The role of the N-terminal acetyltransferase NatA in transcriptional silencing in *Saccharomyces cerevisiae*

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Antje Geißenhöner

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Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jürgen Mlynek

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Michael Linscheid

Gutachter / innen: 1. Prof. Dr. Harald Saumweber
2. PD Dr. Ann Ehrenhofer-Murray
3. Prof. Dr. Jörn Walter

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Zusammenfassung

N^{α}-Acetylierung, eine der häufigsten eukaryontischen Proteinmodifikationen, wird von N-terminalen Acetyltransferasen (NATs) katalysiert. NatA, die bedeutendste NAT in *Saccharomyces cerevisiae,* besteht aus den Untereinheiten Nat1, Ard1 und Nat5, und ist am *silencing*, d.h. am Aufbau repressiver Chromatinstrukturen an Telomeren und den Paarungstyp-Loci *HML* und *HMR* beteiligt. Die vorliegende Arbeit demonstriert eine Rolle von NatA auch beim rDNA-*silencing*, und zeigt erstmals, dass die *silencing*-Faktoren Orc1 und Sir3 funktionell von der N^{α}-Acetylierung durch NatA abhängen.

Orc1, die größte Untereinheit des *origin recognition complex* (ORC), wurde *in vivo* durch NatA N^{α}-acetyliert. Mutationen, die dies verhinderten, bewirkten eine starke telomerische Derepression. NatA wirkte genetisch über die ORC Bindungsstelle des *HMR*-E-*silencers*. Die artifizielle Bindung von Orc1 an *HMR*-E machte *HMR-silencing* NatA-unabhängig. Auch die synthetische Letalität von *nat1*Δ *orc2-1* Doppelmutanten wies auf eine funktionelle Verbindung zwischen NatA und ORC hin.

Als weiteres NatA-Substrat wurde Sir3 identifiziert, dessen zelluläre Lokalisierung von NAT1 abhing. Die schwächeren silencing-Defekte der unacetylierten orc1 sir3 Doppelmutante im Vergleich zu nat1Δ implizierten allerdings, dass noch weitere silencing-Proteine die N^{α}-Acetylierung für ihre Funktion bedürfen.

Weitere Ergebnisse dieser Arbeit belegen eine Funktion N-terminalen 100 Aminosäuren von Orc1 im *silencing*. Deletionen innerhalb dieses Bereichs erzeugten *silencing*-Defekte. Das Fehlen von 51 Aminosäuren vom N-Terminus von Orc1 unterbrach die Interaktion mit Sir1, verstärkte aber auch den *silencing*-Defekt von *sir1*Δ. Dies ergibt ein Model, in dem Orc1 neben Sir1 ein weiteres *silencing*-Protein rekrutiert, das zu seiner Bindung einen intakten, acetylierten N-Terminus von Orc1 benötigt.

Zusammenfassend sprechen die Ergebnisse für eine Rolle der N^{α}-Acetylierung durch NatA bei der Modellierung der Chromatinstruktur.

Schlagworte:
Chromatin, Genregulation, Silencing, Nat1, Orc1

Abstract

N^{α}-acetylation, one of the most abundant eukaryotic protein modifications, is catalyzed by N-terminal acetyltransferases (NATs). NatA, the major NAT in *Saccharomyces cerevisiae*, consists of the subunits Nat1, Ard1 and Nat5 and is necessary for the assembly of repressive chromatin structures at the silent mating type loci and telomeres. This thesis shows that NatA also acts in rDNA repression and it provides the first direct evidence for the functional regulation of the silencing factors Orc1 and Sir3 by NatA-dependent N^{α}-acetylation.

Orc1, the large subunit of the origin recognition complex (ORC), was N^{α}-acetylated *in vivo* by NatA. Mutations that abrogated this acetylation caused strong telomeric derepression. NatA functioned genetically through the ORC binding site of the *HMR*-E silencer. Direct tethering of Orc1 to *HMR*-E circumvented the requirement for NatA in silencing. The synthetic lethality of *nat1*Δ *orc2-1* double mutants further supported a functional link between NatA and ORC.

Sir3 was also indentified as a NatA substrate. Its localization to perinuclear foci was *NAT1* dependent. Unacetylated *sir3 orc1* double mutants did not resemble the *nat1*Δ silencing phenotype. Thus, we suggest that further silencing components require NatA-dependent N^{α}-acetylation for their function.

We further identified the N-terminal 100 amino acids of Orc1 to be important for silencing, since truncations within this region impaired silencing. The deletion of 51 amino acids from the Orc1 N-terminus interrupted the interaction with Sir1 and also reduced silencing in *sir1*Δ strains. We thus propose that the silencing function of Orc1 is not restricted to Sir1 recruitment, but also comprises the interaction with another protein. The silencing function of this hypothesized interaction partner may depend on the N^{α}-acetylation and integrity of the N-terminus of Orc1.

In summary, we propose that N^{α}-acetylation by NatA represents a protein modification that modulates chromatin structure in yeast.

Keywords:
chromatin, gene regulation, silencing, Nat1, Orc1

Contents

1 Introduction

1.1 N-terminal acetylation of proteins

In eukaryotic cells, proteins undergo a number of co- and posttranslational modifications that extend the range of their possible molecular structure beyond the limits of the encoding amino acids and thus amplify their functional potential. This provides the bases for complex cellular mechanisms such as differentiation and gene regulation.

Among protein modifications, the acetylation of the α-amino group at the initiating amino acid, referred to as N-terminal acetylation or N^{α}-acetylation, is a major modification type. 80-90% of the mammalian cytosolic proteins and 50% of those in yeast are estimated to be N^{α}-acetylated (Polevoda and Sherman 2003b). In a cotranslational process, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl coenzyme A to nascent polypeptides of 20 to 50 amino acids when they are just protruding from the ribosome (Driessen et al. 1985) (Fig. 1.1). In proteins with small penultimate amino acids (1.29 Å or less radii of gyration), acetylation is preceded by the removal of the initial methionine residue by means of specific amino peptidases (Bradshaw et al. 1998). Notably, N^{α}-acetylation is irreversible and thus functionally distinct from the reversible posttranslational acetylation of ε-amino groups (N^{ε}-acetylation) of internal lysines in histones, transcription factors (Cheung et al. 2000b), nuclear receptors and import factors (Bannister et al. 2000; Soutoglou et al. 2000).

The substrate specificity of NATs is not determined by a simple consensus motif, but rather is supposed to emerge from degenerate signals within the N-terminal 50 amino acids (Polevoda and Sherman 2003a) of the substrate. The penultimate amino acid has a profound, although no absolute, effect on N^{α}-acetylation. Proteins with methionine, alanine or serine termini are the most frequently acetylated, the latter two contributing more than 74% of all N^{α}-acetylated proteins in the budding yeast *Saccharomyces cerevisiae* (Polevoda and Sherman 2003b).

Whereas N^{α}-acetylation is one of the most common protein modifications in eukaryotes, it occurs only rarely in prokaryotes and archea. In *Escherichia coli*, RimI, RimJ, and RimL specifically N^{α}-acetylate ribosomal proteins, apparently in a posttranslational manner (Tanaka et al. 1989). In general, N^{α}-acetylation in prokaryotes and archea is thought to differ fundamentally from the process in eukaryotes (Polevoda and Sherman 2003b).

In eukaryotic organisms, the same system of N^{α}-acetylation may operate in all species, since sequence homologs to subunits of yeast NAT's exist in the genomes of all model organisms,

e.g. *Caenorhabditis elegans, Drosophila melanogaster, Arabidopsis thaliana, Xenopus laevis*, *Mus musculus,* and in humans (Polevoda and Sherman 2003b). Moreover, the acetylation patterns of N^{α}-acetylated proteins are very similar in yeast and mammals, suggesting that they are evolutionary conserved. Interestingly, N^{α}-acetylation is more frequent in mammals compared to yeast, which may point to some form of selection for this modification during evolution.

Nevertheless, the number of the N^{α}-acetylated proteins characterized so far is limited, and only a few examples demonstrate the biological significance of this modification. It was originally suggested that N^{α}-acetylation generally acts as protection from degradation, but this hypothesis is no longer favored (Mayer et al. 1989). In the current model, the biological importance of N^{α}-acetylation varies with the particular protein. Accordingly, some proteins require N^{α}-acetylation for their function and stability, whereas others do not. Tropomyosin, for example, depends on N^{α}-acetylation for normal binding and stabilization of filamentous actin in yeast and vertebrate muscle cells (Urbancikova and Hitchcock-DeGregori 1994; Singer and Shaw 2003). In addition, N^{α}-acetylated rat α-melanotropin induces increased pigment-producing effects and enhanced activity in behavioral tests compared to the unacetylated form (Smyth et al. 1979; O'Donohye et al. 1982). N^{α}-acetylation of the major coat protein *gag* of the L-A double-stranded RNA virus in *S. cerevisiae* is essential for the assembly of virus particles (Tercero and Wickner 1992). Recently, At*MAK3,* a homolog of yeast Mak3 in *A. thaliana*, was found to acetylate core proteins of photosystem II, which was necessary for the formation of thylacoid complexes and plant growth (Pesaresi et al. 2003). Furthermore, N^{α}-acetylation can also affect the thermal stability of proteins, as observed for the NADP-specific glutamate dehydrogenase of *Neurospora crassa* (Siddig et al. 1980).

Importantly, not only the lack of N^{α}-acetylation can result in various defects, but abnormal acetylation can likewise prevent regular protein function. In yeast, the catalytic α-amino groups of some 20S proteasome subunits have to be protected from N^{α}-acetylation to preserve their peptidase activity (Arendt and Hochstrasser 1999). This is realized by N-terminal propetides, which become removed from the subunits during proteasome assembly. As another example, hemoglobin Lyon-Bron displays decreased oxygen affinity due to N^{α}-acetylation, which consequently causes anemia. In this α2-globin variant, the penultimate amino acid is mutated from valine to alanine, which converts the protein into a NAT substrate (Lacan et al. 2002).

Altogether, these examples demonstrate the biological significance of N^{α}-acetylation in diverse organisms. In spite of this, the knowledge of mechanisms and players of this frequent modification to date is only marginal.

1.2 N^{α}-acetyltransferases in *S. cerevisiae*

Insight into the function of N^{α}-acetylation comes from the analysis of NATs in *S. cerevisiae*. Here, three NAT complexes exist that are known as NatA, NatB and NatC according to their substrate specificity (Tbl. 1.1). NatA accounts for the majority of N^{α}-acetylated proteins in yeast, and acetylates proteins with alanine or serine, and occasionally with glycine or threonine termini. The other two NATs act on the N-terminal methionine when the second residue either is glutamate or aspartate, asparagine or methionine (NatB substrates), or else isoleucine, leucine, tryptophan or phenylalanine (NatC substrates) (Polevoda and Sherman 2003b). Interestingly, whereas all observed proteins with Met-Asp and Met-Glu N-termini were N^{α}-acetylated, only half of the potential NatC substrates were actually modified *in vivo*.

In addition to the aforementioned substrate types, a special subclass of NatA substrates with Ser-Glu, Ser-Asp, Ala-Glu or Gly-Glu termini was designated NatD substrates (Arnold et al. 1999). In systematic analyses for substrate specificities of NATs, these proteins were found to require not only NatA activity for N^{α}-acetylation, but also the integrity of NatB and NatC. As a possible interpretation, it was suggested that the acetylation of NatD proteins requires auxiliary factors to NatA, which in turn are substrates of the other two NATs (Polevoda et al. 1999).

Recently, the novel GNAT (GCN5-related *N*-acetyltransferase) homolog Nat4 was identified to specifically acetylate histones H2A and H4 (Song et al. 2003). Interestingly, this protein is well conserved from yeast to mammals not only in the GNAT domain, indicating a role in histone acetylation also for its homologs. However, mechanistic details or interaction partners of Nat4 are currently not known. Given the importance of the charge of histones for their association with DNA (see below), it is surprising that *nat4*Δ mutants displayed no detectable phenotype, although each nucleosome contained four extra positive charges due to the missing N^{α}-acetylation. Thus, the relevance of N^{α}-acetylation for histone function remains subject to further investigation.

The three NAT complexes, NatA, NatB and NatC, not only differ in substrate specificity, but also in subunit composition. Significantly, they all contain a catalytic subunit homologous to

the GNAT superfamily of acetyltransferases (Tbl. 1.1). Besides NATs, this superfamily contains several histone acetyltransferases (HATs) and proteins involved in gene regulation and diverse other functions, such as detoxification and drug resistance (Neuwald and Landsman 1997). The members of the GNAT superfamily are characterized by a remarkably conserved binding motif for the donor substrate acetyl CoA and exhibit a highly consistent protein topology (Dyda et al. 2000).

Table 1: Characteristics of the three NAT complexes in *S. cerevisiae**

	NatA	**NatB**	**NatC**
Catalytic subunit	Ard1	Nat3	Mak3
Auxiliary subunit	Nat1 Nat5	Mdm20	Mak10 Mak31
Substrate** termini	Ser Ala Gly Thr	Met-Glu Met-Asp Met-Asn Met-Met	Met-Ile Met-Leu Met-Trp Met-Phe
Selected substrates	ribosomal subunits (SU) S1,2,5,7, 11,14,15,16,18,20,24 and L1,4,11,16,33,36; 19S proteasomal SU: Rpt4,5,6 and Rpn2,3,5,6,8; 20S proteasomal SU: Scl1, Pup3 and Pre6,8,9,10	Tropomyosin, actin, ribosomal SU S21 and S28, 19S proteasomal SU Rpt3 and Rpn11, 20S proteasomal SU Pre1	*gag* protein of L-A virus, 20S proteasomal SU Pup2 and Pre5
Deletion mutant phenotypes	Slow growth; temperature and osmotic sensitivity; deficiency in utilizing non-fermentable carbon sources; inability to enter G_0; inability to sporulate; chromosomal instability; derepression of silent loci	Slow growth; temperature and osmotic sensitivity; deficiency in utilizing non-fermentable carbon sources; defects in vacuolar and mitochondrial inheritance; random polarity in budding; reduced mating efficiency; sensitivity to antimitotic drugs and DNA damaging agents	Temperature sensitivity; deficiency in utilizing non- fermentable carbon sources
Characterized homologs	Homologs of Ard1: ARD1 (*T. brucei*), TE2 (human), Xat-1 (*X. laevis*); Homolog of Nat5: SAN (*Drosophila*); Homologs of Nat1: NATH (human), NARG1, tbdn-1, Tbdn100 (all mouse),	No homologs have been characterized at present	At*MAK3* (*A. thaliana*)

* References are given in chapters 1.2 and 1.3.
* * Acetylation occurs only on subclasses of proteins containing the indicated termini, except for Met-Glu and Met-Asp.

In addition to the catalytic subunit, each NAT contains auxiliary components that are required for enzymatic activity (Polevoda and Sherman 2003b) (Tbl. 1.1). The loss of NAT activity generally results in multiple effects in yeast, among them temperature sensitivity and diverse growth defects. Mutations of NatB subunits display the most severe phenotypes, apparently associated with the partial loss of function of unacetylated actin and tropomyosin in these mutants (Polevoda and Sherman 2001; Polevoda et al. 2003). The phenotypic characteristics of NatC were hypothesized to stem from affected mitochondrial substrates (Tercero and Wickner 1992; Polevoda and Sherman 2001), whereas NatA acetylation is important for growth and cell cycle control. Notably, *nat* mutations are not lethal, suggesting that among the various substrates there is no essential protein depending absolutely on N^{α}-acetylation. The hitherto known substrates of the individual NATs were mainly identified in systematic analyses of ribosomal (Arnold et al. 1999) and proteasomal proteins (Kimura et al. 2000; Kimura et al. 2003). Predominant methods applied were mass spectrometry and 2D protein migration analysis.

1.3 NatA – the major N^{α}-acetyltransferase complex of *S. cerevisiae*

NatA is the major NAT in yeast, accounting for most of the N^{α}-acetylated proteins. Given the portion of NatA targets on the total NAT substrates known to date, NatA acetylates potentially 2500 yeast proteins. About 140 NatA substrates have been identified so far, the list including ribosomal and 26S proteasomal subunits as well as some abundant proteins (Polevoda and Sherman 2003b) (Tbl. 1.1). NatA has the most degenerate substrate specificity of all NATs. Approximately 90% and 30%, respectively, of the tested serine and alanine termini, and only one fourth of the glycine and threonine proteins tested, were actually acetylated by NatA (Polevoda and Sherman 2003b).

NatA is not only the predominant, but also the best characterized NAT. The trimeric complex consists of the subunits Ard1, Nat1 and Nat5, which are present in a 1:1:1 stoichiometric ratio (Gautschi et al. 2003). Interestingly, in a tandem affinity purification (TAP) analysis, several other proteins, namely Asc1, Eno1, Mis1, Myo1 and YYGR090w, were co-purified with Ard1, and the ribosomal protein Asc1 (Inada et al. 2002) also associated with Nat1 (http://yeast.cellzome.com/). However, the question whether these proteins were present in stoichiometric amounts was not answered. Therefore, it remains unclear whether Asc1 or the other co-purified proteins are required for NatA's function.

According to the current model, the NatA complex resides at the ribosome, close to the polypeptide tunnel exit (Fig. 1.1). Nat1 (N-terminal acetyltransferase 1) mediates the stable interaction of NatA with the large ribosomal subunit. In addition, it can be crosslinked to nascent polypeptides, and is thus predicted to contact the nascent chains in order to present them to the catalytically active Ard1. This interaction is probably mediated by five to eight tetratricopeptide repeats (TPR) clustered in the first third of the 854 amino-acid protein Nat1 (Gautschi et al. 2003). TPR motifs are evolutionarily ancient protein-protein interaction modules consisting of two antiparallel α-helices that generate a super-helix with an amphipathic channel (Blatch and Lassle 1999). TPR clusters exist in a number of functionally distinct proteins and are important for the function of e.g. chaperones or protein transport complexes (Gatto et al. 2000). In addition to TPR repeats, Nat1 contains highly charged regions between amino acids 550 and 670 with predicted coiled-coil structures. These are proposed to mediate the interaction of Nat1 to other subunits of the complex. Interestingly, a nuclear localization signal (NLS) is likewise predicted for Nat1 between amino acids 648-665. Its functional significance remains unclear, since Nat1 acts in the cytoplasm (Polevoda and Sherman 2003a).

The catalytic subunit Ard1 (arrest defective) is a protein of 238 amino acids and carries the acetyl CoA binding GNAT domain in the N-terminal part between amino acids 3-175 (Neuwald and Landsman 1997). Ard1 contacts the ribosome not directly, but probably binds to Nat1 via a C-terminal coiled-coil (Park and Szostak 1992; Gautschi et al. 2003) (Fig. 1.1).

Recently, the GNAT-family acetyltransferase Nat5 (also termed Rog2), another putative catalytic subunit, was found to be associated with NatA. Currently, it is unclear how Nat5 is bound. In addition, its function within the complex remains to be characterized, since *nat5*Δ mutants display no obvious phenotype (Gautschi et al. 2003). The question remains why the complex should contain a second catalytic subunit. Gautschi et al. (2003) propose that Nat5 may be responsible for the N^{α}-acetylation of a small subset of proteins that are not involved in mating-type silencing or affected at elevated temperature. In an earlier study, the deletion of Nat5 suppressed the temperature sensitivity of the double mutants involved in ubiquitin-dependent protein degradation, *mck1 mds1* and *bul1 bul2* (Andoh et al. 2000). Moreover, the Nat5 homolog SAN in *Drosophila* (Accession GI 6980078) was found to play a role in sister chromatid cohesion. The interaction of SAN with homologs of Nat1 and Ard1 suggests that a NAT complex similar to NatA exists in *Drosophila* (Polevoda and Sherman 2003a).

So far, Nat1 and Ard1 homologs have been implicated in development and cellular proliferation of higher eukaryotes. For instance, Xat-1, a Nat1 homolog in *X. laevis*, was

isolated in a screen for stage-specific gene expression during early embryogenesis (Choi et al. 2001). Likewise, *NARG1* and *tubedown-1*, two mouse Nat1 homologs, were found highly expressed in certain embryonic tissues (Gendron et al. 2000; Sugiura et al. 2001). In *Trypanosoma brucei*, *ARD1* was essential in mammalian and insect-stage cells (Ingram et al. 2000).

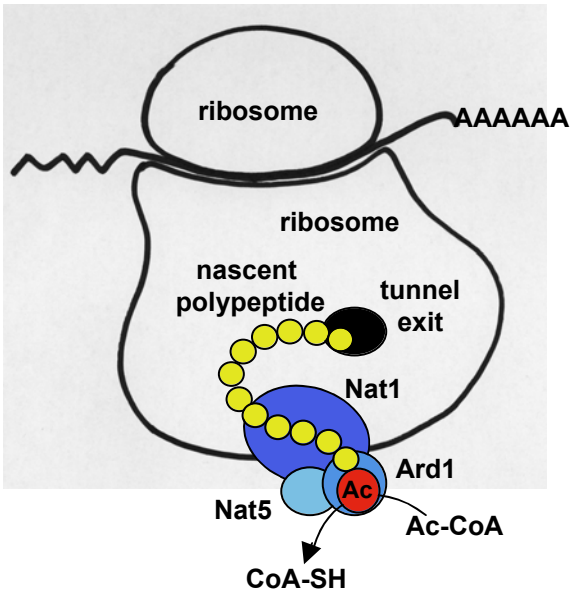


Fig. 1.1: The NatA complex is associated with the ribosome.
In the current model, the non-catalytic subunit Nat1 mediates the stable contact of NatA with the large ribosomal subunit. Nat1 interacts with the nascent polypeptide chain that emerges from the tunnel exit and guides it to the catalytic subunit Ard1, which transfers an acetyl moiety from acetyl coenzyme A to the N-terminal amino acid of NatA substrates. The putative catalytic subunit Nat5 is also associated with the complex. (adapted from Gautschi et al. 2003)

Interestingly, there are data suggesting that the function of at least some of the NatA homologs in mammals has diverged from that of their yeast counterparts. As an example, mouse ARD1, which is 57% homologous to the yeast protein, acetylates lysine 532 of the hypoxia-inducible factor HIF-1α, a protein involved in adaptation to changes in oxygen availability (Jeong et al. 2002). Thereby, mARD1 regulates the protein stability of HIF-1α, since the acetylation is critical to its proteasomal degradation. Notably, in contrast to yeast Ard1, mARD1 acts alone and appears not to require a complex. As a second striking difference, mARD1 shows ε-N-acetyltransferase activity, which is functionally distinct from N^{α}-acetylation. It remains to be determined whether mARD1 can acetylate N^{α}-termini as well. In a possible scenario, mARD1 may perform both modifications in conjunction with different

partner proteins. Intriguingly, provided that yeast NATs are comparable to the evolutionary ancestors of NATs of higher organisms, the substrate shift of mammalian NAT proteins may serve as an example for evolutionary processes on conserved protein modifications.

Overexpression of both Nat1 and Ard1 is required to increase the *in vivo* activity of NatA (Park and Szostak 1992). Interestingly, similarly to *nat1*Δ, the overexpression of Nat1 results in chromosome loss, presumably due to a dominant negative effect on NatA integrity (Ouspenski et al. 1999). Deletions of *NAT1* or *ARD1* display the same pleiotropic phenotypes of slow growth, temperature sensitivity, chromosomal instability, inability to enter G_0 and failure to sporulate as homozygous diploids (Tbl. 1.1) (Whiteway and Szostak 1985; Mullen et al. 1989). Importantly, NatA also functions in transcriptional repression, since *nat1*Δ and *ard1*Δ cause strong derepression of the *HML* silent mating-type locus and subtelomeric reporter genes (Mullen et al. 1989; Aparicio et al. 1991). This suggests that one or several proteins require N^{α}acetylation by NatA in order to function in transcriptional silencing.

1.4 Chromatin and gene regulation

Eukaryotic DNA is packed into a nucleoprotein structure called chromatin. This facilitates the compaction as well as the regulation of genetic information. Compaction is necessary in order to adapt the size of the DNA molecule to the nuclear dimensions. For instance, the human genome comprises about 12 000 Mbp, which corresponds to a molecule length of four meters. This size is scaled down to 10 μm by complex packaging mechanisms, resulting in a compact higher-order structure of chromatin. In addition to this spacial role, the dynamic nature of chromatin plays a crucial role in central genetic processes such as transcription, replication, recombination and repair.

The fundamental chromatin unit is the nucleosome, which is composed of two copies each of the four core histones H2A, H2B, H3 and H4 and approximately 146 bp of DNA wrapped in two turns around the histone octamer (Luger et al. 1997) (Fig. 1.2). This complex is repeated every 200$\pm$40 bp, thereby creating a “pearls on a string” structure of 11 nm width. With the aid of additional proteins, including histone H1 in mammals (Contreras et al. 2003), the nucleosomal array is further packaged into a 30 nm fiber of a spiral, or solenoid, structure with six nucleosomes per turn. This structure has to be unfolded to allow the access of regulatory proteins to the DNA. Thus, the dynamic feature of chromatin is a prerequisite for various processes on the genetic material.

In eukaryotes, chromatin is organized in two types of domains, namely euchromatin and heterochromatin. Euchromatic domains define transcriptionally active portions of the genome, whereas heterochromatin is largely inactive for gene expression (Grewal and Moazed 2003). The repressive character of heterochromatin is accompanied by several other features, such as a highly ordered nucleosomal array, reduced accessibility to restriction nucleases and other DNA altering enzymes (Wallrath and Elgin 1995), replication late in S-phase (Ferguson et al. 1991) and the tendency to localize to perinuclear regions (Andrulis et al. 1998; Feuerbach et al. 2002). Originally, heterochromatin was defined in cytological experiments as chromosomal blocks that remained condensed throughout the cell cycle (Heitz 1928).

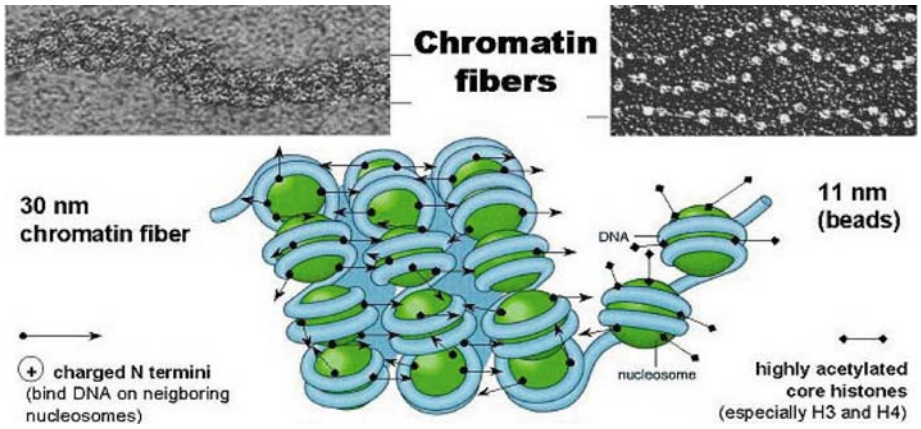


Fig. 1.2: The basic structure of chromatin.
The 11 nm fiber consists of DNA wrapped in two turns around histone octamers (nucleosomes) at intervals of about 200 bp along the DNA. Further folding creates a spiral structure, the 30nm fiber. Positively charged (deacetylated) histone tails (arrows) facilitate higher-order folding, whereas the acetylation of histone tails (bars) promotes the unfolded state corresponding to active chromatin. The two chromatin states are well-defined in electron micrographic images. (adapted from http://sgi.bls.umkc.edu/waterborg/chromat/chroma09.html)

Transcriptional repression in heterochromatin occurs in a sequence-independent fashion, making the chromosomal context in which a gene is located crucial for its transcriptional activity. In this context, a phenomenon called position-effect variegation (PEV) was revealed by pioneering experiments in *Drosophila* about 70 years ago. Muller (1930) described radiation-induced translocations that displaced the *white*$^+$ (w^+) eye color gene from its normal euchromatic location in the vicinity of heterochromatin (Muller 1930). This resulted in a clonally inherited pattern of (w^+) expression in some cells but not in others, thus causing a mosaic eye color phenotype.

Variegated position effects on translocated euchromatic genes are based on the ability of heterochromatin to spread in *cis* from a nucleation site into adjoining regions, which is probably aided by *trans*-interactions between different heterochromatic blocks (Wakimoto 1998).

Large heterochromatic blocks generally surround centromeres and telomeres of eukaryotic chromosomes (Perrod and Gasser 2003). Centromeres consist of large arrays of unspecific, often repetitive sequences in higher eukaryotes and facilitate proper sister-chromatid cohesion and chromosome segregation (Karpen and Allshire 1997). There is growing evidence that this function depends on the heterochromatic structure of centromeres, since mutations in heterochromatic components, like the histone lysine methyltransferase Clr4 in *Schizosaccharomyces pombe*, also interfere with chromosome segregation (Ekwall et al. 1996).

Heterochromatin generally stabilizes repetitive chromosomal regions by inhibition of recombination between homologous repeats. Besides centromeres and telomeres, this is especially important in mammalian genomes, which consist to a great portion of repetitive, non-coding sequences (Wichman et al. 1992). Presumably, over 90% of the mammalian genome is transcriptionally silent in differentiated tissues (Perrod and Gasser 2003). However, this high percentage is not only due to repetitive regions, but also reflects a role of heterochromatin in gene regulation during development and cellular differentiation.

