligonucleotides used for molecular cloning, tagging, knockout or mutagenesis

Oligonucleotide	Oligonucleotide sequence (in 5' to 3' direction)[a]
Rpd3Δ::HisMX fw	ACAATTGCGCCATACAAAACATTCGTGGCTACAACTCGATATCCGTGCAGCGTACGCTGCAGGTCGAC
Rpd3Δ::HisMX rv	TTCTTTTGTTTCACATTATTTATATTCGTATATACTTCCAACTCTTTTTTATCGATGAATTCGAGCTCG
his5 S. pombe up	CTATGGGTAACTTTGCCGG
Rpd3-XbaI.fw	CGGATTCAtctagaATGGTATATGAAGCAACACCTTTTGATCCG
Rpd3-SpeI.rv	CGGATTCAactagtTCAATAGAATTCATTGTCATGCTCAACATGTAGG
Rpd3-XhoI.rv	CGGATTCActcgagTCAATAGAATTCATTGTCATGCTCAACATGTAGG
Rpd3-SpeI.fw	CGGATTCAactagtATGGTATATGAAGCAACACCTTTTGATCCG
dot1Δ::KanMX.fw	AAGGAGGTCACCAGTAATTGTGCGCTTTGGTTACATTTTGTTGTACAGTACGTACGCTGCAGGTCGAC
dot1Δ::KanMX.rv	TATTTCTACTTAGTTATTCATACTCATCGTTAAAAGCCGTTCAAAGTGCCATCGATGAATTCGAGCTCG
dot1(-300).fw	CGGCTAAAGACCGTAGAGTG
dot1(+300).rv	GGTGAGGAGAAATTATCTGCTCC

sin3Δ::KanMX.fw	ATTTGGAAAAGGACAAAATATCTAAGAAACAAGTTATACATTGTACAAAACGTACGCTGCAGGTCGAC
sin3Δ::KanMX.rv	AAAGACCCTGTCGTACTAAAGATTTTTGTTCTAAATCTAGTTAAAACTACATCGATGAATTCGAGCTCG
sin3(-300).fw	CCAGTACGGGTAATGATATCGG
sin3(+300).rv	CGACTGGGGTGAATCACTAGACG
Sir3 (WalkerA).fw	AAACTATTTTATATTTTCCAGCTTGTGAATGATGTAATGG
Sir3 (WalkerA).rv	CATTCACAAGCTGGAAAATATAAAATAGTTTATTTTGGGAAGAC
Sir3 (315).fw	CGAGgtcgacATCGATAAGC
Sir3 (315).rv	GCTCCACCGCGGTGgcggccgc
Gal4-Rpd3.fw	AGTGgtcgacATATGGTATATGAAGCAACACCTTTTG
Gal4-Rpd3.rv	AGTGgtcgacATTCAATAGAATTCATTGTCATGCTCAAC
Gal4-Hos1.fw	AGTGgtcgacATATGTCGAAATTGGTCATATCAACG
Gal4-Hos1.rv	AGTGgtcgacATTTACAGTTCGTAAAACTTCATAAGTTCG
Gal4-Hos2.fw	AGTGgtcgacATATGTCTGGAACATTTAGTTATGATGTG
Gal4-Hos2.rv	AGTGgtcgacATCTATGAAAAGGCAATCAATCCACTG
Gal4-Hos3.fw	AGTGggatccATATGTCTTCCAAGCATTCAGATCC
Gal4-Hos3.rv	AGTGggatccTCACCATCTTCCACCACTTCTTGTTG
Gal4-Hda1.fw	AGTGgtcgacATATGGATTCTGTAATGGTTAAGAAAGAAG
Gal4-Hda1.rv	AGTGgtcgacATTCATTCTTCATCACTCCATTCTTC
Gal4-Hst1.fw	AGTGgtcgacATATGAACATATTGCTAATGCAACGG
Gal4-Hst1.rv	AGTGgtcgacATTTACTGTTGTTTCTTTCGTGGCTG
Gal4-Hst2.fw	AGTGgtcgacATATGTCTGTTTCTACCGCCTCTAC
Gal4-Hst2.rv	AGTGgtcgacATTTATTCTTTAGCGGCTTTTTGTGAAGAAG
Gal4-Hst3.fw	AGTGgtcgacATATGACTTCAGTATCGCCCTCGCC
Gal4-Hst3.rv	AGTGgtcgacTTATGAGGCTTGGTTGTCACCG
Gal4-Hst4.fw	AGTGgtcgacATATGAAGCAAAAATTTGTACTACCGATCACC
Gal4-Hst4.rv	AGTGgtcgacCTATAATAATGAGGTAACATGCTGAC
Rpd3 H188A.fw	CCAGAGTTCTGTATATTGATATTGATG**ggg**ccATGGTGATGGTGTAGAGG
Rpd3 H188A.rv	CCTCTACACCATCACCATgg**g**cc**c**CATCAATATCAATATACAGAACTCTGG

[a] Sites for restriction endonucleases are shown in lower case letters, sequences complementary to the manipulated genes are underlined, bold letters indicate nucleotide changes according to the wt allele.

2.6 Chromatin immunoprecipitations

Chromatin immunoprecipitations **(**ChIPs) were performed essentially as described (Rusche and Rine 2001), except that protein-G Sepharose beads were used. Crosslinking was carried out by adding formaldehyde (1% final concentration) directly to the exponentially growing culture and incubating for 30 min at room temperature. Yeast extracts were prepared by disrupting 100 OD of cells in 500 µl lysis buffer (50 mM HEPES pH7.5, 140 mM NaCl, 1mM EDTA, 1% Triton X-100, 0,1% Na-Deoxycholate, 1x "complete" proteinase inhibitor [Roche Diagnostics]) with glass beads for 5 min at 4°C using a vortex mixer. The lysate was cleared by centrifugation for 10min at 4°C. Precipitations were performed with antibodies against acetylated lysines (α-H4-K5Ac, α-H4-K12Ac, α-H4-K16Ac (Suka et al. 2001) [Upstate]), α-H4 (Abcam) or α-myc [Invitrogen]). Samples were analyzed by quantitative real-time PCR using the Rotor Gene 3000 (Corbett Research). The PCR reactions contained real master mix (Eppendorf) with SYBR green. Samples were cycled 45 times for 15 sec at 94°C, 30 sec at 56°C and 40 sec at 68°C. The C_t value for each reaction was determined, and a standard curve of input samples was used to calculate the amounts of DNA precipitated during the ChIP experiment relative to input DNA. The amount of DNA precipitated with the anti-acetyl-lysine antibodies was given relative to the amount of histone H4 precipitated for the respective regions or were given relative to the acetylation of a control region (*SPS2*). Samples were analyzed in triplicate for two independent ChIPs and standart deviations were calculated. The oligonucleotide sequences used for quantitative PCR are listed in **Table 4**.

Table 4 Oligonucleotides used for ChIP analysis

Oligonucleotide	Oligonucleotide sequence (in 5' to 3' direction)
INO1 (-0,25).fw	GGGGTTGGATGCGGAATCG
INO1 (-0,25).rv	CATTGCCGCCAACGCAGAGG
INO1 (-128).fw	GAGACGTATATAAATTGGAGCTTTCG
INO1 (131).rv	CGGCCACTAGCTGTCTTCG
INO1 (0,5).fw	GGTTGGGACATCAATAACGC
INO1 (0,5).rv	GGATATCGCGTCTGATGCG
INO1 (1).fw	GGGTACATTCATTGCGGG
INO1 (1).rv	GATGACAATGCAGTGGTCAAC
INO1 (1375).fw	GTGTCCTATAAGAAGGTGGAC
INO1 (1637).rv	GCGGAAAAAGAAAAAGAGAGTCG
Hxt6-3'.fw	GCCATTGTACAAGAGAATGTTCAGC
Hxt6-3'.rv	GCGCCTACTTCGCTTCTAG
Hxt6-3'+1000.fw	CGCAGTACCAGAAAGCCGC
Hxt6-3'+1000.rv	CTCTTCTTTTTACTACGTGTCCTGC
Hxt6-3'+2000.fw	GGAACAACCATCACCCCACAC
Hxt6-3'+2000.rv	CTGTCTTGTTGAAGCTCAGATTGCTC
Hxt7-5'-1000.fw	GCATCTCGAAAAATTTCATTCACACGG
Hxt7-5'-1000.rv	GCCCTCTAGTAAAGCGTTATGGG
Hxt7-5'.fw	GCCAATACTTCACAATGTTCGAATC
Hxt7-5'.rv	GAGACAGTGACATAGGCAGAAGC
Gat1-3'.fw	CCCCCAAAAAAAAAAAGTACTCGC
Gat1-3'.rv	CCCAACATCTATTGCGGCGG
Gat1-3'+450.fw	CGTCACTAGAAGCTCAGTAAGAGC
Gat1-3'+450.rv	GGTCCCGCTTTTACACATCAC
Gat1-3'+950.fw	CGCTGTGTACCGCAATATCC
Gat1-3'+950.rv	CCTTCCGATTCTGAAGGTGG
Gat1-3'+1400.fw	CCAAAAGTTGGGTTTGCTCACG
Gat1-3'+1400.rv	GGTGCCTATCCGAAGGTTCGC
Pau5-3'.fw	CCGAACTCCGAGATTCTTGC
Pau5-3'.rv	CCCTTAGGGATAAAATGTGATACG
ars603.fw	GGCTTTTATTCTATTAAAATGATCTATCATAGACAC
ars603.rv	CGAGGCTAAATTAGAATTTTTGAAGTCCC
ars607.fw	GGTGATATAAACACTACATTCGC
ars607.rv	GCTTTCTAGTACCTACTGTGC
Aap1-5'.fw	CGTTGTTTGGCAGTACTTCACG
Aap1-5'.rv	CCCACATTTTTTGAACTCGTTGTG
Aap1-5'-350.fw	CGCCTAGAAAACAGCGTTTGC
Aap1-5'-350.rv	CCGACGCTAACTTTGTTCGAGC
Aap1-5'-900.fw	GGTTCTACAAAAACACAAATATCCGG
Aap1-5'-900.rv	CCTCATCTTACCGCAGTGC
Aap1-5'-1650.fw	GCAAGGGAGAACTTAGCAGC
Aap1-5'-1650.rv	CCTGCCGTGCGTTGATGACC
YHR048W-5'-900.fw	GCGTACTGTGTAGTATGCGC
YHR048W-5'-900.rv	GGCGCAGCTCTTCTGTTAGC
YHR048W-5'-350.fw	GGAAATGTCATGTTGCGCAGG
YHR048W-5'-350.rv	CCAACACTAAATCCGTAGGACC

YHR048W-5'.fw	GGAGATTAGCACGCTTTTCGC
YHR048W-5'.rv	GCACTCGCTATTTGAAATTCTGC
TelVIR 0,5 up	CCTTTGACAATAGCCTTTCAAAGC
TelVIR 0,5 up (2)	CGAGTGGATGCACAGTTCAGAG
TelVIR 0,5 down	CGCGTTATGACAATTTTATGTAGATATCC
Tel VI R 1 (2) up	GTTATGTTAGAGATAACTGTGAG
Tel VI R 1 down	GCTTGTTAACTCTCCGACAG
Tel VIR (2,5).up	GCAATGAATCTTCGGTGCTTGG
Tel VIR (2,5).down	CCATACCAATATCAACTTCACGG
Tel VIR (5).fw	CCCCGCCTTTGAAGATTGTCCC
Tel VIR (5).rv	CGAGACCCACTTGTATTCTTAGTGC
TelVIR (7,5).fw	CCTCTATAGGACCTGTCTCATGG
TelVIR (7).rv	GGAAGTCTACACTAATAGCTATGCG
Tel VIR (15).up	GCGCAATATATAGCAGAAGAGC
Tel VIR (15).down	CAATTCGTCGATAAAGTGC
HMR up(3)	CTATCAGTGTTTTCAATTTTTTATTAAACAATG
HMR-E down (2)	GATGGATAGCTCTGGTAATTTCTAG
HMR.fw	CCCATCAACCTTGAAAAAAAGTAGAAACG
act1 (1372).fw	GCCGGTGACGACGCTCCTCGTGC
act2 (1922).rv	CAGCAGTGGTGGAGAAAGAG
Sps2(-0,25).fw	GTGTGTATATGCATGAGTTTTTGTTTTCC
Sps2(0,25).rv	GGATCGTTGCATTAGTGTTAACC
Sps2 (117).rv	GTTCAACTGGTTCAACATGCTATCC
Sps2 (5).rv	GGCATTTTCTTTTATAGTCTAAGTAATGCC

2.7 Quantitative reverse transcriptase PCR (qRT-PCR)

The expression of subtelomeric genes was determined by reverse transcription followed by quantitative real-time PCR. Total RNA from 0.5 OD units of yeast cells was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using 50 µM oligo$(dT)_{20}$ in a 20 µl reaction. Quantitative real-time PCR was performed as described for the ChIP experiments. Results are given as expression of subtelomeric genes relative to *SPS2* expression. The oligonucleotide sequences used for qRT-PCR are listed in **Table 5**.

Table 5 Oligonucleotides used for gene expression analysis

Oligonucleotide	Oligonucleotide sequence (in 5' to 3' direction)[a]
SOR1.up	GTAGTTCTAGAGAAAGTCGGCG
SOR1.down(2)	GGCGCCTTCAATATGTACTTACC
ACT1 up(2)	GGTGATGGTGTTACTCACGTCG
act2 (1922).rv	CAGCAGTGGTGGAGAAAGAG
COS8.fw	CCGTTCTACCTCAAGATGTTTTCCG
COS8.rv	CCAGGAACAGGACAAGAAGTGAAAC
Sps2 (5).fw	GCCAATTTGGAAAACACAAACATTCTTCAC
Sps2 (0,25).rv	GGATCGTTGCATTAGTGTTAACC
IRC7.fw	CCCCGCCTTTGAAGATTGTCCC = Tel VIR (5).fw
IRC7.rv	CCCAATTGCAAAACTTTCATCCC = YFR055W.down
YCR106W (561).fw	CGCCTTAGGGTTATTATACAATGC
YCR106W (748).rv	CGCTCTCAAAGAGTGAAATGTCC

YDR541C (26).fw	GCGCTTCAGGTTTTATTGCCTTGC
YDR541C (221).rv	CCACGTTTCTGCAGAACCTTATCG
HXK1.fw	CCAAATCAATGGCCAAATAGTTACC
HXK1.rv	GGTTCCATGGCTGATGTGCC
SIR2.fw	GCACACTAAAGCTGCGCTCGG
SIR2.rv	GGGTTTTTACTGATTATGATTGGCC
Sir3 (3).fw	GGCTAAAACATTGAAAGATTTGGACG
Sir3 (214).rv	GGATCAAGTAGACAGAATATGTTTCC

2.8 Synthetic lethal screen

The synthetic lethal screen with *sas2*Δ was performed by searching for non-sectoring derivatives of an *ade2 ade3 sas2*Δ*::KanMX* strain carrying p*ADE3-URA3-SAS2* (AEY3533) (Bender and Pringle 1991). Mutants were characterized by genetic crosses, and the mutant gene was cloned by complementation using a *LEU2*-CEN genomic library. For details, see 3.1.1.

2.9 Yeast genetic assays

Telomeric silencing was tested using either a telomeric *ADE2* reporter (Gottschling et al. 1990) or a *URA3* reporter system with or without Gal4 binding sites between *URA3* and telomeric heterochromatin (Jacobson and Pillus 2004). Strains carrying telomeric *ADE2* (Gottschling et al. 1990)) were used to screen for telomeric silencing in wt and *rpd3*Δ strains. The *ADE2* phenotype allowed to visually screen for the strength of silencing by the colony colour, in that cells that stay red (due to the *ade2* mutation) effectively repress telomeric *ADE2*.

Targeted boundary function was tested with a reporter system in that the telomere VII-L was replaced by an *URA3* gene and a Gal4 *UAS* between telomeric heterochromatin and the *URA3* reporter (see 3.1.11, (Jacobson and Pillus 2004)). Putative boundary factors were recruited to this construct as Gal4-fusion proteins.

HM silencing was monitored by measuring the mating ability of the candidate strains. For this purpose, 2.5 OD units of the mating test strains AEY264 (*MAT***a** *his4*) and AEY265 (*MAT*α *his4*) were plated on a YM plate. 1:6-fold serial dilutions of 0.5 OD of the candidate strains were spotted onto the test lawns and incubated for 2 days at 30°C. Since HM derepression leads to pseudodiploid yeast cells with lower mating efficiency (see 1.8.1), the yield of diploid cells was taken as indicator for *HM* silencing.

2.10 Protein-protein interaction assays

2.10.1 Yeast-two-hybrid assay

Two-hybrid interactions were tested by co-transforming the two-hybrid test strain (AEY1280 or AEY3055) with the bait and prey vectors (pBTM117c + pACT2 or pGAD + pGBD, (James 2001)). The reporter strain(s) contained two reporters under control of the Gal4 promoter, one *HIS3* gene and a *lacZ* reporter. Activation of the *HIS3* reporter was tested by plating serial dilutions of the strains on plates lacking histidine and incubating them for two to three days at 30°C. Activation of the *lacZ* reporter was tested by transferring streaks of the strains on a nitrocellulose membrane (Breeden and Nasmyth 1985). The membrane was then frozen in liquid nitrogen and subjected to the colour reaction using bufferZ + X-Gal (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0 + freshly added 0,1% X-Gal). Activation of the *lacZ* gene is given as estimation in tables (- no activation; ++ activation; +++ strong activation).

2.10.2 Co-Immunoprecipitation

Co-immunoprecipitation (CoIP) experiments were used to directly test for protein-protein interactions *in vivo*. For this purpose, one protein was precipitated from cell extracts and the resulting precipitate was probed for the occurrence or the amount of the other co-precipitated protein. Per CoIP-reaction, 40 OD of yeast cells were lysed

in 150 mM NaCl, 50 mM Tris-HCl pH 7.6, 0.05% Nonidet-P40, 1 mM DTT, freshly supplemented with protease inhibitors (Roche) and 1 mM PMSF. Cells were disrupted by vortexing with glass beads (0,5 mm in diameter).

2.11 Yeast protein extracts for SDS-PAGE and Western Blotting

Approximately 10 OD of yeast cells were harvested with 3000×g and washed once in PBS+P_i (phosphate buffered solution as described in (Sambrook 1989) supplemented with protease inhibitors, consisting of 1 µg/ml aprotinine, 1 µg/ml leupeptine, 1 µg/ml pepstatine, 1 µg/ml TPCK and 0,2 mM PMSF). Then the cells were resuspended in PBS+P_i (according to 0.1 OD/µl) and glass beads (0.5mm in diameter) were added slightly below liquid level. Cells were disrupted by vortexing seven times for 30 sec followed by 30 sec incubation on ice. The resulting lysate was mixed with the appropriate amount of 4 x Laemmli buffer, incubated for 5 min at 70°C. Protein extracts were cleared off the glass beads and centrifuged for 10 minutes at 14,000 rpm in a 5417C Eppendorf table top centrifuge. The supernatant was collected and 1.6-16 µl were applied on SDS gels. Alternatively, the samples were stored at -80°C for later analysis.

2.12 SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE in Tris-glycine buffer according to standard methods (Laemmli 1970). Transfer to nitrocellulose membranes (Pharmacia) was accomplished by blotting with the BIO-RAD Tank Transfer System with 5,5 mA×h/cm^2 in 25 mM Tris; 52 mM glycine; 10% methanol. The nitrocellulose membrane was subsequently blocked for 1 hour at RT in TBS-T, 5% milk (50 mM Tris-HCl pH7.5; 150 mM NaCl; 0,1% Tween-20, 5% milk powder). After incubation over-night at 4°C with the primary antibody (concentrations see below) in TBS-T, 5% milk, the blot was washed 2 times for 5 minutes with TBS-T. It was then incubated with the appropriate secondary antibody (concentrations see below) in TBS-T, 5% milk for 30 to 60 minutes at RT. After washing 4 times for 5 minutes with TBS-T, the Amersham ECL™ Western Blotting Analysis System (GE healthcare) was used to visualize Western blot signals on Amersham Hyperfilm™ ECL chemiluminiscence films (GE healthcare).

Anti-epitope antibodies were purchased from Invitrogen (α-myc; 1:5,000) and Sigma (α-HA 1:1000). Secondary antibodies conjugated to horseradish peroxidase were purchased from Sigma (sheep α-mouse; 1:1000 and goat α-rabbit; 1:1000). Antibodies were used for Western blotting in the concentrations as indicated.

2.13 Computational modelling of the Sir3 structure

The sequence of Sir3 of yeast was taken from SwissProt (http://www.expasy.ch/sprot/) and submitted to mGenThreader (McGuffin et al. 2000). The best sequence-structure alignment was obtained between residues 532 and 834 of the sequence of Sir3 and the structure of Cdc6/Cdc18 (Liu et al. 2000), a protein of the AAA+ family. Thus, the molecular structure of Cdc6/Cdc18 was taken from the Protein Databank PDB (Berman et al. 2000), entry 1fnn, and used as main template in the structural modelling. The structures of two loops that were not covered by this template were drawn from the structures of Cdc6/ORC from *Aeropyrum pernix* (Singleton et al. 2004) (PDB entry 1w5s) and the delta' subunit of the clamp-loader complex of *Escherichia coli* DNA polymerase III (PDB entry 1a5t). Using these three templates, the structure of residues 532–834 of Sir3 was modelled with Modeller 9v2 (Marti-Renom et al. 2000). The model was visualized using PyMOL (DeLano Scientific LLC, San Francisco, CA, USA. Pymol, 2006).

3 Results

3.1 Restriction of heterochromatin spreading by the HDAC Rpd3

3.1.1 Deletion of *RPD3* was synthetically lethal in the absence of the histone acetyltransferase complex SAS-I

Posttranslational modifications of histones create an extra level of information that is used by eukaryotic cells to control the level of gene expression. Histone acetylation and the corresponding enzymes, for instance the HAT Sas2, have historically been linked to chromatin opening and therefore are viewed as transcriptional activators. The absence of Sas2 is not lethal to yeast cells, but causes specific defects in *HM*, telomeric and rDNA silencing (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). To increase our knowledge about the SAS-I complex, we sought to identify factors that become essential for yeast survival in the absence of Sas2, by performing a synthetic lethal screen with *sas2*Δ cells. Synthetic lethal screens allow the identification of genetic interactions between factors that share similar biological function. This screen uses the principle that the loss of one factor leads to a mild phenotype, but that the absence of a second parallel pathway causes lethality in the double mutant. To identify factors that were synthetically lethal with *sas2*Δ, we used an *ade2 ade3 sas2*Δ::*TRP1* strain that carried a p*ADE3-URA3-SAS2* plasmid (AEY3533) (Bender and Pringle 1991). This strain was transformed with a transposon library to generate random mutations by transposon insertion within the yeast genome. The experimental setup allowed the identification of mutants that were unable to lose the plasmid borne *SAS2* gene by a visual screen. Due to the *ade2* mutation, cells accumulate an intermediate of the adenine biosynthesis pathway, which is visible as red pigments. Accumulation of the colour is suppressed by an additional *ade3* mutation. Therefore, cells that were able to lose the p*ADE3-URA3-SAS2* plasmid showed white sectors within red colonies, whereas cells that could not lose the *SAS2* plasmid stayed red. We found a set of colonies that were unable to lose the p*ADE3-URA3-SAS2* plasmid, as indicated by red colonies. The inability of one the nonsectoring colonies to lose the p*ADE3-URA3-SAS2* plasmid was abrogated by transformation of the strain with a p*LEU2-SAS2* plasmid but not a p*LEU2* control plasmid, indicating that the dependence for the plasmid in this candidate was due to the *SAS2* gene. Genetic analysis of this candidate indicated that a recessive mutation caused synthetic lethality with *sas2*Δ.

To identify the mutation that caused lethality with *sas2*Δ in this candidate, the strain was transformed with a p*LEU2*-CEN plasmid library containing fragments of the yeast genome, and transformants were screened for their ability to lose the p*ADE3-URA3-SAS2* plasmid by screening for 5-FOA resistance. One complementing library plasmid was isolated, and the sequence of the genomic insert was determined. This plasmid contained a DNA fragment of chromosome XIV, representing a sequence close to the telomere XIV-L, ranging from the telomeric repeats up to the *PEX6* gene (20 kb away from telomere XIV-L). This sequence contained six genes, including *RPD3.*

To test whether *RPD3* complemented the synthetic lethal defect, a plasmid containing only *RPD3* was constructed and transformed into the candidate strain. Importantly, the candidate strain with this plasmid was able to lose the p*ADE3-URA3-SAS2* plasmid, showing that the *RPD3* gene complemented the lethality of the double mutant candidate. This suggested, that mutation of *RPD3* was synthetically lethal in the absence of Sas2 (data not shown).

We further tested whether a complete deletion of *RPD3*, rather than a mutant allele, was synthetically lethal with *sas2*Δ by performing a cross of isogenic strains deleted for *RPD3* and *SAS2*. To this end, W303 yeast strains deleted either for *RPD3* or *SAS2* were crossed, and diploids were sporulated. After dissection of the ascospores, the analysis of the tetrads revealed that strains deleted for both *RPD3* and *SAS2* showed severe growth defects and lethality for most of the double mutants (**Figure 7A**). At 37°C, *sas2*Δ *rpd3*Δ cells were fully inviable (not shown). This data demonstrated synthetic lethality between *sas2*Δ and *rpd3*Δ and suggested unexpected parallels in the function of the two enzymes.

Our above results indicated that strains deleted for *RPD3* and *SAS2* were not viable. A further test of this notion would be to inhibit the activity of one enzyme in strains deleted for the other. As a member of the class of NAD^+-independent HDACs, Rpd3 can be inhibited by the HDAC inhibitor Trichostatin A (TSA). NAD^+-independent HDACs show high TSA sensitivity, whereas other HDACs, most notably the Sir2-like HDACs, are insensitive to TSA (Yoshida et al. 1990; Bernstein et al. 2000). Since *sas2*Δ cells were unable to survive in the absence of Rpd3, we hypothesized that they would also be sensitive to inhibition of Rpd3 by TSA. Significantly, *sas2*Δ cells showed a zone of growth inhibition around TSA-containing filter discs, whereas wild-type (wt) cells were only mildly inhibited at the highest TSA concentration (**Figure 7B**). This indicated that inhibition of HDACs, including Rpd3, by TSA

was lethal in combination with *sas2*Δ, further supporting the notion that Rpd3 became necessary for the survival of yeast cells in the absence of Sas2.

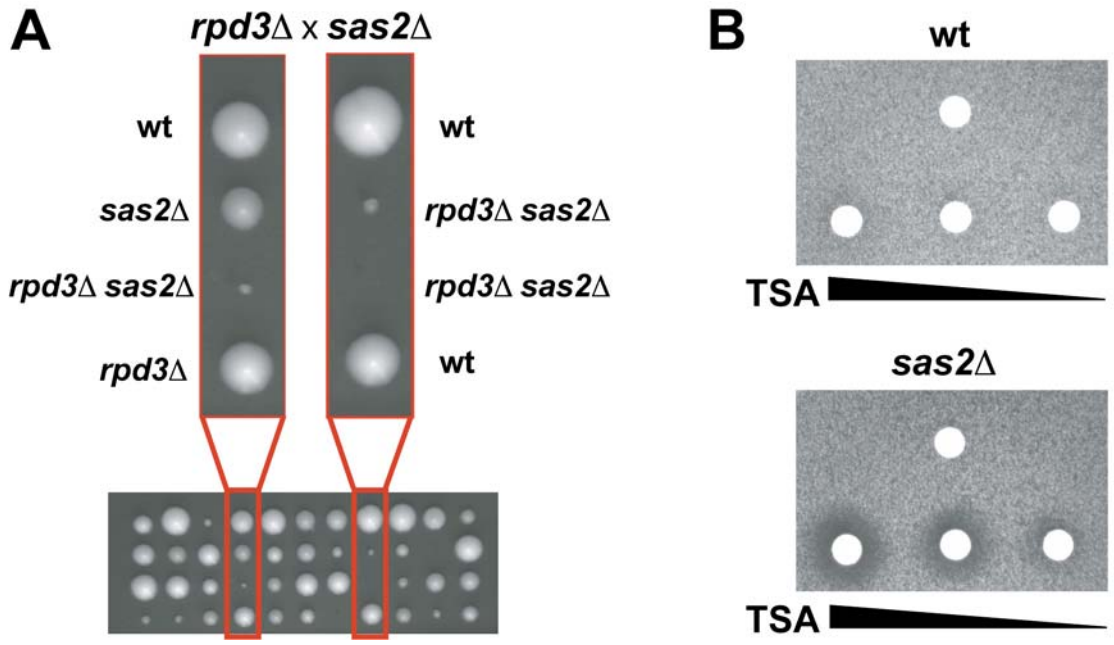

Figure 7 The deletion of *RPD3* was lethal in the absence of Sas2.
(A) Tetrad dissection of a cross between isogenic *sas2*Δ and *rpd3*Δ strains. The four tetrads from one ascospore were dissected in vertical lines. Cells were grown for two days at 30°C and photographed. The genotype of the two enlarged tetrads is indicated, showing a strong synthetic growth defect and lethality of the *sas2*Δ *rpd3*Δ double deletion. wt, wildtype. (B) Growth inhibition of *sas2*Δ cells in the presence of the HDAC inhibitor Trichostatin A (TSA). Filter discs with increasing amounts of TSA (DMSO control in the upper row; 16, 12 and 8 µg of TSA in the lower row) were placed on lawns of 2×10^5 cells of a wt or *sas2*Δ strain. Plates were incubated for one day at 30°C.

