Contribution of histones to nucleosome stability, DNA accessibility, and higher order chromatin structures in yeast

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Contribution of Histones to Nucleosome Stability, DNA Accessibility, and Higher Order Chromatin Structures in Yeast

A dissertation submitted to
ETH ZURICH

For the degree of
Doctor of Sciences

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2007
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## Abbreviations

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<tr>
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>$A_{600}$</td>
<td>absorbance at a wavelength of 600 nm</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom, $10^{-10}$ metres</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CPD</td>
<td>$cis$-$syn$ cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>CPT</td>
<td>camptothecin</td>
</tr>
<tr>
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<td>cytosine ribonucleotide triphosphate</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>DNA double-strand break</td>
</tr>
<tr>
<td>DTE</td>
<td>1,4-dithioerythritol</td>
</tr>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>E. coli</td>
<td>eubacterium <em>Escherichia coli</em></td>
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<td>EDTA</td>
<td>ethylene diamine tetra acetate</td>
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<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethyl)-N,N',N'-tetraacetic acid</td>
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<td>ethidium bromide</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>F</td>
<td>farad, the SI unit of capacitance</td>
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<tr>
<td>G</td>
<td>guanine</td>
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<tr>
<td>GTP</td>
<td>guanosine ribonucleotide triphosphate</td>
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<tr>
<td>GuHCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>HCl</td>
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<td>HTO1/HTA2</td>
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<td>HHF1/HHF2</td>
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<td>histone H1 homologue gene</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>KCl</td>
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</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
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</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LiOAc</td>
<td>lithium acetate</td>
</tr>
<tr>
<td>lrs</td>
<td>loss of rDNA silencing (histone mutation)</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>m</td>
<td>milli, $10^{-3}$</td>
</tr>
<tr>
<td>µ</td>
<td>micro, $10^{-6}$</td>
</tr>
<tr>
<td>mA</td>
<td>milli ampère</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methanesulfonate</td>
</tr>
<tr>
<td>MNase</td>
<td>micrococcal nuclease</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholino propanesulfonic acid</td>
</tr>
<tr>
<td>n</td>
<td>nano, $10^{-9}$</td>
</tr>
<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NCP</td>
<td>nucleosome core particle</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>Ω</td>
<td>ohm, the SI unit of electrical impedance or resistance</td>
</tr>
<tr>
<td>p</td>
<td>pico, $10^{-12}$</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pH</td>
<td>$-\log[H_3O^+]$</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethane sulfonyl fluoride</td>
</tr>
<tr>
<td>PR</td>
<td>photoreactivation</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase A</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>budding yeast Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SD</td>
<td>synthetic dextrose</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHL</td>
<td>superhelix location</td>
</tr>
<tr>
<td>sin</td>
<td>switch-independent (histone mutation)</td>
</tr>
<tr>
<td>Sir proteins</td>
<td>silent information regulator proteins (Sir1p, Sir2, Sir3, and Sir4p)</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine ribonucleotide triphosphate</td>
</tr>
<tr>
<td>U</td>
<td>units, defined according to enzymatic reaction</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activation sequence</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>(6-4) PP</td>
<td>pyrimidine (6-4)-pyrimidone photoproduct</td>
</tr>
<tr>
<td>V</td>
<td>volt, the SI derived unit of electric potential difference</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast growth medium containing glucose</td>
</tr>
<tr>
<td>X. laevis</td>
<td>African clawed frog Xenopus laevis</td>
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Summary

In the nucleus of eukaryotic cells the genome is packaged into chromatin. Chromatin regulates DNA dependent processes, including transcription, replication, recombination, and DNA repair. The structure of chromatin is based on repeating nucleosomes, octamers of the highly conserved histone proteins H2A, H2B, H3, and H4, around which DNA is wrapped in approximately two turns. Nucleosomes line up along the DNA in nucleosomal arrays and associate with linker histones and non-histone proteins, forming higher order chromatin structures. Chromatin structure is modulated by posttranslational modifications of histones, chromatin remodelling activities, and the binding of non-histone proteins, which affects DNA accessibility to regulatory proteins.