Since the heterochromatic state is stably inherited through many cell divisions, it is suitable for long-term inactivation of large regions of the genome. One example is the stable inactivation of developmental regulators, such as the homeotic gene clusters in *Drosophila*. These genes are expressed only in precise spatially restricted patterns during development and are silenced in other parts of the embryo by means of Polycomb-Group proteins (see below) (Bienz and Muller 1995; Jones et al. 2000). Another prominent example for long-term gene repression by heterochromatin is the X chromosome dosage compensation in female mammals. Here, one of the two X chromosomes is inactivated in somatic cells in order to ensure equivalent levels of gene expression from sex chromosomes in males and females (Avner and Heard 2001).

Altogether, heterochromatic domains regulate gene expression in an epigenetic manner, meaning that heritable changes in gene activation occur without a corresponding change of the primary DNA sequence. Notably, epigenetic gene regulation is not only important to maintain certain expression states in differentiated cells, it also has a tremendous impact on processes that change global patterns of gene expression during development. For instance,

the genome-wide DNA-methylation status undergoes dynamic changes during early embryogenesis of mammals (Li 2002), and thereby influences the organization and compartmentalization of the genome during tissue development. The disruption of this patterning may result in global genomic deregulation, since the inactivation of responsible DNA methyltransferases causes early embryonic lethality (Okano et al. 1999).

In contrast to heterochromatin, the less densely packaged structure of euchromatin makes it more accessible to proteins (Fig. 1.2). This may underlie the early time point of euchromatic replication during S-phase, which may in turn propagate the continuity of the open chromatin structure (Gilbert 2002). Accessibility is also the basis for the transcriptional activity of euchromatin. However, transient local chromatin remodeling processes are also required in euchromatin to deal with the general repressive character of nucleosomes. For instance, nucleosomes can occupy binding sites for transcription factors within promoter regions. In this case, nucleosomal repositioning is facilitated by specialized ATP-dependent chromatin remodeling complexes (Becker and Horz 2002). A prominent example is the SWI/SNF complex in yeast, which mediates the sliding of nuleosomes along the DNA template and also recruits further transcription-activating factors (Whitehouse et al. 1999; Krebs et al. 2000).

1.5 Chromatin modifying processes

In light of the above, the dynamic chromatin structure is a prerequisite for both global and local regulation of gene expression. Correspondingly, principle chromatin modulating mechanisms are conserved among eukaryotes, although there are differences in the components between the species (Moazed 2001).

One such mechanism specific to higher eukaryotes is DNA methylation. In mammals, DNA is methylated predominantly at cytosines of CpG dinucleotides and occurs in temporally and spatially variable patterns (Bird 2002). In essence, DNA methylation induces transcriptional repression, either by blocking transcription activators from binding or by recruiting repressive chromatin-remodeling proteins such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) (Li 2002). DNA methylation is further involved in stable X chromosome inactivation in female mammals, along with a non-coding RNA (*Xist*) (Plath et al. 2002). Interestingly, non-coding RNA is also involved in dosage compensation in *Drosophila*. Here, rather than inactivating one X chromosome in females, the single X chromosome in males is hypertranscribed by the association of MSL (male-specific lethal) complexes that contain at least two *roX* RNAs (Park and Kuroda 2001).

Besides these mechanisms, a pivotal role in chromatin regulation in eukaryotes is played by histones and their posttranslational modifications. Each core histone is a small, highly basic protein composed of a globular histone fold domain, which interacts with the other histones of the nucleosome, and a N-terminal "tail" that extends outwards from the nucleosome. Conserved histone tails, particularly those of H3 and H4, are subject to a variety of posttranslational modifications (Fig. 1.3). In the current model, these modifications not only modulate the strength of interaction between the histones and the DNA, and thereby the packaging state of chromatin, but they also serve as markers for specific histone binding proteins that further regulate the chromatin structure.

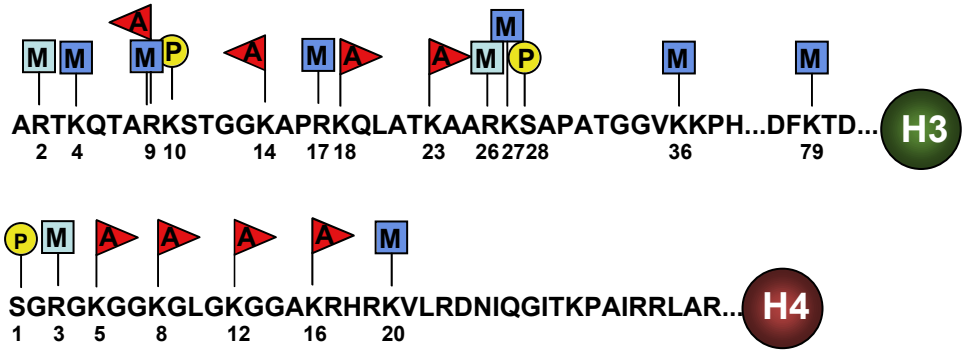


Fig. 1.3: Histone tail modifications.
The amino termini of core histones contain diverse posttranslational modifications. The diagram indicates known modifications at specific residues of human histones H3 and H4. M = methylation, A = acetylation, P = phosphorylation. (adapted from Lachner et al. (2003))

Histone modifications identified so far include methylation, phosphorylation, ubiquitination and ADP ribosylation (Rea et al. 2000; Sun and Allis 2002; Garcia-Salcedo et al. 2003). Among these, the ε-N-acetylation of lysine residues is the most prominent modification and a universal epigenetic mark in eukaryotes. Different conserved HAT and HDAC complexes change the acetylation state of histones in a dynamic manner (Dutnall and Pillus 2001; Carrozza et al. 2003). Generally, histone acetylation correlates with transcriptional activity, whereas histone hypoacetylation is a conserved hallmark of heterochromatin. In theory, acetylation affects transcription by neutralizing the histone charges, which weakens histone-DNA and internucleosomal contacts, thereby reducing chromatin compaction (Workman and Kingston 1998). In addition, the presence or absence of acetylation at specific lysines provides recognition sites for factors involved in activation or repression of gene expression. For example, deacetylation of lysine 16 on histone H4 (H4K16) by the NAD^+-dependent

HDAC Sir2 facilitates the binding of the silencing proteins Sir3 and Sir4 to nucleosomes and the spreading of the silencing complex in *S. cerevisiae* (see chapter 1.6) (Hoppe et al. 2002). In addition, X-chromosome upregulation in males of *Drosophila* requires an increased level of H4K16 acetylation on this chromosome (Akhtar and Becker 2000).

Another conserved histone modification is lysine methylation (Lachner and Jenuwein 2002). Like hypoacetylation, H3K9 methylation generally correlates with heterochromatin in higher eukaryotes. The responsible HMT Su(var)3-9 was identified to suppress PEV in *Drosophila* (Tschiersch et al. 1994), and its homologs in human (SUV39H1) and fission yeast (Clr4) act likewise in transcriptional repression (Nakayama et al. 2001; Peters et al. 2001). As in *Drosophila*, this occurs by the association of the structural heterochromatin protein HP1, which specifically interacts with methylated H3K9 and Su(var)3-9 via conserved chromo- and chromoshadow domains (Bannister et al. 2001; Yamamoto and Sonoda 2003). Similarly, SUV39H1 and Clr4 recruit HP1 homologs and build SUV39H1/HP1 and Clr4/Swi6 methylation systems (Lachner et al. 2001).

Budding yeast also owns HMTs with a catalytic SET domain as in their metazoan counterparts. Moreover, methylation is equally associated with transcriptional repression, for instance in case of H3K4 and H3K36 methylation by Set1 and Set2, respectively (Briggs et al. 2001; Strahl et al. 2002). Recent studies demonstrated that Set2 methylates H3K36 in the coding region of actively transcribed genes (Krogan et al. 2003). In addition, its interaction with RNA polymerase (Pol) II implicates a role for Set2 in transcription elongation. The involvement of HMTs in a dynamic process such as transcription is particularly interesting given the present model that histone methylation is an irreversible modification.

Multiple covalent modifications occur at the same time on histone tails, and there are several examples for their interplay. For instance, on mammlian histone H3, the methylation of lysine 9 interferes with the phosphorylation of serine 10 (Rea et al. 2000), which is in turn synergistically coupled to the acetylation of lysines 4 and 9 (Cheung et al. 2000a; Clayton et al. 2000). Moreover, other findings suggest a link between two different epigenetic marks, namely DNA methylation and histone methylation (Tamaru et al. 2003).

The fact that posttranslational histone modifications can influence each other either positively or negatively gave rise to the hypothesis that they create a combinatorial code. This specific histone code may induce the recruitment of a certain set of chromatin-associated proteins that eventually dictate the particular state of gene expression (Strahl and Allis 2000). The hypothesis further states that protein motifs may have evolved that recognize histone modifications. In line with this, several proteins associated in gene regulation or

heterochromatic silencing were shown to share conserved domains, such as the acetyl-lysine binding bromodomain or the methyl-lysine binding chromodomain (Owen et al. 2000; Bannister et al. 2001). The histone code may also explain why certain types of histone modifications, e.g. methylation, can be involved in transcriptional activation as well as repression. It might further have the potential to reveal the mechanisms behind the maintenance of epigenetic chromatin marks throughout the cell cycle. To date, this process is poorly understood, although some first data exist. For instance, interactions between histone modifying enzymes and proteins of the replication machinery, e.g. the HAT Sas2 and the chromatin assembly factor subunit Cac1 in yeast (Meijsing and Ehrenhofer-Murray 2001) may contribute to the reestablishment of a given histone code on freshly replicated DNA. As another example, the *Drosophila* Fab-7 chromosomal element, the binding site for Polycomb and trithorax proteins to regulate homeotic genes, could convey the maintenance of an active chromatin state during mitosis and meiosis, possibly with H4 hyperacetylation as a heritable tag of the activated element (Cavalli and Paro 1999). Still, these are only first insights into different aspects of the complex, yet important, field of epigenetic inheritance.

1.6 Silencing in *S. cerevisiae*

Silenced chromatin in *S. cerevisiae* is akin to heterochromatin in higher organisms and shares main characteristics, such as general inaccessibility of DNA, hypoacetylated nucleosomes and late replication (Loo and Rine 1995; Lustig 1998). In addition, the overall pathway of assembly of silent chromatin appears to be similar in yeast and multicellular eukaryotes, since silencing proteins in yeast are functionally related to heterochromatic components in metazoans, such as Sir1 and HP1. Similar to higher organisms, budding yeast exhibits histone modifications, which are in parts species specific, such as H4K12 acetylation (Lachner et al. 2003). In light of these similarities, silenced loci in *S. cerevisiae* provide an excellent system to study the mechanisms of heterochromatin in eukaryotes.

In *S. cerevisiae*, silencing is facilitated by *cis*-acting elements and *trans*-acting proteins. There are three silenced loci known within the yeast genome, namely the silent mating-type loci *HML* and *HMR*, the rDNA array and the telomeres. Among these, the silent mating-type loci are the best characterized.

The silent mating-type loci HML and HMR

Haploid yeast cells exist as either **a** or α mating-type, which is determined by the *MAT* locus located near the centromere of chromosome III (Fig. 1.4). In *MAT***a** cells, the *MAT* locus encodes the proteins Mat**a**1/Mat**a**2, and in *MAT*α cells it encodes Matα1/Matα2, which are proteins that regulate the transcription of mating-type specific genes and therewith enable the cell to mate (Herskowitz et al. 1992). During mating, cells of opposite mating-type fuse to form **a** /α diploids, which in turn can undergo meiosis and sporulation to generate haploid progeny. Under certain conditions, haploid cells can switch their mating-type. This is possible, since two additional copies of *MAT* are present on the left and on the right arm of chromosome III, namely *HML* (*homothallic mating left*) containing α-information, and *HMR* (*homothallic mating right*) containing the **a**-information. Mating-type switches occur via specific recombination events between *MAT* and the *HM* loci, but are inhibited in laboratory strains due to the deletion of the responsible *HO*-endonuclease (Strathern et al. 1982).

Given the existence of genes of both mating-types in the yeast genome, cells must ensure that only the *MAT* locus is employed in order to preserve their mating ability. Thus, the silent mating-type or homothallic (*HM*) loci are transcriptionally silenced. Silencing defects at *HML* or *HMR* cause the derepression of these loci and subsequently a pseudodiploid state, which prevents the cells from mating. Thus, the silencing state of a single *HM* locus can be determined by testing the mating ability of a strain of opposite mating-type.

HM silencing is achieved by the presence of silencer elements on each side of the loci, the so-called E and I silencers. These are *cis*-acting, regulatory elements consisting of binding sites for the DNA binding proteins Rap1, Abf1 and the origin recognition complex, ORC (Fig. 1.4). Whereas number and orders of the different binding sites vary, an ORC binding site is present in all silencers.

Besides its role in silencing, the ORC complex has a well-conserved function in replication initiation (see chapter 1.7). However, this function appears not to play a role in silencing, since although its establishment requires S-phase passage, silencing does not require replication initiation or replication fork passage through the silencers (Kirchmaier and Rine 2001). In addition, the ORC binding sites do not need to be active replication origins to act in silencing. For example, the ORC binding sites within the *HML* silencers are no active origins, although they can serve as origins on plasmids (Sharma et al. 2001). In contrast, *HMR*-I and *HMR*-E are chromosomal origins of replication (Rivier et al. 1999), though the latter is inefficient.

Presumably, it is the tight binding of ORC to *HMR*-E that enhances its silencer activity but decreases its origin potential (Palacios DeBeer et al. 2003).

The four *HM* silencers do not only vary in their composition, but they are also of different importance for the silencing state of the respective locus. At *HML*, either E or I individually are sufficient to maintain the silencing state (Mahoney and Broach 1989). In contrast, at *HMR*, E is essential but I is dispensable for silencing (Rivier et al. 1999). The elements of *HMR*-E are functionally redundant, meaning that the absence of at least two of them is necessary to cause the loss of silencing. This redundancy is lost at the synthetic *HMR*-E silencer, which is engineered of minimal binding sequences for Rap1, Abf1 and ORC (McNally and Rine 1991).

Interestingly, all three binding proteins act individually also elsewhere in the genome without initiating transcriptional repression. For instance, Rap1 (Repressor/Activator Protein 1) and Abf1 (ARS Binding Factor 1) have essential functions as transcriptional activators of diverse genes (Lieb et al. 2001; Miyake et al. 2002). Thus, the ability of *HM* silencers to initialize silencing appears not just to be the sum of the silencing abilities of their single elements. It may rather be the combination of the elements and their close proximity that allows the recruitment of further silencing components, the Sir proteins, which eventually induce the formation of silent chromatin (Lustig 1998).

The four Sir (silent information regulator) proteins are *trans*-acting silencing factors. Sir2, Sir3 and Sir4 are the structural components of silenced chromatin and essential for silencing, but non-essential for growth (Rine and Herskowitz 1987). The formation of silenced chromatin is associated with the polymerizing Sir complex that interacts with nucleosomes and thereby spreads outward from its nucleation site at the silencers. The assembly of this complex is hypothesized to occur stepwise (Hoppe et al. 2002). At first, Sir2/Sir4 heterodimers bind via interactions of Sir4 with Sir1 and Rap1 to the silencer. At the same time, Sir3 binds independently via interactions with Rap1 and Sir4. The next step requires the enzymatic activity of Sir2, a NAD^+-dependent histone deacetylase specific to H3K9, H3K14 and H4K16 (Imai et al. 2000). Histone deacetylation by Sir2 facilitates the binding of Sir3 and Sir4 to hypoacetylated histones and the recruitment of new Sir2/Sir4 (Rusche et al. 2002). Repetitions of these modification/binding cycles eventually result in multimerization and spreading of the complex along the chromosome (Fig. 1.4). This model is based on several individual observations of genetic or physical interactions between the different components (Hecht et al. 1995; Gasser and Cockell 2001; Rusche et al. 2002).

Sir1 is no part of the multimeric Sir complex, but is rather proposed to facilitate the establishment of silencing due to its ability to interact with ORC and Sir4 (Triolo and

Sternglanz 1996). This is supported by ChIP data of Rusche et al. (2002), who found Sir1 located primarily to the silencers and not distributed over the whole *HM* loci as the other Sir's. In contrast to them, Sir1 is not vital to silencing (Rusche et al. 2002).

Although the silencing complex spreads in both directions along the chromosome, it is stopped from propagation into adjacent regions that are kept transcriptionally active by DNA elements interposed between silenced and active chromatin domains (Dhillon and Kamakaka 2002). Such so-called boundary elements have been identified at either side of *HMR*, as well as in subtelomeric regions, and are also known in other species (Gombert et al. 2003; Parnell et al. 2003). The boundary at the telomeric proximal side of *HMR* is a tRNA gene, which requires an intact transcriptional potential for its barrier capacity (Donze and Kamakaka 2001). This implicates that it functions passively by a stably bound protein complex (e.g. the RNA Pol III pre-initiation complex), which interferes physically with the spread of the silenced complex. Alternatively, boundaries may be active enzymatic barriers on the bases of associated HATs and chromatin remodeling enzymes, which oppose the propagation of hypoacetylated silenced chromatin (Suka et al. 2002).

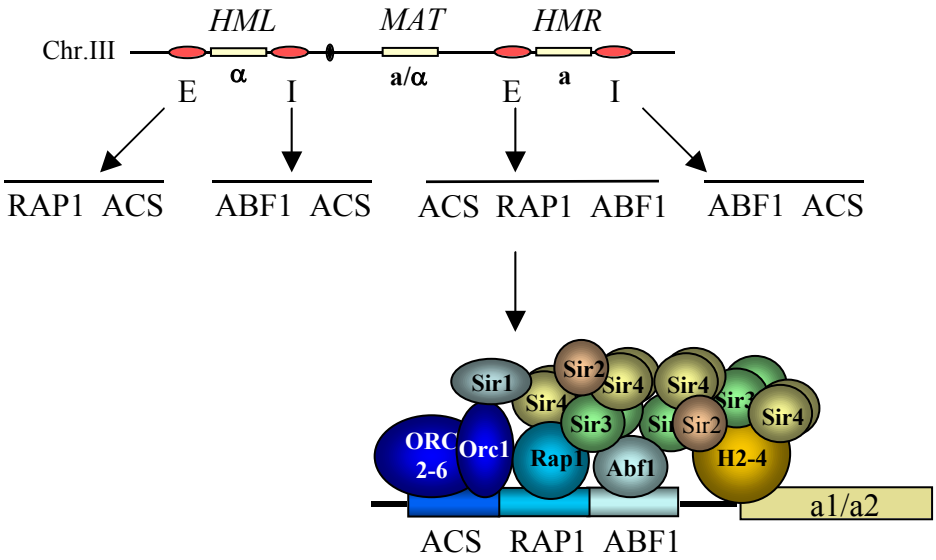


Abb. 1.4: Mating-type loci and *HM* silencers.
The mating-type loci *MAT*, *HML* and *HMR* are localized on chromosome III of *S. cerevisiae*. *HML* and *HMR* are repressed due to the nearby silencers E and I, which consist of binding sites for ORC, Rap1 and Abf1. The silencers are nucleation sites for silencing complexes, as depicted for *HMR*-E. The Sir complex interacts with nucleosomes and spreads into the *HMR* locus thereby creating a silenct chromatin structure.

The spread of the silencing complex from *HML*-I is stopped by the *CHA1* promoter, which locates about two kb downstream of the silencer (Donze and Kamakaka 2001), whereas the *YCL069w* locus is probably the boundary of *HML*-E (Lieb et al. 2001).

How does silenced chromatin inhibit transcriptional activity? Its compact structure was formerly proposed to prevent the access of transcription enzymes to promoters (Loo and Rine 1994). However, more recent data suggest that silenced chromatin prevents the elongation step rather than the recruitment of RNA Pol II, since factors of the transcriptional machinery cohabitate with Sir proteins at promoters of silenced chromatin (Sekinger and Gross 2001).

From a mechanistic point of view, chromatin silencing can be subdivided into three distinct processes, namely establishment, maintenance and inheritance. *Establishment* refers to the *de novo* generation of repression at active loci. Besides S-phase passage, this requires Sir1, since *sir1*Δ strains are mixed populations of cells whose *HM* loci are either completely repressed or completely derepressed (Pillus and Rine 1989). To *maintain* the silenced state throughout one cell cycle, structural components of the Sir complex as well as intact histone tails are required (Cheng and Gartenberg 2000). In addition, mutations in subunits of CAF-I give rise to unstable *HML* repression (Enomoto and Berman 1998). Thus, CAF-I may not only assemble newly synthesized histones onto freshly replicated DNA, but also help to reassociate the Sir complex. As aforementioned, *inheritance* refers to the propagation of silencing to subsequent cell cycles. It requires the silencers as epigenetic markers (Rusche et al. 2002).

The telomeres

Telomeres are protected from exonucleolytic degradation, end-to-end fusion and recombination by their heterochromatic structure (Grunstein 1998; Stevenson and Gottschling 1999). In *S. cerevisiae*, telomeres consist of approximately 300 bp of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats, which build nucleosome-free areas with multiple binding sites for Rap1 (Fig. 1.5) (Sandell and Zakian 1993). In the current view, Rap1 recruits Sir2/Sir4 and Sir3, which then form a complex in a similar mode as at the *HM* loci: after getting in contact with nucleosomes of the adjacent chromatin region, Sir3 and Sir4 interact with histone tails deacetylated by Sir2 and the polymerizing complex spreads inwards the chromosome thereby silencing subtelomeric genes (Luo et al. 2002).

Notably, telomeric silencing occurs discontinuously and is enhanced around subtelomeric CoreX-elements (Fourel et al. 1999; Pryde and Louis 1999). These elements are part of all

chromosomes and contain an ORC binding site (ACS; ARS consensus sequence), often coupled with an Abf1 binding site. The silencing maximum around CoreX depends on the ACS and, additionally, on *SIR2*, *SIR3* and *SIR4*. Notably, the deletion of *SIR1* also causes partial derepression at native telomeres, whereas mutations in the ORC subunits *ORC2* and *ORC5* have no effect (Pryde and Louis 1999). In the current model, silenced chromatin at telomeres is organized by interactions of telomeric Rap1-Sir complexes with Sir proteins bound to CoreX under formation of a loop structure, which may further stabilize the heterochromatin-like complex (Strahl-Bolsinger et al. 1997; Pryde and Louis 1999).

Interestingly, Sir3 was found to be limiting for the propagation of the silencing complex (Renauld et al. 1993), and its overexpression extended the silent domain from 2-4 kb to up to 16 kb away from the telomeric repeats (Hecht et al. 1996). This extension coincided with the spread of Sir3, whereas the amount of Sir2 and Sir4 was reduced in telomere-distal chromatin (Strahl-Bolsinger et al. 1997).

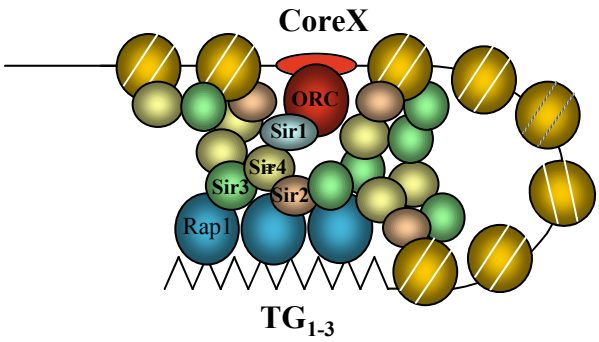


Fig. 1.5: Silent chromatin at a yeast telomere.
The telomeric (TG_{1-3}) repeats provide binding sites for Rap1, which recruits the Sir complex. The subtelomeric CoreX element contains a binding site for ORC and acts likewise as a nucleation site for the Sir complex. Due to interactions of the silencing proteins the telomere folds back and forms a loop, which further stabilizes the chromatin structure.

Rap1, Sir3, Sir4 and clusters of telomeric DNA were observed to colocalize in foci at the nuclear periphery (Gotta et al. 1996). These foci may be tethered to the nuclear envelope through interactions with the nuclear pore complex (Galy et al. 2000). To date it is not clear whether this perinuclear position is the cause or the consequence of telomeric silencing. Feuerbach et al. (2002) have demonstrated a repression-dependent, physical relocation of telomeres from variable intranuclear positions to perinuclear silent domains (Feuerbach et al. 2002). Hence, they hypothesized that the expression state of telomeres is determined by spatial positioning. In contrast, Tham and co-workers found no correlation between

transcriptional silencing of telomeres and their localization to the nuclear periphery (Tham et al. 2001)

Telomeric silencing is usually investigated in subtelomeric reporter strains. For this purpose, reporter genes were inserted at artificially truncated chromosome ends that lack the CoreX element (Gottschling et al. 1990). In these constructs, heterochromatin spreads continuously from the (TG_{1-3}) repeats towards the centromere (Renauld et al. 1993). Subtelomeric reporter genes are subject to epigenetic switches between transcriptional repression and expression (Chien et al. 1993). Since this variegation resembles position effects in *Drosophila*, it is referred to as telomeric position effect (TPE) (Gottschling et al. 1990). TPE may originate from a weaker establishment potential of silencing at the truncated ends due to the lack of Sir1, since *sir1*Δ does not affect the silencing of subtelomeric reporter strains (Fox et al. 1997). Interestingly, the conditional mutant alleles *orc2-1* and *orc5-1* caused silencing defects the truncated reporter constructs, in contrast to the missing effects at native telomeres (Fox et al. 1997).

The common components of silent chromatin at telomeres and the *HM* loci suggest a competition for limiting factors between them. Consistent with this idea, increased telomeric silencing goes along with decreased silencing at *HMR* (Buck and Shore 1995).

The rDNA locus

In the nucleolus, ribosomal DNA (rDNA) sequences are present in a tandem array of 100-200 copies of a 9.1 kb repeat (Fig.1.6). Each repeat encodes a 5S RNA, transcribed by RNA Pol III, 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 (Smith and Boeke 1997).

The highly repetitive nature of the rDNA array necessitates the formation of silenced chromatin to avoid recombination events. Consequently, only about half of the repeats are active at a given time point, whereas the other half is transcriptionally silent (Warner 1989). In addition, Pol II reporter genes inserted into the rDNA become also metastably repressed (Smith and Boeke 1997). Although the mechanism behind this repression is currently not well understood, it is known that rDNA silencing is mediated by a protein complex called RENT (regulator of nuceolar silencing and telophase). RENT contains the subunits Net1, Sir2, and Cdc14 (Shou et al. 1999), and was recently shown to localize to two distinct regions within the rDNA repeats (Huang and Moazed 2003). It binds to NTS1 via Fob1, which surprisingly is also

required for rDNA recombination (Kobayashi and Horiuchi 1996), and to NTS2 around the Pol I promoter.

Each repeat also contains an ACS site in NTS2. However, only about 20% of them are active origins. These are clustered along the rDNA array and separated by large regions where replication initiation is suppressed in a *SIR2*-dependent manner (Pasero et al. 2002). Therefore, like transcription, rDNA replication is under epigenetic control. Deletions of *SIR2* shorten the life span of yeast cells, whereas its overexpression causes cells to live longer (Kaeberlein et al. 1999). This role of *SIR2* as an anti-aging factor was found to be connected with its function in rDNA silencing. Loss of Sir2 results in reduced rDNA silencing and hence in increased recombination between the repeats, which eventually causes the accumulation of extrachromosomal rDNA circles (ERCs). These ERCs cause aging presumably because they titrate components of the replication or transcription machinery from the genomic DNA (Sinclair and Guarente 1997). Interestingly, calorie restriction also leads to life span extension on the basis of reduced rDNA recombination. Here, the activity of Sir2 may be increased due to the higher concentration of NAD^+ in calorie restricted cells (Lin et al. 2000).

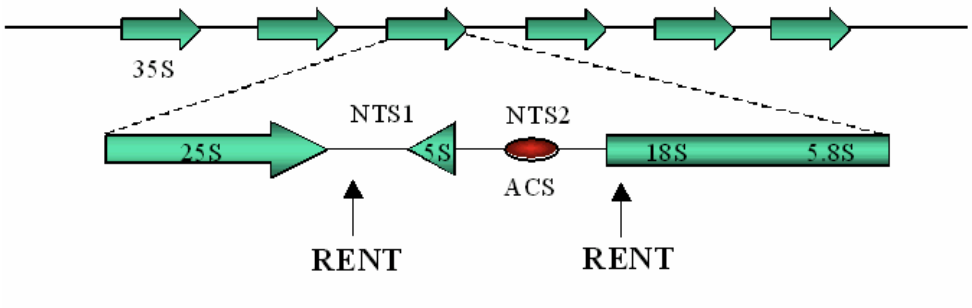


Fig. 1.6: Schematic structure of the rDNA array in *S. cerevisiae*.
The rDNA locus is an array of tandemly repeating units containing the coding regions for ribosomal RNA seperated by non-transcribed spacer regions NTS1 and NTS2. The latter holds a binding site for ORC. Binding sites for the silencing RENT complex are depicted by arrows. (adapted from Huang and Moazed (2003))

Althogether, the mode of rDNA silencing is different from that of *HM* loci and telomeres, since it requires only Sir2, but not the other Sir proteins. Nevertheless, all silenced loci may be linked by competition for limiting amounts of Sir's. In line with this model, rDNA silencing is negatively regulated by the telomeres, which titrate Sir2 out of the nucleolus and sequester it via interactions with Sir4 (Smith et al. 1998).

1.7 Silencing proteins investigated in this thesis

The following section provides additional information on those silencing proteins that were in the focus of this study.