3.1.2 The catalytic activity of the SAS-I complex was required for the survival of *rpd3*Δ cells

Sas2 belongs to the class of the MYST family HATs and thus contains two recognizable protein motifs, an acetyl-CoA binding site and an atypical zinc finger. Mutations in the acetyl-CoA binding site (P213A/P214V, HAT $^-$) or the zinc finger (C106L, Zn$^-$) lead to the loss of Sas2 function (Meijsing and Ehrenhofer-Murray 2001). To test whether the lethality between *sas2*Δ and *rpd3*Δ depended on the catalytic activity of Sas2, we asked if catalytically inactive versions of Sas2 were able to support the growth of *sas2*Δ *rpd3*Δ strains. Therefore, *sas2*Δ *rpd3*Δ strains containing a wt copy of *SAS2* on *URA3*-marked plasmids were transformed with integrating versions of *SAS2* (*SAS2* wt, *sas2* mutated in the acetyl-CoA binding site (P213A/P214V, HAT $^-$) or *sas2* mutations in the zinc finger (C106L, Zn$^-$). Remarkably, only strains carrying wt *SAS2* were able to lose the p*URA3-SAS2* plasmid on *URA3*-

counterselective medium containing 5-fluoro-orotic acid (5-FOA) (**Figure 8**). This indicated that both the acetyl-CoA binding motif and the zinc finger were required to prevent lethality of *sas2*Δ with *rpd3*Δ, suggesting that the lethality depended on the catalytic activity of Sas2.

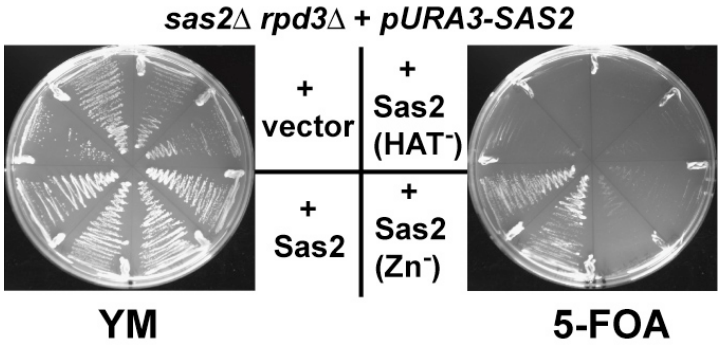

Figure 8 The lethality between *sas2*Δ and *rpd3*Δ depended on the catalytic activity of Sas2.
A *sas2*Δ *rpd3*Δ strain carrying p*URA3-SAS2* (AEY 3923) was transformed with additional *sas2* alleles on *HIS3*-marked integrating plasmids. Cells were grown on minimal plates (YM, growth assay) and on plates containing the *URA3*-counterselective compound 5-FOA to select against *URA3* plasmids. Cells were grown for two days at 30°C.

3.1.3 The lethality between *rpd3*Δ and *sas2*Δ depended on the whole SAS-I complex

The SAS-I complex, consists of the subunits Sas2, Sas4 and Sas5 (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001; Sutton et al. 2003). To test if the whole SAS-I complex is involved in the lethality between SAS-I and Rpd3, we tested whether *rpd3*Δ was also lethal with *sas4*Δ or *sas5*Δ. Isogenic crosses, followed by tetrad dissection and analysis of the outcoming strains, revealed that *sas4*Δ and *sas5*Δ were also synthetically lethal with *rpd3*Δ, thus showing that the whole SAS-I complex was involved in the lethality with *rpd3*Δ (**Figure 9**).

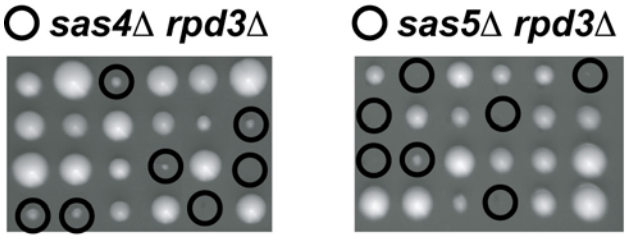

Figure 9 Synthetic lethality between *rpd3*Δ and *sas4*Δ or *sas5*Δ.
Tetrad dissection of a cross of *sas4*Δ or *sas5*Δ with an *rpd3*Δ isogenic W303 strain. The four spores from individual asci were aligned in vertical lines. Double mutants are marked with circles.

3.1.4 The lethality between *rpd3*Δ and *sas2*Δ involved the Rpd3 (L) complex

The HDAC Rpd3 is present in the two functionally and structurally distinct complexes, Rpd3 (L) and Rpd3 (S) (Carrozza et al. 2005; Keogh et al. 2005). Rpd3 (L) is targeted to gene promoters, thus establishing promoter deacetylation and gene silencing, whereas Rpd3 (S) prevents intragenic transcription via deacetylation of histones within the body of genes (Carrozza et al. 2005; Keogh et al. 2005). Therefore, we next asked which of the two complexes was involved in the lethality with *sas2*Δ. To this end, we tested *dep1*Δ and *sds3*Δ (components of Rpd3 (L)) and *rco1*Δ and *eaf3*Δ (components of Rpd3 (S)) for lethality in the absence of Sas2, by performing isogenic crosses followed by dissection and analysis of the tetrads or by selecting against p*URA3-SAS2* on *URA3*-counterselective 5-FOA plates.

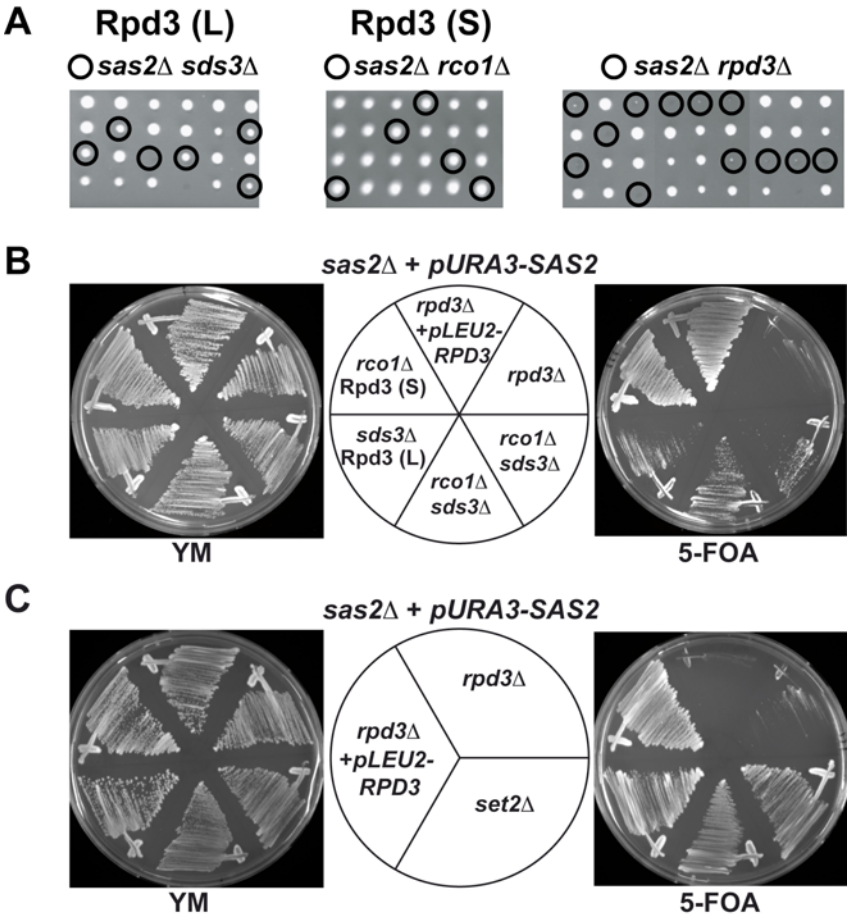

Figure 10 Synthetic lethality between the Rpd3 (L) and the SAS-I complex.
(A) Tetrad dissection of a cross between *sas2*Δ and deletions of components of the Rpd3 (L) complex, represented by *sds3*Δ, and the Rpd3 (S) complex, represented by *rco1*Δ. Synthetic growth defects are compared to the effect of *rpd3*Δ. Double mutants are indicated. (B) The lethality between *rpd3Δ* and *sas2Δ* was specific for the Rpd3 (L) complex. *sas2*Δ cells with *pURA3-SAS2* and additional deletions of *RCO1, SDS3* or both were incubated on supplemented minimal medium (YM) or on URA3-counterselective 5-FOA medium to select against p*URA3-SAS2*. (C) *set2*Δ did not cause synthetic lethality in *sas2*Δ. Cells were tested as in (B). The experiments in (B) and (C) are courtesy of Prof. Ann Ehrenhofer-Murray.

Significantly, we found defects when *sas2*Δ was combined with the absence of Rpd3 (L) components, *sds3*Δ and *dep1*Δ (**Figure 10A-B, Table 6**). Conversely, we observed no defects of *sas2*Δ *rco1*Δ or *sas2*Δ *eaf3*Δ (**Figure 10A-B**, **Table 6**, components of Rpd3 (S)), and deletion of *RCO1* did not exacerbate the growth defect of *rpd3*Δ *sds3*Δ cells, suggesting that the synthetic lethality of *rpd3*Δ *sas2*Δ double mutants was caused by the absence of the Rpd3 (L) complex. However, the growth defect of *sas2*Δ *sds3*Δ (or *dep1*Δ) cells was less pronounced than for *sas2*Δ *rpd3*Δ cells (**Figure 10A**), suggesting that the deletion of *SDS3* or *DEP1* did not have the same impact on the activity of the Rpd3 (L) complex as the absence of the catalytic subunit Rpd3.

Histone methylation by Set2 recruits Rpd3 (S) to the body of genes to prevent intragenic transcription (Keogh et al. 2005), but Set2 also has Rpd3 (S)-independent functions at telomere boundaries (Tompa and Madhani 2007). However, *set2*Δ did not cause a growth defect in *sas2*Δ cells (**Figure 10C**), indicating that neither of the two Set2 functions were involved in the synthetic lethality between *sas2*Δ and *rpd3*Δ, and further supporting the notion that the lethality between *rpd3*Δ and *sas2*Δ involves the Rpd3 (L) complex.

3.1.5 The lethality between *rpd3*Δ and *sas2*Δ was specific for those two enzymes

The observation of combined lethality between the absence of a HAT and an HDAC raised the question whether this lethality was specific for *sas2*Δ and *rpd3*Δ, or whether the lethality was caused by a general imbalance in histone acetylation or transcriptional misregulation. The latter case might be caused by defects in transcriptional regulation in the absence of one activating and one repressive mechanism. If this were the case, the deletion of another HAT might be able to phenocopy the effect of *sas2*Δ, and thus be synthetically lethal with *rpd3*Δ. To this end, we tested other HAT deletions for lethality in the absence of Rpd3 by performing crosses of isogenic yeast strains deleted for *RPD3* with a strain deleted for another HAT. Since Sas2 belongs to the class of MYST family HATs (Carrozza et al. 2003)), other HATs belonging to this class, Esa1 and Sas3, are putative candidates for phenocopying *sas2*Δ. As a component of the NuA4 complex, Esa1 primarily acetylates histone H4 (Smith et al. 1998a; Clarke et al. 1999), whereas Sas3, a component of the NuA3 complex, preferentially acetylates histone H3 (John et al. 2000). However, neither disruption of *SAS3*, nor a mutation in *ESA1* showed a growth defect in combination with the absence of Rpd3 (**Table 6**),

suggesting that the lethality between *sas2*Δ and *rpd3*Δ was not a simple property of the loss of a MYST family HAT in *rpd3*Δ cells.

Beyond the MYST family of HATs, subunits of the SAGA complex (with Gcn5 as the catalytic subunit), an example for the so-called GNAT (Gcn5 related) family of HATs, have been investigated for synthetic genetic interactions. Curiously, one study reported a synthetic growth defect between *rpd3*Δ and *spt3*Δ or *spt8*Δ, which are SAGA components (Keogh et al. 2005). In contrast, another study reported no evident growth defect for an *rpd3*Δ *gcn5-21* strain (Burgess et al. 1999). Therefore it seems that synthetic genetic interactions between *rpd3*Δ and SAGA are not strong and may depend on differences in strain backgrounds.

Table 6 Summary of synthetic genetic interactions between HATs and HDACs

Deleted genes	Protein function	Viability of double mutants with	
		*sas2*Δ	*rpd3*Δ
RPD3	HDAC	-	/
SDS3	subunit of Rpd3 (L)	(-)	+
DEP1	"	(-)	+
RCO1	subunit of Rpd3 (S)	+	+
EAF3	"	+	ND
SAS2	subunit of SAS-I	/	-
SAS4	"	+	-
SAS5	"	+	-
SAS3	HAT	+	+
*ESA1**	HAT	+	+
HAT1	HAT-B	+	+
SIR2	HDAC	+	+
HST1	"	+	ND
HST2	"	+	+
HST3	"	+	ND
HST4	"	+	ND
HOS1	"	+	ND
HOS2	"	+	ND
HOS3	"	+	ND
HDA1	"	+	ND

* *esa1 L327S ts* allele (Clarke et al. 1999);
ND, not determined; / not applicable; +, double mutants viable; -, double mutants not viable

Another member of the GNAT family of HATs is the cytoplasmatic HAT1 complex (Parthun et al. 1996; Ruiz-Garcia et al. 1998). HAT1 is classified as a HAT-B complex and mainly

acetylates free histones prior to their incorporation into chromatin (Parthun et al. 1996). We found that a deletion of *HAT1* was not synthetically lethal with *rpd3*Δ (**Table 6**).

Altogether, none of the tested HAT deletions except *sas2*Δ were synthetically lethal in the absence of Rpd3 (**Table 6**), indicating that the lethality in the absence of these two factors was specific for *sas2*Δ.

In a next set of experiments, we asked whether the lethality between *sas2*Δ and *rpd3*Δ was specific for *rpd3*Δ, or whether other HDACs showed lethality with *sas2*Δ. This was done by testing whether other HDAC deletions were synthetically lethal with *sas2*Δ. The HDACs are classified into different families that are distinguished by the requirement for NAD^+ as a cofactor (see **Introduction**). Rpd3 belongs to the class of NAD^+-independent HDACs, such that we first asked whether the lethality between *sas2*Δ and *rpd3*Δ might be a property of the loss of an NAD^+-independent deacetylase. However, none of the deletions of known NAD^+-independent HDAC (*hos1*Δ, *hos2*Δ, *hos3*Δ, *hda1*Δ) were lethal with *sas2*Δ (**Table 6**). Furthermore, no deletion within the class of NAD^+-dependent HDACs (*sir2*Δ, *hst1*Δ, *hst2*Δ, *hst3*Δ, *hst4*Δ) was lethal with *sas2*Δ, indicating that the lethality between *sas2*Δ and *rpd3*Δ was specific for these two enzymes and was not due to a general imbalance between acetylation and deacetylation activities.

3.1.6 *rpd3*Δ and *sas2*Δ changed subtelomeric gene expression

Histone acetylation is generally described as being linked to chromatin opening, whereas histone deacetylation is thought to act as a condensing mechanism, thus leading to heterochromatin formation. The observation that the simultaneous loss of an opening and a condensing factor is lethal to yeast cells is counterintuitive, because a synthetic lethal screen is generally used to reveal genetic interactions between factors that share the same or similar biological functions. Sas2 functions in the restriction of telomeric heterochromatin spreading by acetylation of H4K16 (Kimura et al. 2002; Suka et al. 2002). Deletion of *SAS2* leads to spreading of SIR proteins towards centromere proximal region, which causes repression of subtelomeric genes (Kimura et al. 2002; Suka et al. 2002). Therefore, the observation of synthetic lethality between *sas2*Δ and *rpd3*Δ suggested a role for Rpd3 in the regulation of heterochromatin formation, similar to that of Sas2. If this were the case, the expression of subtelomeric genes should also be repressed by *rpd3*Δ. Conversely, because HDACs are generally correlated with gene repression, one would expect increased gene expression in

*rpd3*Δ cells. In contrast to the general expectation, it was found more than ten years ago that deletion of *RPD3* leads to improved silencing at telomeres in Drosophila (De Rubertis et al. 1996), as well as in yeast (Sun and Hampsey 1999). To evaluate the effects of *sas2*Δ or *rpd3*Δ on gene expression, we compared the gene expression profile of *sas2*Δ cells (M. Horikoshi, personal communication) with the profile of *rpd3*Δ cells (downloaded from the yeast expression database: http://salt2.med.harvard.edu/cgi-bin/ExpressDByeast/). To this end, we chose genes from the microarray data that were up- or downregulated by more than 50% in both strains. We plotted the resulting 47 genes in a graph that compares the effects of *sas2*Δ with the effect of *rpd3*Δ. In contrast to the general expectation, genes were not globally downregulated upon loss of the HAT Sas2 or globally upregulated upon loss of the HDAC Rpd3 (**Figure 11**). Significantly, the genes that were downregulated in *sas2*Δ as well as in *rpd3*Δ cells, were subtelomeric genes or genes close to *HMR* (YCR099C) (**Figure 11**). This showed that *rpd3*Δ had similar effects on telomeric gene expression as compared to the effects of *sas2*Δ, suggesting an unexpected role for Rpd3 in the prevention of downregulation, rather than assistance in downregulation of genes adjacent to heterochromatin.

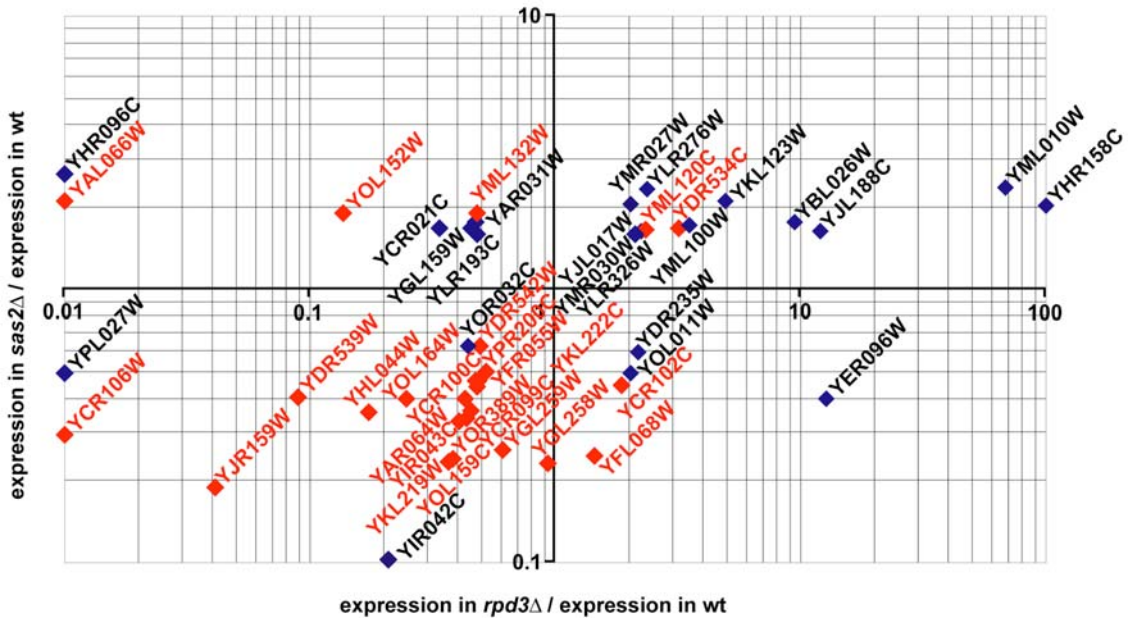

Figure 11 Subtelomeric genes showed similar expression profiles in *sas2*Δ and *rpd3*Δ cells.
The microarray data from yeast cells deleted for *RPD3* or *SAS2* were compared (*rpd3*Δ, data from yeast expression database, http://salt2.med.harvard.edu/cgi-bin/ExpressDByeast/; *sas2*Δ, gift from M. Horikoshi). The diagram shows the x-fold expression in *rpd3*Δ cells compared to the x-fold expression in *sas2*Δ cells for the respective genes. Genes indicated, are at least 50% up- or downregulated in *sas2*Δ and *rpd3*Δ cells. Subtelomeric genes are highlighted in red.

3.1.7 The lethality between *rpd3*Δ and *sas2*Δ was suppressed by *sir2*Δ, *sir3*Δ and *sir4*Δ

The experiments above suggested a function for Rpd3 in restricting SIR proteins to heterochromatin, similar to that of the SAS-I complex (Kimura et al. 2002; Suka et al. 2002). Thus, the parallel loss of two restricting activities might enhance mislocalization of SIR proteins. The combined effect of SIR spreading by *rpd3*Δ and *sas2*Δ might be increased to a degree that is lethal to the cells. If this were the case, then relieving telomeric silencing by deleting one of the SIR components should abrogate the *sas2*Δ *rpd3*Δ lethality. To this end, we performed isogenic crosses that revealed that *sas2*Δ *rpd3*Δ *sir2*Δ strains were fully viable, even at 37°C (data not shown), indicating that the synthetic lethality of *sas2*Δ *rpd3*Δ strains was abrogated by *sir2*Δ. To test whether the suppression of the lethality involves the whole SIR-complex, we tested the ability of *sas2*Δ *rpd3*Δ cells additionally deleted for *SIR1, SIR2, SIR3* or *SIR4,* to lose a *URA3*-marked *pURA3-SAS2* plasmid on *URA3* counterselective 5-FOA media. Remarkably, double mutant *sas2*Δ *rpd3*Δ strains were only able to lose the *SAS2* plasmid if *SIR2, SIR3* or *SIR4* was additionally deleted (**Figure 12A**, **Table 7**). This showed that the deletion of *SIR2, SIR3* or *SIR4* completely suppressed the *sas2*Δ *rpd3*Δ lethality, indicating that the inability of these strains to grow was caused by inappropriate SIR spreading to a degree that is lethal to the cells.

In line with this, the growth inhibition of *sas2*Δ cells by TSA (**Figure 9B**) was abrogated in *sas2*Δ *sir2*Δ cells (data not shown), suggesting that the growth inhibition of *sas2*Δ cells in the presence of TSA was also caused by inappropriate SIR spreading.

Binding of the SIR complex to the telomeres depends on the acetylation state of histones, in particular H4 K16 (Rusche et al. 2003). In fact, binding of Sir3 is strongest to deacetylated histone H4 tails (Liou et al. 2005). Since mutations of critical residues within histones abrogate silencing (Meijsing and Ehrenhofer-Murray 2001), one could speculate that this effect might suppress the *sas2*Δ *rpd3*Δ lethality comparable to *sir2*Δ. Indeed, mutation of the lysine residues to glutamine, which mimics acetylated state, resulted in suppression of the lethality between *rpd3*Δ and *sas2*Δ. The same was seen for mutations within the H3 tail (**Figure 13**), suggesting that factors that reduce binding of the SIR complex to the subtelomeric regions, through acetylation as well as through mutation of histones, are able to suppress the *sas2*Δ *rpd3*Δ lethality.

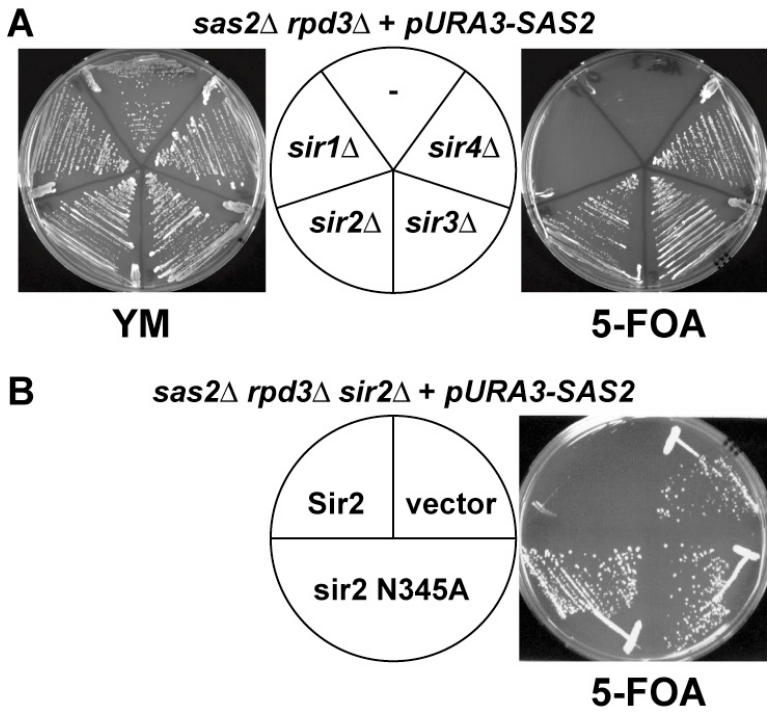

Figure 12 The lethality between *sas2*Δ and *rpd3*Δ was suppressed by *sir2*Δ, *sir3*Δ and *sir4*Δ.
(A) *sas2*Δ *rpd3*Δ strains with p*URA3-SAS2* (AEY 3923) were additionally deleted for *SIR1, SIR2, SIR3* or *SIR4*. Cells were grown on minimal plates (YM, growth assay) and on plates, containing the *URA3*-counterselective 5-FOA to select against *URA3* plasmids. Cells were grown for two days at 30°C. (B) Catalytic inactive Sir2 is unable to cause the lethality between *sas2*Δ and *rpd3*Δ. A *sas2*Δ *rpd3*Δ *sir2*Δ strain with p*URA3-SAS2* (AEY3931) was transformed with the indicated additional *SIR2* alleles on *LEU2* marked plasmids. Cells were grown as in A.

Interestingly, the H4 K16R also suppressed the lethality between *rpd3*Δ and *sas2*Δ. This seemed counterintuitive because a K16R mutation, that is generally used to mimic deacetylation, exhibits the same effect on silencing as the *SAS2* deletion and supposedly represents the acetylation state that is generated by Sir2 itself. In contrast to this assumption, K16R leads to loss of telomeric and *HM* silencing (Meijsing and Ehrenhofer-Murray 2001). This suggests that an arginine residue is not a functionally equivalent to a deacetylated lysine. In contrast to H4 K16, single or double mutations within other lysine within the histone tails did not abrogate the *sas2*Δ *rpd3*Δ lethality, suggesting that the impact of these residues on SIR binding is not as significant as the influence of H4 K16.

Taken together, these experiments showed that mutations that abrogate SIR dependent repression suppressed the lethality between *sas2*Δ and *rpd3*Δ, further strengthening the notion that the lethality was caused by mislocalized SIR proteins.

Histone deacetylation through Sir2 involves consumption of NAD^+ as a cofactor (Imai et al. 2000) and binding of NAD^+ to a binding pocket (Zhao et al. 2003). In fact, mutation of N345A, which is located within this binding pocket (Zhao et al. 2003), abrogates the

deacetylation activity of Sir2 (Imai et al. 2000). To test whether the lethality of *sas2*Δ *rpd3*Δ strains depended on the Sir2 deacetylation activity, we asked if this catalytically inactive mutant of Sir2 (*sir2-N345A*) was able to restore the *sas2*Δ *rpd3*Δ lethality in a *sas2*Δ *rpd3*Δ *sir2*Δ strains. Significantly, *sir2-N345A* was unable to do so (**Figure 12B**), indicating that the ability of the SIR complex to cause this lethality required the enzymatic activity of Sir2.

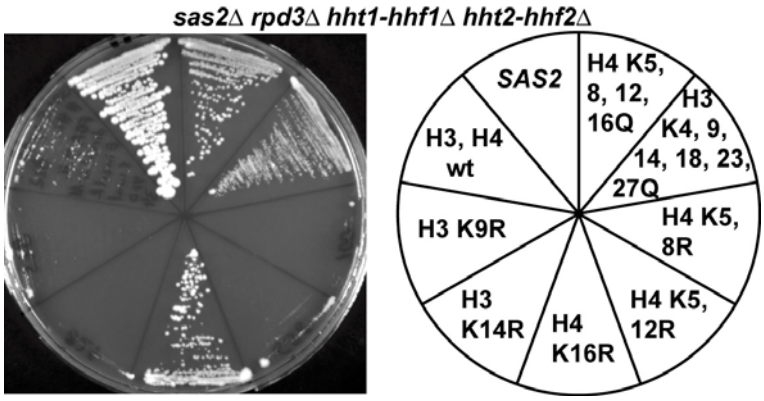

Figure 13 Suppression of the lethality between *sas2*Δ and *rpd3*Δ by histone mutants.
Yeast strains deleted for *SAS2, RPD3* and the wt histone genes were selected for the indicated histone mutants (see Material & Methods for details). To indicate growth differences between the mutants, cell were grown for two days on YPD at 34°C after the selection.

Next, we wanted to know whether the suppression of the *sas2*Δ *rpd3*Δ lethality was specific to disruption of the telomeric SIR complex. Sir2 has four homologs in *S. cerevisiae*, of which Hst2 has been described to have similar substrate specificity as Sir2, namely H4 K16 (Vaquero et al. 2006). In contrast to Sir2, Hst2 and the human Hst2 homolog SirT2 are located in the cytoplasm during most of the cell cycle, except during mitosis (Vaquero et al. 2006). Since the specificity of Sir2 and Hst2 is similar, we asked whether *hst2*Δ was also able to suppress the growth inability of *sas2*Δ *rpd3*Δ cells. However, this was not the case (**Table 7**), showing that the suppression was specific to Sir2.

The formation of heterochromatin is accompanied by chromatin compaction. Therefore, we asked whether deletions of compacting factors like the yeast linker histone (Hho1) or other SIR recruiting factors (Ku70) may reduce the SIR dependent repression of subtelomeric genes and therefore also suppress the lethality between *sas2*Δ and *rpd3*Δ. However, neither *hho1*Δ nor *hdf1*Δ (the gene encoding yeast Ku70) were able to restore the growth of *rpd3*Δ *sas2*Δ strains (**Table 7**).

Table 7 Suppression of the lethality between *sas2*Δ and *rpd3*Δ

Deleted genes	Protein function	Viability of triple mutants with *sas2*Δ *rpd3*Δ
SIR1	subunit of SIR at *HM* loci	-
SIR2	catalytic subunit of SIR	+
SIR3	essential subunit of SIR	+
SIR4	essential subunit of SIR	+
HST2	HDAC, Sir2 homolog	-
HAT1	HAT-B	-
HDF1	yeast Ku 70	-
HHO1	linker histone	-
HMR	silent mating-type locus	-

* *esa1 L327S ts* allele (Clarke et al. 1999); +, double mutants viable; -, double mutants not viable

Deletion of *RPD3* causes higher global acetylation of H4 K5 and H4 K12 (Robyr et al. 2002), acetylation sites that are established by the cytoplasmatic HAT Hat1, which acetylates free histones prior to their incorporation into chromatin (Ruiz-Garcia et al. 1998). To ask if a loss of cytoplasmatic H4K5 and H4K12 acetylation in *hat1*Δ cells might be able to suppress the lethality of *sas2*Δ *rpd3*Δ strains, we tested the viability of *sas2*Δ *rpd3*Δ *hat1*Δ cells. Significantly, the additional *hat1*Δ was not able to restore the growth of *sas2*Δ *rpd3*Δ cells (**Table 7**), suggesting that the lethality between *sas2*Δ and *rpd3*Δ was not caused by a simple change in H4K5 and H4K12 acetylation levels.

Heterochromatic regions under control of SIR include the telomeres, the mating-type loci (*HML* and *HMR*) and rDNA loci (see Introduction). Since *SIR* deletions also cause a change in cell type and cause *HM* derepression (Rusche et al. 2003), it was also possible that this rather than a loss of telomeric silencing was responsible for the suppression. *HM* silencing requires an additional subunit of the SIR complex, Sir1 (Rine et al. 1979). If the suppression is due to a loss of *HM* silencing, *sir1*Δ should also abrogate the lethality between *sas2*Δ and *rpd3*Δ. However, the deletion of *SIR1*, which affects *HM* but not telomeric silencing, did not suppress the lethality (**Figure 12**, **Table 7**). Another possibility to test whether the suppression of the *sas2*Δ *rpd3*Δ lethality through SIR deletion is caused by a change in the cell type is to delete the respective *HM* locus that causes this change (*HML* in *MAT***a**, or *HMR* in *MAT*α cells). Significantly, deletion of *HMR* in a *MAT*α *sas2*Δ *rpd3*Δ *sir2*Δ strain, which reverses the pseudo-diploid cell type, did not abrogate the viability of the strain (**Table 7**), demonstrating that the SIR-dependent repression was not due to cell-type effects and that the *sas2*Δ *rpd3*Δ lethality was caused by SIR proteins spreading into subtelomeric regions.

In summary, these experiments showed that the lethality between *sas2*Δ and *rpd3*Δ was due to increased SIR-dependent repression of subtelomeric regions and further suggested that Rpd3, as Sas2, exerted a boundary function at telomeres.

3.1.8 *rpd3*Δ caused increased SIR spreading at telomeres

Our above results suggested that the deletion of *RPD3* caused increased spreading of SIR complexes into subtelomeric regions. If this were the case, physical enrichment of SIR-proteins should be detectable in Chromatin immunoprecipitation (ChIP) experiments. To this end, we measured Sir2 levels at the telomeres in ChIPs with antibodies against myc-tagged Sir2 in *rpd3*Δ and *sas2*Δ compared to wt at the telomere VI-R region The absence of Rpd3 lead to more Sir2 protein bound to telomeres and the presence of more Sir2 in centromere-proximal regions (**Figure 14**).

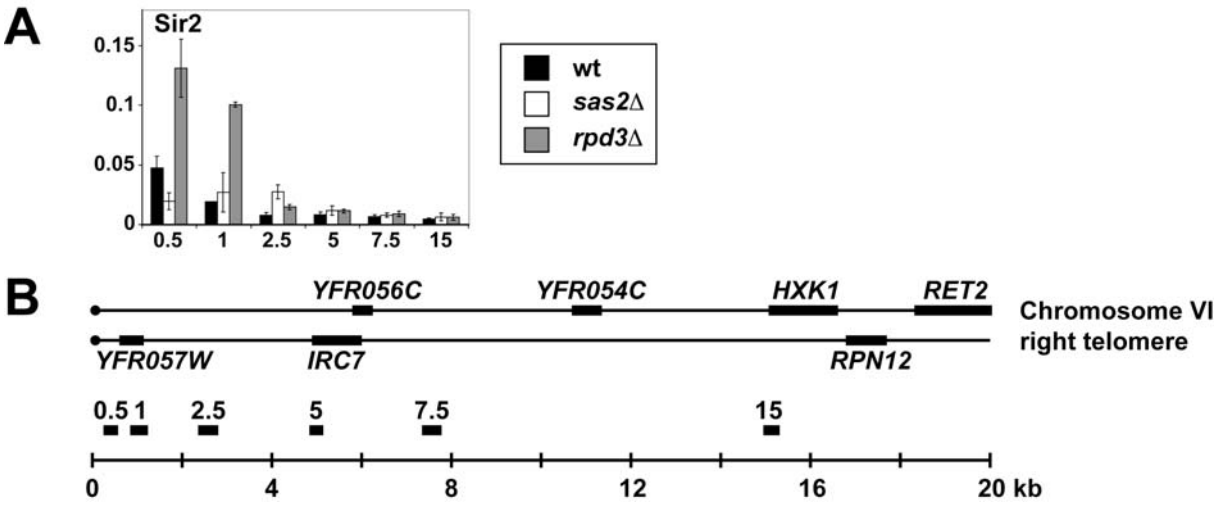

Figure 14 Sir2 was mislocalized to subtelomeric regions in the absence of Rpd3.
(A) Sir2 binding at the right telomere of chromosome VI is shown as enrichment in ChIP experiments relative to the input DNA. The amount of enrichment is given as a function of the distance to the telomere end in kbs in strains with the indicated genotype. ChIPs were performed with antibodies against myc-tagged Sir2. Error bars give standard deviations (see Material & Methods). (B) Schematic representation of the telomere VI-R with ORFs and fragments amplified in quantitative real-time PCR is shown in the lower panel.

In *sas2*Δ cells, SIR proteins are less enriched at the telomere ends, but rather are shifted to more centromere-proximal regions. At 0.5 kb, less Sir2 was bound than in wt, but at 2.5 kb in *sas2*Δ cells, more Sir2 was enriched than in *rpd3*Δ (**Figure 14**). Thus, the effect of *rpd3*Δ was distinct from that of *sas2*Δ, in that *rpd3*Δ led to enrichment of SIR proteins, whereas *sas2*Δ led to a shift of Sir2 towards centromere-proximal sequences and less Sir2 at sequences close

to the telomere ((Kimura et al. 2002; Suka et al. 2002), **Figure 14**). This was in agreement with the observation that *sas2*Δ causes a loss of silencing at reporter genes inserted close to the telomere (Reifsnyder et al. 1996; Meijsing and Ehrenhofer-Murray 2001), whereas *rpd3*Δ causes improved silencing (De Rubertis et al. 1996). This indicates that the observed effects of subtelomeric repression in *rpd3*Δ cells might be due to SIR spreading. Thus, our data showed that Rpd3 was required to restrict Sir2 levels and localization to sequences closest to the telomere. Importantly, the ability of SIR proteins to spread in *rpd3*Δ was not due to increased Sir2 or Sir3 expression in *rpd3*Δ cells (as measured by immunoblotting for Sir2 and RT-PCR for Sir2 and Sir3 (**Figure 15**)).

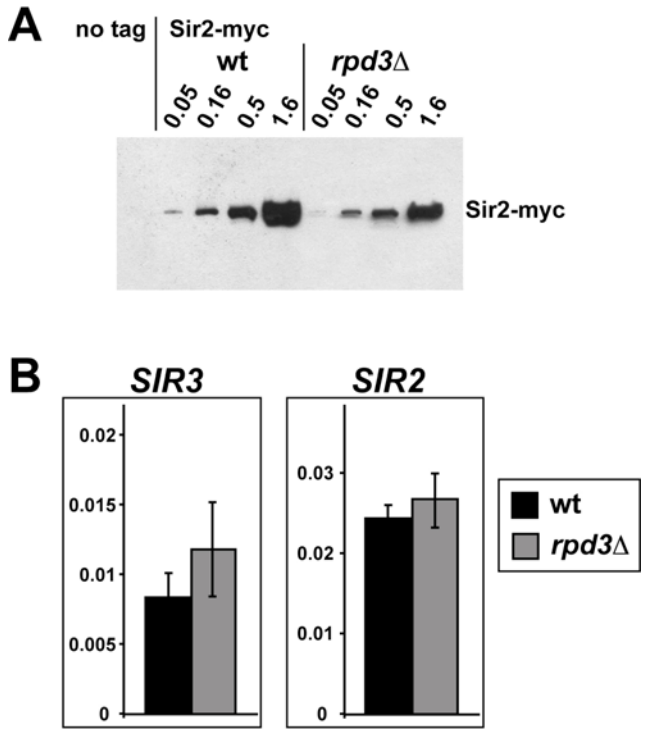

Figure 15 The expression of Sir proteins was not increased in the absence of Rpd3.
(A) Level of Sir2 protein in *rpd3*Δ cells compared to wt as shown by Western blotting of myc-tagged Sir2 protein. The amount of cell extract loaded is given as OD units. (B) Amount of Sir2 and Sir3 transcripts as measured by qRT-PCR in *rpd3*Δ cells compared to wt. The diagram shows the amount of cDNA as X-fold expression level relative to *ACT1*. Error bars give standard deviations of at least three PCR analyses from at least two independent experiments.

3.1.9 *rpd3*Δ caused increased silencing in subtelomeric regions by SIR spreading

The latter analysis revealed an enrichment of Sir proteins in subtelomeric regions in *rpd3*Δ cells, suggesting that SIR spreading caused repression at subtelomeric ORFs, similar to what is seen in *sas2*Δ cells (Kimura et al. 2002; Suka et al. 2002). To ask whether SIR spreading in

*rpd3*Δ strains is coupled to gene repression, we first asked if a telomeric *ADE2* reporter was repressed in the absence of Rpd3. In agreement with previous reports (Sun and Hampsey 1999), loss of *RPD3* led to more red colonies. This indicated that *rpd3*Δ led to repression of the telomeric *ADE2* gene, thus revealing the red colour, typical for *ade2* strains (**Figure 16A**).

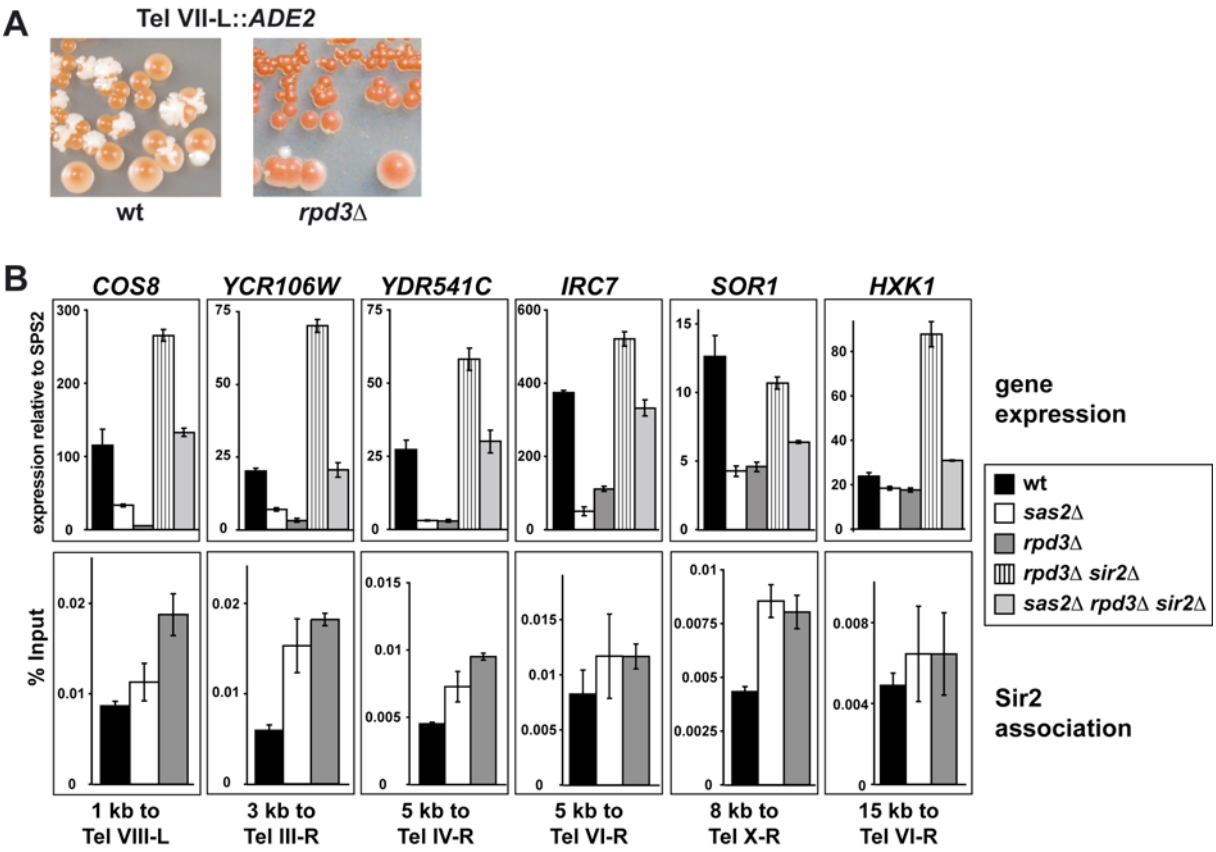

Figure 16 Subtelomeric genes were repressed in *rpd3*Δ cells in a SIR-dependent fashion.
(A) Telomeric *ADE2* was repressed in *rpd3*Δ strains. wt and *rpd3*Δ strains carrying telomeric *ADE2* were grown for two days at 30°C. Expression of *ADE2* is shown as white sector within one colony. Red colonies correspond to improved silencing of the *ADE2* gene. (B) Rpd3-dependent repression of subtelomeric genes. The upper row of diagrams shows the amount of cDNA of selected subtelomeric genes in the indicated strains as X-fold expression level relative to *SPS2*. The location and distance of the selected genes at their telomere is indicated. Error bars give standard deviations of at least three PCR analyses from at least two independent reverse transcriptase reactions. The lower row of diagrams shows the amount of Sir2 at the respective regions and is represented as in **Figure 14**.

Furthermore, we tested the expression level of subtelomeric genes in *rpd3*Δ cells by reverse transcription of RNA followed by quantitative real-time PCR analysis. The telomere VI-R shows a relatively low gene density and additionally, however, most of the genes at Tel VI-R (*YFR057W, YFR056C or YFR054C*) revealed to be expressed at very low levels, where no significant amount of mRNA could be measured or they were no true genes. Thus, we extended our analysis to genes on other chromosomes representing different distances to their

telomere end. We tested six genes that represent regions where more Sir2 is enriched in *rpd3*Δ (close to the telomeres) or regions where more Sir2 is bound in *sas2*Δ (approx. 2.5 to 5 kb away from the telomere). Genes closest to the telomeres, like *COS8* (1 kb from Tel VIII-L) or *YCR106W* (3 kb from Tel III-R) showed strong repression in *rpd3*Δ cells, whereas *IRC7* (5 kb from Tel VI-R) showed stronger repression in *sas2*Δ cells (**Figure 16B**). All repression effects were abrogated by additional *SIR2* deletion, indicating that the repression was due to more SIR proteins mislocalized to the respective regions.

Significantly, *SOR1* (8 kb from Tel X-R), *HXK1* (15 kb to Tel VI-R) and *YDR541C* (5 kb from Tel IV-R) were as strongly repressed in *rpd3*Δ cells as in *sas2*Δ cells (**Figure 16B**), indicating that in regions were the SIR proteins were not so severely enriched, the genes are not so dramatically downregulated. In general, the gene expression reflected the amount of SIR proteins bound, i.e. the more SIR was bound the more the gene became repressed (**Figure 16B**). This showed that SIR spreading in *rpd3*Δ cells caused increased repression of subtelomeric genes. Thus, Rpd3 was required to prevent inappropriate gene repression in subtelomeric regions by delocalized SIR complexes.

3.1.10 Subtelomeric acetylation was reduced in *rpd3*Δ cells

Since the repression of subtelomeric genes in *rpd3*Δ cells is due to the spreading of SIR proteins, this raises the question whether the histone acetylation in these regions is changed by the presence of more SIR proteins. Generally, the encroachment of SIR silencing complexes into euchromatin is associated with chromatin deacetylation through the HDAC activity of Sir2 (Rusche et al. 2003). Thus, SIR spreading in *rpd3*Δ cells may cause less acetylation in subtelomeric regions. In contrast to this, Rpd3 has a global acetylation activity, and loss of Rpd3 leads to global increases in H4 K5 and K12 acetylation (Vogelauer et al. 2000). It was therefore difficult to predict how *rpd3*Δ proteins would influence telomeric acetylation patterns. To test this, we performed ChIP experiments using antibodies against acetylated lysines of H4. In agreement with general higher acetylation in *rpd3*Δ cells, H4K5 and H4K16 became more acetylated for the majority of regions tested (**Figure 17**). In contrast to this, H4K12 became less acetylated at the telomeres in *rpd3*Δ cells (**Figure 17**). These experiments suggested a direct influence of Rpd3 on telomeric acetylation levels.

The latter experiments showed that the loss of Rpd3 changes telomeric acetylation. However, this influence was a mixture of higher acetylation, as expected for the loss of an HDAC, and

lower acetylation, contrary to increased acetylation in *rpd3*Δ cells. This suggested that acetylation in the latter case was influenced by Sir2 spreading into subtelomeric regions. To ask whether the inappropriate presence of Sir2 influences acetylation at subtelomeres in *rpd3*Δ cells, we measured acetylation levels after disruption of *SIR2*. Significantly, the deletion of *SIR2* abrogated the lower H4K12 acetylation at telomeres in *rpd3*Δ cells (**Figure 18**), indicating that the decrease in histone acetylation in *rpd3*Δ cells was caused by SIR proteins spreading into the region.

In summary, this analysis revealed that the chromatin acetylation pattern at telomeres in *rpd3*Δ cells was the result of two conflicting activities, namely chromatin deacetylation by spreading SIR complexes and the global increase of acetylation in the absence of Rpd3.

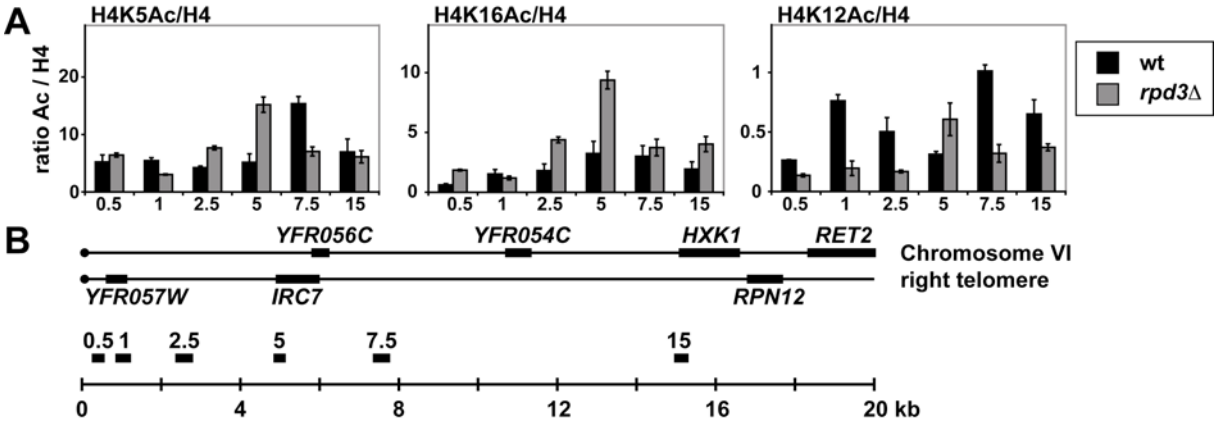

Figure 17 Subtelomeric acetylation is reduced in *rpd3*Δ cells.
(A) Histone acetylation at the right telomere of chromosome VI is shown as ratio from ChIP experiments with anti acetyl lysine antibodies relative to the amount of H4. The amount of acetylation is given as a function of the distance to the telomere end in kbs in wt and *rpd3*Δ strains. ChIPs were performed with antibodies against acetylated lysines and α-H4. Error bars give standard deviations (see Material & Methods). (B) Schematic representation of the telomere VI-R with ORFs and fragments amplified in quantitative real-time PCR.

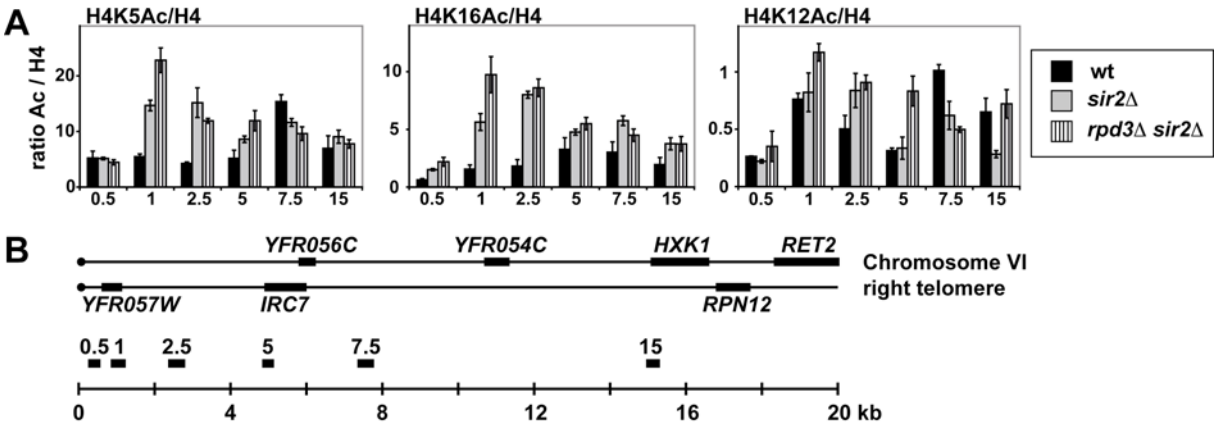

Figure 18 Subtelomeric acetylation was influenced by SIR.
Histone acetylation at Tel VI-R in *sir2*Δ and *rpd3*Δ *sir2*Δ cells, compared to wt, shown as in **Figure 17**.

3.1.11 Targeted Rpd3 established a boundary at telomeres

Our above results suggested that Rpd3 was required to restrict SIR proteins to the telomeres and thus had a boundary function. As a further, stringent test of this notion, we wished to determine whether Rpd3 was capable of creating a boundary when targeted to a normally silenced gene. To this end, we used a reporter system at telomere VII-L. In this assay, the spreading of telomeric heterochromatin into centromere-proximal regions causes repression of the integrated *URA3* gene, resulting in the ability to grow on 5-FOA. Conversely, tethering of a boundary element to the Gal4 binding sequence between the telomere and *URA3* stops the spreading of telomeric heterochromatin, resulting in subtelomeric *URA3* expression and inability to grow on 5-FOA (**Figure 19**, schematic representation) (Jacobson and Pillus 2004). To test whether Rpd3 had the ability to actively create a boundary between telomeric heterochromatin and subtelomeric *URA3*, we fused Rpd3 to the Gal4 DNA binding domain (GBD-Rpd3) and monitored URA3 expression. Importantly, when a Gal4 binding site was present between the telomere and the reporter, the expression of a GBD-Rpd3 fusion disrupted *URA3* silencing as measured by the inability of the strain to grow on 5-FOA medium, whereas the expression of GBD alone caused *URA3* to be silenced by telomeric heterochromatin (**Figure 19**). The effect of GBD-Rpd3 depended upon the presence of the Gal4 binding site, showing that the loss of silencing was not due to a targeting-independent effect of GBD-Rpd3 (**Figure 19**). Furthermore, the effect of targeting GBD-Rpd3 was not a complete loss of silencing, as compared to the complete loss of silencing after *SIR2* or *SIR3* deletion in this assay (data not shown).

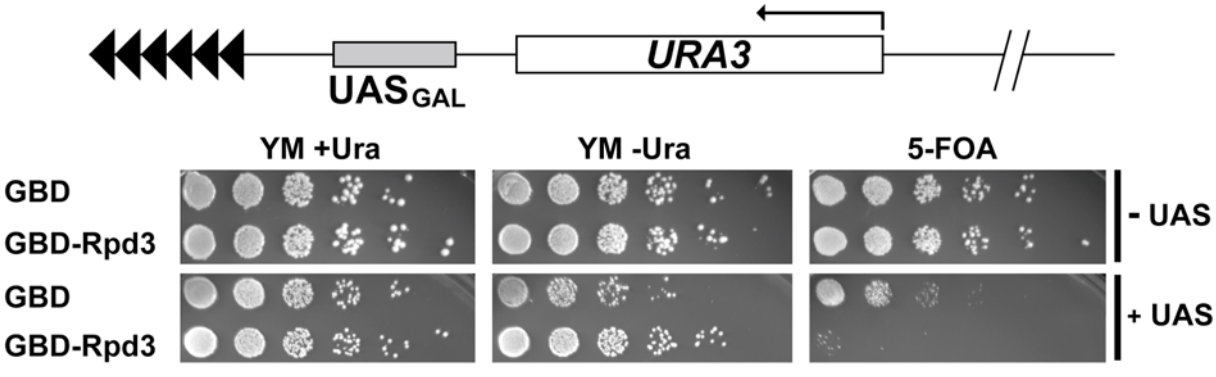

Figure 19 Tethered Rpd3 created a boundary at the telomere.
Cells with *URA3* inserted at TEL VII-L and with (+UAS) or without a Gal4 binding site at the telomere-proximal side (-UAS) (schematic drawing), were transformed with plasmids carrying the *RPD3* gene fused to the *GAL4* DNA binding domain (GBD-Rpd3) or with the vector control (GBD). Repression of *URA3* was tested by growth on *URA3*-counterselective 5-FOA plates. Cells were grown for two days at 30°C. Telomeric heterochromatin is shown with triangles.

Taken together, this showed that targeting of the HDAC Rpd3 to telomeres caused the establishment of a heterochromatin-euchromatin boundary. This was surprising, because so far only HATs and chromatin remodelling complexes, but not HDACs, are known to create boundaries (Jacobson and Pillus 2004; Oki et al. 2004; Oki and Kamakaka 2005).

Rpd3 works in two distinct complexes ((Carrozza et al. 2005; Keogh et al. 2005), see 3.1.4). Both complexes work together with Sin3 as an essential component, and therefore build a large protein complex (Kasten et al. 1997). To test whether the boundary effect of targeted Rpd3 was caused by a large sterically hindering complex, we tested the effect of *sin3*Δ. Significantly, deletion of *SIN3* did not abrogate the boundary function of Rpd3 (**Figure 20A**). Although loss of Sin3 alone lead to better telomeric silencing, targeting of GBD-Rpd3 even upon disruption of the Rpd3-Sin3-HDAC complex was capable of establishing a boundary against heterochromatin spreading (**Figure 20A**).

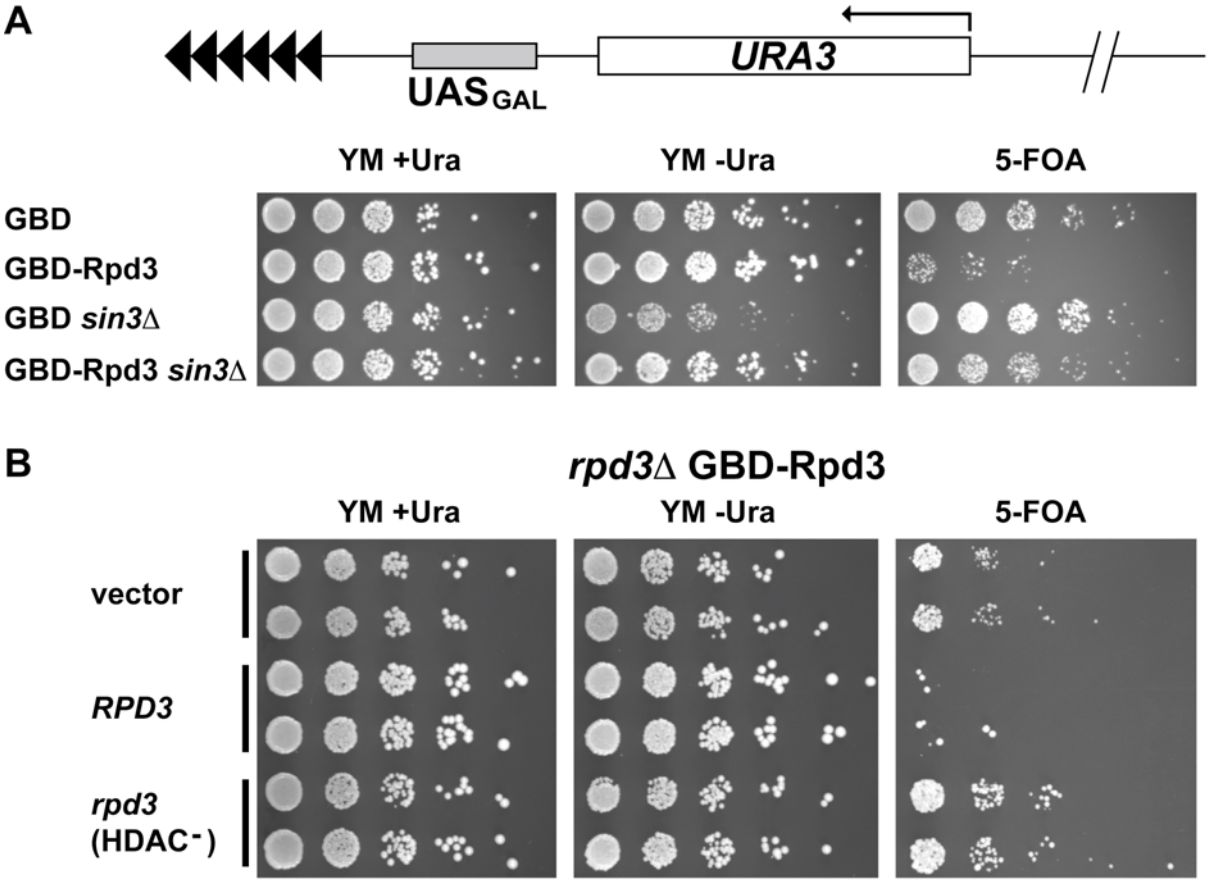

Figure 20 The targeted boundary function of Rpd3 required the HDAC activity of Rpd3.
(A) Cells with *URA3* inserted at TEL VII-L and with a Gal4 binding site at the telomere-proximal side (schematic drawing), were transformed with plasmids carrying the *RPD3* gene fused to the *GAL4* DNA binding domain (GBD-Rpd3) or with the vector control (GBD). Repression of *URA3* was tested by growth on *URA3*-counterselective 5-FOA plates. Cells were grown for two days at 30°C. (B) The targeted boundary function of Rpd3 depended on its catalytic activity. Cells with a Gal4 UAS between telomeric heterochromatin and a subtelomeric *URA3* reporter (as in A) were disrupted for endogenous *RPD3* and transformed with *RPD3* or a catalytically dead *rpd3* allele (*rpd3*-H150:151A, referred to as *rpd3*-HDAC$^-$).