ORC and its largest subunit Orc1

The ORC complex consists of six subunits named Orc1 to Orc6 in order of their decreasing mass (Li and Herskowitz 1993; Bell et al. 1995; Loo et al. 1995a). All subunits are essential for the conserved function of ORC as the eukaryotic replication initiator complex (Bell et al. 1993; Gavin et al. 1995). Homologs of ORC subunits have been found implicated in replication also in *S. pombe*, *D. melanogaster*, *X. laevis*, and human cells (Carpenter et al. 1996; Grallert and Nurse 1996; Landis et al. 1997; Dhar et al. 2001).

In yeast, ORC binds to ACS sites of origin sequences, which are evenly distributed in the genome, and where ORC remains bound throughout the cell cycle (Tanaka et al. 1997). DNA binding requires the coordinate action of all ORC subunits except Orc6 (Lee and Bell 1997). In addition, it requires the binding, but not the hydrolysis, of ATP by Orc1 (Klemm and Bell 2001). To initiate replication, ORC recruits a multifactor prereplicative complex (pre-RC) during G_1. Thereby, the direct binding of ORC to Cdc6 is the first and a key step, and is presumably mediated by ATP-bound Orc1 (Saha et al. 1998; Mizushima et al. 2000).

Due to their vital function, no deletion mutants of ORC subunits are available. Instead, the conditional mutant alleles *orc2-1* and *orc5-1* are frequently used for genetic analysis. Both mutants share phenotypes of impaired replication, including temperature sensitivity, elevated plasmid loss rate and reduced replication initiation. Moreover, double mutants are inviable (Liang et al. 1995; Loo et al. 1995a). It was furthermore demonstrated that the ORC complex is unstable and affected in DNA binding in these mutants (Bell et al. 1993). In addition to the replication defect, telomeric and *HM* silencing was also affected in *orc2-1* and *orc5-1* mutants (Loo et al. 1995a; Fox et al. 1997).

In contrast, a N-terminally truncated *orc1* mutant displayed no combined replication/silencing phenotype, but was impaired in silencing. Replication appeared unaffected, since *orc1*Δ*1-235* could still complement an *orc1*Δ strain for growth and displayed only a 2-fold reduction in plasmid stability, compared to a 20 to 40-fold reduction in *orc2-1* and *orc5-1* strains (Bell et al. 1995). In this *orc1* mutant, silencing was affected at a *HMR* sensitized to defects in ORC function by the lack of the Rap1 binding site in the E silencer (*HMR*-E Δ*RAP*).

In the current model, the N-terminal domain of Orc1 is responsible for the silencing function of ORC, which is the recruitment of Sir1 to silencers (Gardner et al. 1999). Recently, an ORC interaction region (OIR) was identified in the C-terminal part of Sir1 to be necessary and sufficient for the Sir1-ORC interaction (Bose et al. 2004). However, stable silencer association of Sir1 required the additional interaction with Sir4, which may consequently confine Sir1-ORC interactions to origins within silencers.

In the 914 amino acid protein Orc1, Sir1 binding occurs via a small non-conserved domain between amino acids 100 and 129 (Zhang et al. 2002). This so-called H-domain is part of the BAH (bromo-adjacent homology) domain, which is a conserved protein-protein interaction module (Callebaut et al. 1999). In addition, Orc1 has an AAA^+ (ATPases associated with a variety of cellular activities) domain between amino acids 443 and 738. This highly conserved module contains ATP binding and hydrolysis-mediating Walker homology motifs (Neuwald et al. 1999). Hence, the AAA^+ domain is essential for replication initiation by ORC, and mutations in this domain are lethal (Klemm and Bell 2001)

Most likely, the role of ORC in repressive chromatin is conserved in all metazoans. In *Drosophila*, ORC was localized to heterochromatin and interacted directly with HP1, probably via DmOrc1 (Pak et al. 1997). Recessive lethal mutations in *DmORC2* are PEV suppressors and disrupt the localization of HP1 to heterochromatin. Likewise, HP1 point mutations that diminish ORC binding, also suppress PEV. In light of these data, Pak et al. (1997) suggested a conserved role of ORC to target non-DNA-binding-factors, such as Sir1 in yeast and HP1 in *Drosophila*, to sites destined to be heterochromatic.

Recently, it was demonstrated that human ORC1 directly interacted with HBO1 (histone acetyltransferase binding to ORC). HBO1 has HAT activity towards free and nucleosomal H3 and H4 (Iizuka and Stillman 1999) and belongs to the same HAT family (MYST) as MOF and Sas2, which affects silencing in yeast (Ehrenhofer-Murray et al. 1997). Thus, although the ORC-HBO1 interaction may be associated with the replication role of ORC (Burke et al. 2001), it may alternatively be another link between ORC and heterochromatin.

Sir3

Sir3 is a key player in TPE and *HM* silencing, but not in rDNA repression (Aparicio et al. 1991; Smith and Boeke 1997; Stone et al. 2000). As a component of the Sir complex, Sir3 contacts several other silencing proteins. Direct interactions have been demonstrated between Sir3 and Sir2, Sir4, Rap1, Abf1, Zds1, Zds2, Rad7, Sir3 itself and the N-termini of histones H3 and

H4 (Gasser and Cockell 2001). Physical interactions with Sir4, Rap1 and hypoacetylated histones occur via the C-terminal half of Sir3 (Hecht et al. 1995; Park et al. 1998; Moretti and Shore 2001), whereas the N-terminus of the 978 amino acid protein modulates the interactions. Notably, the simultaneous expression of both halves of *SIR3* in *trans* partially complemented the *sir3*Δ mating defect, suggesting that the two domains can function independently (Gotta et al. 1998).

The N-terminal 214 amino acids of Sir3 are very similar to Orc1 (50% identity, 63% similarity) (Bell et al. 1995) and also contain a BAH domain between amino acids 48 and 189, although the H-domain is missing (Zhang et al. 2002). Point mutations within the BAH domain lead to *eso* (enhancers of the *sir one* mutant mating defect) phenotypes (Stone et al. 2000). They disrupted *HM* silencing in *sir1*Δ strains and additionally disrupted TPE as single mutants. Interestingly, *nat1*Δ also enhanced the *sir1*Δ mating defect, and this effect was epistatic with some of the *sir3-eso* mutations.

In line with the high degree of sequence similarity, the N-terminal domains of Sir3 and Orc1 were functionally interchangeable for mating-type silencing when tethered to the C-terminus of the other protein (Bell et al. 1995). Notably, they did not substitute each other in telomeric silencing, pointing to distinct functions of the proteins at the telomeres (Stone et al. 2000). Given the potential of the Sir3 N-terminus to replace the Orc1 N-terminus in *HM* silencing, it appears paradox that Sir3 cannot interact with Sir1 because of the missing H-domain. Thus, Stone et al. (2000) proposed that the BAH domain of Sir3, when tethered to Orc1, may promote silencing in a Sir1 independent manner.

Consistent with the view that the Sir3 N-terminus is a regulatory domain, there is evidence that its phosphorylation enhances TPE (Stone and Pillus 1996).

Sum1-1

Due to a single missense mutation in the C-terminal part, *SUM1-1* is a dominant altered function allele, which can bypass the need for Sir proteins in *HM* silencing and increase telomeric repression in *SIR* wild-type strains (Laurenson and Rine 1991).

Although *SUM1-1* is a suppressor of *HM* silencing defects, the wild-type gene product of *SUM1* appears not to be a direct silencing component. Instead, Sum1 acts as transcriptional repressor of middle sporulation genes during mitosis and vegetative growth and binds specifically to MSE (middle sporulation element) sites in the promoter regions of its target genes (Xie et al. 1999). For this, Sum1 recruits Hst1 (Homologous of Sir Two), a NAD^+-

dependent deacetylase with sequence homology to Sir2. The deletion of *SUM1* had only minor effects on *HM* silencing and did not restore silencing in the absence of Sir proteins (Chi and Shore 1996).

For a long time it remained unclear how the Sum1-1 mediates silencing in the absence of the Sir complex, which is normally essential for silencing. (Sutton et al. 2001) found that Sum1-1 also requires Hst1 and its NAD^+-dependent deacetylase activity as well as ORC for its silencing function. In fact, the *orc1Δ1-235* allele eliminated *SUM1-1* mediated silencing (Rusche and Rine 2001). In the present model, Sum1-1 is bound by ORC (Orc1?) to the silencers and recruits Hst1, whose deacetylase activity leads to hypoacetylated nucleosomes and consequently to a condensed, silenced chromatin structure at the *HM* loci (Rusche and Rine 2001).

1.8 Outline of this thesis

The aim of this study was to determine the role of the N^{α}-acetyltransferase complex NatA in transcriptional silencing in *S. cerevisiae.* Deletions of the NatA subunits *NAT1* or *ARD1* both result equally in impaired *HML* silencing and reduced TPE, suggesting the functional dependence of a silencing protein on N^{α}-acetylation by NatA (Mullen et al. 1989; Aparicio et al. 1991).

So far, some genetic interactions between *NAT1* and genes that encode silencing components have been identified. For instance, the *nat1Δ* mutant displayed an *eso*-phenotype, which was not enhanced in combination with certain *sir3-eso* alleles. Likewise, overexpressed Sir1 suppressed the *nat1Δ ard1Δ* silencing defect at the *HMR*-E *ΔRAP* silencer (Stone et al. 1991 and 2000). However, neither Sir3 nor Sir1 have been directly implicated in NatA-dependent silencing. In addition, histone H2B is a known NatA substrate but the deletion of its N-terminus has no effect in silencing (Kayne et al. 1988).

To date, no significant silencing substrates of NatA have been found and the mechanism by which NatA is involved in silencing remains unclear. It is further not known whether NatA plays a role in rDNA silencing.

In this study, we found that NatA was required for all forms of silencing in *S. cerevisiae*. We further obtained evidence that Orc1 is a NatA substrate and its N^{α}-acetylation is required for telomeric silencing. Genetically, *nat1Δ* functioned through the ORC binding site of the *HMR*-E silencer. The requirement for NatA in silencing could be bypassed by artificially tethering

Orc1, but not the other Orc proteins, to the silencer, thus suggesting that Orc1 was a silencing-relevant NatA target. We found Orc1 to be fully N^{α}-acetylated in wild-type and completely unacetylated in *nat1*Δ strains. Mutations in the penultimate residue of Orc1 that abrogated its ability to be acetylated by NatA caused a severe loss of telomeric silencing, as does the deletion of *NAT1*. The lack of acetylation did not affect the interaction of Orc1 with Sir1, since *HM* silencing was not impaired in the *orc1* mutants and still depended on functional *SIR1* in *nat1*Δ strains.

Genetic interactions further supported a functional link between NatA and ORC in replication, since *nat1*Δ was synthetically lethal with the replication-defective *orc2-1* mutation. Notably, unacetylated *orc1* mutants grew normally suggesting that another subunit of ORC requires N^{α}-acetylation for its function in silencing. Furthermore, *nat1*Δ displayed synthetic lethality with *SUM1-1*. Intriguingly, this lethality was suppressed by a deletion in the N-terminus of Orc1, thus suggesting that N^{α}-acetylation regulated the interaction of Orc1 with Sum1-1.

Furthermore, we found that the N-terminal 100 amino acid region of Orc1 was dispensable for growth, but had a function in silencing. Increasing deletions within this region disrupted silencing at the synthetic *HMR* locus and telomeres, and also reduced the α-factor sensitivity of the mutants. In contrast to earlier proposals (Zhang et al. 2002), we found that the N-terminal 50 amino acids of Orc1 were required for the interaction with Sir1, since the two-hybrid interaction with Sir1 was interrupted in the *orc1*Δ*1-51* mutant. However, this mutant further affected silencing in *sir1*Δ. Therefore, we suggest that the N-terminal 100 amino acids of Orc1 are not only required for Sir1 interaction, but also for the recuitment of another, yet unknown silencing factor.

Furthermore, we present evidence that Sir3 is also N^{α}-acetylated by NatA. Since previous work (Stone et al. 2000) showed that the mutation of the penultimate amino acid of Sir3 causes silencing defects, we likewise propose that Sir3's silencing function is regulated by NatA-dependent N^{α}-acetylation and we further demonstrate that the localization of Sir3 to perinuclear foci depends on *NAT1*.

In addition, we report on a screen for a multicopy suppressor of the *nat1*Δ mating defect. This unbiased approach proved to be ineffective, since we isolated only indirect suppressors of the mating defect, but not of the *HM* silencing defect of *nat1*Δ.

In summary, our data further specify the role of NatA in transcriptional silencing. For the first time, we provide evidence of the functional dependence of two silencing proteins, Orc1 and Sir3, on N^{α}-acetylation by NatA. We propose a model, by which N^{α}-acetylation regulates the

binding of silencing factors to the N-terminus of Orc1 and Sir3 to recruit hetrochromatic factors and establish repression. Thus, N^{α}-acetylation represents a protein modification that modulates chromatin function in *S. cerevisiae*.

2 Materials and Methods

2.1 Materials

2.1.1 *E. coli* strains

TOP10 F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
(chemically or electro-competent; Invitrogen)

DH5α F⁻ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44λ- *thi*-1 *gyr*A96 *rel*A1 (chemically competent; Gibco)

2.1.2 Yeast strains

Table 2.1: Yeast strains used in this study.

Strain	Genotype	Source*
AEY1	*MAT*α *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)	
AEY5	*MAT*α *HMR SS* Δ*I*	
AEY24	*MAT***a** *orc2-1 rho°*	J. Rine
AEY71	*MAT*α *HMR*-E Δ*300-256 (*Δ*ABF)*	A. Brand
AEY80	*MAT***a** *nat1-5::LEU2*	R. Sternglanz
AEY81	*MAT*α *HMR*-E Δ*331-324 (*Δ*RAP)*	A. Brand
AEY84	*MAT*α *HMR*-E Δ*352-358 (*Δ*ACS)*	A. Brand
AEY1017	*MAT*α TEL VII-L::*URA3*	J. Berman
AEY1224	*MAT***a** *SUM1-1*	D. Shore
AEY1227	*MAT*α *nat1-5::LEU2*	
AEY1273	*MAT*α *HMR SS* Δ*I nat1*Δ *::LEU2*	
AEY1275	*MAT*α *HMR SS* Δ*I 5xGal4-RAP-ABF*	
AEY1276	*MAT*α *HMR SS* Δ*I 5xGal4-RAP-ABF nat1*Δ*::LEU2*	
AEY2144	*MAT*α *HMR*-E Δ*331-324 (*Δ*RAP) nat1*Δ*::LEU2*	
AEY2146	*MAT*α *HMR*-E Δ*352-358 (*Δ*ACS) nat1*Δ*::LEU2*	
AEY2148	*MAT*α *HMR*-E Δ*300-256 (*Δ*ABF) nat1*Δ*::LEU2*	
AEY2371	*MAT*α TEL VII-L::*URA3 nat1*Δ*::LEU2*	
AEY2947	AEY1276 *sir1*Δ ::*kanMX*	
AEY3008	*MAT*α *sum1*Δ*::URA3 nat1*Δ*::LEU2*	
AEY3068	MAT**a** *ORC1-HA-URA3*	
AEY3070	MAT**a** *nat1-5::LEU2 ORC1-HA-URA3*	
AEY3134	*MAT***a** *ADE2 lys2*Δ *nat1*Δ *::LEU2*	
AEY3161	*MAT***a** *orc2-1 nat1*Δ *::LEU2* pRS316-*ORC2*	

Table 2.1 (continued)

Strain	Genotype	Source*
AEY2864	*MAT*α *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100* (=W303-1B) *HMR SS abf1*$^{-}$ Δ*I orc1*Δ::*HIS5*-GFP pAE405	
AEY2866	*MAT*α *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100* (=W303-1B) *HMR SS* Δ*I orc1*Δ::*HIS5*-GFP pAE405	
AEY2867	*MAT***a** *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100* (=W303-1A) *HMR SS* Δ*I orc1*Δ::*HIS5*-GFP pAE405	
AEY2877	AEY2866 *orc1*Δ*1-10::LEU2* without pAE405	
AEY2879	AEY2866 *orc1*Δ*1-51::LEU2* without pAE405	
AEY2880	AEY2866 *orc1*Δ*1-100::LEU2* without pAE405	
AEY2883	AEY2864 *orc1*Δ*1-10::LEU2* without pAE405	
AEY2887	AEY2867 *orc1*Δ*1-10::LEU2* without pAE405	
AEY2888	AEY2867 *orc1*Δ*1-51::LEU2* without pAE405	
AEY2889	AEY2867 *orc1*Δ*1-100::LEU2* without pAE405	
AEY2903	AEY2866 *orc1-A2V::LEU2* without pAE405	
AEY2904	AEY2864 *orc1*Δ*1-51::LEU2* without pAE405	
AEY2905	AEY2864 *orc1*Δ*1-100::LEU2* without pAE405	
AEY2907	AEY2866 *orc1*Δ*1-28::LEU2* without pAE405	
AEY2908	AEY2864 *orc1*Δ*1-28::LEU2* without pAE405	
AEY2910	AEY2864 *orc1*Δ*29-51::LEU2* without pAE405	
AEY2911	AEY2867 *orc1*Δ*29-51::LEU2* without pAE405	
AEY2912	AEY2867 *nat1*Δ *::kanMX*	
AEY2913	AEY2867 *orc1-A2V::LEU2* without pAE405	
AEY2916	AEY2866 *nat1*Δ *::kanMX*	
AEY2937	AEY2867 *orc1*Δ*1-28::LEU2* without pAE405	
AEY3000	AEY2867 *sir1*Δ::*kanMX*	
AEY3002	AEY2888 *sir1*Δ::*kanMX* (transformant #1)	
AEY3003	AEY2888 *sir1*Δ::*kanMX* (transformant #2)	
AEY3031	AEY2887 TEL VII-L::*URA3*	
AEY3032	AEY2888 TEL VII-L::*URA3*	
AEY3034	AEY2889 TEL VII-L::*URA3*	
AEY3036	AEY2911 TEL VII-L::*URA3*	
AEY3038	AEY2913 TEL VII-L::*URA3*	
AEY3040	AEY2937 TEL VII-L::*URA3*	
AEY3102	AEY2867 *orc1-A2P::LEU2* without pAE405	
AEY3103	AEY2866 *orc1-A2P::LEU2* without pAE405	
AEY3105	AEY3102 TEL VII-L::*URA3*	
AEY3144	AEY2867 *sir3-A2T::TRP1*	
AEY3145	AEY2866 *sir3-A2T::TRP1*	
AEY3147	AEY3102 *sir3-A2T::TRP1*	
AEY3148	AEY2913 *sir3-A2T::TRP1*	
AEY3149	AEY3103 *sir3-A2T::TRP1*	
AEY3151	AEY2903 *sir3-A2T::TRP1*	
AEY743	*MAT***a** *ade2*Δ*::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 orc1*Δ*::TRP1 HIS3::HMR-URA3*$_P$*-ADE2-E* pSPB162 *(pURA3 ORC1)*	S. Bell
AEY2333	AEY743 *orc1*Δ*1-51*::*LEU2* without pSPB162	
AEY2335	AEY743 *orc1*Δ*1-100*::*LEU2* without pSPB162	
AEY2587	AEY743 *orc1*Δ*1-10*::*LEU2* without pSPB162	
AEY2589	AEY743 *orc1*Δ*1-28*::*LEU2* without pSPB162	
AEY2721	AEY743 *orc1-A2V::LEU2* without pSPB162	

Table 2.1 (continued)

Strain	Genotype	Source*
AEY2760	AEY743 *orc1Δ29-51*::*LEU2* without pSPB162	
AEY3101	AEY743 *orc1-A2P::LEU2* without pSPB162	
AEY3109	AEY743 *nat1Δ::LEU2*	
AEY1558	*MAT***a** *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112*	E.W. Jones
AEY2719	AEY1558 *ORC1*(1-250)-*TAP::URA3*	
AEY2758	AEY1558 *nat1Δ::kanMX orc1(*1-250*)-TAP::URA3*	
AEY3107	AEY1558 *orc1-A2P*(1-250)-*TAP::URA3*	
AEY3110	AEY1558 *orc1-A2V*(1-250)-*TAP::URA3*	
AEY3171	AEY1558 *SIR3*(1-235)-*TAP::URA3*	
AEY3173	AEY1558 *nat1Δ::kanMX SIR3*(1-235)-*TAP::URA3*	
AEY160	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ633 met15 Δ1 RDN::Ty1::MET15*	J. Boeke
AEY2786	AEY160 *nat1Δ::kanMX*	
AH109	*MAT***a** *ade2-101 trp1-901 his3-Δ200 leu2-3 met* MATCHMAKER Two Hybrid strain with reporter genes *ADE2, HIS3, lacZ, MEL1*	Clontech
AEY3028	AH109 pAE951 pAE952	
AEY3099	AH109 pAE966 pAE952	

* Unless indicated otherwise, strains were constructed during the course of this study or were from the laboratory strain collection. Groups of strains between horizontal lines are isogenic.

2.1.3 Growth conditions and media

E.coli strains used for plasmid amplification were cultured according to standard procedures (Sambrook et al. 1989) at 37°C in Luria Bertani (LB) medium supplemented with either 100 μg/ml ampicillin or 50 μg/ml kanamycin. *S. cerevisiae* strains were cultured according to standard procedures (Guthrie and Fink 2002) either in complete (YPD) or minimal (YM) medium supplemented as appropriate with 20 μg/ml adenine, uracil, tryptophan, histidine and methionine or 30 μg/ml leucine and lysine. Strains were grown at 30°C, unless otherwise noted.

Media

LB	10 g/l caseinpeptone, 5 g/l yeast extract, 5 g/l NaCl
SOC	2 g/l tryptone, 500 mg/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
YPD	10 g/l yeast extract, 20 g/l peptone, 2 g/l glucose
YM	6.7 g/l yeast nitrogen base w/o amino acids, 2 g/l glucose
CA	YM medium with 40 g/l casamino acids
5-FOA	14 g/l yeast nitrogen base w/o amino acids, 4 g/l glucose, 2 g/l 5-FOA, 40 mg/l uracil
Lead medium	0.3% peptone, 0.5% yeast extract, 4% glucose, 0.02% (w/v) ammonium acetate, 0.1% $Pb(NO_3)_2$
Sporulation medium	19 g/l KAc, 0.675 mM ZnAc

(For plates, 20 g/l agar was added to liquid media.)

2.1.4 Plasmid constructions

Plasmids used in this study are listed in Table 2.2. Cloning strategies and selection markers for bacteria and yeast are added in brackets. Cloning details are described in chapter 2.2.2.

Table 2.2: Plasmids used in this study.

Plasmid	Description / Construction / Markers*	Source**
YEp24	2μ-based genomic library (Amp, URA)	(Carlson and Botstein 1982)
pAE100	pRS316 $ADH1_P$ - *GAL4*(1-147)-*SIR1* (CEN; Amp; URA)	J. Rine
pAE108	pRH98-1 GPD_P – *GAL4*(1-147)-*ORC2* (CEN; Amp, URA)	J. Rine
pAE109	pRH98-1 GPD_P – *GAL4*(1-147)-*ORC5*	J. Rine
pAE303	YCp50 *NAT1* (CEN; Amp, URA)	J. Rine
pAE405	pRS316 *ORC1* (*Bam*HI-*Xho*I *ORC1*-fragment of pAE246)	
pAE408	pTT64 *GAL4*(1-147)-*ORC1*(5-267) (CEN; Amp, HIS)	R. Sternglanz
pAE516	pRH98-1 GPD_P – *GAL4*(1-147)-*ORC6* (*ORC6*-ORF *Bgl*II-*Sal*I PCR fragment cloned into *Bam*HI-*Sal*I cut pRH98-1)	
pAE580	pRS316 *SIR3*-*GFP*	D. Shore
pAE595	pRH98-1 GPD_P – *GAL4*(1-147)-*ORC3* (*ORC3*-ORF amplified from pAE338; cloned as *Bam*HI-*Sal*I fragment into *Bam*HI-*Sal*I cut pRH98-1)	
pAE597	pRH98-1 GPD_P – *GAL4*(1-147)-*ORC4* (*ORC4*-ORF amplified from pAE349; cloned as *Bam*HI-*Sal*I fragment into *Bam*HI-*Sal*I cut pRH98-1)	
pAE866	pRH98-3 *ORC1* (*ORC1*-ORF amplified from pAE 246; cloned as *Bam*HI-*Sal*I fragment into *Bam*HI-*Sal*I cut pRH98-3) (2μ; Amp, URA)	
pAE877	pRS306 *ORC1*(1-250)-*TAP* *ORC1*(1-250)-*TAP* amplified by PCR sewing; cloned as *Bam*HI-*Sal*I fragment into *Bam*HI-*Sal*I cut pRS306 (integrating; Amp, URA)	
pAE951	pGADT7 *ORC1* (2μ; Amp; LEU)	B. Stillman
pAE952	pGBKT7 *SIR1*(346-678) (2μ; Kan, TRP)	B. Stillman
pAE953	pGADT7	Clontech
pAE964	YEplac112 *SSB1* (*SSB1* as *Bam*HI-*Pst*I fragment from pAE963; cloned into *Bam*HI-*Pst*I cut YEplac112) (2μ; Amp, TRP)	
pAE966	pGADT7 *ORC1*(52-235) *ORC1*(52-235) amplified from pAE246; subcloned into pCR-Blunt II-TOPO; cut out as *Eco*RI fragment and cloned into *Eco*RI cut pAE953	
pAE989	pRS306 *orc1-A2P*(1-250)-*TAP* *orc1-A2P*(1-250) as *Bam*HI-*Hind*III fragment from pAE971; cloned into *Bam*HI-*Hind*III cut pAE877	
pAE990	pRS306 *orc1-A2V*(1-250)-*TAP* *orc1-A2V*(1-250) as *Bam*HI-*Hind*III fragment from pAE881; cloned into *Bam*HI-*Hind*III cut pAE877	
pAE1001	YIplac204 *sir3-A2T*(1-504) *sir3-A2T* as *Kpn*I-*Hind*III fragment from pAE997; cloned into *Kpn*I-*Hind*III cut YIplac204 (integrating, Amp, TRP)	
pAE1007	pRS306 *SIR3*(1-235)-*TAP* *sir3*(1-235)*TAP* amplified by PRC sewing; cloned as *Bam*HI-*Sal*I fragment into *Bam*HI-*Sal*I cut pRS306	

* Amp = ampicillin^R, Kan = kanamycin^R, URA = *URA3*, HIS = *HIS3*, LEU = *LEU2*, TRP = *TRP1*
** Unless indicated otherwise, plasmids were constructed during the course of this study or were taken from the laboratory plasmid collection.

2.1.5 Oligonucleotides

PCR primers were designed using sequence data of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). For cloning of PCR fragments, restriction sites were inserted into primer ends. All oligonucleotides used in this study were synthesized by metabion GmbH.

2.1.6 Buffers

Tris-glycine buffer	25 mM Tris, 192 mM glycine, 0.1% SDS
Blot buffer	25 mM Tris, 192 mM glycine, 10% methanol
TBS-T	20 mM Tris pH 7.5, 500 mM NaCl, 0.05% Tween20
SDS sample buffer	50 mM Tris pH 6.8, 100 mM dithiothreitol; 2% SDS, 0.1% bromphenol blue, 10% glycerol
Zymolyase buffer	1 M sorbitol, 0.1 M NaCitrate; 60 mM EDTA pH 8.0; 5 mg/ml zymolyase (Seikagaku corp., Tokyo)
Zymolyase solution	1.2 M sorbitol, 0.1 M KPO_4 pH 7.5, 400 μg/ml zymolyase
Buffer A	20 mM Tris pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 pill *Complete* (Roche) (protease inhibitor cocktail) ad 50 ml buffer
IPP150	10mM Tris-Cl pH 8.0, 150mM NaCl
TEV cleavage buffer	10mM Tris-Cl pH 8.0, 150mM NaCl, 0.5 mM EDTA, 1 mM DTT
IPP150 Calmodulin binding buffer	10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM MgAcetate, 1 mM imidazole, 2 mM $CaCl_2$
IPP150 Calmodulin elution buffer	10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM MgAcetate, 1 mM imidazole, 2 mM EGTA

2.2 Methods

2.2.1 Yeast strain construction

Strains used in this study were generated either by direct deletion or by chromosomal integration of the gene of interest. Alternatively, strains were derived from crosses between strains from the laboratory stock.

Crossing, sporulation and the dissection of asci

For crosses, some cell material of the 2 parental strains grown over night was smeared together in a drop of water. After 8 h of incubation at 30°C (23°C for *ts* strains) on a YPD plate, the smear was streaked out on selective medium to isolate diploids.

To induce sporulation, the diploids were plated on sporulation plates and incubated at 30°C for 2-3 d or at 23°C for 3-4 d. For dissection, a loopful of asci was incubated in 10 μl zymolyase buffer for 6-10 min at RT. The reaction was stopped by adding 250 μl H_2O. Ascospores were subsequently dissected using a micromanipulator (Narishige) connected to

a Zeiss Axioscope FS microscope. Plates were incubated at 30°C or 23°C for 2-5 d. Marker segregation was followed by standard genetic techniques (Guthrie and Fink, 2002).
The suppression of the *nat1*Δ *SUM1-1* synthetic lethality by *orc1*Δ*1-235* was determined as follows: Strain JRY7176 (Rusche and Rine 2001) was transformed with an *URA3-SIR2* plasmid in order to give the strain mating ability and to create diploids with AEY3134. The *URA3-SIR2* plasmid was then lost from the diploid by counter-selection on 5-FOA containing media. The diploid was sporulated, tetrads were dissected and segregants were analyzed for their genotype. Segregants that were Trp^+ and Leu^+, genotypically were *orc1*Δ*::TRP1* and also *LEU2::orc1*Δ*1 235*, because *orc1*Δ alone is lethal. To select segregants among these with *nat1*Δ::*LEU2*, the fact was exploited that *SIR2* and *NAT1* are neighboring genes within the yeast genome, making recombination between them highly unlikely. Thus, His^- segregants from the cross by interference were also *nat1*Δ*::LEU2*. Ten such segregants were chosen, proteins extracted and submitted to SDS-PAGE and Western blotting with α-myc antibody to determine their *SUM1-1* status. Several segregants were identified that showed a strong signal, and they were presumed to have the genotype *orc1*Δ*::TRP1 LEU2::orc1*Δ*1-235 nat1*Δ*::LEU2 7myc-SUM1-1*.

Gene disruption

Endogenous *ORC1* was disrupted in a diploid strain carrying pAE405 and the two *HMR* alleles *HMR SS* Δ*I and HMR SS abf1*$^-$Δ*I* (AEY2729) using the PCR-mediated knockout technique. In brief, the complete open reading frame of *ORC1* plus 200 bp of upstream sequence was replaced by a fragment containing *SpHIS5-GFP* amplified from pAE913. Haploid *orc1*Δ strains were obtained by sporulation and tetrad dissection. *NAT1* and *SIR1* were disrupted by replacing them with the *kanMX* cassette using the PCR knockout strategy according to the guidelines for EUROFAN (Wach et al. 1994).
For both deletion protocols, integrants were selected for by standard genetic techniques and the correct integration was verified by PCR.

Chromosomal integrations

For *orc1-A2V*, *orc1-A2P* and the *orc1* N-terminal deletion strains, mutant *orc1* alleles were created by site-directed mutagenesis and cloned into an integrative plasmid (pAE785). These constructs were *Kpn*I-linearized and introduced into the *LEU2* locus of AEY2866, AEY2867 and AEY743, followed by elimination of pAE405 on 5-FOA medium. Endogenous *ORC1* was HA-tagged in strains AEY3068 and AEY3070 by duplicative integration using *Xba*I-linearized pSB991(pRS306-ORC1-HA/C; S.Bell). *sir3-A2T* strains were constructed by integrative transformation of *Pst*I-linearized pAE1001, which carries a *Kpn*I/*Hin*dIII fragment of *sir3-A2T* from pLP189 (Stone et al. 2000). Chromosomal integrations of the TAP-tagged versions of *ORC1* and *SIR3* into the *URA3* locus of AEY1558 and AEY2706 were achieved by transforming the strains with the *Nco*I-linearized plasmids pAE877, pAE989, pAE990 and pAE1007. Integrants were selected using standard genetic techniques and were verified by Western blotting. Telomeric *URA3* was inserted into the appropriate strains by transforming *Sal*I/E*co*RI-linearized pVII-L URA3-TEL (Gottschling et al. 1990).

2.2.2 Molecular cloning techniques

Standard molecular cloning techniques were performed according to (Sambrook et al. 1989). Chemicals, kits and enzymes were purchased by NEB, Invitrogen, Qiagen, Roche, Bio-Rad, Promega and Stratagene, and were applied according to the guidelines.

Transformation of DNA in *E. coli* and *S. cerevisiae*
DNA was transformed into competent *E. coli* cells (TOP10 or DH5α) according to the protocol of the manufacturers. Competent yeast cells were created and transformed as described by (Klebe et al. 1983) and (Ito et al. 1983).

Preparation of genomic and plasmid DNA
Plasmid DNA was extracted from *E. coli* by the alkaline lysis procedure (Sambrook et al. 1989), and further purification using the Qiagen plasmid kits. Plasmids were isolated from yeast strains according to the protocol of Jaques Paysan: 1.5 ml yeast culture grown to saturation were pelleted and resuspended in 200 µl zymolyase solution. After 2 hours of incubation at 37°C, plasmids were isolated by alkaline lysis with the Qiagen plasmid kit starting with 400 µl of buffer 2. Genomic DNA from yeast was prepared as described in (Hoffman and Winston 1987).

PCR reactions
As a standard PCR protocol, reactions were carried out in 50 µl volume containing 2.5 U *Taq*-Polymerase (Promega) or 0.5 U VENT Polymerase (NEB), 5 µl of the respective 10x polymerase buffer, 30 pmol of each of the two primers and 0.2 mM of each dNTP. Mg^{2+}ions and template DNA was added in variable concentrations (by default 1.5 mM Mg^{2+} and 100 pg DNA). Standard amplification reaction: 5' 95°C, 23 – 30 cycles [30'' 95°C, 30'' annealing temperature (according to the primers), elongation time (according to the fragment length) at 72°C], 5' 72°C. As benchmark, 1' was given for the elongation of 1 kb sequence.
ORC1(1-250*)-TAP* (and likewise *orc1-A2P-TAP, orc1-A2V-TAP* and *sir3*(1-235)-*TAP*) fusions were created by PCR sewing in two steps: Fragments of the TAP-tag and the respective fusion protein, which were overlapping at the projected fusion site were amplified separately. In a second PCR, the two overlapping fragments were joined using the outer primers of the first reaction. The obtained fragments contained restriction sites at their ends (added by the primer) and were directly digested and cloned into the integrative vector pRS306. In other cases, PRC fragments with primer based restriction sites at their 5' and 3' ends were at first subcloned into the pCR-Blunt II TOPO vector (Invitrogen) and then excised and inserted into their ultimate plasmid. N-terminal deletion alleles of *ORC1* were also created by PRC sewing. Thereby, the deletion region was excised from the *ORC1*-ORF by amplification and subsequent joining of the surrounding sequences. The fused PCR fragment contained the deletion and primer based restriction sites at the 5' and 3' ends, and was cloned into an integrative plasmid (pAE785).

Site-directed mutagenesis
In order to create *orc1-A2P* and *orc1-A2V* alleles, point mutations were introduced into the second codon of *ORC1* using the Quick Change® site-directed mutagenesis strategy (Stratagene). In brief, the *ORC1* encoding plasmid pAE246 was amplified with complementing

primer pairs, whose sequences included the point mutation. Newly synthesized plasmids were selected for by *Dpn*I digestion, which is specific to the methylated parental templates, and were verified by sequencing. For subsequent genomic integration of the mutant alleles, *Nde*I-*Nco*I fragments of the mutagenized plasmids were exchanged for the *Nde*I-*Nco*I fragment of an integrative plasmid containing *orc1Δ1-100* (pAE787).

Sequencing of DNA

Sequencing PCR reactions were performed according to the ABI PRISM® Big Dye™ Terminator Cycle Sequencing protocol. The reaction mix contained 1 µl BD Terminator mix, 1-8 µl template DNA, 2 mM primer, ad 10 µl H_2O. The cycling profile was: 1' 96°C, 35 cycles [20'' 96°C, 10'' annealing temperature, 4' 60°C]. The reaction was then precipitated and submitted for sequencing to the service group of the institute.

2.2.3 Silencing assays

Mating assays

Mating assays were performed using AEY264 (*MAT***a** *his4*) and AEY265 (*MAT*α *his4*) as mating-type tester strains. For qualitative mating assays (patch-mating), strains were grown on plates over night and replica-plated with a lawn of the respective tester strain on YM medium, which was selective for diploids. After 2-4 d of incubation, the yield of diploids indicated the mating efficiency of the strain. Quantitative mating assays were performed as described (Ehrenhofer-Murray et al. 1997).

MET15 colony color silencing assays

Silencing of the *MET15* reporter gene integrated at the rDNA locus was monitored on lead containing plates (Smith et al. 1999). On this medium, strains that silence the reporter gene become darkly pigmented, whereas strains expressing the gene are white. Photographs were taken after 5 d using a Leica stereoscopic microscope equipped with a Sony DXC-9100p CCD color video camera.

URA3 silencing assays

Silencing of the TEL-VIIL::*URA3* (Gottschling et al. 1990) gene was measured by the ability of strains to grow on plates containing 5-fluoroorotic acid (5-FOA), which is counter-selective for *URA3* expressing cells (Guthrie and Fink 2002). Test strains were scraped from fresh plates, resuspended in 0.5 ml sterile water and diluted to an OD_{600} of 0.3. 6-fold serial dilutions thereof were spotted with a cell spotter on plates containing 5-FOA and incubated for 2-3 d at 30°C. As a control for cell viability, the serial dilutions were also spotted onto supplemented minimal medium.

HMR::ADE2 silencing

Silencing of the *ADE2* gene inserted at the *HMR* locus was measured by the ability of strains to grow on medium lacking adenine. For this, serial dilutions of the strains were applied as described for the *URA3* silencing assays.

α-factor response assays

The α-factor response of *MAT***a** *HML*α strains was measured by spreading them on YPD plates containing 40 μg/ml α-factor and segregating 100 individual cells per strain using a micromanipulator. After 17 h of incubation at 23°C, cells were scored according to their response to α-factor. Schmoo: Individual cells that formed a mating projection and remained arrested. Schmoo cluster: Individual cells that formed multiple mating projections and eventually divided at least once. Colony: Cells that formed colonies of round cells and thus did not respond to α-factor.

2.2.4 Two-hybrid assay

The yeast two-hybrid assay was carried out using the MATCHMAKER system (Clontech). A pGBKT7 plasmid encoding Sir1(346-678) (Triolo and Sternglanz 1996) was used as bait. Orc1(1-235) and Orc1(52-235) were cloned into pGADT7 as prey. The Sir1(346-678) and Orc1(1-235) plasmids, as well as the AH109 tester strain, are courtesy of B. Stillman. Two-hybrid interactions were tested in strains cotransformed with bait and prey by plating them in serial dilutions on YM medium lacking adenine and histidine, respectively, followed by 2-3 d of incubation at 30°C. The dilution protocol is described with the *URA3* silencing assays.

2.2.5 Immunofluorescence on yeast cells

Cells carrying the sequence of a Sir3-GFP fusion protein under the control of the natural *SIR3* promoter on a CEN-based plasmid (pAE580) were grown to logarithmic phase in liquid selective medium. 1 ml of cell culture was spun down, washed once with distilled water, and then resuspended in 500 µl of water. DNA was stained by adding 1 μl of Hoechst (1µg/ml). Images were captured with a fluorescence microscope (Axioplan 2, Zeiss) using the FITC filter for GFP.

2.2.6 Biochemical techniques

Yeast protein extract preparation

For Western blotting, crude extracts were prepared according to a protocol from Sigrid Schaper. Strains were grown in selective liquid medium to midlog phase (OD_{600} = 0.5-1). For each probe, 1.5 ODs of cells were harvested and centrifuged for 2' at 6500 rpm on a table-top centrifuge. The pellet was resuspended in 30 μl of buffer A (modified from TAP protocol). After the addition of 70 μl of SDS sample buffer and acid washed glass beads, cells were broken by vortexing at full speed for 1', 5' boiling at 95-100°C, cooling down at RT, and again vortexing for 1'. 15-20 µl of these probes were applied on SDS gels, alternatively, they were stored at –80°C.

For TAP and IEF experiments, protein extracts were prepared according to the TAP protocol (Rigaut et al. 1999). 2 l of cell culture grown in YPD medium at 30°C to an OD_{600} of 1.5-2 were spun at 5000 rpm for 20' at 4°C, washed with cold water, spun again, resuspended in 50 ml in a Falcon tube and spun at 3000 rpm for 15' at 4°C. The pellet was frozen in –80°C (without shock freezing in liquid nitrogen). For protein extract preparation, the pellet was resuspended at RT in one volume of buffer A, and then kept at 4°C. Cells were broken in 3 French press

passages. Then, 0.2 mM KCl was added and the suspension was ultracentrifuged at 21,000 rpm for 30' in a Sw40Ti or 70 Ti rotor (Beckman). The supernatant was centrifuged at 34,000 rpm for 2 h at 4°C. The protein concentration of the obtained extract was determined with the method of Bradford (Bradford 1976), and aliquots were frozen in 17% glycerol in liquid nitrogen and kept at –80°C.

SDS page and Immunoblot

Proteins were separated by SDS-PAGE in Tris-glycine buffer according to standard methods (Sambrook et al. 1989). They were then transferred to nitrocellulose by blotting with the BIO-RAD Tank Transfer System according to the manufacturers guidelines. Mostly, the blot occurred for 2 h at 70V in blot buffer. The nitrocellulose membrane (Pharmacia) was subsequently blocked for 1 h at RT in 5% milk/ TBS-T. After an overnight incubation at 4°C with the primary antibody in 5% milk/ TBS-T, the blot was washed twice for 10' each with TBS-T. Next, the blot was incubated with the appropriate secondary antibody in 5% milk/ TBS-T for 1 h at RT. After washing 3 times for 10' with TBS-T, the SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for immunochemical detection.
Antibodies used were PAP (Peroxidase anti-peroxidase) (Sigma P2026), α-HA (Sigma), α-myc (Invitrogen), α-Tub27 (Babco), and α-Orc1 (Santa Cruz Biotechnology).

Isoelectric focusing

Proteins were separated by IEF-PAGE using precast *ready* gels (pH 3-10) from BIO-RAD. Gels were run according to the suppliers instructions for 1h at 100V, 2 h at 250V, and 30' at 500V using the Pharmacia Power Supply EPS 3500 XL. For immunoblotting, gels were equilibrated in blot buffer for 2 h, and then immuno-blotted like SDS gels. The theoretical p*I* was calculated using (http://us.expasy.org/tools/pi_tool.html).

Tandem affinity purification (TAP)

TAP-tagged proteins were purified according to the TAP protocol of the Séraphin laboratory (http://www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/seraphin/TAP.html), except for omitting of NP40. To prepare a sample for subsequent MALDI-TOF analysis, protein extract of a 4 l cell culture was applied. The TAP tag consists of a calmodulin binding peptide (CBP) and *Staphylococcus aureus* protein A, separated by a tobacco etch virus (TEV) cleavage site. Therefore, the purification occurred in three steps. Firstly, 250 µl of IgG agarose beads (Sigma A2909), washed beforehand with 15 ml of IPP150 buffer, were added to 10 ml of protein extract together with 100 µl of 1 M Tris-Cl pH 8.0 and incubated under rotation for 2 h at 4°C in a Poly-Prep® chromatography column (BIO-RAD). Then, the solution was removed through the column and the remaining beads were washed with 30 ml IPP150 and 10 ml TEV cleavage buffer. Next, 100 units of TEV protease (Invitrogen) were added in 1 ml TEV cleavage buffer, and the protein was eluated from the beads during 2 h of rotation at 16°C. The eluate (1 ml) was recovered from the column under addition of 200 µl TEV cleavage buffer, and supplied with 3 ml calmodulin binding buffer and 3 µl 1 M $CaCl_2$. The mix was added to the second affinity column with 250 µl of a calmodulin beads suspension, washed beforehand with 7.5 ml of IPP150 calomdulin binding buffer, and rotated for 1 h at 4°C. After removal of the solution, the beads were washed with 30 ml of IPP 150 calomdulin binding buffer and eluted with 1 ml of IPP150 calmodulin elution buffer containing EGTA. For precipitation, 5 volumes of ice-cold acetone were added and the sample was incubated at –20°C for 20'. After 30' centrifugation at full speed in a table-top centrifuge at

4°C, the supernatant was removed and the protein pellet was resuspended in 20 µl SDS sample buffer for application on a SDS gel.

In-gel digestion and peptide mass fingerprinting

TAP-purified Orc1 was separated from a 10% acrylamide gel and visualized by Coomassie G-250 staining. The protein band was excised and divided into two probes. The probes were cleaved *in situ* as described previously (Shevchenko et al. 1996) using either AspN (37°C) or GluC (25°C) protease (both Roche, Mannheim) at a final concentration of 11.7 ng/µl or 25 ng/µl, respectively. The reduction and carbamidomethylation step was omitted.

The digest supernatant (0.5 µl) was applied on a fast-evaporation nitrocellulose/ α-cyano-4-hydroxycinnamic acid layer (Vorm et al. 1994) and analyzed by MALDI-TOF mass spectrometry using a Bruker Reflex mass spectrometer (Bruker Daltonics, Bremen) in the reflector mode equipped with pulsed-ion extraction and a nitrogen laser (337nm). For selected peptides, the amino acid sequence was determined by analysis of fragment ions generated by post-source decay (Chaurand et al. 1999) using the FAST™ method (Bruker).

3 Results

3.1 Nat1 was required for repression of the *HM* loci, telomeres and the rDNA locus

The deletion of *NAT1* was previously described to cause pronounced derepression at the natural *HML* locus and at marker genes inserted in subtelomeric regions (Fig. 3.1A, 3.1C) (Mullen et al. 1989) (Aparicio et al. 1991). In contrast, due to functional redundancy within the *HMR*-E silencer, wild-type *HMR* is not affected by *nat1*Δ unless it is weakened by the deletion of the Rap1 binding site (Stone et al. 1991). To further evaluate the role of NatA in silencing, we tested its effect on the synthetic *HMR*-E silencer (*HMR SS* Δ*I*). This silencer variant consists solely of minimal binding sites for ORC, Rap1 and Abf1 and lacks much of the functional redundancy of natural *HMR* (McNally and Rine 1991). Significantly, *nat1*Δ caused complete derepression at *HMR SS* Δ*I*, as monitored by the loss of mating ability due to the coexpression of **a** information in the *MAT*α strain (Fig. 3.1A). This supported the notion that NatA had a function at *HMR* that was masked by the functional redundancy of the natural *HMR*.

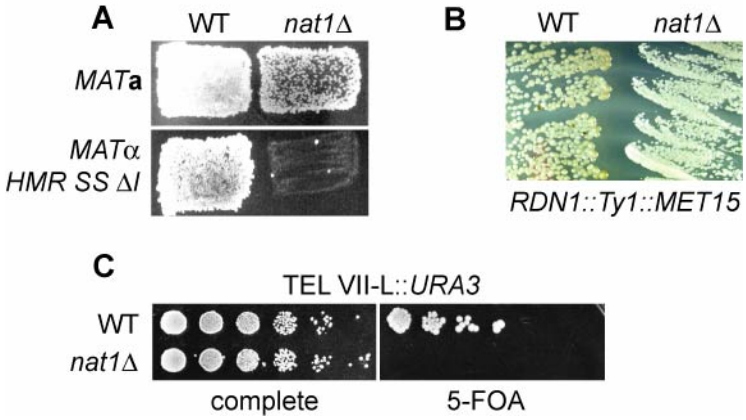


Abb. 3.1: NatA activity was required for *HM*, telomeric and rDNA silencing.
(A) The deletion of *NAT1* resulted in derepression of *HML* and *HMR SS* Δ*I*, as measured by the reduced mating ability of *MAT*a and *MAT*α strains, respectively. Patch-mating assays were performed with *MAT*a strains AEY2 (WT) and AEY80 (*nat1*Δ), and *MAT*α *HMR SS* Δ*I* strains AEY5 (WT) and AEY1273 (*nat1*Δ). (B) Silencing of *MET15* inserted into the rDNA locus was impaired by *nat1*Δ, as indicated by the brighter colony color of strain AEY 2786 (*nat1*Δ) compared to AEY160 (WT) on lead indicator medium. (C) Silencing of *URA3* inserted near the left telomere of chromosome VII depended on functional NatA. Serial dilutions of strains AEY1017 (WT) and AEY2371 (*nat1*Δ) were assayed on 5-FOA containing medium counterselecting for *URA*3-expressing cells.

We next asked whether NatA also functioned in rDNA silencing. To this end, we tested the effect of *nat1*Δ on the expression of a *MET15* reporter gene integrated at the rDNA locus, whose expression can be monitored on lead indicator medium (Smith and Boeke 1997). *nat1*Δ strains showed a brighter colony color than wild-type strains on this medium, indicating that *MET15* was derepressed by *nat1*Δ (Fig. 3.1B).

Together, NatA functioned in all forms of silencing in *S. cerevisiae*, suggesting that one or more silencing factor(s) common to all three silenced regions is the target of NatA.

3.2 Orc1 required N^{α}-acetylation by NatA for its function in telomeric silencing

3.2.1 Tethering of Orc1 or Sir1 to the silencer bypassed the requirement for NatA in silencing

The involvement of NatA in all three classes of silencing in yeast indicated that one or more silencing factors common to all silenced loci depended upon N^{α}-acetylation for proper function. In order to narrow down the number of potential candidates, we sought to genetically characterize the precise role of *nat1*Δ in silencing. We first asked through which of the *HMR*-E silencer elements *nat1*Δ functioned. For these experiments, we exploited the fact that derepression at natural *HMR* requires the loss of at least two of the three silencer elements ORC, RAP1 and ABF1. This can be achieved either by deleting the binding site in *cis*, or by mutating the respective protein in *trans*. We reasoned that measuring the effect of *nat1*Δ on individual *cis* deletions would indicate which *trans* factor it affected. Interestingly, silencing was completely abrogated in *nat1*Δ strains with *HMR*-E lacking the Rap1 binding site, thereby suggesting ORC or Abf1, but not Rap1, as NatA targets (Fig. 3.2A). In contrast, *nat1*Δ did not cause significant derepression when the ORC or Abf1 binding sites were deleted, showing that NatA functioned via these elements (Fig. 3.2A). Since the Abf1 binding site plays a minor role in silencing and the penultimate amino acid of Abf1 is an aspartate, which makes it unlikely to be a NatA substrate, we focused on ORC and asked whether it was a target of NatA.

We therefore sought to dissect through which of the six ORC subunits NatA functioned in silencing. For these experiments, we took advantage of the fact that silencing at *HMR* can be achieved by replacing the ORC binding site of the synthetic *HMR*-E silencer by unrelated Gal4 binding sites and expressing fusions of the ORC subunits or of Sir1 to the Gal4-DNA binding

domain (Fox et al. 1997). This so-called tethered silencing approach circumvents the functional complexity of silencing and allowed us to dissect the contributions of the individual ORC subunits to *NAT1*-dependent silencing. Tethering of Gal4-Sir1 bypasses the requirement for ORC in silencing (Fox et al. 1997), which supports the notion that ORC recruits Sir1 to the silencer. Importantly, Gal4-Sir1 mediated silencing was independent of *NAT1* (Fig. 3.2B), indicating that NatA functioned upstream of Sir1, and hence through ORC, in silencing.

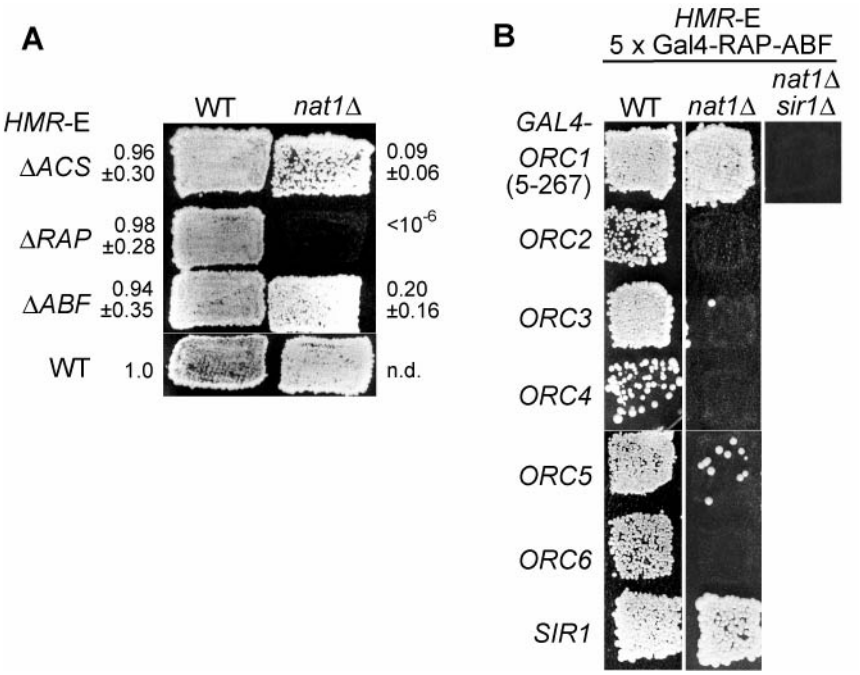


Fig. 3.2: The silencing function of NatA was genetically linked to *ORC1*.
(A) The deletion of the binding site for Rap1, but not for ORC or Abf1, from *HMR*-E disrupted *HMR* silencing in *nat1*Δ mutants. *HMR* silencing was tested by the α-mating ability of wild-type and *nat1*Δ strains with *HMR*-E lacking the binding site for ORC (AEY84, AEY2146), Rap1 (AEY81, AEY2144) and Abf1 (AEY71, AEY2148). Results from quantitative mating assays are given relative to a value of 1.0 for AEY2. (B) Tethered silencing by Orc1, but not the other ORC subunits was independent of *NAT1* and required *SIR1.* In *MAT*α strains AEY1275 (WT), AEY1276 (*nat1*Δ) and AEY 2947 (*nat1*Δ *sir1*Δ), the ORC binding site of the synthetic *HMR-E* silencer was replaced by five Gal4-binding sites (*HMR SS* Δ*I,* 5xGal4-RAP-ABF). The strains carried plasmids encoding the Gal4 DNA binding domain fused N-terminally to Orc1 (5-267aa) (pAE408), Orc2 (pAE108), Orc3 (pAE595), Orc4 (pAE597), Orc5 (pAE109), Orc6 (pAE516) and Sir1 (pAE100) and were tested for *HMR* silencing in patch-mating assays.

We next tested whether the tethering of individual ORC subunits required *NAT1* to establish silencing. The rationale of these experiments was that if N-terminal acetylation were required for an ORC subunit, direct tethering of this subunit to the silencer by an N-terminal fusion to Gal4 would relieve its requirement for NatA. Significantly, we found that tethered silencing of

all subunits except the Orc1 N-terminus (amino acids 5 to 267) was disrupted in *nat1*Δ strains (Fig. 3.2B), whereas tethered Orc1 was able to provide silencing in the absence of *NAT1*. Interestingly, this silencing still depended upon Sir1, since the NatA independent Gal4-Orc1 mediated silencing was abrogated in a *sir1*Δ strain (Fig. 3.2B). These observations indicated that Orc1 needed the N-terminal acetylation in order to fulfill its function in silencing and that the acetylation did not affect Orc1's ability to interact with Sir1. Consistent with this, Orc1 carries an alanine at the penultimate position, making it a likely candidate for N^{α}-acetylation by NatA.

3.2.2 Orc1 was N-terminally acetylated by NatA

Since the above genetic experiments strongly suggested Orc1 as a silencing-relevant substrate of NatA, we directly tested whether Orc1 was N-terminally acetylated in a NatA dependent fashion. For this purpose, a fusion of the first 250 amino acids of Orc1 to the Tandem Affinity Purification (TAP) tag (Orc1-TAP) was introduced into wild-type and *nat1*Δ strains. The TAP tag allows the fast and simple purification of large amounts of the tagged protein by three successive steps: affinity chromatography on IgG agarose is followed by tobacco etch virus (TEV) protease cleavage and purification with calmodulin-coated beads (applied below) (Rigaut et al. 1999).

Since N^{α}-acetylation shifts the isoelectric point (p*I*) of a given protein towards a more acidic pH (Kimura et al. 2000), we used isoelectric focussing gels to determine whether *nat1*Δ altered the p*I* of Orc1-TAP. Significantly, Orc1-TAP migrated at a more basic p*I* when isolated from a *nat1*Δ strain as compared to a wild-type strain (Fig. 3.3A), suggesting that Orc1 was acetylated by NatA.

It has previously been proposed that NATs can also provide ε-N-acetylation (Polevoda and Sherman 2003a). Therefore, to test whether the IEF band shift corresponded to N^{α}-acetylation of Orc1, we used mass spectrometry to measure differences in acetylation in N-terminal peptides derived from Orc1-TAP that was isolated from wild-type or *nat1*Δ strains. Acetylation extends the mass of NAT substrates by 42 Dalton (Da), which is the size of the bound acetyl group (Polevoda and Sherman 2001). Orc1-TAP samples purified with the TAP protocol from wild-type or *nat1*Δ strains were digested individually with AspN and GluC endopeptidases in order to obtain N-terminal peptides of a suitable size. We obtained a set of two different protein solutions of the wild-type and the *nat1*Δ derived samples, which were examined in

independent experiments. In the subsequent analysis, the measured mass of the N-terminal peptide from the wild-type and the *nat1*Δ probe was compared to the calculated value on the basis of the amino acid sequence (Fig. 3.3B).

In the AspN as well as the GluC cleaved sample, the measured mass of the wild-type N-terminal peptide was larger by 42 Da than the calculated value (Fig. 3.3C). However, in both cases this size increase was not found in the *nat1*Δ strain (Fig. 3.3D). Furthermore, neither the wild-type nor the *nat1*Δ strain-derived N-terminal fragments matched the calculated mass of a peptide containing the initial methionine (Fig.3.4A). This supported the notion that the initiator methionine was removed from proteins with alanine at the penultimate position.

The mass 560.47 of the AspN-cleaved *nat1*Δ probe was assigned to the N-terminal peptide AKTLK. To further verify this assignment, the peptide was sequenced by Post-Source Decay MALDI analysis (Chaurand et al. 1999) (Fig. 3.4B). Here, the peptide was degraded into fragments containing different numbers of amino acid residues and the fragment spectrum was recorded. The joined fragment data resulted in the sequence of the complete peptide AKTLK and thus confirmed it to be the unmodified form of the N-terminal peptide of Orc1.