The observation that GBD-Rpd3 alone was able to create a boundary raised the question whether this observation depended on the deacetylation activity of Rpd3. To this end, we asked whether a targeted Rpd3 complex with catalytically inactive Rpd3 (De Nadal et al. 2004) was unable to induce a boundary. We found this to be the case (**Figure 20B**). Specifically, we found that the boundary function of Rpd3 required the endogenous *RPD3* gene. However, GBD-Rpd3 was unable to complement *rpd3*Δ in boundary formation (**Figure 20B**, vector control). This suggested that the Gal4 DNA binding domain was required to target GBD-Rpd3 to the Gal4 UAS, but the deacetylation that established the boundary was catalyzed by chromosomally encoded Rpd3, which likely was targeted to the boundary through GBD-Rpd3. Significantly, upon reintroduction of mutant *rpd3* (*rpd3* HDAC$^-$) into *rpd3*Δ, GBD-Rpd3 cells were unable to restore the boundary, whereas reintroduction of *RPD3* restored boundary formation (**Figure 20B**). This further strengthened the notion that it was direct deacetylation at the boundary by Rpd3 that established a boundary against heterochromatin spreading.

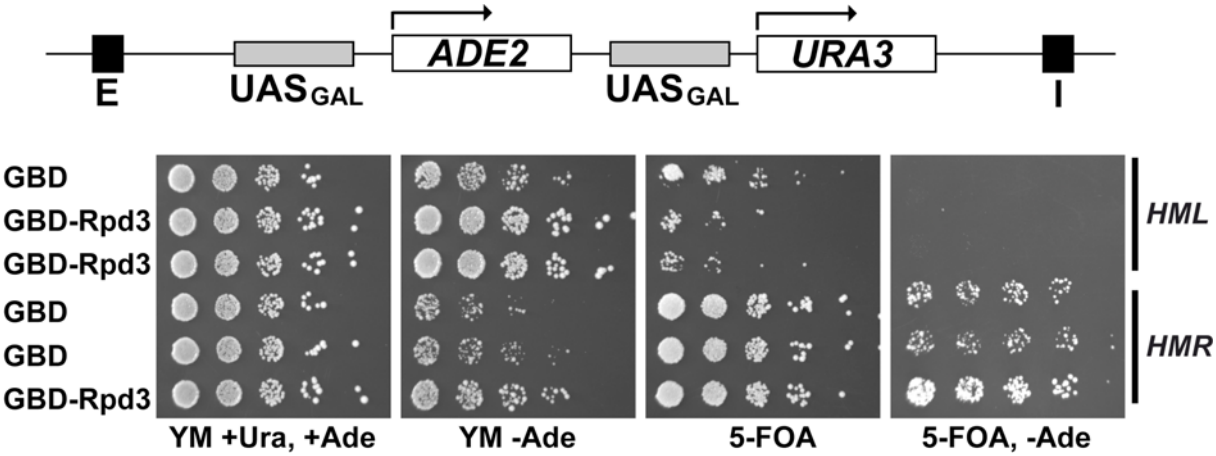

Figure 21 Tethered Rpd3 disrupted silencing at *HML* and had insulating activity at *HMR*.
Cells with *URA3* and *ADE2* flanked from Gal4 binding sites inserted between the *HML* or *HMR* E and I silencers (schematic drawing), were transformed with plasmids carrying the *RPD3* gene fused to the *GAL4* DNA binding domain (GBD-Rpd3) or with the vector control (GBD). Repression of *URA3* was tested by growth on *URA3*-counterselective 5-FOA plates, derepression of *ADE2* was tested by growth on Minimal plates omitting adenine (YM –Ade), insulating activity was tested by growth on 5-FOA omitting adenine (5-FOA, –Ade). Cells were grown for two days at 30°C. The experiments are courtesy of Prof. Ann Ehrenhofer-Murray.

We next asked whether the ability of Rpd3 to induce a boundary was specific for the telomeres, or whether Rpd3 also could stop SIR spreading at the *HM* loci. To this end, we used a strain where an *ADE2* gene, flanked by Gal4 DNA binding sites and an *URA3* gene were inserted between the *HMR* or *HML* silencers (**Figure 21**). Recruitment of a boundary element to the Gal4 DNA binding sites normally abrogates spreading of SIR proteins from the

E and I silencers into the *ADE2* gene but not into the *URA3* gene. This construct allows screening for factors that can insulate *ADE2* from the surrounding heterochromatin without influencing spreading into *URA3*. Significantly, Rpd3 had anti-silencing activity at the *HML* construct, as shown by expression of both reporters, *ADE2* and *URA3* (**Figure 21**), indicating that the silencing disrupting activity of Rpd3 was not restricted to the telomeres. Furthermore, at *HMR*, Rpd3 had true insulating activity, as monitored by full *ADE2* expression and no *URA3* expression (**Figure 21**), indicating that Rpd3 functioned as a "true barrier" factor that can insulate *ADE2* from the surrounding heterochromatin without "desilencing" of *URA3*. In summary, these observations were similar to previous results with factors that have been described to have boundary activity (Valenzuela et al. 2008).

3.1.12 The boundary function of Rpd3 did not depend on subsequent chromatin modifying or remodelling activities

The observation that opposing enzymatic activities act in the same biochemical pathway raised the question how the restriction of heterochromatin by Rpd3 is achieved. In general, histone deacetylation is described as being necessary, rather than inhibitive for heterochromatin formation. One consideration is that histone deacetylation can also act as prerequisite for lysine methylation or even ubiquitination (van Leeuwen et al. 2002; Santos-Rosa et al. 2004). Furthermore, both modifications depend on histone ubiquitination by Rad6 (Briggs et al. 2002). Since the onset of these modifications requires a deacetylated lysine, one could speculate that deletion of these factors might phenocopy the deletion of *RPD3*, assuming that Rpd3 deacetylates H3K4, H3K79 or H2BK123 (target of Rad6 dependent ubiquitination (Robzyk et al. 2000)) during boundary formation. However, deletion of *DOT1*, *SET1* or *RAD6* was not lethal in the absence of Sas2 (**Table 8**). Also, deletions of other histone methyltransferases (HMTases) showed no synthetic growth defect with *sas2*Δ (**Table 8**), suggesting that the boundary function of Rpd3 was not mediated by lysine methylation or ubiquitination.

Acetylation of a lysine residue also influences the ability of kinases to phosphorylate nearby serine residues. For instance, phosphorylation of H2B S10 by Ste20 requires deacetylation of the neighboring H2B K11 by Hos3 (Ahn et al. 2006). We therefore tested the growth of strains deleted for *SAS2* and kinases, revealing no synthetic growth defect (**Table 8**). This indicated that the lethality between *sas2*Δ and *rpd3*Δ was not due to histone phosphorylation. Furthermore, mutations in the chromatin remodelling complex, *isw2*Δ

(subunit of ISWI chromatin remodelling complex), the absence of the yeast linker histone Hho1 or other known boundary factors were not lethal together with *sas2*Δ (**Table 8**). This suggested that the boundary function of Rpd3 did not work through a mechanism that modifies a lysine after deacetylation through Rpd3.

Since the onset of global H4 K16 acetylations is coupled to chromatin assembly factors (Meijsing and Ehrenhofer-Murray 2001; Osada et al. 2001), one could speculate that disruption of chromatin assembly factors might also influence H4 K16 acetylation, and therefore be synthetically lethal with *rpd3*Δ. To this end, we also tested synthetic genetic interactions between *rpd3*Δ and *cac1*Δ, *asf1*Δ or *rtt106*Δ (another histone chaperone (Huang et al. 2005)). However, none of the chromatin assembly factor deletions showed lethality with *rpd3*Δ. This suggested that a reduction of H4K16 acetylation in *cac1*Δ or *asf1*Δ might cause reduced viability (**Table 8**), but not to the degree of *sas2*Δ *rpd3*Δ (also see 3.2.4).

Table 8 Summary of synthetic genetic interactions

Deleted genes	Protein function	Viability of double mutants with	
		*sas2*Δ	*rpd3*Δ
RTT106	chromatin assembly factor	+	+
ASF1	"	+	(+)
CAC1	"	+	+
DOT1	HMTase	+	+
SET1	"	+	+
SET2	"	+	ND
SET3	"	+	ND
SET4	"	+	ND
SET5	"	+	ND
SET6	"	+	ND
SET7	"	+	ND
BDF1	boundary function	+	+
BDF2	"	+	+
RAD6	Ubiquitin-conjugating enzyme	+	(+)
ESC2	establishes silent chromatin	+	ND
HTZ1	histone variant	+	-
HHO1	linker histone	+	+
SNF1	kinase	+	ND
CTK1	kinase (RNA Pol II)	+	ND
CKA1	kinase (RNA Pol I / III)	+	ND
ISW2	subunit of ISWI chromatin remodelling complex	+	ND

ND, not determined; / not applicable; +, double mutants viable; (+), growth defect of double mutants; -, double mutants not viable

H4 K16 acetylation in subtelomeric regions through Sas2 mediates the incorporation of the histone variant H2A.Z (encoded by the *HTZ1* gene) (Shia et al. 2006). If the role of SAS-I in boundary formation is to mediate Htz1 incorporation into chromatin, one would expect that *htz1*Δ, like *sas2*Δ, is synthetically lethal with *rpd3*Δ. We and others found this to be the case (Keogh et al. 2005). However, unlike for *sas2*Δ *rpd3*Δ, the lethality between *htz1*Δ and *rpd3*Δ was not suppressed by *sir3*Δ (data not shown), suggesting that the lethality between *htz1*Δ und *rpd3*Δ was not (solely) due to inappropriate SIR spreading, and that the loss of Htz1 incorporation in *sas2*Δ cells was not the only cause for the lethality between *sas2*Δ and *rpd3*Δ. Taken together, these experiments showed that the lethality between *sas2*Δ and *rpd3*Δ and the boundary function of Rpd3 was not based on a mechanism that acts through the onset of modifications on a lysine deacetylated through Rpd3.

3.1.13 Enhanced SIR binding through enhanced acetylation in *rpd3*Δ mutants?

As another possibility how Rpd3 might work as a boundary element, one could speculate that one particular residue might be acetylated to facilitate SIR binding. If this residue is normally deacetylated by Rpd3, the absence of Rpd3 would lead to more SIR binding. In consequence, mutation of the residue that has to be deacetylated by Rpd3 to inhibit SIR binding, should also abolish the boundary function of Rpd3. The inability of Rpd3 to deacetylate this target residue should lead to spreading of telomeric heterochromatin independently of the presence of targeted Rpd3. Conversely, histone mutations that lead to SIR mislocalization should lead to telomeric derepression that might even be enhanced in the presence of targeted Rpd3.

To test this hypothesis, we asked whether histone mutations that imitate the acetylated state of a residue (lysine to glutamine) were capable of abrogating Rpd3's boundary function. To this end, we introduced histone mutations into the boundary reporter strain and measured *URA3* expression in the presence or absence of GBD-Rpd3.

Significantly, histone mutants that were found to partly restore the growth of *sas2*Δ *rpd3*Δ cells, also showed telomeric derepression in the absence of targeted Rpd3, indicating that the suppression of the lethality was due to reduced SIR binding in these mutants. However, none of the histone mutations abrogated the boundary function of Rpd3 (**Figure 22**). The mutations lead to decreased silencing of the *URA3* reporter, as seen by reduced FOA resistance, but they also decreased silencing in the absence of GBD-Rpd3 (**Figure 22**, upper panel). This indicated that the mutants tested influenced SIR binding, but had no effect on the boundary activity of targeted Rpd3. This in essence suggested that the model of enhanced SIR

binding to a acetylated residue, normally deacetylated through Rpd3, seemed unlikely as a mechanism for Rpd3 to inhibit SIR spreading.

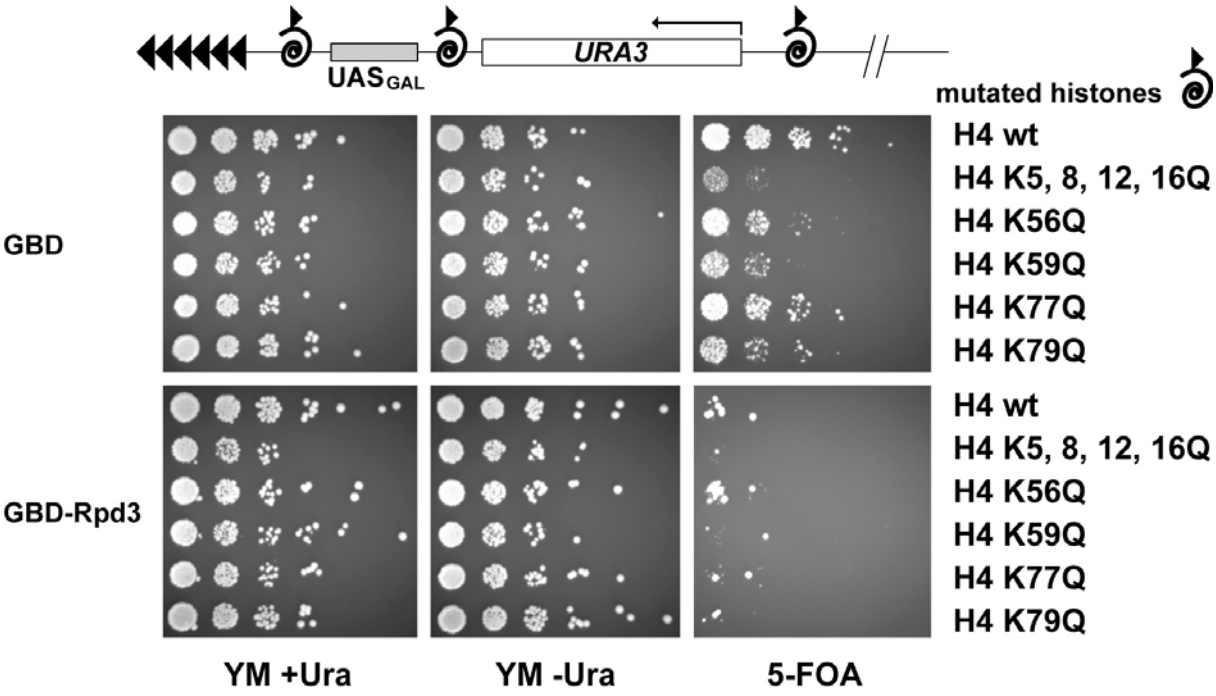

Figure 22 Histone mutants lead to telomeric derepression.
Yeast strains with the same telomeric reporter system as in **Figure 19** were transformed with additional variants of histones (indicated on the right) and with plasmids carrying the *RPD3* gene fused to the *GAL4* DNA binding domain (GBD-Rpd3) or with the vector control (GBD). Strains were grown for two days at 30°C on the indicated media.

3.1.14 A boundary function for Rpd3 through removal of Sir2 substrates

As a third hypothesis for how Rpd3 might work as a boundary element, we reasoned that deacetylation itself through Rpd3 might be prohibitive for the formation of heterochromatin. This model makes the prediction that the deacetylation through Sir2 that helps the SIR complex to propagate along the chromatin fibre is stopped by prior removal of the acetyl group through Rpd3. According to this model, one could postulate that other HDACs also should be able to create a boundary by deacetylating a target residue of Sir2. To this end, we targeted other HDACs to the telomeric reporter system by fusing them to the Gal4 DNA binding domain and tested their ability to create a boundary. We found that targeting the HDAC Hos2 (Wang et al. 2002) formed a boundary to telomeric heterochromatin (**Figure 23**), indicating that deacetylation by other HDACs in principle could also form a boundary to telomeric silencing. Conversely, other HDACs, for instance Hos1, Hos3, Hda1, Hst1, Hst3 or Hst4 (Ekwall 2005) did not show this activity (**Figure 23**). Furthermore, targeting of Hst2

even supported heterochromatin spreading into the reporter gene, shown as an inability to grow on plates lacking uracil due to *URA3* repression. GBD-Hos1 led to a growth defect on plates lacking uracil that did not correspond to an influence on boundary formation (**Figure 23**). Taken together, this analysis showed that other HDACs in principle could also have boundary activity and further suggested that the ability of HDACs to induce a boundary to heterochromatin depended on the particular deacetylation activity of the particular HDACs (see Discussion).

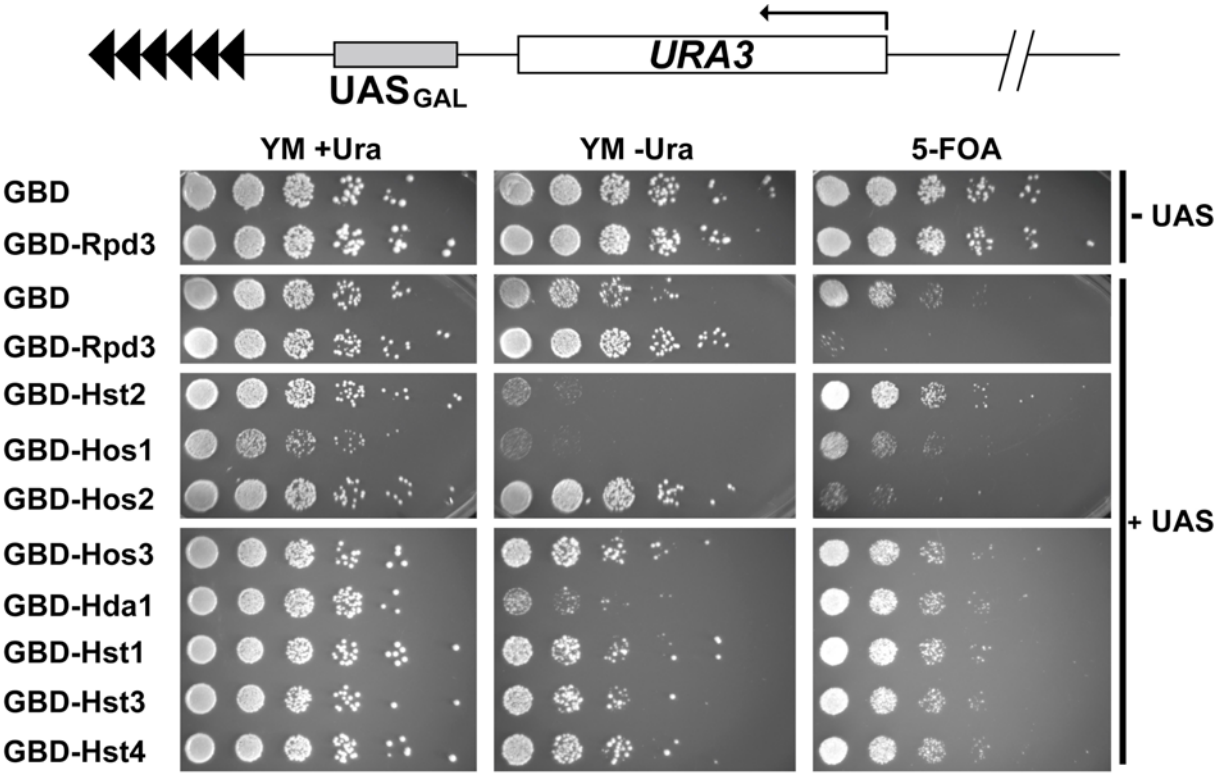

Figure 23 Targeted boundary function of yeast HDACs depended on their substrate specificity.
The telomeric reporter system of **Figure 19** was used to test yeast HDACs for their ability to form a boundary to heterochromatin.

3.1.15 Disruption of the AAA+ domain within Sir3 abrogated SIR spreading

The above observation that some HDACs can create a boundary, whereas others can not, raised the question how one but not another HDAC inhibits SIR spreading. Importantly, the HDACs that stopped heterochromatin (Rpd3 and Hos2) are NAD^+-independent, whereas the HDAC that supported heterochromatin formation (Hst2) is NAD^+-dependent (Ekwall 2005). One important difference between NAD^+-dependent and independent deacetylation reaction is the production of the metabolite O-acetyl-ADP-ribose (OAADPR) during the NAD^+-

dependent deacetylation reaction (Tanner et al. 2000). This metabolite is bound by the SIR complex and has been proposed to be one of the driving forces in the polymerization of the SIR complex on chromatin (Liou et al. 2005). According to this model, the removal of Sir2 substrates through previous deacetylation renders Sir2 unable to produce OAADPR, which then reduces SIR propagation along the chromatin fiber and stops heterochromatin spreading. This makes the prediction that a SIR complex that is unable to bind OAADPR through mutation in the OAADPR binding site within the SIR complex, should be unable to fulfil its role in spreading and silencing. Sir3 is a likely candidate for OAADPR binding, because Sir3 contains a domain that bears similarity to the nucleotide (ATP) binding domain of AAA+ ATPases (Gasser and Cockell 2001). To get further insight into this domain of Sir3, we performed an *in silico* analysis of the Sir3 structure (collaboration with Prof. Dr. Daniel Hoffmann and Nikolaj Dybowski from the bioinformatics department at the University of Duisburg-Essen). This modelling of Sir3 on the structure of AAA+ ATPases shows that the Sir3 model contains an additional cavity as compared to other ATPases that may accommodate the O-acetyl-ribose moiety of OAADPR (**Figure 24**).

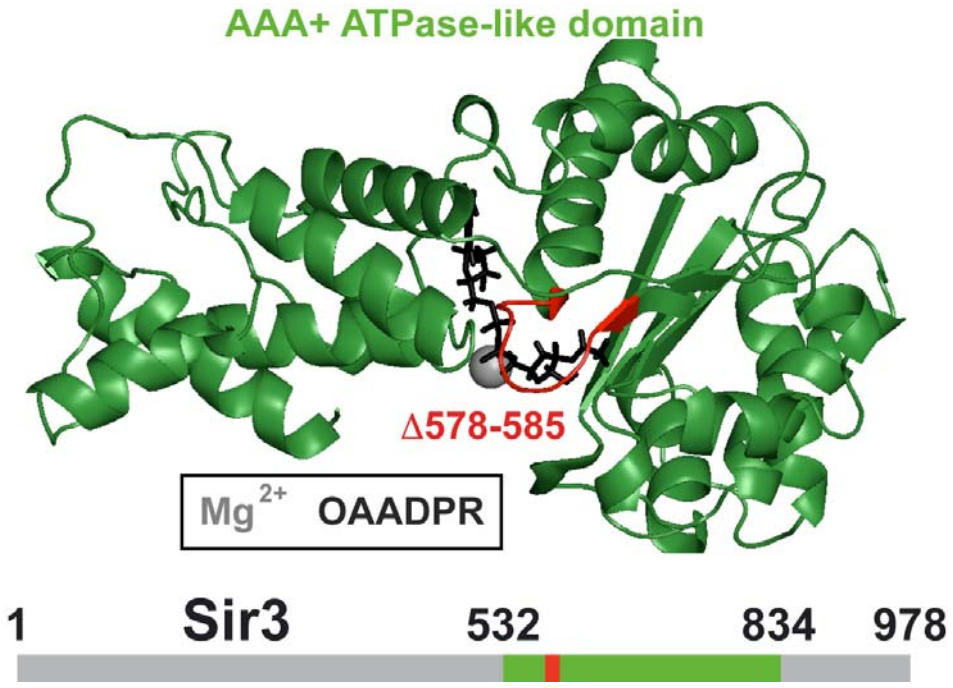

Figure 24 Computational modelling of Sir3 revealed a possible OAADPR binding pocket.
Model of the AAA+ ATPase-like domain of Sir3. For a computational modelling of Sir3, the primary sequence of Sir3 AAA+ domain and structures of other proteins of the AAA+ family were subjected to a sequence-structure alignment, using Modeller 9v2 (see Material and Methods for details). The model was visualized using PyMOL. The position of the putative OAADPR binding pocket is marked by OAADPR. The mutation of Sir3 used in this study is indicated in red. The structural information is courtesy of Prof. Daniel Hoffmann.

According to the observation that OAADPR is necessary to rearrange the SIR complex (Liou et al. 2005), a mutation that abolishes OAADPR binding to SIR presumably should abrogate the ability of the SIR complex to spread, and therefore should also abrogate the *sas2*Δ *rpd3*Δ lethality. Since deletion of *SIR3* abrogated the *sas2*Δ *rpd3*Δ lethality, functional *SIR3* alleles should restore lethality in this background. To test this, we constructed an allele of *SIR3* that deleted amino acids 578 to 585 in the Sir3 protein and therefore affected the putative OAADPR binding site. This allele was unable to restore the lethality in *sas2*Δ *rpd3*Δ *sir3*Δ cells (**Figure 25A**), and it was unable to support *HM* silencing (data not shown), indicating that it had lost functionality, while being expressed at levels comparable to endogenous Sir3 (**Figure 25B**). This showed that the AAA+ domain of Sir3 was important for its silencing function, suggesting that OAADPR binding to Sir3 was critical for heterochromatin formation.

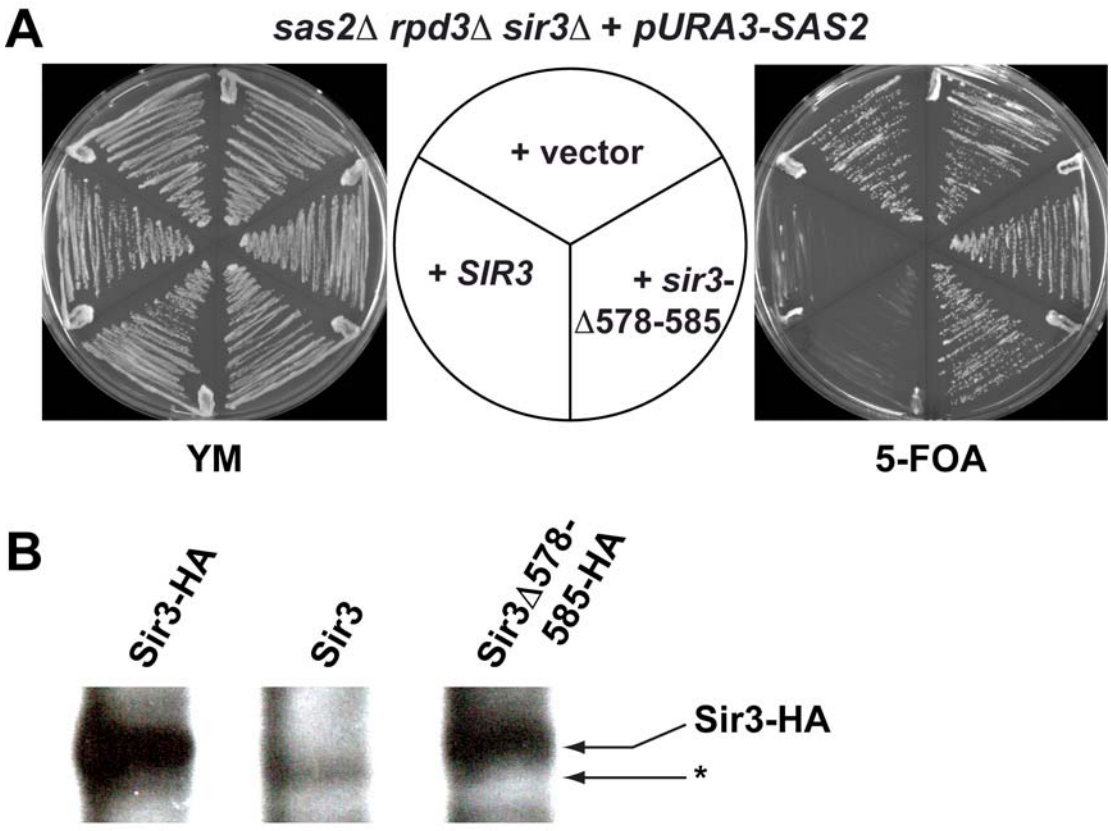

Figure 25 The putative OAADPR binding domain of Sir3 was necessary for its function in silencing.
(A) Mutation of the AAA+ domain of Sir3 abrogated its ability to spread at telomeres. *rpd3*Δ *sas2*Δ *sir3*Δ p*URA3-SAS2* strains carrying the indicated *sir3* allele were tested for their ability to lose the *SAS2* plasmid on 5-FOA. (B) The inability of *sir3*-Δ578-585 was not due to protein degradation. Protein level of HA tagged Sir3 and Sir3-Δ578-585 as measured by Western Blot using antibodies against the HA epitope. The asterisk indicates an unspecific band.