In summary, the mass spectrometric data demonstrated that Orc1 was N-terminally acetylated in the presence of Nat1 and not acetylated in its absence, strongly suggesting that is was a direct target of NatA.

Mass spectrometry was performed by Christoph Weise (FU Berlin).

3.2.3 Unacetylated *orc1* mutants displayed telomeric derepression

We next asked whether the observed N-terminal acetylation of Orc1 was of significance for its silencing function. To this aim, we generated *orc1* alleles in which the penultimate amino acid was changed from alanine to valine or proline, and tested their effect on silencing. Proline as well as valine promote the cleavage of the initiator methionine, but prevent N-terminal acetylation (Huang et al. 1987). In order to test whether the respective mutants were acetylated or not, we tested the isoelectric properties of the TAP variants Orc1-A2P and Orc1-A2V that were constructed analogous to wild-type Orc1-TAP. Significantly, the isolelectric point of Orc1-A2P-TAP and Orc1-A2V-TAP was at a more basic pH than wild-type Orc1, although the calculated p*I* was roughly the same for all Orc1 versions (Fig. 3.3A). The shift was comparable to that of wild-type Orc1-TAP in the *nat1*Δ background, showing that the mutations to valine or proline had abrogated the ability of Orc1 to be acetylated by NatA.

We then asked whether these mutations had an impact on telomeric silencing, since the deletion of *NAT1* strongly affects silencing of subtelomeric genes (Fig. 3.1C).

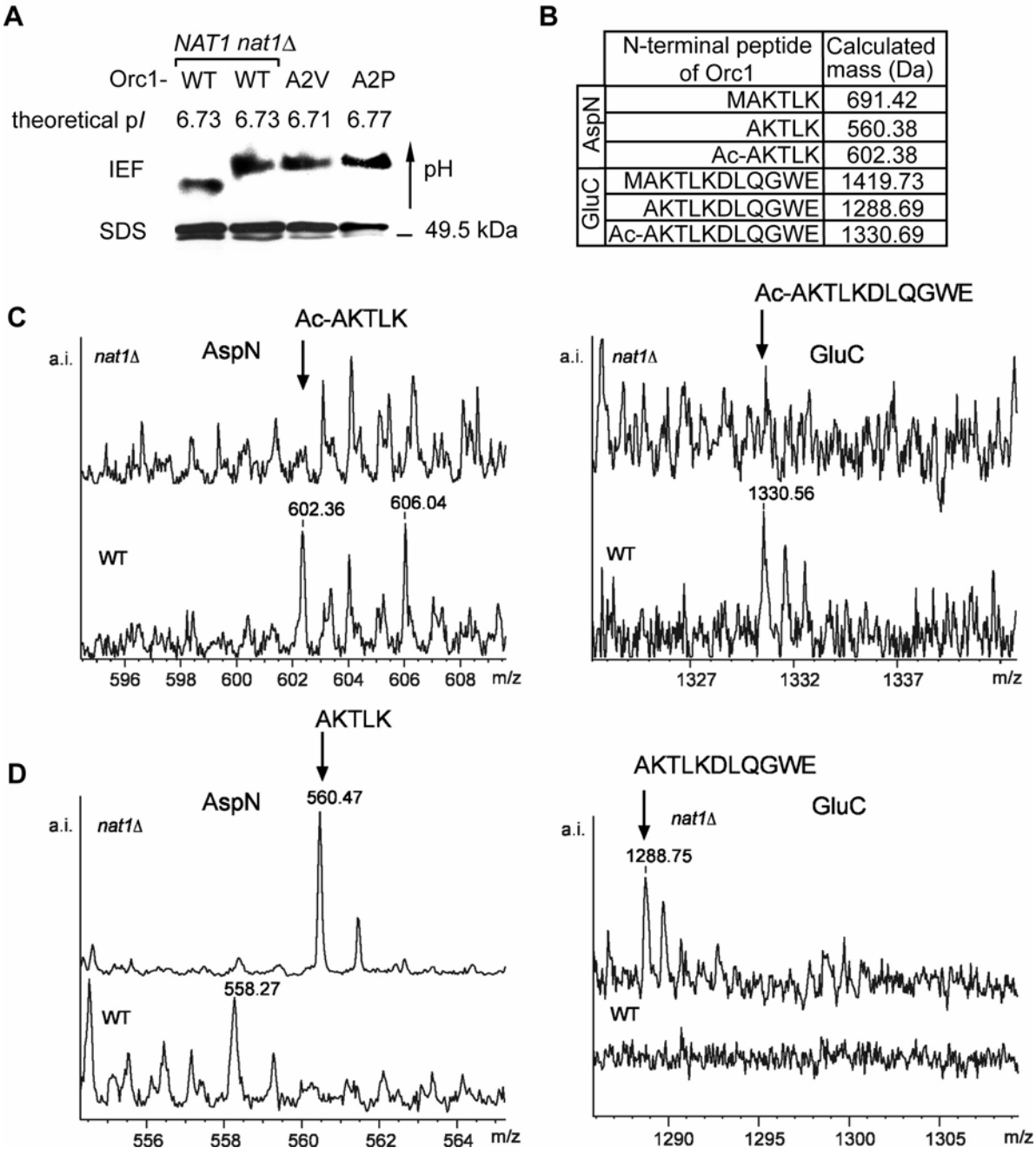


Fig. 3.3: Orc1 was N-terminally acetylated by NatA.
(A) The isoelectric point (p*I*) of the Orc1 N-terminus shifted to a more basic pH either by the deletion of *NAT1* or by the mutation of the penultimate residue alanine to valine or proline. Whole cell protein extracts of strains AEY2719 (WT), AEY2758 (*nat1*Δ), AEY3107 (*orc1-A2P*) and AEY3110 (*orc1-A2V*) were applied to IEF and SDS gels. TAP-tagged Orc1 (amino acids 1-250) was detected in subsequent immunoblots using the PAP antibody. The faster migrating band in

the SDS gel was identified as Orc1 by MALDI-TOF analysis and probably is a proteolytic fragment. (B) Theoretical molecular mass of N-terminal peptides of Orc1 generated by proteolysis with AspN or GluC endopeptidase. The molecular mass as calculated using (http://us.expasy.org/tools/peptide-mass.html) increases by 42 Da due to N^{α}-acetylation. (C) MALDI time-of-flight mass spectra of Orc1-TAP derived from a wild-type, but not from a *nat1*Δ strain, identified the mass of an acetylated N-terminal peptide of Orc1. Orc1-TAP was purified for MALDI-TOF analysis from AEY2719 (WT) and AEY2758 (*nat1*Δ). Data obtained from the AspN and GluC cleaved samples were consistent for each strain with minimal differences to the theoretical value due to the precision of measurements. (D) The MALDI-TOF spectrum of Orc1-TAP from the *nat1*Δ strain, but not from wild-type strain, contained the mass of an unacetylated N-terminal Orc1 peptide. Analysis was performed as in Fig. 3.3C.

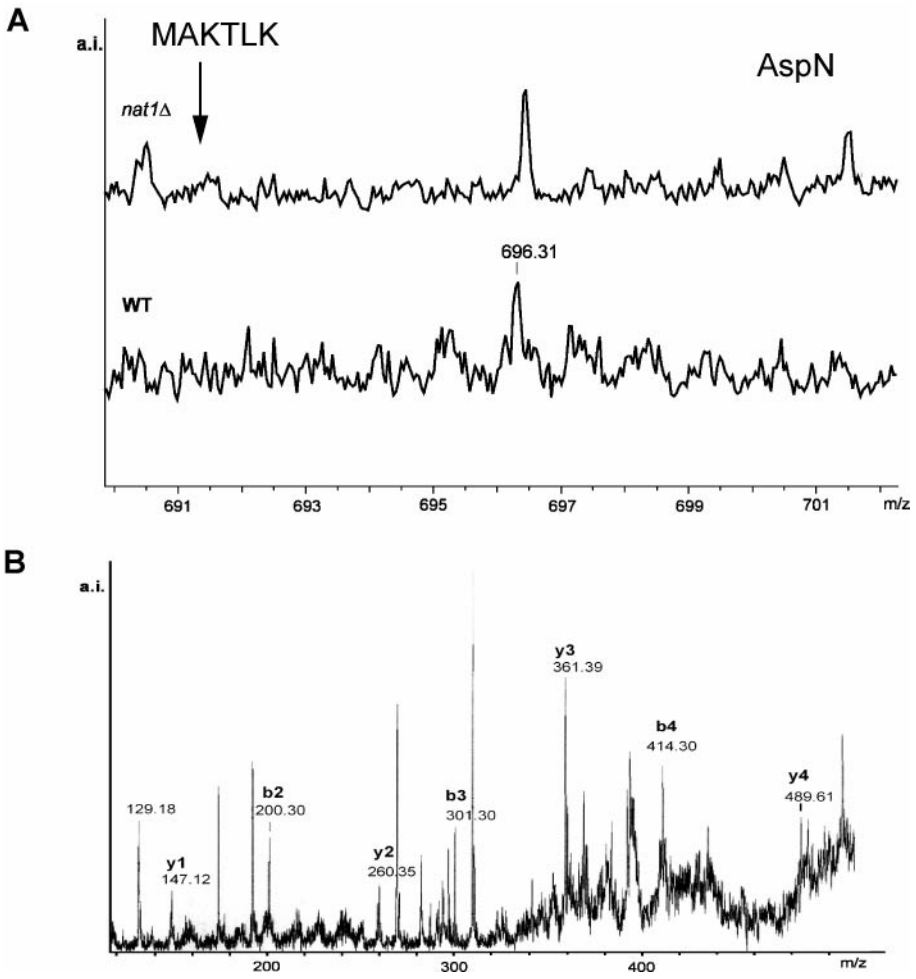


Fig. 3.4: The N-terminal peptide of Orc1, whose identity was verified by fragmentation, lacked the initial methionine.
(A) A mass corresponding to the Orc1 N-terminal peptide including the initial methionine was detected neither in the wild-type nor in the *nat1*Δ derived probe. MALDI-TOF spectra of AspN cleaved Orc1-TAP were obtained as in Fig. 3.3C. The result was confirmed by the data of the GluC cleaved samples (not shown). (B) The sequence of the *nat1*Δ-derived 560.47 Da peptide corresponded to the N-terminus of Orc1. The peptide was sequenced by fragmentation in post-source decay MALDI analysis. The detected N-terminal sequence ions AK (b2=200), AKT (b3=301), AKTL (b4=414), and C-terminal sequence ions K (y1=147), LK (y2=260), TLK (y3=361) and KTLK (y4=489) added up to the amino acid sequence AKTLK of the Orc1 N-terminus.

For this purpose, we monitored the repression of an *URA3* reporter gene inserted in the subtelomeric region of chromosome VII-L (Gottschling et al. 1990). Comparable to *nat1*Δ, *orc1-A2P* and *orc1-A2V* caused a strong derepression of the subtelomeric *URA3* reporter as indicated by diminished growth on *URA3*-counterselective 5-FOA medium (Fig. 3.5A). This showed that the loss of N-terminal acetylation of Orc1 compromised its function in telomeric silencing

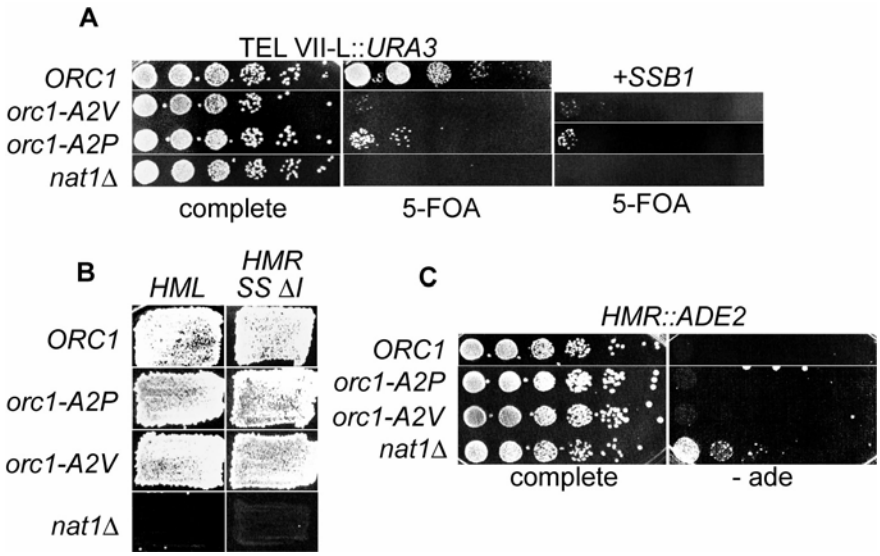


Fig. 3.5: N$^{\alpha}$-acetylation of Orc1 was essential for telomeric silencing.
(A) A *URA3* gene inserted near the left telomere of chromosome VII was derepressed in unacetylated *orc1-A2P and orc1-A2V* mutants. In these mutants as well as in the *nat1*Δ mutant, the telomeric effect was not suppressed by the overexpression of *SSB1*. *URA3* expression was tested in serial dilution assays of strains AEY1017 (*ORC1*), AEY3038 (*orc1-A2V*), AEY3105 (*orc1-A2P*), and AEY2371 (*nat1*Δ) on 5-FOA containing medium. For *SSB1* overexpression, strains were transformed with pAE964. (B) The loss of N$^{\alpha}$-acetylation of Orc1 did not impair silencing of *HML* and *HMR SS* Δ*I.* Patch-mating assays were performed to test *HML* silencing using *MAT*a strains AEY2867 (ORC1), AEY3102 (*orc1-A2P*), AEY2913 (*orc1-A2V*), and AEY2912 (*nat1*Δ), and to test *HMR SS* Δ*I* silencing using *MAT*α strains AEY2866 (ORC1), AEY3103 (*orc1-A2P*), AEY2903 (*orc1-A2V*), and AEY2916 (*nat1*Δ). (C) *nat1*Δ, but not unacetylated *orc1*, caused the slight derepression of *ADE2* inserted at the *HMR* locus. Serial dilutions of strains AEY743 (WT), AEY3101 (*orc1-A2P*), AEY2721 (*orc1-A2V*) and AEY3109 (*nat1*Δ) were grown on medium lacking adenine.

We next tested whether this defect was suppressed by *SSB1* overexpression. In the *nat1*Δ mutant, defective *HML* silencing and temperature sensitivity were suppressed by overexpression of the gene encoding the ribosome-bound chaperone Ssb1 (Gautschi et al. 2003). This Hsp70 homolog (and the 99% identical Ssb2), like NatA, is located close to the tunnel exit of the large ribosomal subunit and cross-links to a variety of nascent polypeptides

(Pfund et al. 1998). Since Ssb1 is assumed to prevent misfolding of newly synthesized proteins, its ability to suppress *nat1*Δ defects suggested that the *nat1*Δ phenotype derives from disturbed protein folding rather than decreased protein stability. As shown in Fig. 3.5A, telomeric silencing was not increased upon *SSB1* overexpression in the unacetylated *orc1* mutants or in the *nat1*Δ strain.

Though we do not understand why *SSB1* overexpression does not suppress the telomeric silencing defect of *nat1*Δ, it prompts the presumption that this silencing defect may not be the result of impaired protein folding of Orc1.

3.2.4 *HM* silencing was not affected by the lack of N-terminal acetylation of Orc1

We next tested whether *HM* silencing was also impaired by the lack of N-terminal acetylation of Orc1. However, in contrast to the strong defect caused by *nat1*Δ, no effect was detectable in the *orc1-A2P and orc1-A2V* mutants at *HML* and the synthetic *HMR SS* Δ*I* (Fig. 3.5B). In addition, the mutants showed no derepression of the sensitive *ADE2* reporter inserted at *HMR*, whereas *nat1*Δ caused a slight effect in this context (Fig. 3.5C). One possible explanation for this result is that *HM* silencing is more robust than telomeric silencing and thus is less sensitive to the *orc1* mutations. Furthermore, this suggested that more NatA silencing targets exist in *HM* silencing.

3.2.5 N^{α}-acetylation was not required for the protein stability of Orc1

Among the known NAT substrates, some require the N^{α}-acetylation for protein stability. For example, there is evidence that the half-life of non-acetylated α-MSH in rabbit plasma is one-third of that of the acetylated form (Rudman et al. 1983).

In order to test whether N^{α}-acetylation was required for the protein stability of Orc1, we compared the abundance of HA-tagged Orc1 in wild-type and *nat1*Δ strains by Western Blot analysis. Since similar amounts of Orc1 were present in whole cell protein extracts of both strains (Fig. 3.6), we concluded that it was not destabilized by the loss of N^{α}-acetylation. This result was consistent with the observation of (Mayer et al. 1989) that N^{α}-acetylation has no general protection function, since it does not prevent proteins from degradation by the ubiquitin system.

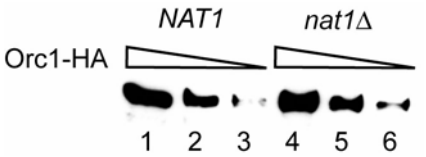


Fig. 3.6: Orc1 was present in equal amounts in a wild-type and a *nat1*Δ strain.
Whole cell protein extracts of strains AEY3068 (*NAT1*) and AEY3070 (*nat1*Δ) expressing HA-tagged *ORC1* were loaded on a SDS gel as samples of 22 μg (lanes 1 and 4), 11μg (lanes 2 and 5), and 5.5μg (lanes 3 and 6) protein. HA-tagged Orc1 was detected in a subsequent Western blot using an α-HA antibody.

3.2.6 NatA activity, but not N^{α}-acetylation of Orc1, was required for replication

Since the ORC complex functions as the eukaryotic replication initiator, we further asked whether N^{α}-acetylation of Orc1 was relevant for its replication function. We therefore tested *orc1-A2P* and *orc1-A2V* strains for temperature sensitivity, a phenotype that is associated with replication defects in *orc2-1* and *orc5-1* mutants (Loo et al. 1995a). Both unacetylated *orc1* mutants grew as well as wild-type strains and were not temperature sensitive, suggesting that replication was not affected (Fig. 3.7A). Therefore, the temperature sensitivity of the *nat1*Δ strain appeared to be based on other defects than the missing N^{α}-acetylation of Orc1.

In order to further evaluate functional links between NatA and the ORC complex, we next investigated genetic interactions between *nat1*Δ and *orc2-1*. Interestingly, we found that *nat1*Δ *orc2-1* double mutants were unable to survive. In crosses between *nat1*Δ and *orc2-1* strains, double mutant segregants did not grow up except for a few cases, where pinprick colonies appeared after prolonged incubation, but which were unable to form colonies when restreaked (Fig. 3.7B). In addition, the viability of *orc2-1 nat1*Δ double mutants was dependent on the presence of an Orc2 encoding plasmid (Fig. 3.7C). Since *orc2-1* affects replication, our results suggested that *nat1*Δ compromised replication even further such that the double mutants were unable to replicate. In summary, we found that the replication function of the ORC complex, but not of its subunit Orc1, was genetically linked to NatA activity.

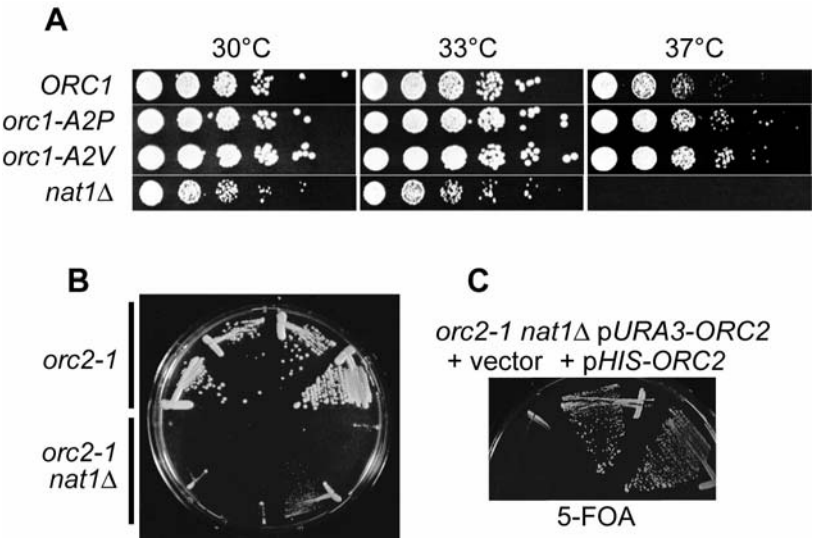


Fig. 3.7: *nat1*Δ affected the replication function of the ORC complex independently of Orc1. (A) Unacetylated *orc1* mutants were not temperature-sensitive and thereby differed from *nat1*Δ. Serial dilutions of strains AEY2866 (*ORC1*), AEY 3103 (*orc1-A2P*), AEY2903 (*orc1-A2V*) and AEY2916 (*nat1*Δ) were grown for two days on complete medium at the indicated temperatures. (B) *orc2-1 nat1*Δ double mutants were not viable. *orc2-1* and *orc2-1 nat1*Δ segregants from an *orc2-1 nat1*Δ double heterozygous cross (AEY24 crossed with AEY1227) were grown for five days on complete medium at 23°C. (C) Viability of the *orc2-1 nat1*Δ double mutant was rescued by plasmid-borne *ORC2*. AEY3161 (*orc2-1 nat1*Δ *pURA3-ORC2*) transformed either with pJR1818 (*pHIS3-ORC2*) (Fox et al. 1997) or with pRS313 (vector) was tested for *ORC2* dependence by counterselection for *pURA3-ORC2* on 5-FOA medium.
Figures B and C are courtesy of Ann Ehrenhofer-Murray.

3.2.7 Synthetic lethality between *nat1*Δ and *SUM1-1* was suppressed by *orc1*Δ*1-235*

We next sought to determine the role of NatA in *SIR* independent, *SUM1-1* dependent silencing. However, in a set of genetic crosses in which *nat1*Δ and *SUM1-1* segregated, we observed synthetic lethality between *nat1*Δ and *SUM1-1* (Fig. 3.8). The segregation of the unmarked *SUM1-1* mutation was determined by following *sum1*Δ::*URA3* in the segregants from *sum1*Δ::*URA3/ SUM1-1* heterozygous diploids. Interestingly, *nat1*Δ was not synthetically lethal with *sum1*Δ (data not shown), suggesting that the lethality was due to novel properties of the mutant Sum1-1 protein.

Since Sum1-1 has been shown to interact with the N-terminus of Orc1 and because NatA acetylates this very N-terminus, we hypothesized that the lethality may be connected to the lack of Orc1 acetylation. The ability of Sum1-1 to function in silencing is abrogated by the deletion of amino acids 1 to 235 of Orc1 (Rusche and Rine 2001). Hence, we tested whether this deletion also abrogated the synthetic lethality of *SUM1-1* with *nat1*Δ.

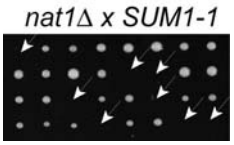


Fig. 3.8: *SUM1-1 nat1*Δ double mutants were inviable.
***SUM1-1 nat1*Δ segregants of tetrads dissected from a cross between *SUM1-1* (AEY1224) and *nat1*Δ (AEY3008) are marked by arrows. Figure is courtesy of A. Ehrenhofer-Murray.**

Significantly, strains with *orc1*Δ*1-235* as the sole source of Orc1 that were both *nat1*Δ and *SUM1-1* were readily recovered from a cross and showed normal growth characteristics (data not shown; see Materials and Methods for experimental details). Thus, the synthetic lethality of *nat1*Δ with *SUM1-1* was abrogated by deletion of the N-terminus of Orc1.
Data from 3.2.6 and 3.2.7 are courtesy of Ann Ehrenhofer-Murray.

3.3 N-terminal deletions of Orc1 caused silencing defects distinct from those of *nat1*Δ

3.3.1 *HMR* silencing was disrupted in N-terminally truncated *orc1* mutants

In previous studies, the silencing function of Orc1 has been shown to depend on the N-terminal region of 235 amino acids, which is capable of binding to Sir1 (Bell et al. 1995). Zhang and colleagues (2002) specified the Sir1 interacting domain of Orc1 to lie within amino acids 100 and 129. Since mutations of the penultimate amino acid of Orc1 affected telomeric silencing in our studies, we wished to determine the functional relevance of the N-terminal 100 amino acids of Orc1. To this end, we constructed a series of *orc1* mutants with N-terminal deletions of increasing size. Strains with *orc1*Δ*1-10*, *orc1*Δ*1-28*, *orc1*Δ*1-51* and *orc1*Δ*1-100* as the sole source of Orc1 were obtained by inserting the respective mutant allele into the *LEU2* locus of a strain whose endogenous *ORC1* gene was disrupted.
Silencing in these mutants was first tested at different sensitized *HMR* versions. *ADE2* inserted at *HMR* was silenced in *orc1*Δ*1-10* and *orc1*Δ*1-28* mutants, but was expressed upon the deletion of 51 or 100 residues from the Orc1 N-terminus (Fig. 3.9A). The phenotypic difference between *orc1*Δ1-*28* and *orc1*Δ*1-51* caused us to examine whether the region between 28 and 52 amino acids was of special significance for the silencing function of Orc1.

Indeed, as shown in Fig. 3.9A, the deletion of this region resulted in complete derepression of *HMR::ADE2*.

Silencing was next tested at *HMR* carrying either a synthetic silencer (*HMR SS ΔI*) or a silencer variant further sensitized by the deletion of the Abf1 binding site (*HMR SS abf1⁻ ΔI*). Both silencers were affected by the deletion of 28 amino acids or more of the Orc1 N-terminus (Fig. 3.9B). In addition, deleting the region of amino acids 29 to 51 also interrupted silencing at *HMR SS abf1⁻ ΔI.*

In contrast to the *HMR* variants, *HML* silencing was not affected by any of the N-terminal Orc1 deletions (Fig. 3.9B). We expect that the more robust wild-type *HMR* silencers are likewise not affected. This would be in agreement with the observation of Bell et al. (1995) that deleting the N-terminal 235 amino acids of Orc1 does not affect the mating ability (and thus the natural *HM* silencers) of an otherwise wild-type strain.

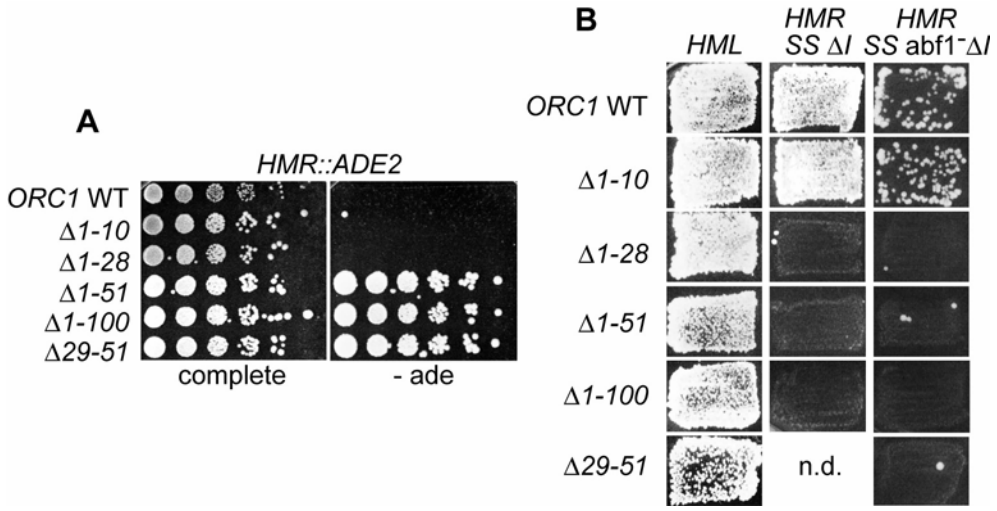


Fig. 3.9: N-terminal truncations of Orc1 impaired *HMR* silencing.
(A) *ADE2* inserted at the *HMR* locus was derepressed when the N-terminus of Orc1 was shortened by 51 or 100 amino acids and when the region between 29 and 51 amino acids was deleted. Serial dilutions of strains AEY743 (WT), AEY2587 (Δ*1-10*), AEY2589 (Δ*1-28*), AEY2333 (Δ*1-51*), AEY2335 (Δ*1-100*) and AEY2760 (Δ*29-51*) were grown on medium lacking adenine to test *ADE2* expression. (B) In contrast to *HML* silencers, synthetic *HMR* silencer variants were affected by the deletion of the N-terminal 28, 51 or 100 amino acids of Orc1, and the region between amino acids 29 and 51. Patch-mating assays were performed to test silencing at *HMR SS ΔI* and *HMR SS abf1⁻ΔI* using *MAT*α strains AEY2866 and 2864 (WT), AEY2877 and 2883 (Δ*1-10*), AEY2907 and 2908 (Δ*1-28*), AEY2879 and 2904 (Δ*1-51*), AEY2880 and 2905 (Δ*1-100*) and AEY2910 (Δ*29-51*). *HML* silencing was tested in patch-mating assays of *MAT*a strains AEY2867 (WT), AEY2887 (Δ*1-10*), AEY2937 (Δ*1-28*), AEY2888 (Δ*1-51*), AEY2889 (Δ*1-100*) and AEY2911 (Δ*29-51*).

3.3.2 Alpha-factor sensitivity was reduced in N-terminally truncated *orc1* mutants

Response to α-mating pheromone (α-factor) is required for the mating ability of haploid *MAT***a** cells and is normally characterized by arrest in late G_1 and the formation of mating projections (so-called shmoos). Derepression of *HML*, however, generates an a/α-diploid phenotype and therefore α-factor resistance of haploid *MAT***a** cells, as indicated by continued divisions in the presence of α-factor. Thus, α-factor sensitivity of *MAT***a** cells can serve as a measure of the silencing status of *HML* (Pillus and Rine 1989).

α-factor response tests are a more sensitive way than the usual patch-mating assays to investigate *HML* silencing, and we therefore employed this method here to measure *HML* silencing in the N-terminally truncated *orc1* mutants. To this end, we examined the morphology of at least 300 individual cells of each *orc1* strain after 18 hours of growth on α-factor containing medium. As for wild-type cells, almost all cells carrying Orc1 lacking 10 or 28 amino acids of the N-terminus formed shmoos, indicating repression of *HML* (Fig. 3.10).

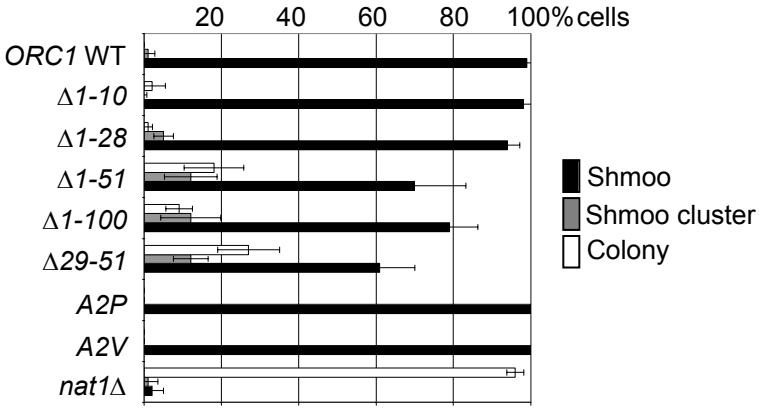


Fig. 3.10: α-factor response was abrogated by *nat1*Δ and diminished in *orc1* mutants lacking 51 or 100 amino acids of the N-terminus or the region of amino acids 29 to 51. 100 cells per strain were analyzed individually after 18 hours of exposure to α-factor. The ability to respond to α-factor was measured by the formation of one mating projection per cell (shmoo), whereas α-factor resistance was indicated by budding and subsequent colony formation. Structures emerging from alternated shmooing and budding are referred to as shmoo clusters. Results of at least three individual experiments per strain are given with respective standard deviations. *MAT*a strains used were depicted in figures 3.5(B) and 3.9(B).

Interestingly, shmoos were also generated by all of the unacetylated *orc1-A2P* and *orc1-A2V* cells, suggesting tight *HML* repression in these mutants. However, α-factor sensitivity was reduced by the deletion of the N-terminal 51 or 100 amino acids or the region between amino acids 29 and 51 of Orc1. In these strains, the shmooing fraction was smaller, whereas a

significant number of cells continued dividing and eventually formed colonies. Interestingly, another portion of these mutants formed shmoo-clusters. Here, shmoo formation alternated with cell divisions, indicating unstable repression of *HML* (Enomoto and Berman 1998).
In summary, the α-factor response tests revealed that *HML* silencing was affected by increasing truncations of the N-terminus of Orc1. As expected, the deletion of *NAT1* resulted in complete α-factor resistance, due to strong *HML* derepression, which was in contrast to the strong α-factor-response of *orc1-A2P* and *orc1-A2V* mutants indicative of full *HML* repression.

3.3.3 N-terminal truncations of Orc1 enhanced the α-factor resistance of *sir1*Δ

*sir1*Δ strains have a characteristic α-factor response phenotype, namely a mixed population of genetically identical cells, with one portion completely repressed and the other completely derepressed at *HML*. Thus, *SIR1* was proposed to function in establishment rather than maintenance of transcriptional repression (Pillus and Rine 1989). The finding that the ORC binding site of *HM* silencers is likewise involved in the establishment of silencing (Sussel et al. 1993) is in accordance with the model that Sir1 is recruited to the silencer by Orc1.

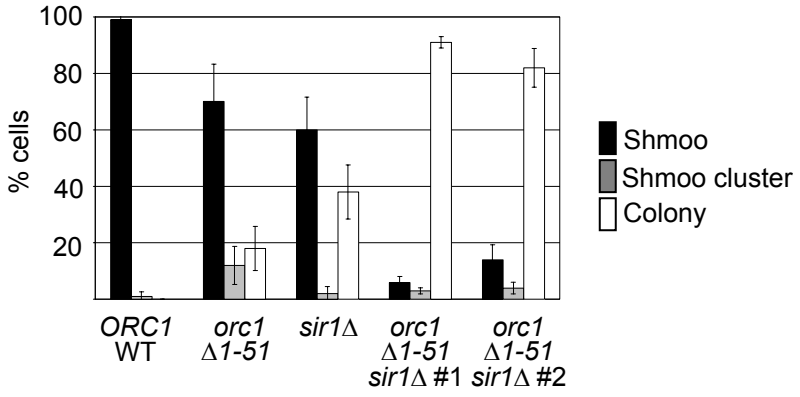


Fig. 3.11: Deletion of 51 amino acids from the Orc1 N-terminus enhanced the α-factor response defect of *sir1*Δ mutants.
The ability to respond to α-factor was tested as described in Fig. 3.10 using *MAT*a strains AEY2867 (WT), AEY2888 (Δ*1-51*), AEY3000 (*sir1*Δ), AEY3002 (*orc1* Δ*1-51 sir1*Δ #1) and AEY3003 (*orc1* Δ*1-51 sir1*Δ #2).

We sought to determine whether the truncation of the very N-terminus of Orc1 would enhance the α-factor response defect in *sir1*Δ cells. To this aim, we combined the deletion of *SIR1* with

the *orc1*Δ*1-51* mutation, which had affected each of the above-tested silencers. Significantly, in two individual double mutants, we found an increased portion of colony-forming cells (Fig. 3.11), indicating further derepression of *HML*. Thus, the silencing defect of *sir1*Δ was enhanced by the deletion of the N-terminal 50 amino acids of Orc1. This effect is surprising in light of the current view that Orc1's sole function in silencing is to recruit Sir1. It rather suggests that Orc1 has a broader task.

3.3.4 Telomeric silencing was affected by N-terminal truncations of Orc1

We next asked whether truncations within the N-terminal 100 amino acids of Orc1 had an impact on telomeric silencing. To this aim, we investigated the expression of a subtelomeric *URA3* reporter gene in the different *orc1* N-terminal mutants. The deletion of 28, 51 or 100 amino acids from the N-terminus, as well as the removal of amino acids 29 to 51, increased the expression of *URA3*, as indicated by diminished growth of these mutants on counterselective 5-FOA medium (Fig. 3.12). This was the first evidence for a function of the Orc1 N-terminus in telomeric silencing.

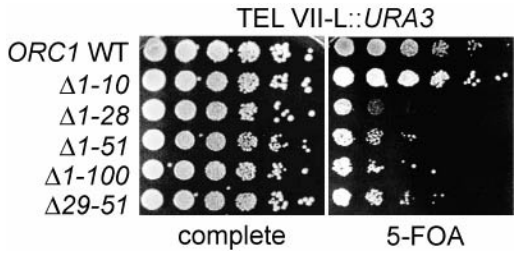


Fig. 3.12: N-terminal truncations of 28, 51 and 100 amino acids, as well as removing the region of amino acids 29 to 51 of Orc1, reduced telomeric silencing. Silencing of *URA3* inserted near the left telomere of chromosome VII was tested in serial dilution assays of strains AEY1017 (WT), AEY3031 (Δ*1-10*), AEY3040 (Δ*1-28*), AEY3032 (Δ*1-51*), AEY3034 (Δ*1-100*) and AEY3036 (Δ*29-51*) on 5-FOA containing medium counterselecting for *URA3* expressing cells.

Interestingly, this phenotype was weaker than that of the N-terminally unacetylated *orc1* mutants. This is surprising given that both mutations should abolish the N^{α}-acetylation of Orc1. In contrast, this result suggests that the N-terminal deletions suppress the defect caused by missing N^{α}-acetylation, implicating that the two types of mutations have different consequences for telomeric silencing.

3.3.5 Replication was not disturbed by N-terminal truncations of Orc1

As shown above, the lack of N^{α}-acetylation of Orc1 appeared to have no impact on its replication function, since unacetylated mutants grew as well as wild-type strains (Fig. 3.7A). To determine whether this was also the case for the N-terminal deletion mutants of Orc1, we tested their growth at different temperatures.

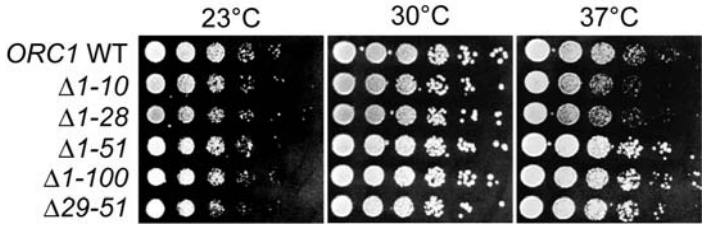


Fig.3.13: N-terminal deletions of up to 100 amino acids of Orc1 did not affect the temperature sensitivity of the respective mutants.
Strains AEY743 (WT), AEY2587 (Δ*1-10*), AEY2589 (Δ*1-28*), AEY2333 (Δ*1-51*), AEY2335 (Δ*1-100*) and AEY2760 (Δ*29-51*) were grown for two days on complete medium at the indicated temperatures.

Significantly, none of the mutants displayed a growth defect or temperature sensitivity (Fig. 3.13), suggesting that the first 100 amino acids of Orc1 were dispensable for its function in replication. This result agreed with the notion of Bell et al. (1995) that the N-terminal 235 amino acids of Orc1 have no function in replication, since their deletion causes only a slight reduction of plasmid stability.

3.3.6 The N-terminal 51 amino acids of Orc1 were required for its two-hybrid interaction with Sir1

The N-terminus of Orc1 (amino acids 5-235) interacts with the C-terminus of Sir1 (amino acids 346-678) in a two-hybrid assay (Triolo and Sternglanz 1996; Gardner et al. 1999). This interaction was interrupted when the region of amino acids 100-129 of Orc1 was substituted with the corresponding region of human Orc1, but remained intact when amino acids 21 to 35 were replaced by four alanines (Zhang et al. 2002). The latter observation implicated that the part before amino acids 100-129 was dispensable for Orc1 to interact with Sir1.

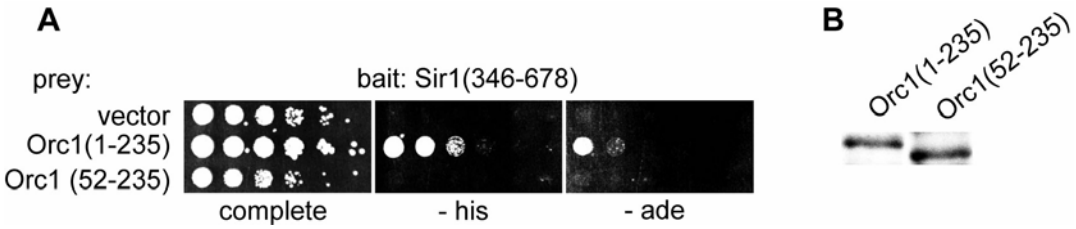


Fig. 3.14: Deletion of the N-terminal 51 amino acids abrogated the ability of Orc1 to interact with Sir1 in a two-hybrid assay.
(A) The reporter genes *ADE2* and *HIS3* were induced in two-hybrid strain AH109 by simultaneous expression of $Gal4_{BD}$-Sir1(346-678) and $Gal4_{AD}$-Orc1(1-235), but not $Gal4_{AD}$-Orc1(52-235). The bait-vector pAE952 was co-transformed with a prey-vector containing either no insert (pAE953), full-length Orc1 (pAE951) or Orc1(52-235) (pAE966). Two-hybrid interaction was tested by monitoring the expression of *HIS3* and *ADE2* in serial dilution assays on media lacking histidine or adenine, respectively. (B) The prey protein of Orc1(52-235) was as abundant as that of Orc1(1-235) in the two-hybrid strains AEY3028 (Orc1(1-235)) and AEY3099 (Orc1(52-235)). A SDS gel of whole cell extracts was analyzed by Western blotting with antibodies against the HA epitope that was part of the prey vector.

We therefore tested whether the deletion of the N-terminal 50 amino acids of Orc1, which affected silencing in our experiments, would disrupt the two-hybrid interaction with Sir1.
Using Sir1(346-678) as bait and Orc1(1-235) as prey, the two-hybrid reporter genes *HIS3* and *ADE2* were only expressed when Orc1 contained its N-terminal 50 amino acids (Fig. 3.14A). To eliminate the possibility that the missing interaction was due to a lower availability of mutant Orc1, we measured its abundance in a Western blot. Here, equal levels of Orc1(1-235) and Orc1(52-235) prey protein were detected in the respective two-hybrid strains (Fig. 3.14B). Although the loss of physical interaction between Sir1 and Orc1(52-235) has to be confirmed *in vivo*, for example by Co-Immunoprecipitation, the disrupted two-hybrid interaction was a first indication that the N-terminal 51 amino acid region of Orc1 is required for its binding to Sir1.

3.4 Sir3 was a substrate of NatA

3.4.1 Sir3 was N^{α}-acetylated by NatA

In a previous study, (Stone et al. 2000) observed decreased telomeric silencing and an enhanced *sir1*Δ mating defect when the penultimate alanine of Sir3 was exchanged for a threonine. This *sir3-A2T* mutation was epistatic to *nat1*Δ, and suggested that N^{α}-acetylation was required for the silencing function of Sir3. We therefore tested Sir3 directly for

N^{α}-acetylation by isoelectric focusing, in analogy to our experiments with Orc1. The isoelectric point of a TAP-tagged N-terminal peptide of Sir3 (amino acids 1 to 235) was more acidic in a wild-type strain than in a *nat1*Δ strain (Fig. 3.15A), suggesting that Sir3 was N^{α}-acetylated by NatA.

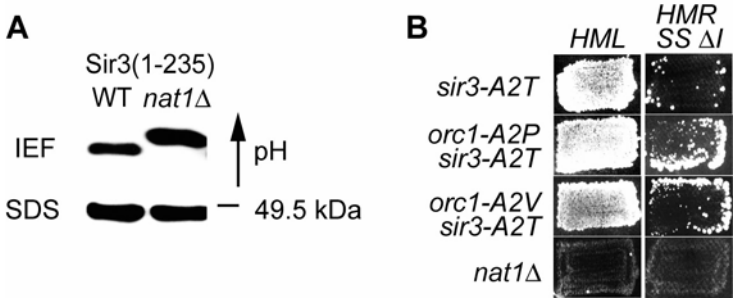


Fig. 3.15: Sir3 was acetylated by NatA.
(A) The isoelectric point of the Sir3 N-terminus became more basic upon the deletion of *NAT1*. Whole cell extracts of strains AEY3171 (WT) and AEY3173 (*nat1*Δ) expressing TAP-tagged Sir3 peptides (amino acids 1-235) were analyzed as described in Fig. 3.3A. (B) The silencing defect at the synthetic *HMR* silencer caused by the mutated penultimate amino acid of Sir3 was not enhanced by missing N^{α}-acetylation of Orc1*. HML* silencing was assayed in patch mating assays of *MAT*a strains AEY3144 (*sir3-A2T*), AEY3147 (*orc1-A2P sir3-A2T*), AEY3148 (*orc1-A2V sir3-A2T*) and AEY2912 (*nat1*Δ). Likewise, synthetic *HMR* silencing was tested in *MAT*α strains AEY3145 (*sir3-A2T*), AEY3149 (*orc1-A2P sir3-A2T*), AEY3151 (*orc1-A2V sir3-A2T*), and AEY2916 (*nat1*Δ).

Since the unacetylated forms of both Orc1 and Sir3 singly had a less pronounced silencing defect than *nat1*Δ*,* we asked whether their combination would enhance the effect on silencing. However, *orc1-A2P sir3-A2T* and *orc1-A2V sir3-A2T* double mutants showed the same amount of *HM* derepression as *sir3-A2T* alone, suggesting that NatA had other targets whose function was required for *HM* silencing (Fig. 3.15B).

3.4.2 NatA activity was required to localize Sir3 to perinuclear foci

NatA is required for silencing of subtelomeric reporter genes (Fig. 3.1C) (Aparicio et al. 1991), and the lack of N^{α}-acetylation of Orc1 and Sir3 resulted in derepression of subtelomeric *URA3* (Fig. 3.5) (Stone et al. 2000). While the insertion of reporter genes generates truncated versions of these telomeres (Gottschling et al. 1990), Sir3 is also required for silencing in a native telomeric context (Vega-Palas et al. 1997; Venditti et al. 1999). Normally, Sir3 colocalizes with Rap1 and Sir4 in perinuclear foci (Grunstein 1998) whose structural integrity was proposed to be a prerequisite for telomeric silencing (Cockell et al. 1995).

In order to determine whether NatA played a role in chromatin organization of native telomeres, we investigated the localization of GFP-tagged Sir3 in wild-type and *nat1*Δ strains. Interestingly, whereas GFP signals in wild-type cells showed the expected perinuclear foci, Sir3 became distributed throughout the nucleus in the absence of *NAT1* (Fig. 3.16).

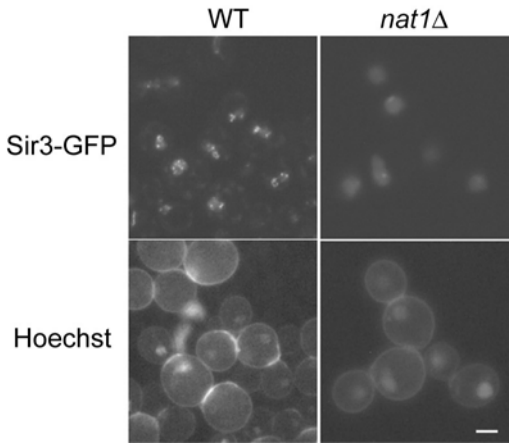


Fig. 3.16: The association of GFP-tagged Sir3 with telomeric foci was abrogated in *nat1*Δ cells. Strains AEY160 (WT) and AEY2786 (*nat1*Δ) transformed with pAE580 were examined by fluorescent microscopy using a FITC filter. Bar, 2 μm.

This suggested that the structure of native chromosomal ends depended on NatA activity. Since GFP was fused to the C-terminus of Sir3, it was probably still N^{α}-acetylated in the wild-type and unacetylated in the *nat1*Δ strain. Thus, it is conceivable, that the missing N^{α}-acetylation caused Sir3 to detach from the perinuclear foci, rather then it was an indirect effect. However, this question was not answered in our experiment and requires further investigation.

3.5 A genetic screen for multicopy suppressors of the *nat1*Δ silencing defect

3.5.1 Screening for restored silencing of *HMR SS* Δ*I in a nat1*Δ strain

The silencing phenotype of unacetylated *orc1 sir3* double mutants suggested that the function of NatA in silencing comprises more than these two substrates. In order to identify more silencing components that require N^{α}-acetylation by NatA, we performed a genetic screen for multi-copy suppressors of the *nat1*Δ silencing defect. This unbiased approach had the

advantage that NatA substrates with so far unknown implication in silencing could be discovered.

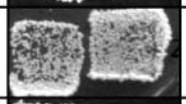
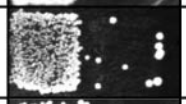
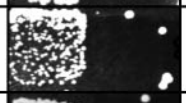
Our experiment based on the assumption that the malfunction of a silencing component provoked by missing N^{α}-acetylation might be compensated for by its overexpression. It should therefore be possible to identify such a NatA substrate by screening for genes that, when overexpressed, are capable of restoring *HM* silencing in a *nat1*Δ strain. For the screen, we used a *MAT*α strain with a synthetic *HMR* silencer (*HMR SS* Δ*I*), which was a complete non-mater due to the deletion of *NAT1* (Fig. 3.1A). In this background, multicopy suppressors of *nat1*Δ should be easily detectable by restored mating of the respective transformants. As a positive control, we expected to isolate *NAT1,* which should suppress its own deletion phenotype.

We transformed the strain with a 2μ-based genomic library (YEp24) (Carlson and Botstein 1982) and tested the mating ability of 30.000 transformants by replica-plating the colonies on *MAT***a** tester plates. The 90 maters identified were verified by repeated patch-mating assays and only those with reproducible results were further tested. Also, candidates that were assigned to be identical according to the restriction pattern of their plasmid were rejected from further tests. Since many of the originally identified maters did not give reproducible results, only 15 candidates remained. Their plasmids were isolated and retransformed into the *HMR SS* Δ*I nat1*Δ strain to confirm their suppression potential. Furthermore, the mating ability of the candidate strains was tested after loss of the *URA3* marked library plasmids on 5-FOA medium. Interestingly, candidate strain number 23 displayed good mating in the absence of the plasmid (Tbl. 3.1), suggesting that its mating ability was reestablished by (an) additional chromosomal mutation(s) rather than the overexpression a suppressor gene. The retransformed plasmids of six out of the 15 candidates could induce mating. The inserts of these plasmids were sequenced and subsequently blasted using the *Saccharomyces* genome database (http://www.yeastgenome.org/) to determine the encoded chromosomal region. Among them were two different *NAT1* containing clones (72 (Tbl. 3.1) and 89 (data not shown)), implicating that the tested number of transformants was sufficient to cover all open reading frames of the genome with the screen.

3.5.2 Overexpression of *SSF2* suppressed the *nat1*Δ mating defect

Each of the remaining four candidate clones encoded a gene whose function has been linked to mating in earlier studies. On candidate clone 22, *RVS161* (reduced viability upon starvation), encodes a protein with a direct role in cell fusion during mating (Brizzio et al. 1998). *RGA1* (rho-type GTPase-activating protein) (on candidate clone 80) was shown to act as a negative regulator of the pheromone response pathway by controlling the activity of Cdc42, a p21 GTPase required for polarity establishment and bud emergence (Stevenson et al. 1995). The overexpression of *SSF2* (suppresor of Sterile Four) (candidate clone 83) was shown to increase the mating efficiency in an earlier study (Yu and Hirsch 1995), and acts directly in RNA processing (J. Hirsch; personal communication). *NPL3* (nuclear protein localization) (candidate clone 84) is also involved in processing and nuclear-cytoplasmatic transport of RNA and is required for silencing of the mating-type loci (Loo et al. 1995b). However, as Npl3 does not act directly at *HMR*-E, this effect was proposed to be indirect.

Table 3.1: Positive candidates from a screen for multi-copy suppressors of the *nat1*Δ mating defect in the *MAT*α *HMR SS* Δ*I* strain AEY1273.

Candidate Clone	Silencing with plasmid	Silencing without plasmid	Genomic region present on plasmid (coordinates in bp)	putative suppressor	Reference
22			Chr. III: 129734 - 139824	*RVS161*	Brizzio et al. 1997
23			—	—	—
72			Chr. IV: 373176 - 385376	*NAT1*	
80			Chr. XV: 556909 - 563749	*RGA1*	Stevenson et al. 1995
83			Chr. IV: 1081417 - 1095238	*SSF2*	Yu and Hirsch 1995
84			Chr. IV: 1324767 - 1331941	*NPL3*	Loo et al. 1995

In order to confirm the four genes as multicopy suppressors of *nat1*Δ, they were cloned in 2μ-based pRS426 vectors and transformed individually into the *HMR SS* Δ*I nat1*Δ strain used above. Notably, only *SSF2* restored mating, suggesting that *RVS161*, *RGA1* and *NPL3* were not responsible for the suppressing effect of the library plasmid they were derived from.

However, further subcloning of these plasmids did not reveal any of the other encoded ORFs to be responsible for the suppressing effect. Thus, it appeared that for unknown reasons *RVS161*, *RGA1* and *NPL3* acted as suppressors of *nat1*Δ specifically in the context of the library vectors.

All four candidate suppressor genes were potential NatA substrates according to their penultimate amino acid, and therefore their dependence on N^{α}-acetylation might have been suppressed by overexpression. However, they appeared to improve mating by processes distinct from *HMR SS* Δ*I* silencing, and therefore their suppressing effect on the *nat1*Δ phenotype was indirect.

In summary, our screen identified one gene, *SSF2*, as a multi-copy suppressor of the *nat1*Δ mating defect. Since the suppression phenotype of *SSF2* appeared to be indirect, the screen failed to identify a NatA substrate directly involved in *HM* silencing.

3.5.3 Overexpression of *ORC1* did not suppress the mating defect caused by *nat1*Δ

We next tested whether overexpression of *ORC1*, which we had earlier identified as a NatA substrate, would suppress the mating defect of the *nat1*Δ mutant. For this, *ORC1* was placed in a 2μ-based plasmid under control of the strong constitutive GPD-promoter to ensure overexpression. This construct was biologically active, since it restored *HMR* silencing in the *orc1*Δ*1-51* mutant (Fig. 3.17A). Moreover, *ORC1* was overexpressed efficiently in the *MAT*α *HMR SS* Δ*I nat1*Δ strain, which was used before in the screen for multicopy suppressors (Fig. 3.17B). However, the mating defect of this strain was not suppressed by *ORC1* overexpression (Fig. 3.17C).

Interestingly, overexpression of *SIR3*, which we had also identified as NatA substrate, also failed to suppress the *nat1*Δ mating defect in an earlier study (Stone et al. 1991).

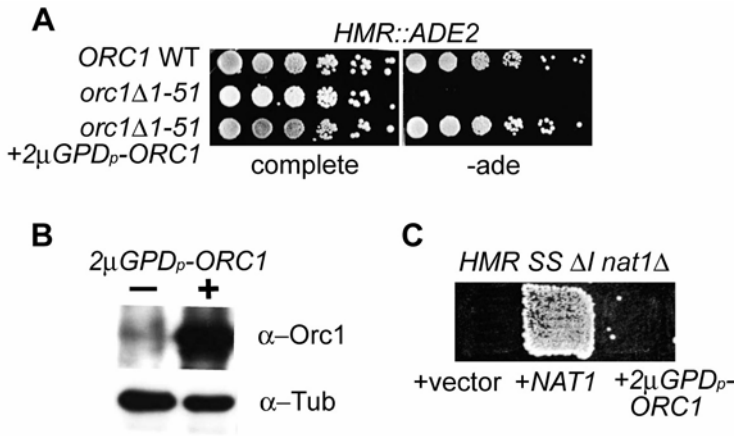


Fig. 3.17: The mating defect of *nat1*Δ was not rescued by overexpressed *ORC1*. (A) *ORC1* expressed under control of the GPD-promoter on a 2μ-based plasmid rescued the silencing defect of *orc1*Δ*1-51* at *HMR::ADE2*. Strain AEY2333 (*orc1*Δ*1-51*) was transformed with pAE866 (p2μ *GPDp-ORC1*) and grown in serial dilutions on medium lacking adenine. Strain AEY743 (WT) was tested in parallel for comparison. (B) In strain AEY1273 (-2μ *GPDp-ORC1),* more Orc1 protein was abundant upon transformation with the overexpressing construct pAE866 (+ 2μ *GPDp-ORC1)*. Equal amounts of protein from whole cell extracts were applied to a SDS gel and subsequently to Western blot analysis using antibodies against Orc1 and β tubuline (as loading control). (C) Overexpression of *ORC1* did not increase silencing of the synthetic *HMR* silencer in a *nat1*Δ background. *HMR* silencing was determined by the mating ability of *MAT*α-strain AEY1273 (*HMR SS* Δ*I nat1*Δ) transformed with pRS316 (vector), pAE303 (*NAT1*) or pAE866 (2μ *GPDp-ORC1*).

4 Discussion

N^{α}-acetylation, a frequent protein modification in eukaryotes, occurs in the yeast *S. cerevisiae* mostly by the N^{α}-acetyltransferase complex NatA. Previous work had revealed a role for NatA in transcriptional silencing of heterochromatin-like regions in the yeast genome, although the mechanism by which NatA functions in silencing had remained unclear.

In this work, we used different approaches to find silencing components that require N^{α}-acetylation by NatA, and both tested silencing proteins directly for N^{α}-acetylation and screened genetically for suppressors of the *nat1*Δ phenotype. With Orc1 and Sir3, we identified two NatA substrates with a direct role in silencing, whose function is partially impaired by the loss of N^{α}-acetylation. However, as inferred from their double mutant phenotype, NatA's role in silencing likely comprises the N^{α}-acetylation of more components.

Interestingly, the subunits of NatA are conserved in higher organisms. Since only preliminary data exist on their function to date, future research is required to reveal their role in cellular processes of complex organisms. For this challenge, the identification of NatA functions in yeast provides a useful basis.

4.1 Relevance of N^{α}-acetylation for Orc1

In this study, we gained genetic and biochemical evidence that Orc1, the biggest subunit of the ORC complex, is a substrate of NatA. Furthermore, we found that N^{α}-acetylation of Orc1 was essential for its function in telomeric silencing.