The observations described above suggest that the loss of OAADPR binding to Sir3 abrogated the Sir3 function in SIR spreading. Although the mutant was expressed at endogenous protein levels (**Figure 25B**), this effect might also result from a loss of protein function due to misfolding or conformational rearrangements of the protein. To test this, we asked whether other typical Sir3 functions were also influenced by our mutation. Since Sir3 *in vivo* forms dimers (McBryant et al. 2006), we first asked in a yeast two-hybrid experiment whether the Sir3 dimerization was affected by disruption of the putative OAADPR binding site of Sir3. Significantly the Sir3 dimerization was reduced (**Figure 26A**) but not completely abolished, indicating that the functionality of Sir3 was diminished by disruption of the AAA+ motif.

The assembly of the SIR complex involves binding of Sir3 and Sir4 to deacetylated histone tails (reviewed in (Rusche et al. 2003)). In a next step, the structural rearrangement that leads to the repressive SIR complex is presumably induced through OAAADPR (Liou et al. 2005). However, this structural change within SIR facilitates or strengthens the interaction between Sir3 and Sir4 (Liou et al. 2005). In addition to the reduction of the Sir3 dimerization activity, we also found that the two-hybrid interaction between Sir3 and Sir4 was abolished in the mutant *SIR3* allele (**Figure 26A**), suggesting that binding of OAADPR to Sir3 is important for the SIR complex integrity.

To further characterize the *sir3* mutant, we asked whether the Sir3-Δ578-585 mutant was able to support SIR spreading *in vivo* by measuring the enrichment of the mutated protein at heterochromatic loci. To this end, we measured the amount of HA-tagged Sir3 and Sir3-Δ578-585 at the right telomere of chromosome VI, as described in **Figure 14** for Sir2. Specifically, the amount of Sir3-Δ578-585 bound to the telomere ends was significantly reduced compared to wt Sir3 (**Figure 26B**). Although the mutant still was able to bind to chromatin to a certain background level (**Figure 26B**, for instance 15 kb away from the telomere end), the inability to become enriched at the telomere ends suggested that the AAA+ domain of Sir3 was important for its function in silencing and heterochromatin spreading.

In summary, this analysis suggested that binding of OAADPR to the SIR complex is a critical step in SIR complex assembly. Therefore, disruption of OAADPR production through removal of acetyl groups prior to Sir2 deacetylation, as disruption of OAADPR binding to Sir3 had the potential to abolish the propagation of the SIR complex along the chromatin fibre.

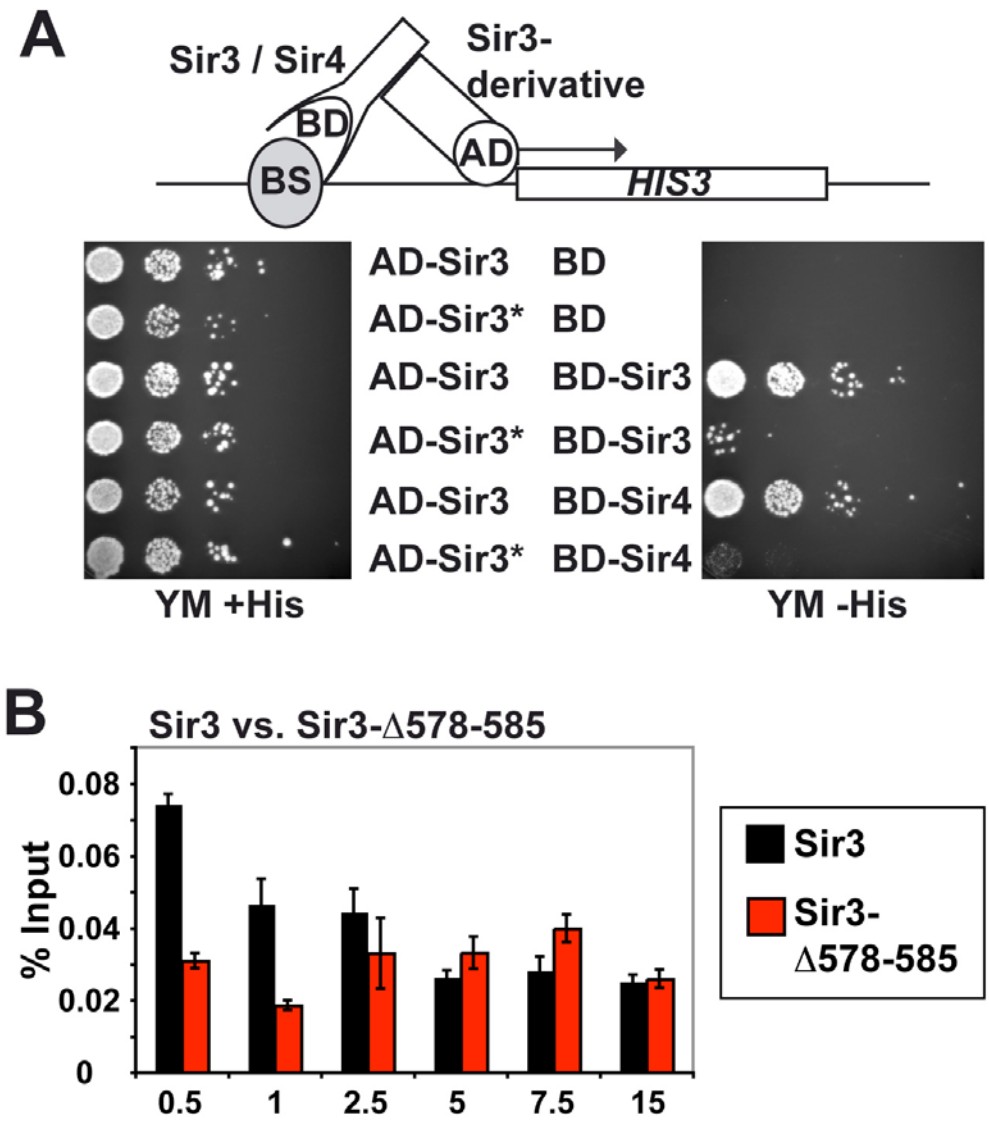

Figure 26 Disruption of the OAADPR binding motif abrogated the function of Sir3.
(A) Sir3-Δ578-585 reduced the Sir3-Sir3 and Sir3-Sir4 interaction. Yeast-two-hybrid interaction assay for specific interactions within the SIR complex. Sir3 and Sir3-Δ578-585 (Sir3*) were fused to the Gal4 activating domain and tested for interaction with Sir3 or Sir4 fused to the Gal4 DNA binding domain. Interaction was monitored by activation of the *HIS3* reporter on plates with or without histidine. (B) *sir3*-Δ578-585 was unable to support SIR spreading. The amount of Sir3 and Sir3-Δ578-585 was monitored by ChIP using HA-antibodies. The data is presented as in **Figure 14**.

Taken together, these experiments suggested that the ability of Rpd3 to antagonize heterochromatin formation worked through removal of acetyl groups in a way that left Sir2 unable to deacetylate these residues and produce OAADPR. This in essence led to the loss of OAADPR production and rendered the SIR complex unable to spread (see Discussion).

3.2 The removal of cytoplasmatic acetylation patterns partially depended on chromatin assembly factors

3.2.1 The *INO1* ORF became more acetylated in *cac1*Δ and *asf1*Δ cells

The duplication of genetic information in eukaryotes implies that the epigenetic information, which is carried on the histones, also has to be duplicated, involving the incorporation of newly synthesized histones (reviewed in (Ehrenhofer-Murray 2004)). Newly synthesized histones carry a typical modification pattern, which is characterized by acetylation of H4 K5 and K12 and is established through the cytoplasmatic HAT-B complex HAT1 (Ruiz-Garcia et al. 1998). Newly synthesized histones are further characterized by the absence of H4 K16 acetylation. Since the re-establishment of global H4 K16 acetylation patterns functions through interaction between SAS-I and chromatin assembly factors (Meijsing and Ehrenhofer-Murray 2001), one might hypothesize that the removal of cytoplasmatic acetylations via a global HDAC might also be coupled to chromatin assembly. One HDAC that leads to a global increase of H4 K5 and K12 acetylation in addition to its local function in gene repression is Rpd3 (Kurdistani et al. 2002). To test whether the absence of one or both chromatin assembly factors influences the global level of H4 K5 or K12 acetylation, we performed ChIP experiments in cells deleted for one or both chromatin assembly factors and compared it to the effect of *rpd3*Δ.

We first measured acetylation differences within the *INO1* gene, a gene that had previously been shown to become more acetylated in *rpd3*Δ cells (Rundlett et al. 1998), and that under normal growth conditions is repressed by Rpd3. As expected, H4 K5 and K12 became more acetylated in *rpd3*Δ cells within the whole region (**Figure 27**, (Rundlett et al. 1998)). In wt cells, the promoter region was most deacetylated, reflecting the repressed state, and the acetylation levels rose throughout the gene (**Figure 27**). Low acetylation of the promoter region was also seen in *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ cells, but within the *INO1* ORF H4 K12 became more acetylated in these cells, although not to the degree of acetylation in *rpd3*Δ. In contrast to the ORF, the acetylation state of the promoter region in *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ cells remained unchanged (**Figure 27**), suggesting that the targeted local function of Rpd3 in gene repression was not influenced by chromatin assembly factors. Unlike H4 K12, acetylation of H4 K5 remained unchanged by chromatin assembly factor deletions (**Figure 27**), suggesting different mechanisms for the removal of the two cytoplasmatic acetylation marks. Taken together, these experiments showed that the targeted

local function of Rpd3 worked independently of chromatin assembly and that the establishment of acetylation patterns was overridden by changes due to transcriptional regulation.

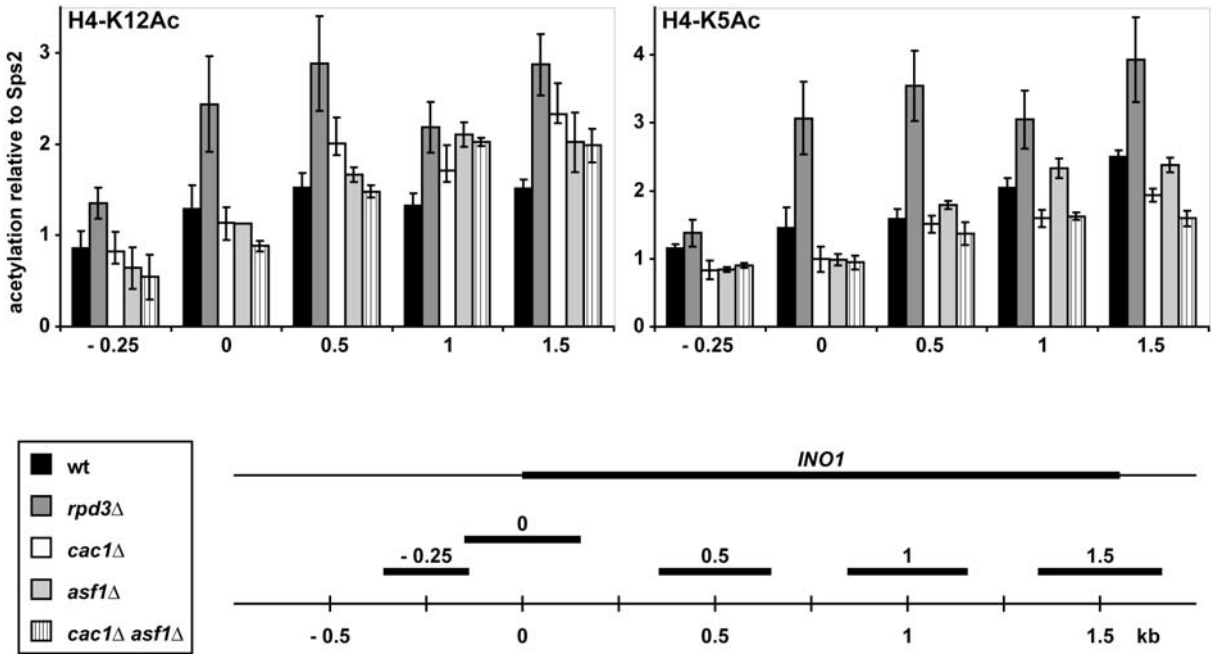

Figure 27 The absence of chromatin assembly factors led to higher H4-K12 acetylation in the body of the *INO1* gene.
Acetylation of the INO1 gene and promoter region was determined in chromatin immunoprecipitation (ChIP) experiments and is shown relative to the acetylation of *SPS2*. Relative acetylation is given as a function of the distance within the *INO1* gene in wild-type (wt) or strains lacking either one or both chromatin assembly factors or the HDAC Rpd3. ChIPs were performed with antibodies against acetylated lysines. Error bars give standard deviations (see Material & Methods). A schematic representation of the INO1 gene and fragments amplified in semi-quantitative PCR is shown in the lower panel.

3.2.2 Late-replicating intergenic regions became more acetylated in *cac1*Δ and *asf1*Δ cells

In addition to its local gene repression function, Rpd3 also controls the timing of replication initiation, especially at late initiating origins (Vogelauer et al. 2002; Aparicio et al. 2004). To ask how the loss of chromatin assembly factors influences the acetylation at origins of replication, we tested one late and one early-initiating origin. The influence of *rpd3*Δ (Aparicio et al. 2004), *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ on the acetylation of the early initiating origin (ars607) was not significant (**Figure 28**), whereas the late replicating origin (ars603) became more acetylated in *rpd3*Δ cells (**Figure 28**, (Aparicio et al. 2004)), as well as in *asf1*Δ and *cac1*Δ *asf1*Δ cells, on H4 K5 and K12 (**Figure 28**). Conversely, the acetylation of H4 K5 and K12 was not changed in *cac1*Δ cells and not further enhanced in *cac1*Δ *asf1*Δ

cells, compared to *asf1*Δ, suggesting that the removal of cytoplasmatic acetylation marks at origins worked through Asf1 but not through CAF-I (**Figure 28**).

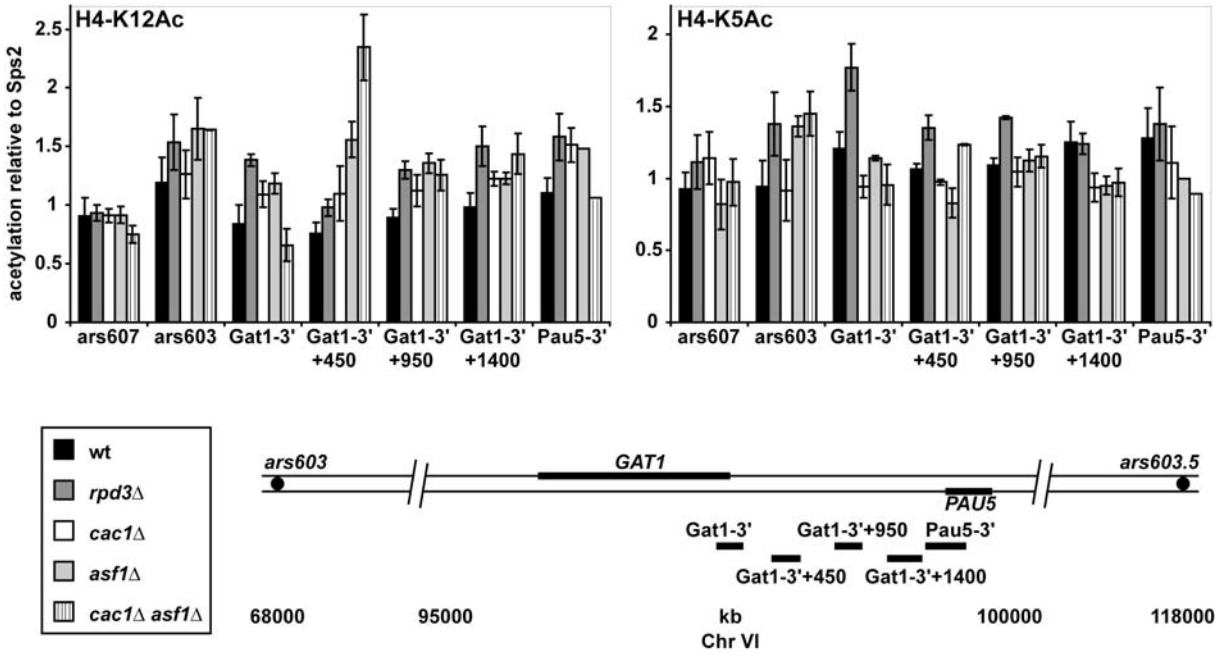

Figure 28 Late replicating intergenic regions became more acetylated at H4-K12 in the absence of chromatin assembly factors.
Acetylation of the *GAT1-PAU5* intergenic region was determined by ChIP and is shown relative to the acetylation of *SPS2*, as in **Figure 27**. A schematic representation of the intergenic region and fragments amplified in semi-quantitative PCR is shown. The intergenic region with opposing 3' ends of *GAT1* and *PAU5* becomes late replicated during S-phase and the next origin of replication is 18 kb away. Additionally acetylation of a late replicating origin (ars603) and an early initiating origin (ars607) is shown.

We next hypothesized that the removal of cytoplasmatic acetylation marks through a global HDAC might be coupled to chromatin assembly. This effect might be more significant in regions where Rpd3 is not recruited to freshly replicated chromatin via transcriptional regulators. Within the *INO1* gene, the acetylation state was mainly influenced by the targeted local repression function of Rpd3 (**Figure 27**). To look for regions where the acetylation differences are not overridden by changes due to transcriptional regulation, intergenic regions were chosen for further analysis. Late replicating regions show higher acetylation differences and earlier replication initiation in *rpd3*Δ cells, compared to wt (**Figure 28**, (Aparicio et al. 2004)). Furthermore, CAF-I was suggested to incorporate H4 acetylated at K5 and K12 into late replicating regions in higher eukaryotes (Taddei et al. 1999), making late replicating regions good candidates for studies of acetylation differences in *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ cells. One candidate region lies on chromosome VI, where a two kb intergenic region separates two genes with opposing 3' ends (**Figure 28**), and chromosome VIII contains a 3.3

kb long intergenic region between genes with divergent promoters (**Figure 29**). To test these regions for acetylation differences in the absence of chromatin assembly factors, we compared the effect of *rpd3*Δ on the acetylation level of the region with the effect of *cac1*Δ, *asf1*Δ and *cac1*Δ *asf1*Δ. As expected, the absence of Rpd3 led to higher H4 K5 and K12 acetylation in both intergenic regions (**Figure 28**, **Figure 29**). In contrast to the origins of replication, the intergenic regions showed only changes of the wt acetylation patterns of H4 K12 in the absence of chromatin assembly factors (**Figure 28**, **Figure 29**). In general, *cac1*Δ and *asf1*Δ cells showed an intermediate H4 K12 acetylation level between the effects of *rpd3*Δ and wt (**Figure 28**, **Figure 29**). Like for the *INO1* gene, the proximity of transcriptional regulators (promoter and start region of *AAP1* or *YHR048W*) weakened the effect of *cac1*Δ and *asf1*Δ on H4 K12 acetylation. In contrast to the Cac1- or Asf1- .independent influence of transcriptional regulators on lysine acetylation, the distance to the next origin of replication had no effect on the acetylation in *cac1*Δ or *asf1*Δ strains (compare **Figure 28** and **Figure 29**), suggesting that the effect of replication initiation was restricted to regions close to the origin itself.

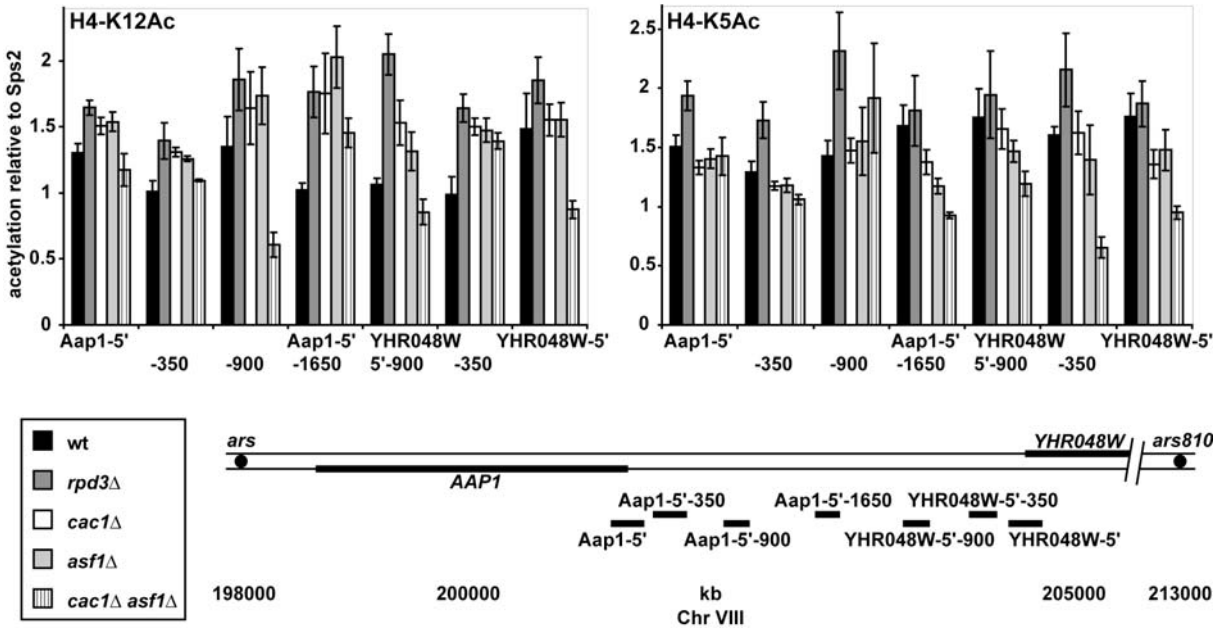

Figure 29 The higher H4-K12 acetylation of intergenic regions was independent of replication initiation.
Acetylation of the *AAP1-YHR048W* intergenic region was determined by ChIP and is shown relative to the acetylation of *SPS2*, as in **Figure 27**. A schematic representation of the intergenic region and fragments amplified in semi-quantitative PCR is shown. The intergenic region with opposing 5' ends of *AAP1* and *YHR048W* becomes late replicated during S-phase and the next origin of replication is 2 kb away.

However, *cac1*Δ *asf1*Δ cells showed no additive effects on histone acetylation compared to the effect of either single deletions, and in most cases acetylation even dropped, suggesting that these cells may not completely assemble nucleosomes like wt cells.

Taken together, *cac1*Δ or *asf1*Δ led to higher H4 K12 acetylation in late replicating intergenic regions, although not to the degree of *rpd3*Δ, suggesting that a mechanism for the removal of cytoplasmatic acetylation marks exists that is not coupled to chromatin assembly and these additional mechanisms might also be responsible for H4 K5 deacetylation.

3.2.3 The higher acetylation in *cac1*Δ cells was based on an interaction between CAF-I and Rpd3

The observation that the HAT complex SAS-I interacts with chromatin assembly factors (Meijsing and Ehrenhofer-Murray 2001), suggested that chromatin assembly factors may serve as a platform to recruit histone modifying enzymes to freshly assembled chromatin. To test whether the influence of chromatin assembly factors on the H4 K12 acetylation was also based on a physical interaction between Rpd3 and CAF-I or Asf1, we performed interaction studies the yeast two-hybrid assay. To this end, the HDACs Rpd3 and Hda1, an HDAC mainly deacetylating HAST domains adjacent to the telomeres, were fused to the Gal4-binding domain (BD), chromatin assembly factors were fused to the Gal4 activating domain (AD), and the resulting strains were subjected to a yeast two-hybrid analysis. The reporter strain (AEY1280) contained two reporters under the control of Gal promoters, a *HIS3* reporter and a *lacZ* reporter. Interaction of the respective construct activates the expression of the reporters through recruitment of the Gal4 binding domain to the Gal4 binding sequence and activation of the reporter through the Gal4 activating domain (**Figure 30**, schematic representation). However, the BD-Rpd3 construct autoactivated the lacZ reporter, but the second reporter was not autoactivated by BD-Rpd3 (**Figure 30**).

Essentially, both reporters revealed two-hybrid interactions between Rpd3 and Asf1 and between Rpd3 and Cac1, the large subunit of CAF-I (**Figure 30**), suggesting that the partial defect in the removal of cytoplasmatic acetylations in strains lacking chromatin assembly factors might be based on an interaction between Rpd3 and chromatin assembly factors. An interaction between chromatin assembly factors and Hda1 was not found (**Figure 30**).

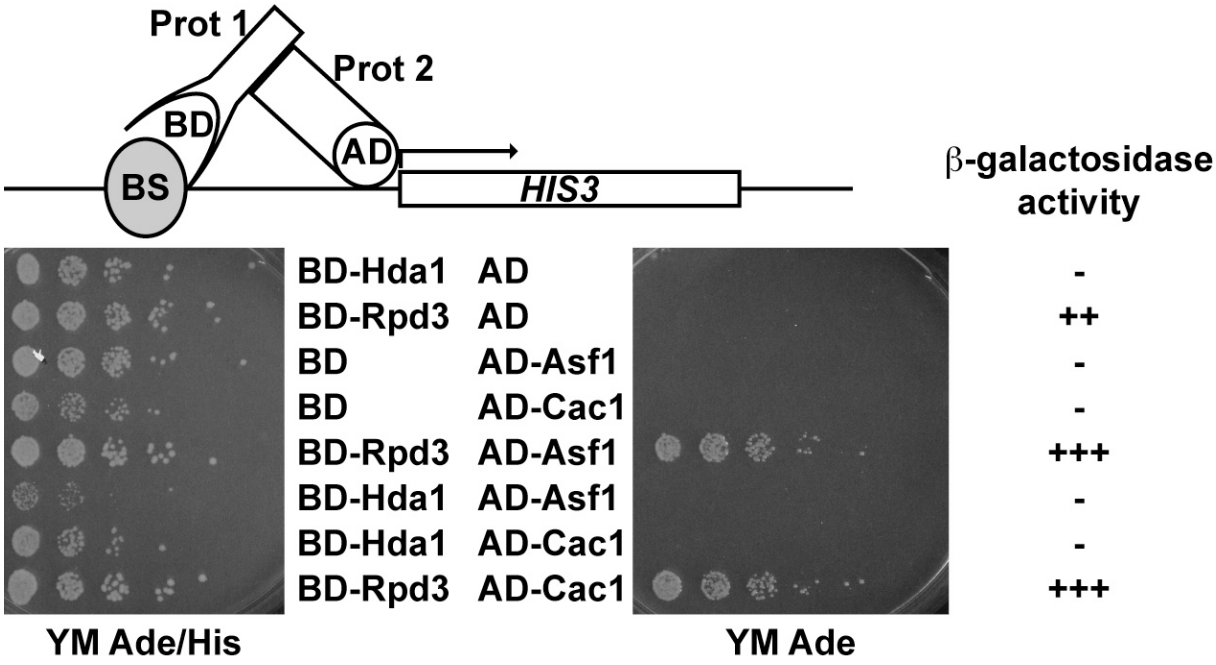

Figure 30 Yeast-two-hybrid interaction between Rpd3 and Asf1 and between Rpd3 and Cac1.
Indicated proteins were fused to the Gal4-binding domain (BD) or to the Gal4-activating domain (AD). The AEY1280 reporter strain was transformed with the indicated combinations of fusion proteins or the domains alone. To test for activation of the *HIS3* reporter, cells were spotted in 6-fold serial dilution and were grown for two days at 30°C on minimal plates with or without histidine. Activation of the lacZ reporter is given in quantification (- no activation; ++ activation; +++ strong activation). A schematic representation of the used two-hybrid system is shown in the upper part. Interaction between the protein fused to the Gal4-activating domain (AD) and the second protein fused to the Gal4-binding (BD) domain enables the binding of the constructs to the Gal4 binding sequences (BS) and activation of the reporter gene.

To test the proteins for direct physical interaction *in vivo*, we performed CoIP experiments with epitope tagged version of the proteins that showed two-hybrid interaction. To this end, the *RPD3* gene was fused to a 9×myc sequence, and the genes encoding the chromatin assembly factors were fused to a 3×HA sequence. The resulting proteins were detected with antibodies against the epitope tag in a Western blot. To test for interaction between the two proteins, we precipitated one protein from cell lysates of strains expressing both (or as control only one) of the fusion constructs and tested whether the second protein was co-precipitated. Therefore, we performed a western blot with an antibody against the potential interaction partner. Significantly, cell lysate containing Rpd3-myc and Cac1-HA showed clear signals for Rpd3-myc after precipitation of Cac1-HA with α-HA antibodies and vice versa (**Figure 31**), revealing a direct physical interaction between the two proteins. Although the Rpd3-myc signal was also detectable in cells that did not contain Cac1-HA, the signal was significantly stronger in cells that contained both proteins (**Figure 31**).

We hypothesized that the interaction between Rpd3 and Cac1 might serve as a platform to couple the removal of cytoplasmatic acetylations to chromatin assembly. Perhaps the

interaction between Rpd3 and Cac1 is most prominent during the S-phase of the cell cycle. To test this, we synchronized cells in the S-phase via treatment with hydroxyurea (HU). HU deprives the cells of nucleotides and thus stalls the replication forks. However, cells that were HU treated and therefore synchronized during S-phase did not show stronger interaction between Rpd3 and Cac1 (data not shown), suggesting that the interaction was strong enough to be even detectable in unsynchronized cultures.