Why was telomeric but not *HM* silencing affected by the lack of N^{α}-acetylation of Orc1? One possible reason is that the semistable manner of telomeric repression makes it more sensitive than stable *HM* silencing to *orc1* mutations. The semistability has been proposed to originate in reduced silencing establishment at telomeres, resulting in the variegated expression of subtelomeric genes (Chien et al. 1993). Another possible explanation for the specific telomeric effect is a difference in structure of the silencing complexes at *HM* loci and chromosomal ends. Thus, N^{α}-acetylation might be of different relevance for the role of Orc1 at the different silenced loci.

How does N^{α}-acetylation affect Orc1's silencing function? So far, the recruitment of Sir1 to the *HM* silencers has been considered the exclusive task of Orc1 in silencing. However, genetic

data indicate that Orc1 has additional silencing functions at the telomeres. Foremost, telomeric silencing completely depends upon ORC while being unaffected by the deletion of Sir1 (Fox et al. 1997).

Several lines of evidence suggest that N^{α}-acetylation of Orc1, although being required for telomeric silencing, has no impact on Orc1's ability to interact with Sir1: Firstly, Orc1-tethered silencing in a *nat1*Δ strain was still dependent on Sir1. Secondly, in a two-hybrid assay, Sir1 interacted with Orc1(5-235), whose N-terminus was blocked to acetylation in that it was N-terminally fused to the Gal4 activation domain. Thirdly, unacetylated *orc1* mutants were fully sensitive to α-factor and did not resemble the typical establishment-defective α-factor response phenotype of *sir1*Δ mutants. Finally, the observation that N^{α}-acetylation is not required by Orc1 for Sir1 binding is in agreement with the observation that *SIR1* overexpression suppressed the *nat1*Δ silencing defect at *HML* (Stone et al. 1991).

Thus, our data propose a novel function of Orc1 in silencing that is in addition to Sir1 recruitment. We hypothesize that the Orc1 amino terminus interacts with an as yet unidentified silencing protein that functions primarily in telomeric silencing, and that this interaction requires N^{α}-acetylation of Orc1 by NatA. Crystallographic data show that the extreme N-terminus of Orc1 is exposed on the surface of the protein in a structure distinct from the Sir1 interaction domain (Zhang et al. 2002) (Fig. 4.1), thus rendering it a potential interaction module for another protein. The novel interaction partner may specifically recognize the N-terminus of Orc1 in its acetylated form. Precedence for modification dependent protein interactions comes from bromodomain and chromodomain proteins that preferentially bind specific acetylated or methylated histone residues, respectively (Jenuwein and Allis 2001).

Further evidence for the hypothesis that N^{α}-acetylation regulates interactions between Orc1 and other proteins comes from our observation of a synthetic lethal interaction between *nat1*Δ and *SUM1-1*, but not *sum1*Δ. Interestingly, the *SUM1-1* mutation confers to the Sum1-1 protein the ability to interact with ORC, but retains the ability to interact with the histone deacetylase Hst1. Thus, Sum1-1 binds to the silencers via ORC, recruits Hst1 to the *HM* silencers and establishes Sir2 independent silencing at the *HM* loci (Rusche and Rine 2001). The binding of Sum1-1 to ORC is abrogated by deletion of the amino-terminal 235 amino acids of Orc1, suggesting that Sum1-1 interacts with the Orc1 N-terminus. Interestingly, the slow growth rate of *SUM1-1* mutants was suppressed by additional *orc* mutations in an earlier study, which implicated that Sum1-1 interferes with DNA replication through ORC (Rusche and Rine 2001). Therefore, one interpretation of the inviability of *nat1*Δ *SUM1-1* strains is that Sum1-1 interacts better (i.e. stronger and at more genomic locations) with Orc1 in its

unacetylated form, and that this inhibits replication initiation, which is ORC's essential function. This hypothesis is supported by the observation that the *SUM1-1 nat1*Δ synthetic lethality was abrogated by the deletion of the amino-terminal 235 amino acids of Orc1. Thus, we postulate that N^{α}-acetylation of Orc1 regulates its ability to interact with Sum1-1 as well as with other proteins.

N-terminal protein acetylation has been hypothesized initially to protect proteins from degradation (Jornvall 1975; Hershko et al. 1984). However, several observations indicate that this does not hold true for the influence of NatA on silencing. First, we found that the level of Orc1 protein was indistinguishable between wild-type and *nat1*Δ strains. Second, the effect of *nat1*Δ on *HML* silencing and temperature sensitivity was suppressed by overexpression of the ribosome-bound chaperone Ssb1 (Gautschi et al. 2003), suggesting that *nat1*Δ caused a defect in protein folding rather than stability. However, *SSB1* overexpression did not suppress the telomeric silencing defect of *nat1*Δ and the *orc1* N-terminal mutants, thus supporting the notion that acetylated Orc1 specifically recruits a novel protein to establish silencing rather than affecting Orc1 folding.

Interestingly, N^{α}-acetylation affects Orc1's function in silencing, but not its function in replication initiation. Together with our observation that N-terminal truncations of up to 100 amino acids from Orc1 have no effect on growth, this confirms the earlier hypothesis of Bell et al. (1995) that the N-terminus of Orc1 has no function in replication. Notably, in contrast to Orc1 acetylation, NatA activity has an impact on ORC's replication function, because *nat1*Δ *orc2-1* double mutants were inviable. This suggested that other ORC subunits, or other replication factors, may be N^{α}-acetylated by NatA, and that this acetylation may impinge upon their ability to initiate replication, perhaps by affecting their ability to interact with other replication proteins.

Future experiments are required to validate our model of a protein interaction specific to N^{α}-acetylated Orc1. One possible approach to identify such an interacting protein is a two-hybrid screen with Orc1 fused C-terminally to the Gal4 binding domain as bait. Those prey proteins that bind to Orc1 in a wild-type but not in a *nat1*Δ strain are good candidates for novel interaction partners of N^{α}-acetylated Orc1. If these interactions can be confirmed by *in vitro* and *in vivo* binding studies, it will be further interesting to determine whether mutations of the candidates mimic the phenotype of the unacetylated *orc1* mutants.

Another future task is to specify the role of NatA in replication. Here, one obvious question is the N^{α}-acetylation of ORC subunits other than Orc1. As judged their penultimate amino acid, Orc3, Orc4 and Orc6 are potential NatA substrates whose N^{α}-acetylation can be investigated

in vivo. Additional candidates for NatA targets in replication are the cell-cycle genes, which also display genetic interactions to ORC alleles (Loo et al. 1995).
Since both Orc1 and the subunits of NatA are evolutionarily conserved, it will be further interesting to test if N^{α}-acetylation plays a role in the function of Orc1 homologs in higher organisms.

4.2 Function of the N-terminal 100 amino acid domain of Orc1

In this study, we found that N-terminal deletions of 50, 100, and in some cases only 28 amino acids from the N-terminus of Orc1 disturbed silencing at the *HM* loci and telomeres. Since this phenotype differed clearly from that of the missing N^{α}-acetylation, different facets of Orc1's function in silencing appear to be affected by the two types of mutations.
The N-terminal 100 amino acids of Orc1 belong to the BAH domain. This domain is found also in a number of other chromatin-associated proteins, such as mammalian DNA (cytosine-5) methyltransferases, components of the RISC chromatin-remodeling complex and histone deacetylase complexes (Callebaut et al. 1999), and was thus proposed to function as a protein-protein interacting module involved in transcriptional regulation and chromatin-mediated gene silencing.
By sequence homology analysis, the BAH domain of Orc1 had been originally located to amino acids 48 to189 (Callebaut et al. 1999). Crystallographic data of Zhang et al. (2002) redefined the region between amino acids 10 and 190 as the BAH core domain, whose secondary structure is mainly composed of β-strands (Fig. 4.1). In addition, the so-called H-domain, a small, non-conserved helical subdomain between amino acids 100 and 129 (β6 and β7), was identified to be necessary and sufficient for the interaction with Sir1. This interaction is supported by the core domain, since a number of amino acid interactions stabilize the position of the H-domain with respect to the core (Zhang et al. 2002). Notably, almost all of the amino acids participating in these interactions lie between amino acids 100 and 190, and the loop between amino acids 21 and 35 (connecting β1 and β2) was dispensable for Sir1 binding. This suggested that the region before amino acid 100 was not important for the function of the BAH domain. In contrast to this notion, we found that the two-hybrid interaction of Orc1 with Sir1 required the first 50 amino acids of Orc1, implicating that this region was also relevant for the BAH function.

Nevertheless, our data propose that the *orc1* N-terminal deletion mutants had further defects than the loss of Sir1 binding. Strikingly, truncated *orc1* mutants did not resemble the α-factor response phenotype of *sir1*Δ, and instead formed shmoo clusters, which indicate defects in the maintenance of silent chromatin rather than its establishment (Enomoto and Berman 1998). In contrast to that, an *orc1* mutant missing the H-domain responded to α-factor similarly to *sir1*Δ (Zhang et al. 2002). Since the deletion of the N-terminal 50 amino acids of Orc1 further decreased the α-factor sensitivity of *sir1*Δ, we propose that the *orc1* mutation caused a structural defect at the *HML* silencer, in addition to ineffective reestablishment of silencing due to the loss of *SIR1*. This structural defect might be based on the loss an interacting partner of Orc1 that is distinct from Sir1. This hypothesis is further supported by the telomeric phenotype of the *orc1* truncation mutants, which was earlier characterized as a Sir1 independent effect.

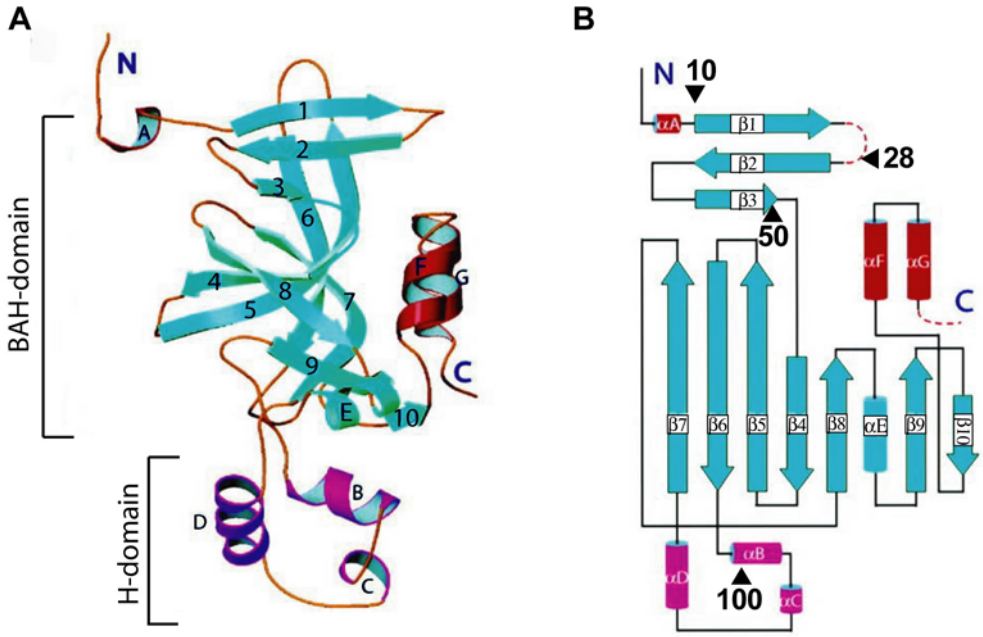


Fig. 4.1: The crystal structure of the N-terminal domain of Orc1.
(A) Ribbon presentation. (B) Topology diagram showing the fold of the structure and deletion sites of the *orc1* mutants investigated in this study. The BAH core structure is colored blue, the H-domain is shown in magenta, N- and C-terminal helices are shown in red. (adapted from Zhang et al. 2002)

Altogether, our data suggest that deletions within the first 100 amino acids of Orc1 not only have a destabilizing effect on the interaction with Sir1, but also impair the interaction of Orc1

with (a) further silencing partner(s). We hypothesize that this protein requires N^{α}-acetylation as well as an intact N-terminal structure of Orc1 for its binding (Fig. 4.2).

Interestingly, the deletion of the region between amino acids 28 and 52 of Orc1 also affected silencing, in contrast to the removal of amino acids 21 to 35 (Zhang et al. 2002). The telomeric phenotype of this mutant points to a special role of this region in the binding of the hypothesized silencing factor, which we propose to act in particular at the telomeres.

Why did the deletion of 10 amino acids from the N-terminus of Orc1 not effect silencing? Whereas the BAH domain should be still functional in this mutant, N^{α}-acetylation by NatA is expected to be interrupted. However, the penultimate amino acid tryptophan makes it to a possible substrate for the N^{α}-acetyltransferase NatC. Thus, one conceivable scenario is that the N^{α}-acetylation of Orc1(Δ1-10) by NatC still enables it to interact with the hypothesized silencing factor and therefore to function like full-length Orc1. This could be tested in an IEF gel with Orc1(Δ1-10) probes derived from wild-type and *natC* mutant strains.

According to our model, the region comprising the first 100 amino acids of Orc1 has a dual function in silencing: supporting the interaction of the H-domain with Sir1 and providing the binding site for a further interacting factor. Further studies will be required to test our hypothesis. For example Co-IPs could test Sir1 binding to the truncated Orc1 versions, and ChIP assays can be used to test its association to silencers in the mutant strains. Moreover, it has to be specified what regions of Orc1 are required for the binding of new interacting partners.

Notably, the BAH domain is conserved in all known Orc1 homologs, posing the question of whether this motif mediates a role of Orc1 in transcripitional repression also in higher eukaryotes. Significantly, Orc1 is associated with heterochromatin in *Drosophila*, where its N-terminus interacts physically with HP1, a central component of heterochromatin. In addition, the amino terminus of Xenopus Orc1 likewise interacts with HP1 homologs (Pak et al. 1997). Although it is not known at present whether the BAH module plays a direct role in this interaction, these data strongly suggest that not only the replication initiator function, but also the silencing function of ORC is evolutionary conserved.

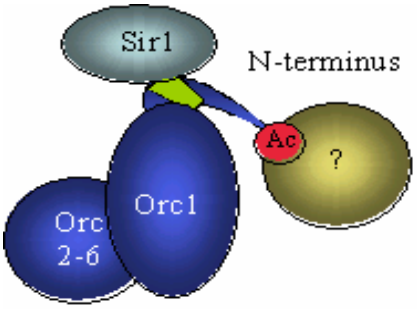


Fig. 4.2: Model of protein interactions at the N-terminus of Orc1.
In addition to Sir1, the Orc1 N-terminus interacts with another, yet unknown protein. This protein requires the N^{α}-acetylation of Orc1 for binding and is specifically recruited in the context of telomeric silent chromatin, when Sir1 is absent. In contrast, Sir1 is recruited via the H-domain of Orc1 specifically to the *HM* silencers. This binding does not require the N^{α}-acetylation of Orc1, but it depends on the integrity of the N-terminal region of 100 amino acids.

4.3 A model of the role of NatA in silencing

In this study, we sought to reveal by which mechanism NatA functions at silenced loci. We identified Orc1 as a NatA substrate, and showed that its N^{α}-acetylation was required particularly for telomeric silencing. Summarizing our data, we propose a novel silencing factor to bind to Orc1 in its N^{α}-acetylated form.

In light of the high similarity of between Orc1 and Sir3 N-termini, it is interesting that we also found Sir3 to be N^{α}-acetylated by NatA. One possibility is that acetylated Orc1 and Sir3 both interact with the same hypothesized silencing factor, which is in agreement with the observation that double *orc1 sir3* unacetylated mutants showed no additional silencing effects. However, these double mutants showed weaker derepression at the *HM* loci than *nat1*Δ, suggesting that further targets of NatA exist that require N^{α}-acetylation for their silencing function.

We therefore propose a model, in which the effect of *nat1*Δ at the *HM* loci is the sum of several proteins lacking acetylation, among them Orc1 and Sir3. The cumulative effect on these proteins causes Sir3 and perhaps other Sir proteins to lose their ability to bind silenced chromatin, and thus causes derepression. Significantly, missing N^{α}-acetylation caused only a partial loss of function in the two substrates here identified. In unacetylated Orc1, Sir1 recruitment was not affected, and unacetylated Sir3 did not completely disrupt silencing as seen in the *sir3* null mutant (Rine and Herskowitz 1987).

Interestingly, the N-terminus of Sir3 interacts in two-hybrid assays with Abf1 (Gasser and Cockell 2001), and it is well conceivable that this interaction depends on N^{α}-acetylation of

Sir3. This may provide an explanation for the missing effect of *nat1*Δ at *HMR*-E lacking the Abf1 binding site.

It would be in line with our model if some of the NatA targets in silencing acted not directly at the silenced loci, but rather would influence silencing indirectly by regulating silencing factors. To date, there exist only preliminary data on the regulation of members of the silencing complex. One example for such a regulation is the Sir3 hyperphosphorylation in response to mating pheromone, heat shock and starvation, which increases silencing (Stone and Pillus 1996). This modification requires an activated MAP kinase cascade, although the detailed mechanism of Sir3 regulation remains unclear (Ai et al. 2002; Ray et al. 2003).

How can further NatA substrates that require N^{α}-acetylation for their silencing function be identified? Besides testing individual silencing components for N^{α}-acetylation, genetic screens provide one possibility to reveal new genes linked to the silencing function of NatA. In light of the results of the multicopy-suppressor screen performed in this study, it is important to strictly focus the screen on those NatA substrates that act in silencing. In other words, when mating ability is used as a sensor for *HM* silencing, no genes involved in other aspects of mating should be isolated. This could be realized, for instance, by screening for mutants which reestablish silencing in a *nat1*Δ background. In addition, the screen has to be sensitive enough to detect slightest improvements of *HM* silencing, since single components have to be isolated out of an orchestra of components that constitute the *nat1*Δ phenotype.

To complete the picture of NatA's silencing function, it is also necessary to uncover its involvement at the other silenced loci. Silencing of subtelomeric reporter genes was completely disrupted by the deletion of *NAT1*, and unacetylated *orc1* and *sir3* mutants displayed the same effect (Stone et al. 2000). At the moment it is not clear, whether the N^{α}-acetylation of Orc1 and Sir3 alone constitutes the role of NatA at telomeres. Further experiments will be required to determine the structural integrity of the perinuclear foci in unacetylated *orc1* and *sir3* mutants and whether the association of other components, such as Rap1, is likewise affected.

In this study, we present evidence that NatA also has a function in rDNA silencing. Future work has to reveal which silencing components at the rDNA require N^{α}-acetylation. Interestingly, both NatA substrates identified here are capable of binding to the rDNA array. Although Sir3 is not required for the repression of rDNA reporter genes (Bryk et al. 1997; Smith and Boeke 1997), it locates in the nucleolus of aging cells (Kennedy et al. 1997). Notably, the N-terminus of Sir3 was shown to contain efficient information for nucleolar targeting (Gotta et al. 1998). Given its high homology to the N-terminus of Orc1, both proteins

might be recruited to the rDNA locus by the same mechanism, and a role of the already hypothesized common interaction partner in this process is conceivable. Alternatively, ORC can bind to the rDNA array via the ACS sites that are part of the NTS2 of each repeat (Huang and Moazed 2003). In order to verify a role of N^{α}-acetylated Orc1 and Sir3 in rDNA silencing, the unacetylated mutants need to be tested for a rDNA phenotype. An interesting observation with regard to the mechanism of NatA's role in rDNA silencing was that GFP-tagged Sir2 was still located in the nucleolus of *nat1*Δ cells (data not shown). This indicated that the integrity of the silencing-mediating RENT complex was not dependent on NatA.

4.4 N^{α}-acetylation as a conserved eukaryotic protein modification

In this study, we provide evidence that N^{α}-acetylation participates in the regulation of chromatin structure in yeast, since the silencing proteins Orc1 and Sir3 depended on this modification to function properly. Thus, we propose that N^{α}-acetylation can be classified as a chromatin regulatory mechanism comparable to acetylation or methylation of ε-N-lysines.
However, in contrast to ε-N-acetylation, which readily can be removed by deacetylases (Dutnall and Pillus 2001), N^{α}-acetylation is irreversible, and N^{α}-deacetylases are hitherto unknown. This raises the question of how the modification can be removed in order to alter protein function upon demand. One possibility is that amino-terminal proteolysis may remove N^{α}-acetylation, as is proposed for histone methylation, which also is irreversible (Jenuwein and Allis 2001). Regulated ubiquitin-based protein processing (Palombella et al. 1994) is a conceivable mechanism for the purpose, since the mutation of a putative ubiquitin-specific protease was demonstrated to specifically enhance PEV in *Drosophila* (Henchoz et al. 1996). Another possibility is the removal of the N^{α}-acetylated amino acid. Interestingly, acylamino acid-releasing enzymes (AARE) have been identified in eukaryotes and an archeon (Ishikawa et al. 1998; Yamauchi et al. 2003), which catalyze the amino-terminal hydrolysis of N^{α}-acylpeptides to release N^{α}-acetylated amino acids. Although AAREs act specifically on short nascent chains of 2-5 amino acids (Krishna and Wold 1992), related enzymes might perform this reaction on N^{α}-acetylated proteins. A further alternative to modulate the function of N^{α}-acetylated silencing proteins may be their removal from chromatin, much like preexisting histone modifications are eliminated by histone replacement during replication. Interestingly, methylated H3 histones are postulated to be exchanged during transcription for the

unmethylated histone variant H3.3, which promotes the generation of active chromatin (Jenuwein and Allis 2001; Ahmad and Henikoff 2002).

We have not investigated whether any of these several theoretical mechanisms regulate the silencing activity of Orc1 or Sir3. In light of our data, we suggest that N^{α}-acetylation may provide a stable, long-term epigenetic mark for maintaining chromatin states.

Intriguingly, N^{α}-acetylation can be found in all kingdoms of life and especially in eukaryotes. The high level of evolutionary conservation suggests that this protein modification is capable of acting in fundamental cellular processes. In higher organisms, homologs of the NatA subunits have been linked to developmental and differentiation processes. Mouse mNAT1 is expressed in the developing brain and is regulated by physiological levels of functional N-methyl-D-aspartate (NMDA) receptor in developing neurons (Sugiura et al. 2001). The mNAT1 homolog *tubedown-1* is expressed highly in developing tissues and down-regulated upon differentiation (Gendron et al. 2000). Furthermore, a *tubedown-1* variant, Tbdn100, was isolated in a transcription regulatory complex, suggesting that it may be a transcriptional co-regulator (Willis et al. 2002). Interestingly, the human homolog NATH also shows high expression in parts of the human brain and is overexpressed in malignant cells, for instance in papillary thyroid carcinomas and several leukemia and carcinoma cell lines (Fluge et al. 2002). It therefore has been hypothesized that NatA overexpression might simply correlate with high transcriptional activity. Even though this may be the case, overdosed N^{α}-acetylation itself could result in deregulated gene expression and tumorgenesis, as overexpression of *NAT1* impaired the stability of chromosomes in yeast (Ouspenski et al. 1999). In light of our findings, it is tempting to speculate that NatA acetylation regulates cell proliferation by modifying ORC function in replication or in the control of gene expression. Thus, it will be interesting to identify chromatin factors in higher eukaryotes whose function depend on N^{α}-acetylation by NatA.

The implication of NatA homologs in cellular differentiation processes provokes the important question whether N^{α}-acetylation plays a role in carcinogenesis. Interestingly, inappropriate regulation of chromatin structure (Singh et al. 2000) and notably mutated histone acetylating and deacetylating enzymes (Borrow et al. 1996; Vaziri et al. 2001) have been revealed to be tumor generating factors. In this respect, to enlighten the work of human N^{α}-acetylating complexes is a challenging future task, which can be facilitated by a comprehensive knowledge of the mechanisms in yeast.

References

Ahmad, K. and Henikoff, S. 2002. The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol Cell* **9**: 1191-1200.

Ai, W., Bertram, P.G., Tsang, C.K., Chan, T.F., and Zheng, X.F. 2002. Regulation of subtelomeric silencing during stress response. *Mol Cell* **10**: 1295-1305.

Akhtar, A. and Becker, P.B. 2000. Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in *Drosophila*. *Mol Cell* **5**: 367-375.

Andoh, T., Hirata, Y., and Kikuchi, A. 2000. Yeast glycogen synthase kinase 3 is involved in protein degradation in cooperation with Bul1, Bul2, and Rsp5. *Mol Cell Biol* **20**: 6712-6720.

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

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**: 1279-1287.

Arendt, C.S. and Hochstrasser, M. 1999. Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly. *Embo J* **18**: 3575-3585.

Arnold, R.J., Polevoda, B., Reilly, J.P., and Sherman, F. 1999. The action of N-terminal acetyltransferases on yeast ribosomal proteins. *J Biol Chem* **274**: 37035-37040.

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

Bannister, A.J., Miska, E.A., Gorlich, D., and Kouzarides, T. 2000. Acetylation of importin-α nuclear import factors by CBP/p300. *Curr Biol* **10**: 467-470.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**: 120-124.

Becker, P.B. and Horz, W. 2002. ATP-dependent nucleosome remodeling. *Annu Rev Biochem* **71**: 247-273.

Bell, S.P., Kobayashi, R., and Stillman, B. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science* **262**: 1844-1849.

Bell, S.P., Mitchell, J., Leber, J., Kobayashi, R., and Stillman, B. 1995. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell* **83**: 563-568.

Bienz, M. and Muller, J. 1995. Transcriptional silencing of homeotic genes in *Drosophila*. *Bioessays* **17**: 775-784.

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

Blatch, G.L. and Lassle, M. 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**: 932-939.

Borrow, J., Stanton, V.P., Jr., Andresen, J.M., Becher, R., Behm, F.G., Chaganti, R.S., Civin, C.I., Disteche, C., Dube, I., Frischauf, A.M., Horsman, D., Mitelman, F., Volinia, S., Watmore, A.E., and Housman, D.E. 1996. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet* **14**: 33-41.

Bose, M.E., McConnell, K.H., Gardner-Aukema, K.A., Muller, U., Weinreich, M., Keck, J.L., and Fox, C.A. 2004. The origin recognition complex and Sir4 protein recruit Sir1p to yeast silent chromatin through independent interactions requiring a common Sir1p domain. *Mol Cell Biol* **24**: 774-786.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Bradshaw, R.A., Brickey, W.W., and Walker, K.W. 1998. N-terminal processing: the methionine aminopeptidase and N^{α}-acetyl transferase families. *Trends Biochem Sci* **23**: 263-267.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F., and Allis, C.D. 2001. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev* **15**: 3286-3295.

Brizzio, V., Gammie, A.E., and Rose, M.D. 1998. Rvs161p interacts with Fus2p to promote cell fusion in *Saccharomyces cerevisiae*. *J Cell Biol* **141**: 567-584.

Bryk, M., Banerjee, M., Murphy, M., Knudsen, K.E., Garfinkel, D.J., and Curcio, M.J. 1997. Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev* **11**: 255-269.

Buck, S.W. and Shore, D. 1995. Action of a *RAP1* carboxy-terminal silencing domain reveals an underlying competition between *HMR* and telomeres in yeast. *Genes Dev* **9**: 370-384.

Burke, T.W., Cook, J.G., Asano, M., and Nevins, J.R. 2001. Replication factors MCM2 and ORC1 interact with the histone acetyltransferase HBO1. *J Biol Chem* **276**: 15397-15408.

Callebaut, I., Courvalin, J.C., and Mornon, J.P. 1999. The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. *FEBS Lett* **446**: 189-193.

Carlson, M. and Botstein, D. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase. *Cell* **28**: 145-154.

Carpenter, P.B., Mueller, P.R., and Dunphy, W.G. 1996. Role for a *Xenopus* Orc2-related protein in controlling DNA replication. *Nature* **379**: 357-360.

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

Cavalli, G. and Paro, R. 1999. Epigenetic inheritance of active chromatin after removal of the main transactivator. *Science* **286**: 955-958.

Chaurand, P., Luetzenkirchen, F., and Spengler, B. 1999. Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry. *J Am Soc Mass Spectrom* **10**: 91-103.

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

Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M., and Allis, C.D. 2000a. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* **5**: 905-915.

Cheung, W.L., Briggs, S.D., and Allis, C.D. 2000b. Acetylation and chromosomal functions. *Curr Opin Cell Biol* **12**: 326-333.

Chi, M.H. and Shore, D. 1996. *SUM1-1*, a dominant suppressor of *SIR* mutations in *Saccharomyces cerevisiae*, increases transcriptional silencing at telomeres and *HM* mating-type loci and decreases chromosome stability. *Mol Cell Biol* **16**: 4281-4294.

Chien, C.T., Buck, S., Sternglanz, R., and Shore, D. 1993. Targeting of *SIR1* protein establishes transcriptional silencing at *HM* loci and telomeres in yeast. *Cell* **75**: 531-541.

Choi, S.C., Chang, J.Y., and Han, J.K. 2001. A novel *Xenopus* acetyltransferase with a dynamic expression in early development. *Biochem Biophys Res Commun* **285**: 1338-1343.

Clayton, A.L., Rose, S., Barratt, M.J., and Mahadevan, L.C. 2000. Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *Embo J* **19**: 3714-3726.

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**: 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**: 8626-8636.

Dhar, S.K., Delmolino, L., and Dutta, A. 2001. Architecture of the human origin recognition complex. *J Biol Chem* **276**: 29067-29071.

Dhillon, N. and Kamakaka, R.T. 2002. Breaking through to the other side: silencers and barriers. *Curr Opin Genet Dev* **12**: 188-192.

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**: 520-531.

Driessen, H.P., de Jong, W.W., Tesser, G.I., and Bloemendal, H. 1985. The mechanism of N-terminal acetylation of proteins. *CRC Crit Rev Biochem* **18**: 281-325.

Dutnall, R.N. and Pillus, L. 2001. Deciphering NAD-dependent deacetylases. *Cell* **105**: 161-164.

Dyda, F., Klein, D.C., and Hickman, A.B. 2000. GCN5-related N-acetyltransferases: a structural overview. *Annu Rev Biophys Biomol Struct* **29**: 81-103.

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**: 923-934.

Ekwall, K., Nimmo, E.R., Javerzat, J.P., Borgstrom, B., Egel, R., Cranston, G., and Allshire, R. 1996. Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. *J Cell Sci* **109**: 2637-2648.

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**: 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**: 507-515.

Feuerbach, F., Galy, V., Trelles-Sticken, E., Fromont-Racine, M., Jacquier, A., Gilson, E., Olivo-Marin, J.C., Scherthan, H., and Nehrbass, U. 2002. Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. *Nat Cell Biol* **4**: 214-221.

Fluge, O., Bruland, O., Akslen, L.A., Varhaug, J.E., and Lillehaug, J.R. 2002. NATH, a novel gene overexpressed in papillary thyroid carcinomas. *Oncogene* **21**: 5056-5068.

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

Fox, C.A., Ehrenhofer-Murray, A.E., Loo, S., and Rine, J. 1997. The origin recognition complex, *SIR1*, and the S phase requirement for silencing. *Science* **276**: 1547-1551.

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**: 108-112.

Garcia-Salcedo, J.A., Gijon, P., Nolan, D.P., Tebabi, P., and Pays, E. 2003. A chromosomal *SIR2* homologue with both histone NAD-dependent ADP-ribosyltransferase and deacetylase activities is involved in DNA repair in *Trypanosoma brucei*. *Embo J* **22**: 5851-5862.

Gardner, K.A., Rine, J., and Fox, C.A. 1999. A region of the Sir1 protein dedicated to recognition of a silencer and required for interaction with the Orc1 protein in *Saccharomyces cerevisiae*. *Genetics* **151**: 31-44.

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

Gatto, G.J., Jr., Geisbrecht, B.V., Gould, S.J., and Berg, J.M. 2000. Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5. *Nat Struct Biol* **7**: 1091-1095.

Gautschi, M., Just, S., Mun, A., Ross, S., Rucknagel, P., Dubaquie, Y., Ehrenhofer-Murray, A., and Rospert, S. 2003. The yeast N^{α}-acetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides. *Mol Cell Biol* **23**: 7403-7414.

Gavin, K.A., Hidaka, M., and Stillman, B. 1995. Conserved initiator proteins in eukaryotes. *Science* **270**: 1667-1671.

Gendron, R.L., Adams, L.C., and Paradis, H. 2000. Tubedown-1, a novel acetyltransferase associated with blood vessel development. *Dev Dyn* **218**: 300-315.

Gilbert, D.M. 2002. Replication timing and transcriptional control: beyond cause and effect. *Curr Opin Cell Biol* **14**: 377-383.

Gombert, W.M., Farris, S.D., Rubio, E.D., Morey-Rosler, K.M., Schubach, W.H., and Krumm, A. 2003. The c-myc insulator element and matrix attachment regions define the c-myc chromosomal domain. *Mol Cell Biol* **23**: 9338-9348.

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**: 1349-1363.

Gotta, M., Palladino, F., and Gasser, S.M. 1998. Functional characterization of the N-terminus of Sir3p. *Mol Cell Biol* **18**: 6110-6120.

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**: 751-762.

Grallert, B. and Nurse, P. 1996. The *ORC1* homolog orp1 in fission yeast plays a key role in regulating onset of S phase. *Genes Dev* **10**: 2644-2654.

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

Grunstein, M. 1998. Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell* **93**: 325-328.

Guthrie, C. and Fink, G.R. 2002. *Guide to Yeast Genetics and Molecular and Cell Biology*. Academic Press, San Diego, CA.

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**: 583-592.

Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. 1996. Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature* **383**: 92-96.

Heitz, E. 1928. Das Heterochromatin der Moose. *Jb Wiss Bot* **69**: 728-818.

Henchoz, S., De Rubertis, F., Pauli, D., and Spierer, P. 1996. The dose of a putative ubiquitin-specific protease affects position-effect variegation in *Drosophila melanogaster*. *Mol Cell Biol* **16**: 5717-5725.

Hershko, A., Heller, H., Eytan, E., Kaklij, G., and Rose, I.A. 1984. Role of the alpha-amino group of protein in ubiquitin-mediated protein breakdown. *Proc Natl Acad Sci U S A* **81**: 7021-7025.

Herskowitz, I., Rine, J., and Strathern, J.N. 1992. *Mating-type determination and mating-type interconversion in Saccharomyces cerevisiae.* (ed. E.W. Jones, J.R. Pringle, and J.R. Broach). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hoffman, C.S. and Winston, F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**: 267-272.

Hoppe, G.J., Tanny, J.C., Rudner, A.D., Gerber, S.A., Danaie, S., Gygi, S.P., and Moazed, D. 2002. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol Cell Biol* **22**: 4167-4180.

Huang, J. and Moazed, D. 2003. Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev* **17**: 2162-2176.

Huang, S., Elliott, R.C., Liu, P.S., Koduri, R.K., Weickmann, J.L., Lee, J.H., Blair, L.C., Ghosh-Dastidar, P., Bradshaw, R.A., Bryan, K.M., and et al. 1987. Specificity of cotranslational amino-terminal processing of proteins in yeast. *Biochemistry* **26**: 8242-8246.

Iizuka, M. and Stillman, B. 1999. Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *J Biol Chem* **274**: 23027-23034.

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**: 795-800.

Inada, T., Winstall, E., Tarun, S.Z., Jr., Yates, J.R., 3rd, Schieltz, D., and Sachs, A.B. 2002. One-step affinity purification of the yeast ribosome and its associated proteins and mRNAs. *Rna* **8**: 948-958.

Ingram, A.K., Cross, G.A., and Horn, D. 2000. Genetic manipulation indicates that ARD1 is an essential N(infinity)-acetyltransferase in *Trypanosoma brucei*. *Mol Biochem Parasitol* **111**: 309-317.

Ishikawa, K., Ishida, H., Koyama, Y., Kawarabayasi, Y., Kawahara, J., Matsui, E., and Matsui, I. 1998. Acylamino acid-releasing enzyme from the thermophilic archaeon *Pyrococcus horikoshii*. *J Biol Chem* **273**: 17726-17731.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**: 163-168.

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

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., and Kim, K.W. 2002. Regulation and destabilization of HIF-1α by ARD1-mediated acetylation. *Cell* **111**: 709-720.

Jones, D.O., Cowell, I.G., and Singh, P.B. 2000. Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* **22**: 124-137.

Jornvall, H. 1975. Acetylation of Protein N-terminal amino groups structural observations on α-amino acetylated proteins. *J Theor Biol* **55**: 1-12.

Kaeberlein, M., McVey, M., and Guarente, L. 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev* **13**: 2570-2580.

Karpen, G.H. and Allshire, R.C. 1997. The case for epigenetic effects on centromere identity and function. *Trends Genet* **13**: 489-496.

Kayne, P.S., Kim, U.J., Han, M., Mullen, J.R., Yoshizaki, F., and Grunstein, M. 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. *Cell* **55**: 27-39.

Kennedy, B.K., Gotta, M., Sinclair, D.A., Mills, K., McNabb, D.S., Murthy, M., Pak, S.M., Laroche, T., Gasser, S.M., and Guarente, L. 1997. Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. *Cell* **89**: 381-391.

Kimura, Y., Saeki, Y., Yokosawa, H., Polevoda, B., Sherman, F., and Hirano, H. 2003. N-terminal modifications of the 19S regulatory particle subunits of the yeast proteasome. *Arch Biochem Biophys* **409**: 341-348.

Kimura, Y., Takaoka, M., Tanaka, S., Sassa, H., Tanaka, K., Polevoda, B., Sherman, F., and Hirano, H. 2000. N^{α}-acetylation and proteolytic activity of the yeast 20 S proteasome. *J Biol Chem* **275**: 4635-4639.

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

Klebe, R.J., Harriss, J.V., Sharp, Z.D., and Douglas, M.G. 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* **25**: 333-341.

Klemm, R.D. and Bell, S.P. 2001. ATP bound to the origin recognition complex is important for preRC formation. *Proc Natl Acad Sci U S A* **98**: 8361-8367.

Kobayashi, T. and Horiuchi, T. 1996. A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells* **1**: 465-474.

Krebs, J.E., Fry, C.J., Samuels, M.L., and Peterson, C.L. 2000. Global role for chromatin remodeling enzymes in mitotic gene expression. *Cell* **102**: 587-598.

Krishna, R.G. and Wold, F. 1992. Specificity determinants of acylaminoacyl-peptide hydrolase. *Protein Sci* **1**: 582-589.

Krogan, N.J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien, V., Richards, D.P., Beattie, B.K., Emili, A., Boone, C., Shilatifard, A., Buratowski, S., and Greenblatt, J. 2003. Methylation of histone H3 by Set2 in *Saccharomyces cerevisiae* is linked to transcriptional elongation by RNA polymerase II. *Mol Cell Biol* **23**: 4207-4218.

Lacan, P., Souillet, G., Aubry, M., Prome, D., Richelme-David, S., Kister, J., Wajcman, H., and Francina, A. 2002. New α2 globin chain variant with low oxygen affinity affecting the N-terminal residue and leading to N-acetylation [Hb Lyon-Bron α 1(NA1)Val --> Ac-Ala]. *Am J Hematol* **69**: 214-218.

Lachner, M. and Jenuwein, T. 2002. The many faces of histone lysine methylation. *Curr Opin Cell Biol* **14**: 286-298.

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**: 116-120.

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

Landis, G., Kelley, R., Spradling, A.C., and Tower, J. 1997. The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the *Drosophila* homolog of yeast origin recognition complex subunit 2. *Proc Natl Acad Sci U S A* **94**: 3888-3892.

Laurenson, P. and Rine, J. 1991. *SUM1-1*: a suppressor of silencing defects in *Saccharomyces cerevisiae*. *Genetics* **129**: 685-696.

Lee, D.G. and Bell, S.P. 1997. Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* **17**: 7159-7168.

Li, E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* **3**: 662-673.

Li, J.J. and Herskowitz, I. 1993. Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science* **262**: 1870-1874.

Liang, C., Weinreich, M., and Stillman, B. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81**: 667-676.

Lieb, J.D., Liu, X., Botstein, D., and Brown, P.O. 2001. Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association. *Nat Genet* **28**: 327-334.

Lin, S.J., Defossez, P.A., and Guarente, L. 2000. Requirement of NAD and *SIR2* for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* **289**: 2126-2128.

Loo, S., Fox, C.A., Rine, J., Kobayashi, R., Stillman, B., and Bell, S. 1995a. The origin recognition complex in silencing, cell cycle progression, and DNA replication. *Mol Biol Cell* **6**: 741-756.

Loo, S., Laurenson, P., Foss, M., Dillin, A., and Rine, J. 1995b. Roles of ABF1, NPL3, and YCL54 in silencing in *Saccharomyces cerevisiae*. *Genetics* **141**: 889-902.

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

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

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**: 251-260.

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**: 1528-1539.

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

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**: 4621-4630.

Mayer, A., Siegel, N.R., Schwartz, A.L., and Ciechanover, A. 1989. Degradation of proteins with acetylated amino termini by the ubiquitin system. *Science* **244**: 1480-1483.

McNally, F.J. and Rine, J. 1991. A synthetic silencer mediates *SIR*-dependent functions in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**: 5648-5659.

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**: 3169-3182.

Miyake, T., Loch, C.M., and Li, R. 2002. Identification of a multifunctional domain in autonomously replicating sequence-binding factor 1 required for transcriptional activation, DNA replication, and gene silencing. *Mol Cell Biol* **22**: 505-516.

Mizushima, T., Takahashi, N., and Stillman, B. 2000. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex *in vitro*. *Genes Dev* **14**: 1631-1641.

Moazed, D. 2001. Common themes in mechanisms of gene silencing. *Mol Cell* **8**: 489-498.

Moretti, P. and Shore, D. 2001. Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast. *Mol Cell Biol* **21**: 8082-8094.

Mullen, J.R., Kayne, P.S., Moerschell, R.P., Tsunasawa, S., Gribskov, M., Colavito-Shepanski, M., Grunstein, M., Sherman, F., and Sternglanz, R. 1989. Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. *Embo J* **8**: 2067-2075.

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

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I. 2001. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292**: 110-113.

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**: 27-43.

Neuwald, A.F. and Landsman, D. 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem Sci* **22**: 154-155.

O'Donohye, T.L., Handelmann, G.E., Miller, R.L., and Jacobowitz, D.M. 1982. N-acetylation regulates the behavioral activity of α-melanotropin in a multineurotransmitter neuron. *Science* **215**: 1125-1127.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**: 247-257.

Ouspenski, II, Elledge, S.J., and Brinkley, B.R. 1999. New yeast genes important for chromosome integrity and segregation identified by dosage effects on genome stability. *Nucleic Acids Res* **27**: 3001-3008.

Owen, D.J., Ornaghi, P., Yang, J.C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P., and Travers, A.A. 2000. The structural basis for the recognition of acetylated

histone H4 by the bromodomain of histone acetyltransferase gcn5p. *Embo J* **19**: 6141-6149.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P., and Botchan, M.R. 1997. Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**: 311-323.

Palacios DeBeer, M.A., Muller, U., and Fox, C.A. 2003. Differential DNA affinity specifies roles for the origin recognition complex in budding yeast heterochromatin. *Genes Dev* **17**: 1817-1822.

Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. 1994. The ubiquitin-proteasome pathway is required for processing the NF-κ B1 precursor protein and the activation of NF-κ B. *Cell* **78**: 773-785.

Park, E.C. and Szostak, J.W. 1992. ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. *Embo J* **11**: 2087-2093.

Park, Y., Hanish, J., and Lustig, A.J. 1998. Sir3p domains involved in the initiation of telomeric silencing in *Saccharomyces cerevisiae*. *Genetics* **150**: 977-986.

Park, Y. and Kuroda, M.I. 2001. Epigenetic aspects of X-chromosome dosage compensation. *Science* **293**: 1083-1085.

Parnell, T.J., Viering, M.M., Skjesol, A., Helou, C., Kuhn, E.J., and Geyer, P.K. 2003. An endogenous suppressor of hairy-wing insulator separates regulatory domains in *Drosophila*. *Proc Natl Acad Sci U S A* **100**: 13436-13441.

Pasero, P., Bensimon, A., and Schwob, E. 2002. Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus. *Genes Dev* **16**: 2479-2484.

Perrod, S. and Gasser, S.M. 2003. Long-range silencing and position effects at telomeres and centromeres: parallels and differences. *Cell Mol Life Sci* **60**: 2303-2318.

Pesaresi, P., Gardner, N.A., Masiero, S., Dietzmann, A., Eichacker, L., Wickner, R., Salamini, F., and Leister, D. 2003. Cytoplasmic N-terminal protein acetylation is required for efficient photosynthesis in *Arabidopsis*. *Plant Cell* **15**: 1817-1832.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. 2001. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**: 323-337.

Pfund, C., Lopez-Hoyo, N., Ziegelhoffer, T., Schilke, B.A., Lopez-Buesa, P., Walter, W.A., Wiedmann, M., and Craig, E.A. 1998. The molecular chaperone Ssb from

Saccharomyces cerevisiae is a component of the ribosome-nascent chain complex. *Embo J* **17**: 3981-3989.

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

Plath, K., Mlynarczyk-Evans, S., Nusinow, D.A., and Panning, B. 2002. Xist RNA and the mechanism of X chromosome inactivation. *Annu Rev Genet* **36**: 233-278.

Polevoda, B., Cardillo, T.S., Doyle, T.C., Bedi, G.S., and Sherman, F. 2003. Nat3p and Mdm20p are required for function of yeast NatB N^{α}-terminal acetyltransferase and of actin and tropomyosin. *J Biol Chem* **278**: 30686-30697.

Polevoda, B., Norbeck, J., Takakura, H., Blomberg, A., and Sherman, F. 1999. Identification and specificities of N-terminal acetyltransferases from *Saccharomyces cerevisiae*. *Embo J* **18**: 6155-6168.

Polevoda, B. and Sherman, F. 2001. NatC N^{α}-terminal acetyltransferase of yeast contains three subunits, Mak3p, Mak10p, and Mak31p. *J Biol Chem* **276**: 20154-20159.

-. 2003a. Composition and function of the eukaryotic N-terminal acetyltransferase subunits. *Biochem Biophys Res Commun* **308**: 1-11.

-. 2003b. N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J Mol Biol* **325**: 595-622.

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

Ray, A., Hector, R.E., Roy, N., Song, J.H., Berkner, K.L., and Runge, K.W. 2003. Sir3p phosphorylation by the Slt2p pathway effects redistribution of silencing function and shortened lifespan. *Nat Genet* **33**: 522-526.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**: 593-599.

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**: 1133-1145.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17**: 1030-1032.

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

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**: 521-529.

Rudman, D., Hollins, B.M., Kutner, M.H., Moffitt, S.D., and Lynn, M.J. 1983. Three types of alpha-melanocyte-stimulating hormone: bioactivities and half-lives. *Am J Physiol* **245**: E47-54.

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

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

Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D., and Dutta, A. 1998. Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol Cell Biol* **18**: 2758-2767.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual* (ed. C. Nolan). Cold Spring Habor Laboratory Press, New York.

Sandell, L.L. and Zakian, V.A. 1993. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* **75**: 729-739.

Sekinger, E.A. and Gross, D.S. 2001. Silenced chromatin is permissive to activator binding and PIC recruitment. *Cell* **105**: 403-414.

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**: 35-45.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**: 850-858.

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* **97**: 233-244.

Siddig, M.A., Kinsey, J.A., Fincham, J.R., and Keighren, M. 1980. Frameshift mutations affecting the N-terminal sequence of Neurospora NADP-specific glutamate dehydrogenase. *J Mol Biol* **137**: 125-135.

Sinclair, D.A. and Guarente, L. 1997. Extrachromosomal rDNA circles--a cause of aging in yeast. *Cell* **91**: 1033-1042.

Singer, J.M. and Shaw, J.M. 2003. Mdm20 protein functions with Nat3 protein to acetylate Tpm1 protein and regulate tropomyosin-actin interactions in budding yeast. *Proc Natl Acad Sci U S A* **100**: 7644-7649.

Singh, H., Sekinger, E.A., and Gross, D.S. 2000. Chromatin and cancer: causes and consequences. *J Cell Biochem Suppl* **Suppl**: 61-68.

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

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

Smith, J.S., Caputo, E., and Boeke, J.D. 1999. A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. *Mol Cell Biol* **19**: 3184-3197.

Smyth, D.G., Massey, D.E., Zakarian, S., and Finnie, M.D. 1979. Endorphins are stored in biologically active and inactive forms: isolation of α-N-acetyl peptides. *Nature* **279**: 252-254.

Song, O.K., Wang, X., Waterborg, J.H., and Sternglanz, R. 2003. A N^{α}-acetyltransferase responsible for acetylation of the N-terminal residues of histones H4 and H2A. *J Biol Chem* **278**: 38109-38112.

Soutoglou, E., Katrakili, N., and Talianidis, I. 2000. Acetylation regulates transcription factor activity at multiple levels. *Mol Cell* **5**: 745-751.

Stevenson, B.J., Ferguson, B., De Virgilio, C., Bi, E., Pringle, J.R., Ammerer, G., and Sprague, G.F., Jr. 1995. Mutation of RGA1, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*. *Genes Dev* **9**: 2949-2963.

Stevenson, J.B. and Gottschling, D.E. 1999. Telomeric chromatin modulates replication timing near chromosome ends. *Genes Dev* **13**: 146-151.

Stone, E.M. and Pillus, L. 1996. Activation of an MAP kinase cascade leads to Sir3p hyperphosphorylation and strengthens transcriptional silencing. *J Cell Biol* **135**: 571-583.

Stone, E.M., Reifsnyder, C., McVey, M., Gazo, B., and Pillus, L. 2000. Two classes of *sir3* mutants enhance the *sir1* mutant mating defect and abolish telomeric silencing in *Saccharomyces cerevisiae*. *Genetics* **155**: 509-522.

Stone, E.M., Swanson, M.J., Romeo, A.M., Hicks, J.B., and Sternglanz, R. 1991. The *SIR1* gene of *Saccharomyces cerevisiae* and its role as an extragenic suppressor of several mating-defective mutants. *Mol Cell Biol* **11**: 2253-2262.

Strahl, B.D. and Allis, C.D. 2000. The language of covalent histone modifications. *Nature* **403**: 41-45.

Strahl, B.D., Grant, P.A., Briggs, S.D., Sun, Z.W., Bone, J.R., Caldwell, J.A., Mollah, S., Cook, R.G., Shabanowitz, J., Hunt, D.F., and Allis, C.D. 2002. Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. *Mol Cell Biol* **22**: 1298-1306.

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

Strathern, J.N., Klar, A.J., Hicks, J.B., Abraham, J.A., Ivy, J.M., Nasmyth, K.A., and McGill, C. 1982. Homothallic switching of yeast mating type cassettes is initiated by a double-stranded cut in the *MAT* locus. *Cell* **31**: 183-192.

Sugiura, N., Patel, R.G., and Corriveau, R.A. 2001. N-methyl-D-aspartate receptors regulate a group of transiently expressed genes in the developing brain. *J Biol Chem* **276**: 14257-14263.

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

Sun, Z.W. and Allis, C.D. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418**: 104-108.

Sussel, L., Vannier, D., and Shore, D. 1993. Epigenetic switching of transcriptional states: *cis*- and *trans*-acting factors affecting establishment of silencing at the *HMR* locus in *Saccharomyces cerevisiae*. *Mol Cell Biol* **13**: 3919-3928.

Sutton, A., Heller, R.C., Landry, J., Choy, J.S., Sirko, A., and Sternglanz, R. 2001. A novel form of transcriptional silencing by *Sum1-1* requires Hst1 and the origin recognition complex. *Mol Cell Biol* **21**: 3514-3522.

Tamaru, H., Zhang, X., McMillen, D., Singh, P.B., Nakayama, J., Grewal, S.I., Allis, C.D., Cheng, X., and Selker, E.U. 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. *Nat Genet* **34**: 75-79.

Tanaka, S., Matsushita, Y., Yoshikawa, A., and Isono, K. 1989. Cloning and molecular characterization of the gene rimL which encodes an enzyme acetylating ribosomal protein L12 of *Escherichia coli* K12. *Mol Gen Genet* **217**: 289-293.

Tanaka, T., Knapp, D., and Nasmyth, K. 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**: 649-660.

Tercero, J.C. and Wickner, R.B. 1992. *MAK3* encodes an N-acetyltransferase whose modification of the L-A gag NH2 terminus is necessary for virus particle assembly. *J Biol Chem* **267**: 20277-20281.

Tham, W.H., Wyithe, J.S., Ko Ferrigno, P., Silver, P.A., and Zakian, V.A. 2001. Localization of yeast telomeres to the nuclear periphery is separable from transcriptional repression and telomere stability functions. *Mol Cell* **8**: 189-199.

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

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G., and Reuter, G. 1994. The protein encoded by the *Drosophila* position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. *Embo J* **13**: 3822-3831.

Urbancikova, M. and Hitchcock-DeGregori, S.E. 1994. Requirement of amino-terminal modification for striated muscle α-tropomyosin function. *J Biol Chem* **269**: 24310-24315.

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**: 149-159.

Vega-Palas, M.A., Venditti, S., and Di Mauro, E. 1997. Telomeric transcriptional silencing in a natural context. *Nat Genet* **15**: 232-233.

Venditti, S., Vega-Palas, M.A., and Di Mauro, E. 1999. Heterochromatin organization of a natural yeast telomere. Recruitment of Sir3p through interaction with histone H4 N terminus is required for the establishment of repressive structures. *J Biol Chem* **274**: 1928-1933.

Vorm, O., Roepstorff, P., and Mann, M. 1994. Improved Resolution and Very High Sensitivity in MALDI TOF of Matrix Surfaces Made by Fast Evaporation. *Anal. Chem.* **66**: 3281-3287.

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**: 1793-1808.

Wakimoto, B.T. 1998. Beyond the nucleosome: epigenetic aspects of position-effect variegation in *Drosophila*. *Cell* **93**: 321-324.

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

Warner, J.R. 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. *Microbiol Rev* **53**: 256-271.

Whitehouse, I., Flaus, A., Cairns, B.R., White, M.F., Workman, J.L., and Owen-Hughes, T. 1999. Nucleosome mobilization catalysed by the yeast SWI/SNF complex. *Nature* **400**: 784-787.

Whiteway, M. and Szostak, J.W. 1985. The *ARD1* gene of yeast functions in the switch between the mitotic cell cycle and alternative developmental pathways. *Cell* **43**: 483-492.

Wichman, H.A., Van den Bussche, R.A., Hamilton, M.J., and Baker, R.J. 1992. Transposable elements and the evolution of genome organization in mammals. *Genetica* **86**: 287-293.

Willis, D.M., Loewy, A.P., Charlton-Kachigian, N., Shao, J.S., Ornitz, D.M., and Towler, D.A. 2002. Regulation of osteocalcin gene expression by a novel Ku antigen transcription factor complex. *J Biol Chem* **277**: 37280-37291.

Workman, J.L. and Kingston, R.E. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* **67**: 545-579.

Xie, J., Pierce, M., Gailus-Durner, V., Wagner, M., Winter, E., and Vershon, A.K. 1999. Sum1 and Hst1 repress middle sporulation-specific gene expression during mitosis in *Saccharomyces cerevisiae*. *Embo J* **18**: 6448-6454.

Yamamoto, K. and Sonoda, M. 2003. Self-interaction of heterochromatin protein 1 is required for direct binding to histone methyltransferase, SUV39H1. *Biochem Biophys Res Commun* **301**: 287-292.

Yamauchi, Y., Ejiri, Y., Toyoda, Y., and Tanaka, K. 2003. Identification and biochemical characterization of plant acylamino acid-releasing enzyme. *J Biochem (Tokyo)* **134**: 251-257.

Yu, Y. and Hirsch, J.P. 1995. An essential gene pair in *Saccharomyces cerevisiae* with a potential role in mating. *DNA Cell Biol* **14**: 411-418.

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

Abbreviations

aa	amino acid
a.i.	arbitrary intensity
ACS	ARS consensus sequence
ARS	autonomous replication sequence
BAH	bromo-adjacent homology
bp	base pair
ChIP	Chromatin Immunoprecipitation
Co-IP	Co-Immunoprecipitation
Da	Dalton
$Gal4_{AD}$	Gal4 activation domain
$Gal4_{BD}$	Gal4 binding domain
HA	hemagglutinin A
HAT	histone acetyltransferase
HDAC	histone deacetylase
HM	homothallic mating (*HML* und *HMR*)
HML	homothallic mating left
HMR	homothallic mating right
HDAC	histone methyltransferase
IEF	isoelectric focusing
IgG	Immune globulin G
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MAT	mating-type locus
NAT	N-terminal acetyltransferase
OD	optical density
ORC	origin recognition complex
ORF	open reading frame
PEV	position effect variegation
p*I*	isoelectric point
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
Sir	silent information regulator
TAP	tandem affinity purification
TPE	telomere position effect
ts	temperature sensitive
WT	wild-type

Curriculum vitae

Antje Geißenhöner

Date of Birth: 12.01.1969

Place of Birth: Suhl

University education

2000 – 2004	Doktorarbeit (PhD thesis project) *The role of the N-terminal acetyltransferase NatA in transcriptional silencing in Saccharomyces cerevisiae* MPI for Molecular Genetics, Berlin Research group of Dr. Ann Ehrenhofer-Murray
1999 – 2000	Diplomarbeit (Graduate thesis project) *Molecular analysis of the digA mutant in Aspergillus nidulans* MPI for Terrestrial Microbiology, Marburg Research group of Dr. Reinhard Fischer
1993 – 2000	Undergraduate studies in biology Philipps Universität Marburg
1996 – 1997	Academic year at the University of Edinburgh, Scotland

Vocational training and occupation

1988 – 1993	Employed as a nurse Klinikum Suhl
1985 – 1988	Vocational training to become a nurse Medizinische Fachschule Suhl

Education

1991 – 1993	Abitur course (A-level) Abendschule an der VHS Suhl
1975 – 1985	primary and secondary schooling Polytechnische Oberschule Erlau

Publications

Geissenhoener, A., Weise, C., and Ehrenhofer-Murray, A.E. 2004. Regulation of ORC silencing function by NatA-dependent N^{α}-acetylation in *Saccharomyces cerevisiae*. *submitted*

Geissenhoner A., Sievers N., Brock M., and Fischer R. 2001. *Aspergillus nidulans* DigA, a potential homolog of *Saccharomyces cerevisiae* Pep3 (Vps18), is required for nuclear migration, mitochondrial morphology and polarized growth. *Mol Genet Genomics* **266:** 672-85.

Poster Abstract

Geissenhoener, A. and Ehrenhofer-Murray, A.E. 2001.
The role of the N-terminal acetyltransferase Nat1/Ard1 in transcriptional silencing in *Saccharomyces cerevisiae*.
C.N.R.S. conference Jaques Monod: Signaling and control of transcription.
June 2001, Aussois, France.

Scientific Talk

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Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 19.06.2002 ist mir bekannt.

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Antje Geißenhöner