Rpd3 also showed a two-hybrid interaction with Asf1 (**Figure 30**). However, the unspecific Asf1-HA signal after myc-IP in cells lacking Rpd3-myc could not be enriched in the presence of Rpd3-myc (**Figure 31**). Also vice versa no interaction between Rpd3 and Asf1 was detectable, suggesting that the two-hybrid interaction between these two proteins was not stable enough to reveal direct physical interaction *in vivo.*

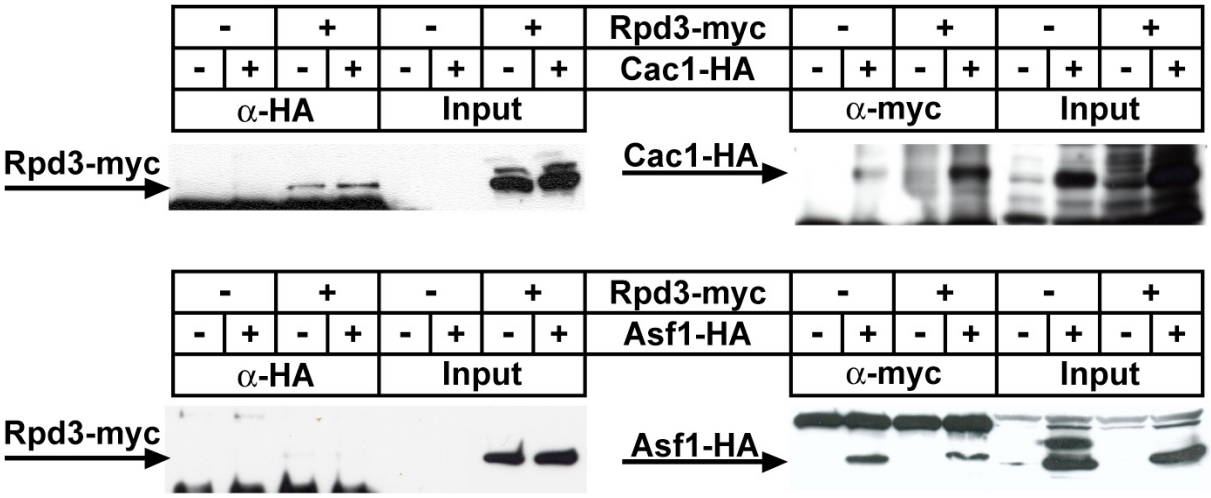

Figure 31 Physical interaction between Cac1 and Rpd3.
Rpd3 was tagged with the 9×myc epitope, Asf1 and Cac1 were tagged with the 3×HA epitope. Lysate of cells that contained (+) or did not contain (-) the epitope tagged proteins was precipitated with the antibodies indicated above. Precipitated proteins were analyzed by western blot. In the two left panels α-HA precipitated cell lysate was stained with α-myc antibodies to visualize Rpd3-myc and in the right panels α-myc precipitated cell lysate was analyzed with α-HA antibodies to detect Cac1-HA or Asf1-HA. Input samples indicate the proteins of interest.

Taken together these interaction studies revealed that Rpd3 and Cac1 interacted *in vivo*, suggesting that the incomplete removal of cytoplasmatic acetylations in *cac1*Δ cells may be due to reduced recruitment of Rpd3 to newly assembled chromatin in cells lacking Cac1. However, we were unable to detect a direct interaction between Rpd3 and Asf1, suggesting that the incomplete removal of cytoplasmatic acetylations in *asf1*Δ cells may not (solely) be due to reduced Rpd3 recruitment.

3.2.4 Rpd3 and Cac1 work epistatic in *HM* silencing

We hypothesized that CAF-I might have a platform function in recruiting histone modifying activities to fresh chromatin. This hypothesis is based on the observation that Sas2 is recruited to fresh chromatin via the chromatin assembly factor CAF-I (Meijsing and Ehrenhofer-Murray 2001). If the onset of H4 K16 acetylation on fresh chromatin depends on the Sas2-CAF-I interaction, a *cac1*Δ strain should show similar silencing defects as a *sas2*Δ strain. Indeed, this was found to be the case (Kaufman et al. 1997; Enomoto and Berman 1998)). Furthermore, the deletion of *SAS2* and the deletion of *CAC1* (or the other subunits of CAF-I) were epistatic in *HML* silencing (Meijsing and Ehrenhofer-Murray 2001), indicating that both factors lead to the same defect.

In analogy to *sas2*Δ, we now asked for effects of *rpd3*Δ combined with *asf1*Δ or *cac1*Δ in *HM* silencing. To this end, we performed quantitative mating assays with all combinations of *rpd3*Δ, *asf1*Δ or *cac1*Δ strains. In this assay, defects in *HML* silencing in a *MAT***a** strain lead to pseudo-diploid yeast cells with lower mating efficiency. Therefore, we created *MAT***a** strains carrying deletions as indicated in **Figure 32** and tested *HML* silencing by measuring the mating efficiency of these strains on lawns of *MAT*α test strains and *HMR* silencing was monitored on *MAT***a** test lawns, respectively. In both cell types, the absence of Rpd3 led to lower mating efficiency, although more significant for *HML* silencing (**Figure 32**). Deletion of *CAC1* or *ASF1* alone led to minor mating defects, whereas *asf1*Δ *cac1*Δ cells showed mating defects similar to *rpd3*Δ. Significantly, the additional deletion of *CAC1* in *rpd3*Δ cells did not enhance the mating defect of *rpd3*Δ (**Figure 32**). In contrast to that, the additional deletion of *ASF1* in *rpd3*Δ cells enhanced the mating defect, suggesting that Asf1 acted in a parallel pathway to Rpd3, whereas Cac1 and Rpd3 were epistatic in *HM* silencing. Additionally, cells deleted for *CAC1*, *ASF1* and *RPD3* showed no additional mating defect compared to *rpd3*Δ *asf1*Δ cells (**Figure 32**).

The growth of *cac1*Δ or *rpd3*Δ cells was not significantly impaired, whereas *asf1*Δ cells showed a weak, but noticeable growth defect (**Figure 32**). Also in this case, deletion of *RPD3* or *CAC1* in *asf1*Δ cells led to additional growth defects, that were comparable between *asf1*Δ *rpd3*Δ and *asf1*Δ *cac1*Δ, whereas *rpd3*Δ *cac1*Δ cells showed no obvious growth defect (**Figure 32**). These combinatorial effects suggested that Rpd3 and Cac1 work in the same biochemical pathway during growth and *HM* silencing, whereas Asf1 works in a parallel pathway. This further supported the notion that Rpd3 and CAF-I have the ability to work together in the replication-coupled removal of cytoplasmatic acetylation marks.

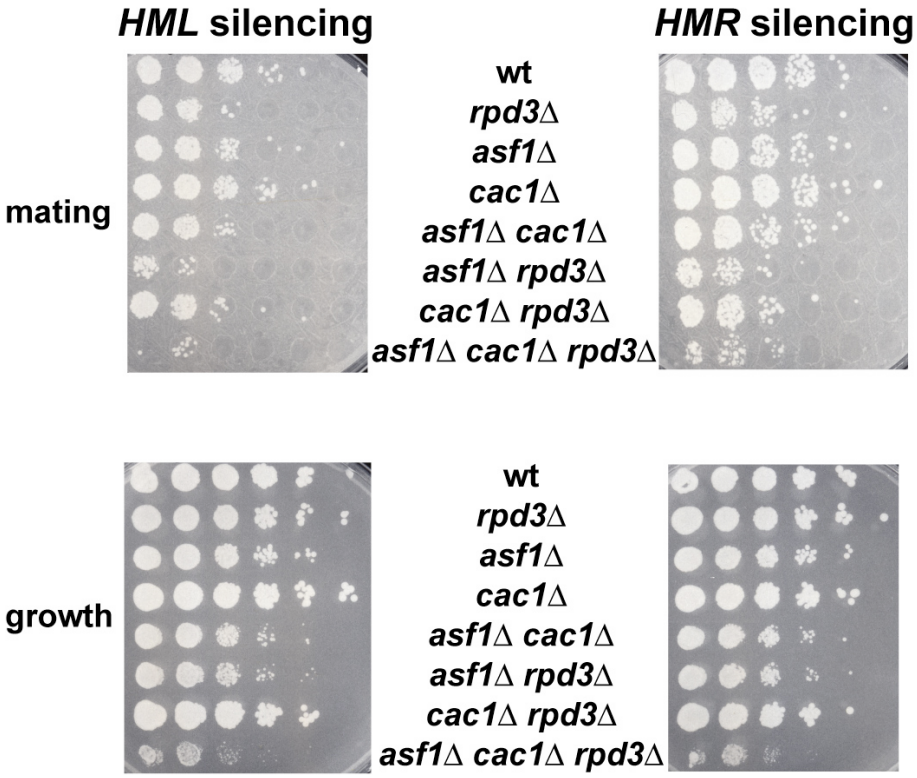

Figure 32 The absence of Rpd3 led to *HM* derepression.
Ten-fold serial dilution of the indicated yeast strains were grown for two days at 30°C on YPD (growth assay) or on YM with test lawns of AEY264 or AEY265. Silencing of *HML* is monitored as mating ability with the α*his4* test strain (AEY265) and *HMR* silencing as ability to mate with **a***his4* (AEY264), respectively.

As described above, Cac1 and Rpd3 work in the same biochemical pathway during growth and HM silencing, leading to comparable growth defect between *asf1*Δ *cac1*Δ, compared to *asf1*Δ *rpd3*Δ cells (**Figure 32**). However, the generation of *rpd3*Δ *cac1*Δ *asf1*Δ cells led to an additional growth defect (**Figure 32**). Since the onset of H4 K16 acetylation through Sas2 is coupled to chromatin assembly factors (Meijsing and Ehrenhofer-Murray 2001), the growth defect of *asf1*Δ *cac1*Δ *rpd3*Δ cells might partially reflect the lethality between *sas2*Δ and *rpd3*Δ. Remarkably, cells deleted for *RPD3*, *ASF1* and *CAC1* showed a growth defect that was not comparable to the lethality between *rpd3*Δ and *sas2*Δ, although Sas2 is recruited to fresh chromatin via chromatin assembly factors. This suggested that the establishment of global H4 K16 acetylation at boundaries is not solely dependent on chromatin assembly factors.

4 Discussion

Barriers between active and inactive chromatin in a variety of organisms have been associated with activating mechanisms, for instance histone acetylation, chromatin remodelling and nucleosome-free regions (Oki and Kamakaka 2002), as well as with the attachment with nuclear pore structures (Ishii et al. 2002). Here, we describe the unexpected finding that an enzymatic activity generally associated with repression, the HDAC Rpd3, was necessary to prevent the spread of heterochromatic SIR proteins into euchromatin at yeast telomeres. Loss of Rpd3 led to SIR spreading and gene repression in subtelomeric regions and caused lethality in cells lacking the HAT complex SAS-I, which are also characterized by inappropriate SIR localization (Kimura et al. 2002; Suka et al. 2002). Importantly, artificially tethering Rpd3 to telomeres or to the *HM* loci created a boundary and relieved repression of an otherwise silenced reporter gene. In summary, these results demonstrated that histone deacetylation by Rpd3 was necessary and sufficient for boundary formation in *S. cerevisiae*. This opens up new insights into the mechanisms for the demarcation of chromatin states.

4.1 Rpd3 formed boundaries against heterochromatin

The unexpected observation that the HDAC Rpd3 antagonizes the SIR complex which itself is characterized by the HDAC Sir2, raised the question how directly this effect of deacetylation through Rpd3 influenced boundary formation, or whether it was a result of a general imbalance in histone acetylation. One possibility to address this issue is to test if the effect of *rpd3*Δ in the *rpd3*Δ *sas2*Δ lethality might be phenocopied by another HDAC deletion, or equivalently, one could ask if other HAT deletions might be able to phenocopy *sas2*Δ in the *sas2*Δ *rpd3*Δ lethality. In essence, we found that the lethality between *sas2*Δ and *rpd3*Δ was specific for these two enzymes, in that no other HDAC deletion was synthetically lethal with *sas2*Δ. However, in addition to Rpd3, Hos2 was also able to actively create a boundary. Since *sas2*Δ *rpd3*Δ is lethal, but not *sas2*Δ *hos2*Δ, we hypothesized that Rpd3 *in vivo* can form boundaries presumably through deacetylation within subtelomeric regions, whereas Hos2 is not normally recruited to subtelomeric regions. This suggested that Rpd3 is the only HDAC in yeast that normally can form a boundary against heterochromatin spreading. A mechanism how Rpd3 and Hos2 create boundaries and how Rpd3 might be targeted to the subtelomeres *in vivo* is discussed below.

The observation that the parallel loss of two boundary factors led to SIR spreading to a detrimental degree is somewhat comparable to the effect of *gcn5*Δ *elp3*Δ strains. In this case, the parallel loss of both acetyltransferases leads to SIR spreading into centromere-proximal regions, which causes a growth defect that can be abrogated by additional deletion of one of the SIR components (Kristjuhan et al. 2003). Although SIR spreading due to the parallel loss of two HATs seems intuitive compared to the loss of an HDAC and a HAT, we hypothesize that we observe a comparable phenomenon, namely SIR spreading due to the loss of two independent boundary factors. Nevertheless, the Kristjuhan et al. study indicated Gcn5 as a boundary element. Surprisingly, the parallel loss of Gcn5 and Rpd3 does not lead to synthetic lethality, as shown by studies that intensively work with *gcn5*Δ *rpd3*Δ strains (Burgess et al. 1999; Biswas et al. 2008). Also, the absence of other HATs that are able to actively create boundaries when targeted to the telomeres, like Sas3 or Esa1 (Jacobson and Pillus 2004; Oki et al. 2004), shows no synthetic growth defect in combination with *rpd3*Δ. This suggests that these HATs do not have the same impact on boundary formation as Sas2 and indicates that Sas2 and Rpd3 are both boundary factors that influence the histone modification state in subtelomeric regions *in vivo* and therefore inhibit SIR spreading.

Taken together, our data indicate that Rpd3, as Sas2, is a boundary element that is required for the restriction of heterochromatin through changing the histone modification state, and by doing so influencing the binding or the assembly of the SIR complex.

4.2 Does boundary formation through Rpd3 require permanent presence of Rpd3 at the subtelomere?

As described above, we observed that Rpd3 could induce boundary formation *in vivo* as well as after targeting to telomeric or *HM* reporter genes. Essentially, we found two HDACs with the ability to actively create a boundary against heterochromatin formation when targeted to the telomeres, Rpd3 and Hos2. We hypothesized that the difference between the two HDACs is that one, Rpd3, *in vivo* deacetylates subtelomeric regions and therefore is a true boundary element, whereas the other, Hos2, could only form a boundary when targeted to the telomere. This suggests that Rpd3 deacetylates the chromatin of subtelomeric regions and raises the question whether Rpd3 does this while permanently being located at the subtelomere, whether the Rpd3 global deacetylation facilitates the boundary function, or whether Rpd3 can be recruited to boundaries. However, an earlier study found no strong binding of Rpd3 to

subtelomeric regions (Kurdistani et al. 2002), suggesting that Rpd3 is not permanently bound to subtelomeric regions to induce boundary formation. We therefore propose that the boundary function for Rpd3 described here reflects a global, untargeted (versus a targeted) role for the Rpd3 (L) complex in establishing histone acetylation patterns at telomeres, because the loss of Rpd3 influenced subtelomeric acetylation, although it was not permanently located there.

The hypothesis that Rpd3 functions in boundary formation through a transient contact to chromatin was further supported by the notion that Rpd3 could be recruited to chromatin via chromatin assembly factors. As described in 3.2, we propose that Rpd3 deacetylates cytoplasmatic acetylation marks on newly synthesized histones when it is associated with the chromatin assembly factor I (CAF-I). To test whether Rpd3 is targeted to chromatin via CAF-I during replication coupled chromatin assembly, we asked whether histone acetylation is influenced by the loss of chromatin assembly factors. Significantly, upon deletion of *CAC1* we found higher acetylation of H4 K12 in regions where Rpd3 is not recruited through other mechanisms, indicating that the removal of this mark is coupled to chromatin assembly. However, the removal of H4 K5 did not depend on Cac1, suggesting that the removal of cytoplasmatic acetylations is not solely dependent on CAF-I, and that additional factors exist that remove these acetylations.

One possible mechanism for the removal of H4 K5 acetylation might be the involvement of the other chromatin assembly factor, Asf1, in the chromatin assembly coupled restoration of parental acetylation marks during replication. However, the absence of Asf1 had no influence on H4 K5 and K12 acetylation, with the only exception at the late initiating origin of replication (ars 603). This suggested that the recruitment of Rpd3 to replication forks was facilitated by Cac1 alone.

To further ask whether the recruitment of Rpd3 to chromatin depends on CAF-I, we tested whether both factors work epistatically in the establishment of heterochromatin. Significantly, we found that the deletions of *RPD3* and *CAC1* were epistatic in the establishment of *HM* silencing, indicating that the deletion of *RPD3* had the same impact on heterochromatin formation at the *HM* loci as deletion of *CAC1*. This strengthened the hypothesis that CAF-I and Rpd3 work together in the removal of cytoplasmatic acetylation marks and reflected a mechanism that possibly recruits Rpd3 to chromatin to facilitate global histone deacetylation. Therefore, coupling of deacetylation to chromatin assembly creates a transient contact of Rpd3 to chromatin, rather then a permanent location of Rpd3 at subtelomeric regions.

4.3 Is the Rpd3 boundary function related to other chromatin modifying or remodelling activities?

How can the HDAC Rpd3 work as a boundary element? A priori, our observation is counterintuitive, because chromatin deacetylation is generally viewed as being necessary for, rather than prohibitive to SIR spreading. Another explanation for the heterochromatin antagonizing activity of Rpd3 might be that the removal of lysine acetylation through Rpd3 enables the onset of another modification. An acetylated lysine, for instance, cannot be methylated and vice versa. In line with this, it was described in *Drosophila* that heterochromatic complexes that establish H3 K9 methylation, also contain RPD3 to deacetylate H3 K9. By doing so, this complex not only prevents the onset of H3 K9 acetylation as a typical euchromatic mark (reviewed in (Ebert et al. 2006)), but also enables the establishment of H3 K9 methylation as a typical heterochromatic signal.

In contrast to H3 K9 methylation in *Drosophila*, lysine methylation in yeast (e.g. H3 K4; H3 K79) can be a typical euchromatic mark. Therefore, one explanation for the boundary function of Rpd3 is that deacetylation of a particular histone residue by Rpd3 would be the prerequisite for another modification of that residue, for instance H3 K4 methylation by Set1 (Santos-Rosa et al. 2004), or H3 K79 methylation by Dot1 (van Leeuwen et al. 2002), which have previously been shown to characterize euchromatin. Since both modifications depend on histone ubiquitination by Rad6 (Briggs et al. 2002), one can speculate that deacetylation by Rpd3 may be required for Rad6-dependent ubiquitination. Furthermore, deacetylation of one residue may be necessary for phosphorylation of S/T residues in the vicinity (Lo et al. 2001). One would then postulate that loss of the respective enzymes should mimic the effect of *rpd3*Δ and be synthetically lethal with *sas2*Δ. However, none of the known histone methyltransferases, none of the tested kinases nor *rad6*Δ were lethal with *sas2*Δ, indicating that the lethality between *rpd3*Δ and *sas2*Δ was not based on the onset of lysine methylation, ubiquitination or phosphorylation in the vicinity of lysines deacetylated by Rpd3.

Similarly, one can hypothesize that deacetylation by Rpd3 might enhance chromatin remodelling, exchange of histone variants or the presence of linker histones. However, *sas2*Δ was not lethal in the absence of Htz1, histone H1 (*hho1*Δ) or ISW2 (**Table 8**), suggesting that the Rpd3 boundary function was not mediated by these mechanisms.

4.4 Relationship between Rpd3 and other boundary factors

A recent study showed that H4 K16 acetylation by SAS-I was necessary for the deposition of the histone variant H2A.Z (Htz1) in euchromatic regions at yeast telomeres (Shia et al. 2006). If the role of SAS-I in boundary formation is to mediate Htz1 incorporation into chromatin, one would expect that *htz1*Δ, like *sas2*Δ, is synthetically lethal with *rpd3*Δ. We and others found this to be the case (Keogh et al. 2005). However, unlike for *sas2*Δ *rpd3*Δ, the lethality between *htz1*Δ and *rpd3*Δ was not suppressed by *sir3*Δ (data not shown), suggesting that the lethality between *htz1*Δ und *rpd3*Δ was not (solely) due to inappropriate SIR spreading, and that the loss of Htz1 incorporation in *sas2*Δ cells was not the only cause for the lethality between *sas2*Δ and *rpd3*Δ.

It has further been suggested that the boundary function at yeast telomeres may be relayed by the bromodomain protein Bdf1, which may protect acetylated H4 from deacetylation by SIR complexes (Ladurner et al. 2003). We found that *bdf1*Δ and *bdf2*Δ were not lethal with *rpd3*Δ, showing that the effect of H4 K16 acetylation by SAS-I in the prevention of SIR spreading was not mediated by Bdf1 or Bdf2.

Another explanation for the boundary function of Rpd3 is that the deacetylation of an as yet unknown histone lysine residue (for instance, in the globular domain of histones) is necessary to prevent SIR binding to chromatin, and thus, that a particular acetylation is required for SIR binding. This is an intriguing possibility, because so far, SIR binding has been thought to require deacetylated lysines, at least in the histone tail domains (Rusche et al. 2003). However, the direct test of this notion in 3.1.13 did not reveal one particular lysine residue that fulfils this criterion.

4.5 Is Rpd3 required for subtelomeric gene activation?

As mentioned above, the observation of an HDAC working against heterochromatin formation is counterintuitive, because in principle one would expect an HDAC to aid in, rather than to prevent the formation of heterochromatin. Surprisingly, Rpd3, as well as the HDAC Hos2, have been implicated in gene activation before (Wang et al. 2002; De Nadal et al. 2004). This observation on its own also seems surprising, but this might reflect an activating mechanism that classically is associated with the restriction of heterochromatin. Despite this gene activating mechanism for these two HDACs, we propose that the effect of Rpd3 at telomeres was distinct from the role of HDACs in gene activation, because the

reduced expression of subtelomeric genes in the absence of Rpd3 was caused by mislocalization of the SIR silencing complex, and could be suppressed by additional *sir* deletion. This was in line with the observation that subtelomeric genes are downregulated by mutants of the Rpd3(L) complex (Bernstein et al. 2000; Keogh et al. 2005). Furthermore, the involvement of the Rpd3 (L) but not the Rpd3 (S) complex in the *rpd3*Δ *sas2*Δ lethality suggests that the boundary function of Rpd3 is coupled to the global deacetylation activity of the Rpd3 (L) complex, rather then to the gene expression coupled activity of the Rpd3 (S) complex. Taken together, our data indicate that the boundary function of Rpd3 was not related to the gene activation activity of Rpd3 at osmo-sensitive genes.

4.6 Boundary formation by HDACs

As discussed in 4.3 and 4.4, the role of Rpd3 in boundary formation is not indirect in that it facilitates lysine methylation on a residue deacetylated through Rpd3, nor is Rpd3 required to deacetylate a lysine residue that possibly is bound by the SIR complex in the acetylated form. This suggested that the deacetylation *per se* might be the mechanism that creates the boundary. Deacetylation through Sir2 is needed for the SIR complex to propagate along the chromatin fibre (1.8.4). In this scenario, deacetylation through an HDAC other than Sir2 might reflect a competition for acetyl groups, in that deacetylation through Rpd3 renders Sir2 unable to deacetylate its target residues. If this were the case, then in principle other HDACs should also be able to create a boundary. Significantly, one other HDAC, Hos2, also was able to actively create a boundary against heterochromatin spreading, whereas all other HDACs tested had no influence on SIR spreading. Our observation that not every HDAC displayed boundary activity suggested that differences in the substrate specificity determine whether an HDAC is capable of boundary activity or not, although it is also possible that some of the HDACs lost their function due to fusion with GBD. Significantly, HDACs that are specialized to deacetylate a small subset of lysines show no boundary function, whereas Rpd3 and Hos2 have a relatively broad substrate specificity (Suka et al. 2001; Wang et al. 2002). A model for how the substrate specificity of an HDAC renders them capable of boundary formation is discussed in 4.7. Taken together, our observations suggested that in principle, the model of deacetylation prior to Sir2 is valid, although it is limited to Rpd3 and Hos2.

Another possibility for how HDACs actively create a boundary against heterochromatin spreading might be that they act as a large protein complex to block the spread of heterochromatin. Therefore, the recruitment of a large, sterically hindering complex to the

Gal4 binding sites at the reporter genes (Jacobson and Pillus 2004) might be able to physically block the spreading of the SIR complex. Since Rpd3 (L) works as a large protein complex (Kadosh and Struhl 1997; Kasten et al. 1997; Carrozza et al. 2005; Keogh et al. 2005), this might be a possibility for Rpd3 to create a boundary. To test whether the deacetylation through Rpd3 or the recruitment of the large complex creates the boundary, we asked whether a catalytically inactive Rpd3 has boundary activity. However, we found that the direct disruption of the catalytic residues within GBD-Rpd3 (Kadosh and Struhl 1997) did not disrupt boundary function (data not shown). Surprisingly, further analysis revealed that deletion of endogenous *RPD3* abrogated the boundary function of GBD-Rpd3, suggesting that wt Rpd3 is required for the boundary activity of GBD-Rpd3. Furthermore, GBD-Rpd3 was unable to complement other *rpd3*Δ phenotypes (not shown), indicating that GBD-Rpd3 itself is inactive and can therefore not induce boundary formation. Since GBD-Rpd3 is required for boundary formation, another interpretation is that GBD-Rpd3 brings wt Rpd3 to the reporter gene, and therefore not GBD-Rpd3 has to be mutated in the catalytic residues, but mutation of endogenous *RPD3* should be able abrogate the boundary activity. Exactly this was the case, indicating that the boundary activity of Rpd3 depended on the deacetylation activity of chromosomally encoded Rpd3. This indicated that the deacetylation activity of the targeted Rpd3 created the boundary, rather than a physical block against the SIR proteins. This supports the model that the removal of acetyl groups through Rpd3 is the driving force in the establishment of a boundary against heterochromatin spreading.

The model suggested above implies that a general removal of acetyl groups prior to Sir2 deacetylation abrogates SIR spreading. However, Hst2, as member of the sirtuin family of HDACs, even enhanced SIR spreading, suggesting that the activity of Hst2 stimulates spreading of the SIR complex. Significantly, Hst2 *in vivo* is located mainly in the cytoplasm and is relocalized to the nucleus only during mitosis (Vaquero et al. 2006), making it unlikely that Hst2 has a function in supporting SIR spreading *in vivo*. This further suggested that although both belong to the sirtuins and have the same substrates, their deacetylation has different consequences *in vivo*, which is supported by the notion that *hst2*Δ, in contrast to *sir2*Δ, was unable to suppress the *sas2*Δ *rpd3*Δ lethality. Taken together, we observed that two HDACs belonging to the class I HDACs, Rpd3 and Hos2, can actively create boundaries, whereas one member of the sirtuins, Hst2, supports SIR spreading. This suggested that the difference between the deacetylation mechanisms of the sirtuins compared to the class I HDACs might classify an HDAC as to whether it is a boundary element or not.

As an extension of this hypothesis one could ask if the deacetylation mechanism, rather than a broad substrate specificity, qualifies an HDAC to be a boundary element. One important difference between deacetylation through class I or class II HDACs and the sirtuins is the requirement of the cofactor NAD^+ during the deacetylation reaction (also see 1.6). Like Sir2, every member of the sirtuin class of HDACs needs NAD^+ and releases OAADPR during the deacetylation reaction. Since the HDACs that can form a boundary are class I HDACs, and one member of the sirtuins even enhances SIR spreading, one could speculate that the mechanisms of NAD^+-dependent deacetylation, especially the production of OAADPR, renders a sirtuin incapable of boundary formation. This goes in line with the theory that disruption of OAADPR production forms a boundary in that the mechanism of NAD^+-independent deacetylation renders the sirtuins unable to deacetylate its target residues and therefore unable to produce OAADPR. A role for OAADPR in SIR complex assembly and possible consequences of the loss of OAADPR production are discussed in 4.8.

However, to date we cannot exclude that the observation of boundary formation through Rpd3 is based on the deacetylation of targets other than histones through Rpd3. This target then might be required in the deacetylated form to restrict SIR proteins together with Sas2. Significantly, our data pointed towards effects of both factors in directly and independently restricting the SIR complexes through modifying histones, because the lethality between *rpd3*Δ and *sas2*Δ can be suppressed by mutating lysine residues within the histone tails. The notion that the boundary is caused by Rpd3 directly acting on histones is further strengthened by the fact that in principle also recruitment of another HDAC, Hos2, is able to create a boundary. Furthermore, the loss of Rpd3 led to a change of subtelomeric histone acetylation. This indicated that Rpd3 works as boundary element by deacetylating histones.

4.7 Which histone residues are important for the Rpd3 boundary function?

The hypothesis that histone deacetylation *per se* is the mechanism that creates a boundary, together with the observation that not every HDAC can form a boundary, raises the question what determines whether an HDAC is capable of boundary formation or not. As discussed above, Rpd3 and Hos2, as HDACs capable of boundary formation, have a relatively broad histone substrate range (Suka et al. 2001; Wang et al. 2002). As a simple explanation, one could say that the more histone residues are deacetylated by an HDAC, the more likely it is to possess boundary activity. Rpd3 deacetylates all sites in the amino-terminal tails of histones, showing a preference for H4 K5 and K12 (Suka et al. 2001). Hos2 is specific for acetylation

sites on H3 and H4, but shows no deacetylation on H2A and H2B (Wang et al. 2002), suggesting that broad deacetylation of H3 and H4 are critical to boundary function of HDACs.

The latter model predicts that HDACs deacetylating various lysines remove the Sir2 targets. It might be a matter of chance that HDACs with a broad substrate range deacetylate one of the main Sir2 targets among various others. Sir2 was described previously to be quite specialized for specific residues, with H4 K16 as the most prominent *in vitro* target (Imai et al. 2000). Significantly, silent chromatin that is characterized by the presence of the SIR complex, is generally deacetylated (Braunstein et al. 1993). This suggested that the substrate range of Sir2 obviously is much wider as preference for H4 K16. Significantly, we found in *rpd3*Δ cells that the acetylation of H4 K5 and K12 in subtelomeric regions was reduced by the SIR complex. This analysis suggested that H4 K5 and K12 are also targets to Sir2 deacetylation, and therefore the residues that become globally more acetylated upon deletion of *RPD3* are now available for Sir2. This in essence creates an important difference between H4 K5 or K12 acetylation, as compared to H4 K16 acetylation. On the one hand, H4 K5 and K12 not deacetylated by Rpd3 create additional Sir2 targets that are conducive for SIR spreading. On the other hand, the onset of H4 K16 acetylation by SAS-I, rather than its deacetylation, creates a boundary (Kimura et al. 2002; Suka et al. 2002). This implies that a functional difference exists between acetylation of H4 K16 and other lysine residues. We propose that acetylated K16, once deposited in chromatin, becomes inaccessible to Sir2 deacetylation, such that it cannot be used by Sir2 for SIR propagation along the chromatin fibre. Perhaps acetylated H4 K16 is inaccessible because it is occluded by the chromatin configuration (Shogren-Knaak et al. 2006), or because of the presence of H2A.Z (Shia et al. 2006) on the chromatin, whereas other residues are amenable to deacetylation by Sir2 and thus contribute to SIR boundary formation if they are deacetylated by another HDAC.

4.8 A role for the Sir2 metabolite OAADPR in heterochromatin spreading

After the recruitment of the Sir2-Sir4 heterodimer to heterochromatic loci, the activity of the SIR complex starts with the deacetylation of histones through Sir2 (1.8.4, reviewed in (Rusche et al. 2003)). One further event in SIR spreading is the binding of Sir3 to the deacetylated histone tails (Rusche et al. 2003). A crucial role within SIR complex assembly is the structural rearrangement that leads to the formation of the heterochromatic complex containing Sir2, Sir3 and Sir4. It has been shown that OAADPR, which is generated during

the NAD^+-dependent deacetylation through Sir2, plays a vital role within this rearrangement (Liou et al. 2005), in that the SIR complex formation *in vitro* is dependent on OAADPR (Onishi et al. 2007). In this scenario, polymerization and spreading of SIR depends on the production of OAADPR. As a synthesis of this scenario and our observation of Rpd3 forming a boundary against heterochromatin spreading, one could hypothesize that the boundary function might be based on the disruption of OAADPR production through the NAD^+-independent deacetylation via Rpd3.

One approach to address how the polymerization of SIR depends on OAADPR is to abrogate binding of OAADPR to SIR. As described in 3.1.15, the assembly of the SIR complex could not only be stopped by removal of the Sir2 substrate, but also through mutation of the AAA+ motif within Sir3. The observation that a mutation within the putative OAADPR binding site of Sir3 was sufficient for the disruption of SIR spreading led us to the hypothesis that this compound, which was previously described as a byproduct of NAD^+-dependent deacetylation, plays a vital role in the establishment of heterochromatin. This strengthened our hypothesis that disruption of OAADPR production (through NAD^+-independent removal of acetyl groups, as well as through disruption of OAADPR binding to the SIR complex) might be the driving force in the establishment of a boundary through HDACs.

Recently, a study described that OAADPR is dispensable for the establishment of silencing (Chou et al. 2008). This study used a fusion protein, containing the NAD^+-independent HDAC Hos3 directly fused to Sir3 (Chou et al. 2008). They concluded that this Hos3-Sir3 hybrid bypassed the requirement of OAADPR, in that this molecule is able to nucleate silencing without the production of OAADPR during the deacetylation reaction (Chou et al. 2008). One possible explanation for a role of OAADPR in the assembly of the SIR complex is to bring all SIRs together (Liou et al. 2005). Therefore, OAADPR facilitates the interaction between the deacetylase Sir2 to Sir3. Generating a fusion of an HDAC directly to Sir3, however, might bridge this role of OAADPR. Essentially, all requirements needed to establish silencing are fulfilled with this molecule, namely generating a deacetylated lysine and thereby facilitating binding of the fusion protein to chromatin through the Sir3 part. The fusion protein can then deacetylate another histone adjacent to the previous one and generate another binding surface. Altogether, this creates a self-propagating system that obviously is sufficient to achieve silencing of the reporter genes tested. The recruitment of the Hos3-Sir3 hybrid to endogenous heterochromatic loci (the *HM* loci) thereby might be facilitated through Sir3 itself (Chou et al. 2008). This led us to the conclusion that OAADPR is not needed to

achieve artificial silencing through bridging the interaction between an HDAC and Sir3 through the generation of a fusion molecule. Our interpretation is that OAADPR is needed to couple deacetylation through Sir2 to the SIR complex assembly that requires Sir3, and therefore that OAADPR is needed for the establishment of the natural SIR complex.

4.9 Is OAADPR transported within the cell?

As shown above, the recruitment of the NAD^+-dependent HDAC Hst2 to the telomeric reporter genes stimulated spreading of the SIR complex. One possible interpretation would be that local production of OAADPR increases spreading of the SIR complex. If this were the case, then in principle other sirtuins should also be able to increase SIR spreading. However, only after recruitment of Hst2, silencing of the reporter gene was enhanced. Again, we cannot exclude that the other Hst proteins have lost their function due to the fusion to GBD. Nevertheless, one can hypothesize that the stimulation of SIR spreading through Hst2 might be based on local production of OAADPR that can then be bound by the SIR complex in the vicinity. This model makes the prediction that the OAADPR molecule can migrate from the place of deacetylation to the SIR complex to induce the reassembly that leads to the chromatin condensing, repressive complex. Indeed, the addition of OAADPR to a mixture containing oligonucleosomes and all SIR components is sufficient to induce this rearrangement *in vitro* (Onishi et al. 2007). If the model of OAADPR migrating through the cells were true, then every NAD^+-dependent deacetylation reaction in the cell could stimulate SIR spreading.

Since Hst2 was able to stimulate SIR spreading when recruited to telomeric reporter genes, we wished to address this issue by asking whether a catalytically inactive Sir2 (Imai et al. 2000) that cannot support SIR spreading and cannot produce OAADPR, can be "reactivated" for SIR spreading by the production of OAADPR in the vicinity by deacetylation through Hst2. However, the complete derepression of telomeric reporters due to disruption of the catalytic activity of Sir2 could not be suppressed by recruitment of Hst2 (data not shown). One interpretation is that the mutation of Sir2 (*sir2*-N345A, (Imai et al. 2000)) is located within the NAD^+ binding pocket and therefore also might lower the affinity to OAADPR. Another interpretation would be that the deacetylation through Sir2 cannot be bypassed by another deacetylase, suggesting that the OAADPR molecule after deacetylation through Hst2 cannot be transferred to the SIR complex. However, Hst2 had the ability to stimulate SIR spreading when artificially brought to the telomeres. Significantly, Hst2, like

Sir2, preferentially deacetylates H4 K16 (Vaquero et al. 2006), although it *in vivo* is mainly located to the cytoplasm. Therefore, one interpretation for the Sir spreading stimulation might be an additional factor that for instance antagonizes the onset of H4 K16 acetylation through Sas2 as a boundary element and therefore unbalances the negotiable boundary at the telomeres (Kimura et al. 2002; Suka et al. 2002).

In summary, our hypothesis predicts that deacetylation through Sir2 generates the deacetylated state of histones that is bound by Sir3 and Sir4. During this step, all Sir proteins are close enough to enable the structural rearrangement that finally leads to SIR dependent chromatin compaction. Finally, we hypothesize here that the place of deacetylation and the place of OAADPR binding to the complex have to be in close proximity, making it unlikely that OAADPR is transported through the cell over distances longer then from one Sir protein to the other. Nevertheless, at this point we cannot exclude that the OAADPR molecule as such remains bound to Sir2 after the deacetylation and changes the confirmation of Sir2 to a state that can be bound by Sir3. Significantly, the prediction of the Sir3 structure revealed a putative binding pocket, for OAADPR. This prompted us to favour the theory of OAADPR leaving Sir2 and becoming bound to Sir3, which is supported by the notion that a subtle mutation within the putative OAADPR binding motif of Sir3 has the same consequence for SIR spreading as preventing deacetylation through Sir2.

4.10 A mechanism for Rpd3 as a boundary element

Taken together, our data indicate that chromatin deacetylation *per se* by Rpd3 is the cause for the establishment of a chromatin boundary. One event in SIR spreading is the binding of Sir3 to hypoacetylated histones, a state that is generated by the HDAC activity of Sir2 (Rusche et al. 2003). It has been suggested that the production of O-acetyl-ADP-Ribose (OAADPR) during NAD^+-dependent deacetylation is one of the driving forces in the polymerization of SIR complexes along chromatin (Liou et al. 2005). Furthermore, Sir3 not only binds deacetylated histone tails, but also is discussed to be the recipient for OAADPR binding to the complex and driving the polymerization (Gasser and Cockell 2001). A putative OAADPR binding region within Sir3, is the AAA+ motif (Neuwald et al. 1999; Gasser and Cockell 2001). As discussed in 4.8, SIR spreading not only can be stopped by removing the Sir2 targets, but also through mutation within the putative OAADPR binding site of Sir3. This indicated a crucial role for OAADPR in SIR complex assembly and suggested that the loss of

this compound by NAD^+-independent removal of Sir2 targets creates a halt to spreading of the SIR complex.

To summarize these observations, we propose the following model (**Figure 33**). The process of SIR spreading involves deacetylation through Sir2, production of OAADPR, binding of Sir3 and Sir4 to the deacetylated histones, OAADPR-induced structural rearrangement of the SIR complex, formation of the chromatin condensing SIR complex. This process is reiterated until it is stopped by a boundary element (**Figure 33A**, reviewed in (Rusche et al. 2003)) Chromatin deacetylation by Rpd3 in the vicinity of telomeres results in hypoacetylation, which thus removes potential acetyl-lysine substrates for the SIR complex. In the absence of substrates, the SIR complex cannot produce OAADPR and thus is hindered in its ability to propagate along the chromatin fibre. Thus, in essence Rpd3 deprives Sir2 of its substrate for OAADPR production, which causes a halt to SIR spreading (**Figure 33**).

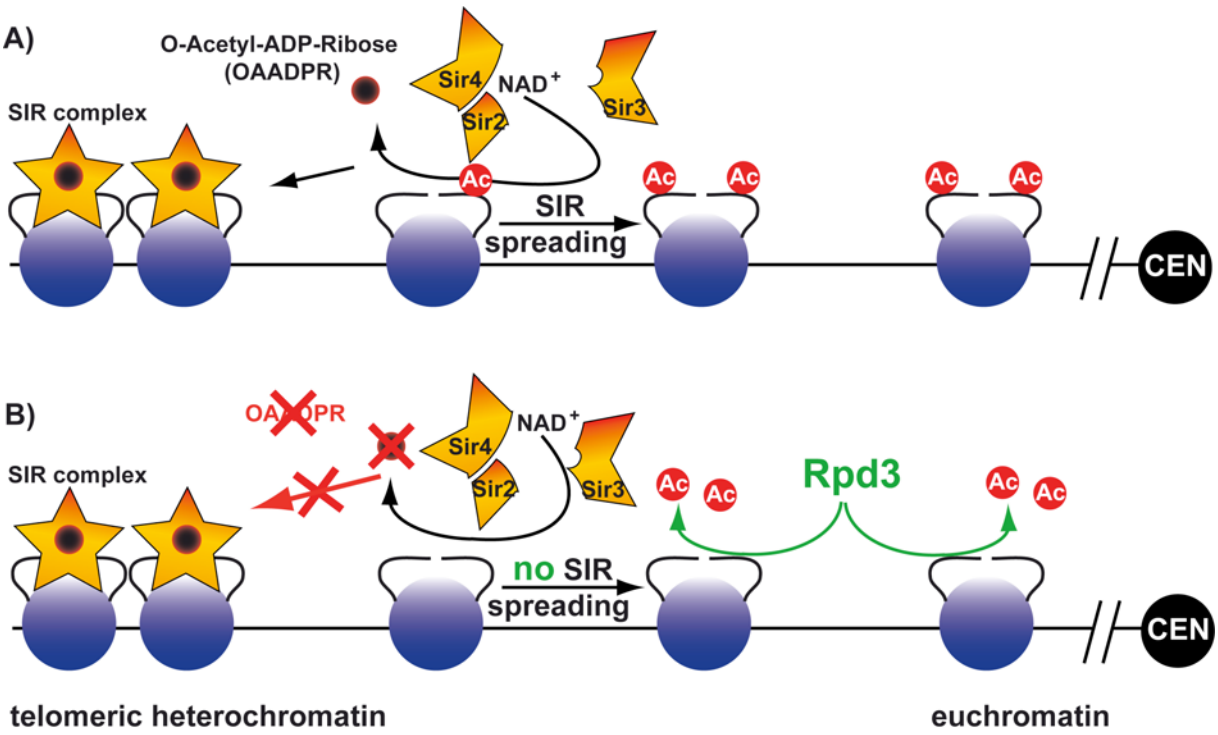

Figure 33 Model for the mechanism of boundary formation by the HDAC Rpd3.
(A) SIR complex propagation on chromatin is driven by the deacetylation activity of Sir2 and concomitant production of OAADPR, which binds via Sir3 to the Sir2/ Sir3/ Sir4 complex (top). (B) Global chromatin deacetylation by Rpd3 results in the removal of acetyl-lysine substrates for Sir2 (red circles), thus halting Sir2 deacetylation, OAADPR production and SIR spreading, and consequently creating a barrier to heterochromatin spreading.

In summary, with this work we have expanded the current view of Rpd3's function in the establishment of global histone acetylation patterns in that we found the Rpd3(L) complex to

restrict heterochromatin to telomeric regions. This boundary activity functioned by a novel mechanism in which the spreading of SIR complexes along the chromatin fiber was halted by prior removal of acetyl-lysine groups on histones by Rpd3. We propose that the deacetylation reaction of Sir2 *per se*, the generation of OAADPR by Sir2 and the binding of the metabolite to Sir3 are essential for SIR spreading and that they are abrogated by the competing histone deacetylation activity of Rpd3 in subtelomeric regions (**Figure 33**).

4.11 Summary and outlook

In this work we describe that spreading of the heterochromatic SIR complex can be stopped by removal of acetyl groups as well as by inhibiting the binding of the metabolite OAADPR to Sir3. In this study, we used an *in silico* structure prediction to determine a possible binding pocket for OAADPR binding. Our analysis showed that mutation within that region abrogated the activity of Sir3 in supporting SIR spreading. Nevertheless, to reveal more information about structural and energetic details of OAADPR binding to Sir3, another analysis will be needed. Although the *in silico* analysis and our data point towards binding of OAADPR to the AAA+ motif of Sir3, a detailed mutagenesis analysis will reveal more detailed information about the protein-ligand interaction. With the aid of the Pymol software, concrete van-der-Waals interactions between OAADPR and Sir3 can be predicted and taken as a starting point to modify single amino acids to influence binding of the metabolite. With the information which mutation leads a phenotype, the *in silico* prediction can be refined and brought towards the real natural conformation of Sir3.

A further possibility to characterize binding of OAADPR to Sir3 is a biochemical *in vitro* analysis of the affinity between Sir3 and OAADPR. To this end we constructed a 6×His-tagged Sir3 AAA+ domain and purified it using affinity chromatographic protein purification approaches. However, the resulting protein was not soluble enough to perform an adequate biochemical analysis that shows binding of the metabolite to the protein. Nevertheless, careful selection of truncation sites along the protein primary sequence can lead to a more soluble protein. With these proteins, the affinity between Sir3 and OAADPR can be measured, for instance by isothermal calorimetric analysis, and concrete binding constants can be calculated. Furthermore, the binding affinity of Sir3 mutations that result in the loss of Sir3 function *in vivo* can be measured by these methods, and the real impact of OAADPR binding on the nature of the SIR complex can be estimated.

Another possibility to reveal the structure of Sir3 would be to crystallize the AAA+ domain and obtain a crystal structure of the domain. However, the evaluation of protein truncations that result in a protein that form crystals and to obtain crystals that reflect the natural protein conformation is a time-consuming process.

Taken together, barriers between chromatin states have been described in a variety of organisms (Oki and Kamakaka 2002). Fundamentally, the functional distinction between active euchromatin and inactive heterochromatin is essential in order to maintain gene expression programs that drive development and differentiation in multicellular organisms. Interestingly, homologs of Rpd3 in larger organisms show strong functional parallels to yeast Rpd3 in gene repression, the control of replication initiation through global histone deacetylation (Aggarwal and Calvi 2004), as well as in aging (Rogina et al. 2002). In the latter case, the reduction of the RPD3 activity extended the lifespan of Drosophila (Rogina et al. 2002). The same lifespan extension was seen by subjecting the flies to caloric restriction, which was known previously to extend the lifespan of several organisms including yeast (Masoro 2000). In light of our data, the observed effect of longevity in RPD3 mutants may be interpreted as an effect of enhanced Sir2 activity. This observation strengthened the theory that, similar to the observations in *S. cerevisiae*, RPD3 works as a boundary element against heterochromatin spreading in *Drosophila*. Furthermore, similar to its boundary function in *S. cerevisiae*, mutation of RPD3 enhances position effect variegation in *Drosophila* (De Rubertis et al. 1996), in that subtelomeric reporters are repressed upon deletion RPD3, thus making it likely that Rpd3's barrier function and the mechanism for boundary formation are conserved in higher eukaryotes.

5 Abstract

In *Saccharomyces cerevisiae*, spreading of the telomeric SIR heterochromatin complexes into centromere-proximal euchromatic regions is prevented by the activity of boundary elements. So far, these boundaries have been associated with chromatin opening activities, like histone acetyltransferases (HATs) or histone methyltransferases. Here, we show that the opposite enzymatic activity, the histone deacetylase (HDAC) Rpd3, was necessary to prevent the encroachment of heterochromatin into euchromatin at telomeres in *S. cerevisiae*.

We found by ChIP analysis that in the absence of Rpd3, the SIR complexes were mislocalized to more centromere-proximal regions, showing that Rpd3 was necessary to restrict SIR complexes to the telomere. Furthermore, quantitative RT-PCR showed that SIR proteins repressed subtelomeric genes in *rpd3*Δ cells, suggesting a role for Rpd3 in the restriction of telomeric heterochromatin. When combined with the absence of the known boundary factor, the HAT SAS-I, *rpd3*Δ caused inappropriate SIR spreading that was lethal to yeast cells. Significantly, the lethality between *sas2*Δ *rpd3*Δ was suppressed by *sir* deletions, suggesting parallel functions for the two enzymes in restricting SIR proteins to heterochromatin despite their opposing enzymatic activity. In addition, Rpd3 was capable of creating a boundary when targeted to the heterochromatic loci, indicating a boundary function for Rpd3. Further analysis showed that Rpd3 in essence functioned by removing acetyl groups, such that they were no longer available for NAD^+-dependent deacetylation via Sir2. This further suggested that prevention of O-acetyl-ADP-ribose (OAADPR) production during deacetylation by Sir2 in effect prevented SIR propagation. This hypothesis was strengthened by the notion that inhibition of OAADPR binding to Sir3 created a halt to SIR spreading. In further experiments, we found that Rpd3 interacted *in vivo* with Cac1, the largest subunit of the chromatin assembly complex CAF-I, suggesting that it deacetylated cytoplasmic histone acetylation marks in a replication-coupled fashion. Thus, Rpd3 likely performed its function in SIR restriction through a transient contact to chromatin, rather than being permanently located at subtelomeric regions.

In summary, our data indicated that Rpd3 effectively removed acetyl groups in subtelomeric regions and therefore deprived Sir2 of its ability to perform the deacetylation reaction and in doing so to produce OAADPR. This in essence prevented SIR propagation and created a boundary against heterochromatin spreading.

6 Zusammenfassung

In *Saccharomyces cerevisiae* wird die Ausbreitung des heterochromatischen SIR-Komplexes vom Telomer in angrenzende euchromatische Bereiche durch sogenannte Boundary-Elemente verhindert. Bisher wurden Boundary-Elemente eher mit Chromatin aktivierenden Faktoren, wie Histon-Acetyltranferasen (HATs) oder Methyltransferasen, in Verbindung gebracht. In dieser Arbeit wird beschrieben, dass auch die entgegengesetzte enzymatische Aktivität, die Histon-Deacetylierung durch Rpd3, in *S. cerevisiae* die Ausbreitung von telomerischem Heterochromatin in aktives Euchromatin verhindert.

Mittels ChIP-Experimenten konnten wir zeigen, dass in Abwesenheit von Rpd3 mehr SIR-Komplexe in subtelomerische Bereiche verlagert wurden. Dies zeigte, dass Rpd3 notwendig war, um SIR-Ausbreitung zu verhindern. Diese Hypothese wurde zusätzlich von der Beobachtung gestützt, dass die Expression subtelomerischer Gene in Abwesenheit von Rpd3 stark SIR-abhängig reprimiert wurde. Deletion von *RPD3* war letal für die Zellen, wenn zusätzlich ein anderes bereits bekanntes Boundary-Element, der SAS-I HAT-Komplex, fehlte. Dieser synthetisch-letale Effekt deutete auf übermäßige SIR-Ausbreitung in Abwesenheit von Rpd3 und SAS-I hin und war unterstützt von der Beobachtung, dass die Letalität durch zusätzliche Deletion von SIR-Untereinheiten aufgehoben wurde. Die Ergebnisse führten zur These, dass sowohl Rpd3 als auch SAS-I die Ausbreitung des SIR-Komplexes verhinderten, obwohl sie entgegengesetzte enzymatische Aktivitäten besitzen. Weiterhin konnte durch Rekrutierung von Rpd3 an die Grenzen heterochromatischer Bereiche aktiv eine Boundary gegen die SIR-Ausbreitung erzeugt werden. Weitere Analysen zeigten, dass die Boundary-Funktion von Rpd3 im Wesentlichen darauf beruht, die NAD^+-abhängige Deacetylierung durch Sir2 zu verhindern. Findet keine Deacetylierung durch Sir2 statt, wird die O-Acetyl-ADP-Ribose (OAADPR) Produktion, ein wichtiger Schritt in der Sir-Komplex-Assemblierung, unterbrochen. Für diese Hypothese spricht zudem die Beobachtung, dass SIR-Ausbreitung gestoppt werden konnte, indem die Bindung von OAADPR an Sir3 verhindert wurde. Weiterhin zeigte sich, dass Rpd3 *in vivo* mit der großen Untereinheit des Chromatin-Assemblierungsfaktors CAF-I interagierte. Somit entfernte Rpd3 cytoplasmatische Histonmodifikationen durch temporären replikationsgebundenen Kontakt mit Chromatin, und nicht durch permanente Anwesenheit am Subtelomer.

Zusammengefasst zeigen die Daten, dass die Deacetylierung durch Rpd3 die Sir2-abhängige Deacetylierung und somit die OAADPR-Produktion unterbrach, und dadurch die Assemblierung und Ausbreitung des SIR-Komplexes verhinderte.

7 Literature Cited

Adams, C.R. and Kamakaka, R.T. 1999. Chromatin assembly: biochemical identities and genetic redundancy. *Curr Opin Genet Dev* **9**(2): 185-190.

Aggarwal, B.D. and Calvi, B.R. 2004. Chromatin regulates origin activity in *Drosophila* follicle cells. *Nature* **430**(6997): 372-376.

Ahn, S.H., Diaz, R.L., Grunstein, M., and Allis, C.D. 2006. Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10. *Mol Cell* **24**(2): 211-220.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. 1998. Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**(6693): 592-595.

Aparicio, J.G., Viggiani, C.J., Gibson, D.G., and Aparicio, O.M. 2004. The Rpd3-Sin3 histone deacetylase regulates replication timing and enables intra-S origin control in *Saccharomyces cerevisiae*. *Mol Cell Biol* **24**(11): 4769-4780.

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* **66**(6): 1279-1287.

Avner, P. and Heard, E. 2001. X-chromosome inactivation: counting, choice and initiation. *Nat Rev Genet* **2**(1): 59-67.

Bender, A. and Pringle, J.R. 1991. Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**(3): 1295-1305.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., and Bourne, P.E. 2000. The Protein Data Bank. *Nucleic Acids Res* **28**(1): 235-242.

Bernstein, B.E., Tong, J.K., and Schreiber, S.L. 2000. Genomewide studies of histone deacetylase function in yeast. *Proc Natl Acad Sci U S A* **97**(25): 13708-13713.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* **16**(1): 6-21.

Bird, A.W., Yu, D.Y., Pray-Grant, M.G., Qiu, Q., Harmon, K.E., Megee, P.C., Grant, P.A., Smith, M.M., and Christman, M.F. 2002. Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. *Nature* **419**(6905): 411-415.

Biswas, D., Takahata, S., and Stillman, D.J. 2008. Different genetic functions for the Rpd3(L) and Rpd3(S) complexes suggest competition between NuA4 and Rpd3(S). *Mol Cell Biol* **28**(14): 4445-4458. Epub 2008 May 4419.

Blander, G. and Guarente, L. 2004. The Sir2 family of protein deacetylases. *Annu Rev Biochem* **73**: 417-435.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L., and Boeke, J.D. 1995. The SIR2 gene family, conserved from bacteria to humans, functions in

silencing, cell cycle progression, and chromosome stability. *Genes Dev* **9**(23): 2888-2902.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev* **7**(4): 592-604.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M., and Broach, J.R. 1996. Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. *Mol Cell Biol* **16**(8): 4349-4356.

Breeden, L. and Nasmyth, K. 1985. Regulation of the yeast HO gene. *Cold Spring Harb Symp Quant Biol* **50**: 643-650.

Briggs, S.D., Xiao, T., Sun, Z.W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D., and Strahl, B.D. 2002. Gene silencing: trans-histone regulatory pathway in chromatin. *Nature* **418**(6897): 498. Epub 2002 Jul 2014.

Burgess, S.M., Ajimura, M., and Kleckner, N. 1999. *GCN5*-dependent histone H3 acetylation and *RPD3*-dependent histone H4 deacetylation have distinct, opposing effects on *IME2* transcription, during meiosis and during vegetative growth, in budding yeast. *Proc Natl Acad Sci U S A* **96**(12): 6835-6840.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.J., Anderson, S., Yates, J., Washburn, M.P. et al. 2005. Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. *Cell* **123**(4): 581-592.

Carrozza, M.J., Utley, R.T., Workman, J.L., and Cote, J. 2003. The diverse functions of histone acetyltransferase complexes. *Trends Genet* **19**(6): 321-329.

Chou, C.C., Li, Y.C., and Gartenberg, M.R. 2008. Bypassing Sir2 and O-Acetyl-ADP-Ribose in Transcriptional Silencing. *Mol Cell* **31**(5): 650-659.

Christman, M.F., Dietrich, F.S., and Fink, G.R. 1988. Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II. *Cell* **55**(3): 413-425.

Clarke, A.S., Lowell, J.E., Jacobson, S.J., and Pillus, L. 1999. Esa1p is an essential histone acetyltransferase required for cell cycle progression. *Mol Cell Biol* **19**(4): 2515-2526.

Cockell, M., Palladino, F., Laroche, T., Kyrion, G., Liu, C., Lustig, A.J., and Gasser, S.M. 1995. The carboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponent complex required for yeast telomeric silencing. *J Cell Biol* **129**(4): 909-924.

Contreras, A., Hale, T.K., Stenoien, D.L., Rosen, J.M., Mancini, M.A., and Herrera, R.E. 2003. The dynamic mobility of histone H1 is regulated by cyclin/CDK phosphorylation. *Mol Cell Biol* **23**(23): 8626-8636.

De Nadal, E., Zapater, M., Alepuz, P.M., Sumoy, L., Mas, G., and Posas, F. 2004. The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. *Nature* **427**(6972): 370-374.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. 1996. The histone deacetylase RPD3 counteracts genomic silencing in *Drosophila* and yeast. *Nature* **384**(6609): 589-591.

Donze, D. and Kamakaka, R.T. 2001. RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. *EMBO J* **20**(3): 520-531.

Driscoll, D.J., Waters, M.F., Williams, C.A., Zori, R.T., Glenn, C.C., Avidano, K.M., and Nicholls, R.D. 1992. A DNA methylation imprint, determined by the sex of the parent, distinguishes the Angelman and Prader-Willi syndromes. *Genomics* **13**(4): 917-924.

Ebert, A., Lein, S., Schotta, G., and Reuter, G. 2006. Histone modification and the control of heterochromatic gene silencing in Drosophila. *Chromosome Res* **14**(4): 377-392.

Ehrenhofer-Murray, A.E. 2004. Chromatin dynamics at DNA replication, transcription and repair. *Eur J Biochem* **271**(12): 2335-2349.

Ehrenhofer-Murray, A.E., Rivier, D.H., and Rine, J. 1997. The role of Sas2, an acetyltransferase homologue of *Saccharomyces cerevisiae*, in silencing and ORC function. *Genetics* **145**(4): 923-934.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V., and Elgin, S.C. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **87**(24): 9923-9927.

Ekwall, K. 2005. Genome-wide analysis of HDAC function. *Trends Genet* **21**(11): 608-615. Epub 2005 Sep 2008.

Enomoto, S. and Berman, J. 1998. Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci. *Genes Dev* **12**(2): 219-232.

Ferguson, B.M., Brewer, B.J., Reynolds, A.E., and Fangman, W.L. 1991. A yeast origin of replication is activated late in S phase. *Cell* **65**(3): 507-515.

Foss, M., McNally, F.J., Laurenson, P., and Rine, J. 1993. Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S. cerevisiae*. *Science* **262**(5141): 1838-1844.

Fourel, G., Revardel, E., Koering, C.E., and Gilson, E. 1999. Cohabitation of insulators and silencing elements in yeast subtelomeric regions. *EMBO J* **18**(9): 2522-2537.

Fuks, F., Hurd, P.J., Wolf, D., Nan, X., Bird, A.P., and Kouzarides, T. 2003. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* **278**(6): 4035-4040. Epub 2002 Nov 4039.

Galy, V., Olivo-Marin, J.C., Scherthan, H., Doye, V., Rascalou, N., and Nehrbass, U. 2000. Nuclear pore complexes in the organization of silent telomeric chromatin. *Nature* **403**(6765): 108-112.

Gasser, S.M. and Cockell, M.M. 2001. The molecular biology of the SIR proteins. *Gene* **279**(1): 1-16.

Gotta, M., Laroche, T., Formenton, A., Maillet, L., Scherthan, H., and Gasser, S.M. 1996. The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type *Saccharomyces cerevisiae*. *J Cell Biol* **134**(6): 1349-1363.

Gottschling, D.E. 1992. Telomere-proximal DNA in Saccharomyces cerevisiae is refractory to methyltransferase activity in vivo. *Proc Natl Acad Sci U S A* **89**(9): 4062-4065.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. 1990. Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* **63**(4): 751-762.

Grewal, S.I. and Moazed, D. 2003. Heterochromatin and epigenetic control of gene expression. *Science* **301**(5634): 798-802.

Grienenberger, A., Miotto, B., Sagnier, T., Cavalli, G., Schramke, V., Geli, V., Mariol, M.C., Berenger, H., Graba, Y., and Pradel, J. 2002. The MYST domain acetyltransferase Chameau functions in epigenetic mechanisms of transcriptional repression. *Curr Biol* **12**(9): 762-766.

Gruss, C., Wu, J., Koller, T., and Sogo, J.M. 1993. Disruption of the nucleosomes at the replication fork. *EMBO J* **12**(12): 4533-4545.

Hall, I.M., Shankaranarayana, G.D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S.I. 2002. Establishment and maintenance of a heterochromatin domain. *Science* **297**(5590): 2232-2237. Epub 2002 Sep 2235.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell* **80**(4): 583-592.

Herskowitz I., S., J. N., Hicks, J. B., Rine, J. 1977. *Mating type interconversion in yeast and its relationship to development in higher eucaryotes*. New York: Academic press, New York.

Huang, S., Zhou, H., Katzmann, D., Hochstrasser, M., Atanasova, E., and Zhang, Z. 2005. Rtt106p is a histone chaperone involved in heterochromatin-mediated silencing. *Proc Natl Acad Sci U S A* **102**(38): 13410-13415. Epub 12005 Sep 13412.

Hyland, E.M., Cosgrove, M.S., Molina, H., Wang, D., Pandey, A., Cottee, R.J., and Boeke, J.D. 2005. Insights into the role of histone H3 and histone H4 core modifiable residues in Saccharomyces cerevisiae. *Mol Cell Biol* **25**(22): 10060-10070.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**(6771): 795-800.

Irlbacher, H., Franke, J., Manke, T., Vingron, M., and Ehrenhofer-Murray, A.E. 2005. Control of replication initiation and heterochromatin formation in *Saccharomyces cerevisiae* by a regulator of meiotic gene expression. *Genes Dev* **19**(15): 1811-1822.

Ishii, K., Arib, G., Lin, C., Van Houwe, G., and Laemmli, U.K. 2002. Chromatin boundaries in budding yeast: the nuclear pore connection. *Cell* **109**(5): 551-562.

Jacobson, R.H., Ladurner, A.G., King, D.S., and Tjian, R. 2000. Structure and function of a human TAFII250 double bromodomain module. *Science* **288**(5470): 1422-1425.

Jacobson, S. and Pillus, L. 2004. Molecular requirements for gene expression mediated by targeted histone acetyltransferases. *Mol Cell Biol* **24**(13): 6029-6039.

James, P. 2001. Yeast two-hybrid vectors and strains. *Methods Mol Biol* **177**: 41-84.

Jeanmougin, F., Wurtz, J.M., Le Douarin, B., Chambon, P., and Losson, R. 1997. The bromodomain revisited. *Trends Biochem Sci* **22**(5): 151-153.

Jenuwein, T. and Allis, C.D. 2001. Translating the histone code. *Science* **293**(5532): 1074-1080.

John, S., Howe, L., Tafrov, S.T., Grant, P.A., Sternglanz, R., and Workman, J.L. 2000. The something about silencing protein, Sas3, is the catalytic subunit of NuA3, a yTAF(II)30-containing HAT complex that interacts with the Spt16 subunit of the yeast CP (Cdc68/Pob3)-FACT complex. *Genes Dev* **14**(10): 1196-1208.

Kadosh, D. and Struhl, K. 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* **89**(3): 365-371.

Kasten, M.M., Dorland, S., and Stillman, D.J. 1997. A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. *Mol Cell Biol* **17**(8): 4852-4858.

Kaufman, P.D., Kobayashi, R., and Stillman, B. 1997. Ultraviolet radiation sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cells lacking chromatin assembly factor-I. *Genes Dev* **11**(3): 345-357.

Keogh, M.C., Kurdistani, S.K., Morris, S.A., Ahn, S.H., Podolny, V., Collins, S.R., Schuldiner, M., Chin, K., Punna, T., Thompson, N.J. et al. 2005. Cotranscriptional Set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. *Cell* **123**(4): 593-605.

Kimura, A. and Horikoshi, M. 2004. Partition of distinct chromosomal regions: negotiable border and fixed border. *Genes Cells* **9**(6): 499-508.

Kimura, A., Umehara, T., and Horikoshi, M. 2002. Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. *Nat Genet* **32**(3): 370-377. Epub 2002 Oct 2015.

Kirchmaier, A.L. and Rine, J. 2001. DNA replication-independent silencing in *S. cerevisiae*. *Science* **291**(5504): 646-650.

Kornberg, R.D. 1974. Chromatin structure: a repeating unit of histones and DNA. *Science* **184**(139): 868-871.

Kristjuhan, A., Wittschieben, B.O., Walker, J., Roberts, D., Cairns, B.R., and Svejstrup, J.Q. 2003. Spreading of Sir3 protein in cells with severe histone H3 hypoacetylation. *Proc Natl Acad Sci U S A* **100**(13): 7551-7556. Epub 2003 Jun 7559.

Kurdistani, S.K., Robyr, D., Tavazoie, S., and Grunstein, M. 2002. Genome-wide binding map of the histone deacetylase Rpd3 in yeast. *Nat Genet* **31**(3): 248-254. Epub 2002 Jun 2024.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**(6824): 116-120.

Lachner, M., O'Sullivan, R.J., and Jenuwein, T. 2003. An epigenetic road map for histone lysine methylation. *J Cell Sci* **116**(Pt 11): 2117-2124.

Ladurner, A.G., Inouye, C., Jain, R., and Tjian, R. 2003. Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries. *Mol Cell* **11**(2): 365-376.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259): 680-685.

Li, B., Gogol, M., Carey, M., Lee, D., Seidel, C., and Workman, J.L. 2007. Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. *Science* **316**(5827): 1050-1054.

Liou, G.G., Tanny, J.C., Kruger, R.G., Walz, T., and Moazed, D. 2005. Assembly of the SIR complex and its regulation by *O*-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. *Cell* **121**(4): 515-527.

Liu, C. and Lustig, A.J. 1996. Genetic analysis of Rap1p/Sir3p interactions in telomeric and HML silencing in *Saccharomyces cerevisiae*. *Genetics* **143**(1): 81-93.

Liu, J., Smith, C.L., DeRyckere, D., DeAngelis, K., Martin, G.S., and Berger, J.M. 2000. Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. *Mol Cell* **6**(3): 637-648.

Lo, W.S., Duggan, L., Emre, N.C., Belotserkovskya, R., Lane, W.S., Shiekhattar, R., and Berger, S.L. 2001. Snf1--a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* **293**(5532): 1142-1146.

Loo, S. and Rine, J. 1994. Silencers and domains of generalized repression. *Science* **264**(5166): 1768-1771.

-. 1995. Silencing and heritable domains of gene expression. *Annu Rev Cell Dev Biol* **11**: 519-548.

Louis, E.J. and Haber, J.E. 1992. The structure and evolution of subtelomeric Y' repeats in Saccharomyces cerevisiae. *Genetics* **131**(3): 559-574.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**(6648): 251-260.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. 2001. Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**(2): 137-148.

Luo, K., Vega-Palas, M.A., and Grunstein, M. 2002. Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes Dev* **16**(12): 1528-1539.

Lustig, A.J. 1998. Mechanisms of silencing in *Saccharomyces cerevisiae*. *Curr Opin Genet Dev* **8**(2): 233-239.

Maas, N.L., Miller, K.M., DeFazio, L.G., and Toczyski, D.P. 2006. Cell cycle and checkpoint regulation of histone H3 K56 acetylation by Hst3 and Hst4. *Mol Cell* **23**(1): 109-119.

Mahoney, D.J. and Broach, J.R. 1989. The *HML* mating-type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. *Mol Cell Biol* **9**(11): 4621-4630.

Marti-Renom, M.A., Stuart, A.C., Fiser, A., Sanchez, R., Melo, F., and Sali, A. 2000. Comparative protein structure modeling of genes and genomes. *Annu Rev Biophys Biomol Struct* **29**: 291-325.

Masoro, E.J. 2000. Caloric restriction and aging: an update. *Exp Gerontol* **35**(3): 299-305.

Mayer, C., Schmitz, K.M., Li, J., Grummt, I., and Santoro, R. 2006. Intergenic transcripts regulate the epigenetic state of rRNA genes. *Mol Cell* **22**(3): 351-361.

McBryant, S.J., Krause, C., and Hansen, J.C. 2006. Domain organization and quaternary structure of the Saccharomyces cerevisiae silent information regulator 3 protein, Sir3p. *Biochemistry* **45**(51): 15941-15948.

McGhee, J.D., Rau, D.C., Charney, E., and Felsenfeld, G. 1980. Orientation of the nucleosome within the higher order structure of chromatin. *Cell* **22**(1 Pt 1): 87-96.

McGuffin, L.J., Bryson, K., and Jones, D.T. 2000. The PSIPRED protein structure prediction server. *Bioinformatics* **16**(4): 404-405.

Meijsing, S.H. and Ehrenhofer-Murray, A.E. 2001. The silencing complex SAS-I links histone acetylation to the assembly of repressed chromatin by CAF-I and Asf1 in *Saccharomyces cerevisiae*. *Genes Dev* **15**(23): 3169-3182.

Meneghini, M.D., Wu, M., and Madhani, H.D. 2003. Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* **112**(5): 725-736.

Moazed, D., Kistler, A., Axelrod, A., Rine, J., and Johnson, A.D. 1997. Silent information regulator protein complexes in *Saccharomyces cerevisiae*: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. *Proc Natl Acad Sci U S A* **94**(6): 2186-2191.

Muller, H.J. 1930. Types of visible variations induced by X-rays in *Drosophila*. *Journal of Genetics* **22**: 299-335.

Nathan, D., Ingvarsdottir, K., Sterner, D.E., Bylebyl, G.R., Dokmanovic, M., Dorsey, J.A., Whelan, K.A., Krsmanovic, M., Lane, W.S., Meluh, P.B. et al. 2006. Histone sumoylation is a negative regulator in *Saccharomyces cerevisiae* and shows dynamic interplay with positive-acting histone modifications. *Genes Dev* **20**(8): 966-976. Epub 2006 Apr 2005.

Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V. 1999. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**(1): 27-43.

Oki, M. and Kamakaka, R.T. 2002. Blockers and barriers to transcription: competing activities? *Curr Opin Cell Biol* **14**(3): 299-304.

-. 2005. Barrier function at *HMR*. *Mol Cell* **19**(5): 707-716.

Oki, M., Valenzuela, L., Chiba, T., Ito, T., and Kamakaka, R.T. 2004. Barrier proteins remodel and modify chromatin to restrict silenced domains. *Mol Cell Biol* **24**(5): 1956-1967.

Onishi, M., Liou, G.G., Buchberger, J.R., Walz, T., and Moazed, D. 2007. Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. *Mol Cell* **28**(6): 1015-1028.

Ornaghi, P., Ballario, P., Lena, A.M., Gonzalez, A., and Filetici, P. 1999. The bromodomain of Gcn5p interacts in vitro with specific residues in the N terminus of histone H4. *J Mol Biol* **287**(1): 1-7.

Osada, S., Sutton, A., Muster, N., Brown, C.E., Yates, J.R., 3rd, Sternglanz, R., and Workman, J.L. 2001. The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1. *Genes Dev* **15**(23): 3155-3168.

Pappas, D.L., Jr., Frisch, R., and Weinreich, M. 2004. The NAD($^{+}$)-dependent Sir2p histone deacetylase is a negative regulator of chromosomal DNA replication. *Genes Dev* **18**(7): 769-781.

Parthun, M.R., Widom, J., and Gottschling, D.E. 1996. The major cytoplasmic histone acetyltransferase in yeast: links to chromatin replication and histone metabolism. *Cell* **87**(1): 85-94.

Petes, T.D. and Botstein, D. 1977. Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast. *Proc Natl Acad Sci U S A* **74**(11): 5091-5095.

Pillus, L. and Rine, J. 1989. Epigenetic inheritance of transcriptional states in *S. cerevisiae*. *Cell* **59**(4): 637-647.

Planta, R.J., Goncalves, P.M., and Mager, W.H. 1995. Global regulators of ribosome biosynthesis in yeast. *Biochem Cell Biol* **73**(11-12): 825-834.

Pryde, F.E. and Louis, E.J. 1999. Limitations of silencing at native yeast telomeres. *EMBO J* **18**(9): 2538-2550.

Qiu, J. 2006. Epigenetics: unfinished symphony. *Nature* **441**(7090): 143-145.

Reifsnyder, C., Lowell, J., Clarke, A., and Pillus, L. 1996. Yeast *SAS* silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. *Nat Genet* **14**(1): 42-49.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. 1993. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by *SIR3* dosage. *Genes Dev* **7**(7A): 1133-1145.

Rine, J. and Herskowitz, I. 1987. Four genes responsible for a position effect on expression from *HML* and *HMR* in Saccharomyces cerevisiae. *Genetics* **116**(1): 9-22.

Rine, J., Strathern, J.N., Hicks, J.B., and Herskowitz, I. 1979. A suppressor of mating-type locus mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating-type loci. *Genetics* **93**(4): 877-901.

Rivier, D.H., Ekena, J.L., and Rine, J. 1999. *HMR*-I is an origin of replication and a silencer in *Saccharomyces cerevisiae*. *Genetics* **151**(2): 521-529.

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S.K., Wang, A., Suka, N., and Grunstein, M. 2002. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell* **109**(4): 437-446.

Robzyk, K., Recht, J., and Osley, M.A. 2000. Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287**(5452): 501-504.

Rogina, B., Helfand, S.L., and Frankel, S. 2002. Longevity regulation by *Drosophila* Rpd3 deacetylase and caloric restriction. *Science* **298**(5599): 1745.

Roth, S.Y., Denu, J.M., and Allis, C.D. 2001. Histone acetyltransferases. *Annu Rev Biochem* **70**: 81-120.

Ruiz-Garcia, A.B., Sendra, R., Galiana, M., Pamblanco, M., Perez-Ortin, J.E., and Tordera, V. 1998. HAT1 and HAT2 proteins are components of a yeast nuclear histone acetyltransferase enzyme specific for free histone H4. *J Biol Chem* **273**(20): 12599-12605.

Rundlett, S.E., Carmen, A.A., Suka, N., Turner, B.M., and Grunstein, M. 1998. Transcriptional repression by *UME6* involves deacetylation of lysine 5 of histone H4 by *RPD3*. *Nature* **392**(6678): 831-835.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. 2002. Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol Biol Cell* **13**(7): 2207-2222.

-. 2003. The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu Rev Biochem* **72**: 481-516. Epub 2003 Mar 2027.

Rusche, L.N. and Rine, J. 2001. Conversion of a gene-specific repressor to a regional silencer. *Genes Dev* **15**(8): 955-967.

Sambrook, J.F., E F; Maniatis, Tom. 1989. *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, New York.

Santos-Rosa, H., Bannister, A.J., Dehe, P.M., Geli, V., and Kouzarides, T. 2004. Methylation of H3 lysine 4 at euchromatin promotes Sir3p association with heterochromatin. *J Biol Chem* **279**(46): 47506-47512. Epub 42004 Jul 47527.

Schalch, T., Duda, S., Sargent, D.F., and Richmond, T.J. 2005. X-ray structure of a tetranucleosome and its implications for the chromatin fibre. *Nature* **436**(7047): 138-141.

Schultz, J. 1936. Variegation in Drosophila and the Inert Chromosome Regions. *Proc Natl Acad Sci U S A* **22**(1): 27-33.

Sharma, K., Weinberger, M., and Huberman, J.A. 2001. Roles for internal and flanking sequences in regulating the activity of mating-type-silencer-associated replication origins in *Saccharomyces cerevisiae*. *Genetics* **159**(1): 35-45.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol* **194**: 3-21.

Shia, W.J., Li, B., and Workman, J.L. 2006. SAS-mediated acetylation of histone H4 Lys 16 is required for H2A.Z incorporation at subtelomeric regions in *Saccharomyces cerevisiae*. *Genes Dev* **20**(18): 2507-2512.

Shibahara, K., Verreault, A., and Stillman, B. 2000. The N-terminal domains of histones H3 and H4 are not necessary for chromatin assembly factor-1- mediated nucleosome assembly onto replicated DNA in vitro. *Proc Natl Acad Sci U S A* **97**(14): 7766-7771.

Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R., and Peterson, C.L. 2006. Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* **311**(5762): 844-847.

Singleton, M.R., Morales, R., Grainge, I., Cook, N., Isupov, M.N., and Wigley, D.B. 2004. Conformational changes induced by nucleotide binding in Cdc6/ORC from Aeropyrum pernix. *J Mol Biol* **343**(3): 547-557.

Smith, E.R., Eisen, A., Gu, W., Sattah, M., Pannuti, A., Zhou, J., Cook, R.G., Lucchesi, J.C., and Allis, C.D. 1998a. *ESA1* is a histone acetyltransferase that is essential for growth in yeast. *Proc Natl Acad Sci U S A* **95**(7): 3561-3565.

Smith, J.S. and Boeke, J.D. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes Dev* **11**(2): 241-254.

Smith, J.S., Brachmann, C.B., Pillus, L., and Boeke, J.D. 1998b. Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. *Genetics* **149**(3): 1205-1219.

Sobel, R.E., Cook, R.G., Perry, C.A., Annunziato, A.T., and Allis, C.D. 1995. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc Natl Acad Sci U S A* **92**(4): 1237-1241.

Sogo, J.M., Stahl, H., Koller, T., and Knippers, R. 1986. Structure of replicating simian virus 40 minichromosomes. The replication fork, core histone segregation and terminal structures. *J Mol Biol* **189**(1): 189-204.

Stavenhagen, J.B. and Zakian, V.A. 1994. Internal tracts of telomeric DNA act as silencers in *Saccharomyces cerevisiae*. *Genes Dev* **8**(12): 1411-1422.

Stone, E.M. and Pillus, L. 1998. Silent chromatin in yeast: an orchestrated medley featuring Sir3p [corrected]. *Bioessays* **20**(1): 30-40.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev* **11**(1): 83-93.

Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. 1999. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* **97**(2): 245-256.

Suka, N., Luo, K., and Grunstein, M. 2002. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat Genet* **32**(3): 378-383. Epub 2002 Oct 2015.

Suka, N., Suka, Y., Carmen, A.A., Wu, J., and Grunstein, M. 2001. Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol Cell* **8**(2): 473-479.

Sun, Z.W. and Hampsey, M. 1999. A general requirement for the Sin3-Rpd3 histone deacetylase complex in regulating silencing in *Saccharomyces cerevisiae*. *Genetics* **152**(3): 921-932.

Sutton, A., Shia, W.J., Band, D., Kaufman, P.D., Osada, S., Workman, J.L., and Sternglanz, R. 2003. Sas4 and Sas5 are required for the histone acetyltransferase activity of Sas2 in the SAS complex. *J Biol Chem* **278**(19): 16887-16892. Epub 12003 Mar 16886.

Taddei, A., Roche, D., Sibarita, J.B., Turner, B.M., and Almouzni, G. 1999. Duplication and maintenance of heterochromatin domains. *J Cell Biol* **147**(6): 1153-1166.

Tamaru, H. and Selker, E.U. 2001. A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. *Nature* **414**(6861): 277-283.

Tanner, K.G., Landry, J., Sternglanz, R., and Denu, J.M. 2000. Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc Natl Acad Sci U S A* **97**(26): 14178-14182.

Tompa, R. and Madhani, H.D. 2007. Histone H3 lysine 36 methylation antagonizes silencing in Saccharomyces cerevisiae independently of the Rpd3S histone deacetylase complex. *Genetics* **175**(2): 585-593. Epub 2006 Dec 2018.

Triolo, T. and Sternglanz, R. 1996. Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. *Nature* **381**(6579): 251-253.

Tyler, J.K., Adams, C.R., Chen, S.R., Kobayashi, R., Kamakaka, R.T., and Kadonaga, J.T. 1999. The RCAF complex mediates chromatin assembly during DNA replication and repair. *Nature* **402**(6761): 555-560.

Valenzuela, L., Dhillon, N., Dubey, R.N., Gartenberg, M.R., and Kamakaka, R.T. 2008. Long-range communication between the silencers of HMR. *Mol Cell Biol* **28**(6): 1924-1935. Epub 2008 Jan 1914.

van Leeuwen, F., Gafken, P.R., and Gottschling, D.E. 2002. Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109**(6): 745-756.

Vaquero, A., Scher, M.B., Lee, D.H., Sutton, A., Cheng, H.L., Alt, F.W., Serrano, L., Sternglanz, R., and Reinberg, D. 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev* **20**(10): 1256-1261. Epub 2006 Apr 1228.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. 2001. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**(2): 149-159.

Vidal, M., Buckley, A.M., Hilger, F., and Gaber, R.F. 1990. Direct selection for mutants with increased K+ transport in *Saccharomyces cerevisiae*. *Genetics* **125**(2): 313-320.

Vogelauer, M., Rubbi, L., Lucas, I., Brewer, B.J., and Grunstein, M. 2002. Histone acetylation regulates the time of replication origin firing. *Mol Cell* **10**(5): 1223-1233.

Vogelauer, M., Wu, J., Suka, N., and Grunstein, M. 2000. Global histone acetylation and deacetylation in yeast. *Nature* **408**(6811): 495-498.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**(5588): 1833-1837. Epub 2002 Aug 1822.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**(13): 1793-1808.

Wallrath, L.L. and Elgin, S.C. 1995. Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev* **9**(10): 1263-1277.

Wang, A., Kurdistani, S.K., and Grunstein, M. 2002. Requirement of Hos2 histone deacetylase for gene activity in yeast. *Science* **298**(5597): 1412-1414.

Wittschieben, B.O., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C.D., Tempst, P., and Svejstrup, J.Q. 1999. A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. *Mol Cell* **4**(1): 123-128.

Wu, J., Suka, N., Carlson, M., and Grunstein, M. 2001. TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. *Mol Cell* **7**(1): 117-126.

Xu, F., Zhang, Q., Zhang, K., Xie, W., and Grunstein, M. 2007. Sir2 deacetylates histone H3 lysine 56 to regulate telomeric heterochromatin structure in yeast. *Mol Cell* **27**(6): 890-900.

Yang, T. and Sauve, A.A. 2006. NAD metabolism and sirtuins: metabolic regulation of protein deacetylation in stress and toxicity. *AAPS J* **8**(4): E632-643.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. 1990. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem* **265**(28): 17174-17179.

Zachariae, W., Shevchenko, A., Andrews, P.D., Ciosk, R., Galova, M., Stark, M.J., Mann, M., and Nasmyth, K. 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. *Science* **279**(5354): 1216-1219.

Zhao, K., Chai, X., and Marmorstein, R. 2003. Structure of the yeast Hst2 protein deacetylase in ternary complex with 2'-O-acetyl ADP ribose and histone peptide. *Structure* **11**(11): 1403-1411.

Danksagung

Mein besonderer Dank gilt Frau Prof. Ann Ehrenhofer-Murray. Ihre exzellente Betreuung, hilfreichen Diskussionen und Ihre experimentelle Unterstützung waren eine große Hilfe bei der Entstehung dieser Arbeit.

Ich bedanke mich bei Jan für die Unterstützung bei einigen Experimenten, besonders während der Paper-Korrektur Phasen. Weiterhin danke ich Daniel Hoffmann und Niko Dybowski für die bioinformatischen Daten. Die Vorhersage der Sir3 Struktur brachte das Projekt entscheidend voran. Jonathan Müller danke ich für die große Mühe bei der (leider nicht erfolgreichen) biochemischen Messung der Bindung zwischen OAADPR und Sir3.

Ich bedanke mich bei allen Mitarbeitern des Genetik Labors: Anke, Jessica, Jan, Martin, Christiane, Franziska, Corinna, Maria, Gesine, Christian, Alexandra und Wolfgang für die angenehme Atmosphäre im Labor. Besonders danke ich Christiane für die Idee die Energie für den Endspurt bei gemeinsamen Yoga-Stunden wieder aufzufüllen. Ich danke Anke für die aufbauenden Gespräche und danke Jessica für Ihre spontane Hilfsbereitschaft. Auch die Beratung der Studenten, Olga, Rosti, Kamilla und Tanja machte großen Spaß.

Senta, Rita, Martina, und Rolf danke ich für die technische und organisatorische Unterstützung. Ich bedanke mich bei den „alten Berlinern“, Jacqueline, Horst, Uta, Antje und Sigrid für die Unterstützung und die unvergesslichen Erinnerungen an die Anfangszeit in Berlin.

Ich bedanke mich bei meiner Mutter und Christian für die erholsame Zeit in der Heimat. Ganz besonders danke ich meinem Großvater, der das Ergebnis seiner langjährigen Unterstützung leider nichtmehr erleben konnte.

Ich bedanke mich bei den Leitern anderer Labore: J. Boeke, D. Gottschling, L. Guarente, R. Kamakaka, M. Keogh, R.Morse L. Pillus, F. Posas, J. Rine and D. Rivier für das zur Verfügung stellen von Hefestämmen und Plasmiden. Die Arbeit wurde unterstützt von der Max-Planck-Gesellschaft, der Justus-Liebig-Universität Giessen, der Universität Duisburg-Essen und der Deutschen Forschungsgemeinschaft (EH 194/ 4-1). Ich danke den Genetik-Studenten der JLU Giessen für die Durchführung des synthetisch-lethal Screens, deren Ergebnisse den Startpunkt für diese Arbeit setzten.

Lebenslauf

Name:	Stefan Ehrentraut
geboren:	22. November 1977 in Lauchhammer
Nationalität:	deutsch
Familienstand:	ledig
Anschrift:	Siemensstr. 25, 45143 Essen
Telefon:	+49 174 35 96 672 +49 201 47 48 295
E-mail Adresse:	StefanEhrentraut@web.de
Schulbildung:	1984 - 1986 POS Senftenberg 1986 - 1990 POS mit erweitertem Sprachunterricht Senftenberg 1990 - 1997 Gymnasium Senftenberg, Abitur im Mai 1997
Sprachkenntnisse:	Englisch (gut, in Wort und Schrift), Russisch (in Wort und Schrift, 11 Schuljahre) Französisch (Grundkenntnisse, 2 Schuljahre)
Wehrdienst:	1. Juli 1997 bis 30. April 1998 Märkische Kaserne Lehnitz
Studium:	Oktober 1998 bis Juni 2003 Studium der Biotechnologie (Grundstudium Chemieingenieurwesen) an der Fachhochschule Lausitz in Senftenberg mit Abschluß „sehr gut“
Diplomarbeit:	Mai 2002 bis April 2003 am Max-Planck-Institut für Molekulare Genetik in Berlin Dahlem in der Arbeitsgruppe Lanka mit dem Inhalt genetischer und biochemischer Untersuchungen zur Enzymologie des DNA-Metabolismus in Prokaryonten, ausgezeichnet mit dem “Gottlob-Schumann-Preis“ für herausragende Diplomarbeiten“
Doktorarbeit:	seit August 2003 beschäftigt als wissenschaftlicher Mitarbeiter am Otto-Warburg-Laboratorium des Max-Planck-Institut für Molekulare Genetik in Berlin Dahlem, seit August 2005 an der Universität Duisburg-Essen, Abteilung für Genetik in der Arbeitsgruppe von Frau Prof. Dr. Ann Ehrenhofer-Murray
Preise:	“Gottlob-Schumann-Preis” für herausragende Diplomarbeiten, Fachhochschule Lausitz, Senftenberg, 2003. Posterpreis während des Forschungstages der Medizinischen Fakultät, Universitätsklinikum Essen, Essen, 2008.

Essen, 09.10.2008

Stefan Ehrentraut

Erklärung:

Hiermit erkläre ich gem. § 6 Abs. 2, Nr. 6 der weiterhin geltenden Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, 09/10/2008 Stefan Ehrentraut

Erklärung:

Hiermit erkläre ich gem. § 6 Abs. 2, Nr. 7 der weiterhin geltenden Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema "The histone deacetylase (HDAC) Rpd3 antagonizes heterochromatn formation at telomeres in *Saccharomyces cerevisiae"* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Stefan Ehrentraut befürworte.

Essen, 09/10/2008 Prof. Dr. Ann Ehrenhofer-Murray

Erklärung:

Hiermit erkläre ich gem. § 6 Abs. 2, Nr. 8 der weiterhin geltenden Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, 09/10/2008 Stefan Ehrentraut