Phosphorylation of yeast histone H2A (in mammals H2A.X) at the C-terminal tail plays a role in DNA double-strand break repair. Mimicking H2A phosphorylation in yeast by replacement of serine 129 with glutamic acid (hta1-S129E) suggested that phosphorylation destabilizes chromatin structures and thereby facilitates access of repair proteins. The C-terminal tail of H2A was proposed to contact the linker histone binding site on the nucleosome and could thereby affect higher order chromatin structures. In S. cerevisiae Hho1p is a candidate linker histone, and it suppresses homologous recombination. Here, we have tested chromatin structures in hta1-S129 mutants, in C-terminal tail deletion strains, and in a HHO1 deletion strain. We show that the hta1-S129E affects neither nucleosome positioning in minichromosomes and genomic loci nor supercoiling of minichromosomes. Moreover, hta1-S129E has no effect on chromatin stability measured by conventional nuclease digestion nor does it affect DNA accessibility and repair of UV-induced DNA lesions by nucleotide excision repair and photolyase. Similarly, deletion of the C-terminal tail or HHO1 has no effect on nucleosome positioning and stability. These data argue against a general role of the C-terminal tail in organizing chromatin structure and suggest that phosphorylated H2A acts by recruitment of repair components and not by disruption of chromatin.

Histone H3 and H4 mutations that affect residues, which cluster in a discrete region on the surface of the (H3·H4)$_2$ tetramer around lysine 79 of H3, impair transcriptional silencing. Lysine 79 of H3 is methylated by the conserved histone methyltransferase Dot1p, and deletion of DOT1 also affects silencing. The nucleosome surface around lysine 79 of H3 has therefore been suggested to be involved in nucleosome-nucleosome contacts or in the interaction of nucleosomes with silencing proteins. In a second part of this thesis we analysed nucleosome positioning, stability, and nucleosome-nucleosome contacts on a minichromosome and a subtelomeric URA3 gene in H3 mutants affecting silencing. Loss of subtelomeric URA3 silencing seems to be caused by a shift of a nucleosome at the promoter. However, these mutations show normal nucleosome positioning and stability on minichromosomes. Thus, these H3 mutations rather impair the recruitment and/or spreading of silencing proteins, than disturb nucleosome-nucleosome contacts.
Zusammenfassung


Mutationen der Histone H3 und H4, die Aminosäuren betreffen, welche in einer Oberflächenregion des (H3·H4)_2 Tetramers um Lysin 79 von H3 angehäuft sind, mindern das Silencing von Genen. Lysin 79 von H3 wird durch die konservierte Histon-Methyltransferase Dot1p methyliert und die Deletion von DOT1 hat auch einen Einfluss auf das Silencing. Aus diesem Grund wurde vorgeschlagen, dass die Oberfläche des Nukleosomal um Lysin 79 von H3 herum an Nukleosom-
Zusammenfassung

Nukleosom-Kontakten oder an der Interaktion von Nukleosomen mit Silencing-Proteinen beteiligt ist.


1 Introduction

The first chapter of this thesis provides an introduction to general concepts of chromatin structures and function. The chapters 2 and 3 deal with specific topics of this work and they contain introductions focused on theses topics in separate sections (2.1 and 3.1).

1.1 Overview of chromatin structure

The term “chromatin” was already proposed at the end of the 19th century by Walther Flemming. He developed methods that allowed him to recognize a fibrous scaffold in the nucleus of mammalian cells, which could easily be stained with basic dyes (Flemming, 1882). Chromatin is the substance of the genetic material in the nucleus of eukaryotic cells; it builds up chromosomes and consists of nucleic acids and a wide variety of proteins, most of which are histones (Felsenfeld and Groudine, 2003). Chromatin packages the long eukaryotic genomic DNA, which is in the range of several centimetres to meters, into the small nucleus that is of some micrometers in diameter (Ball, 2003). The structure of chromatin has a hierarchical organization (Woodcock and Dimitrov, 2001). On the lowest level the structure is based on repeating units, the nucleosomes. The nucleosome is composed of a 160–200 bp long DNA segment that is wrapped in approximately two turns around a disc-shaped octamer of two each of the core histone proteins H2A, H2B, H3 and H4 (Kornberg and Thomas, 1974). Intense digestion of chromatin with micrococcal nuclease (MNase) releases a particle containing the four core histones and about 146 bp of DNA, the nucleosome core particle. Stretches of linker DNA that are variable in length (~10–90 bp) connect nucleosome core particles.

At least in higher eukaryotes, a fifth histone, H1 (also called “linker histone”), protects additional 20 bp of DNA beyond the core particle from limited MNase digestion. Linker histones bind to nucleosomes near the entry and exit site of DNA, but the precise structural role remains to be determined (Robinson and Rhodes, 2006).

Beyond the string of nucleosomes chromatin is further organized into higher order structures. At low ionic strength chromatin appears as a zigzag structure, referred to as nucleosome filament. At higher ionic strengths, and particularly in the presence of divalent cations, the nucleosome filament folds into a more compact fibre with a diameter of about 30 nm. Linker histones can interact with the ends of nucleosomal DNA and stabilize the 30-nm fibres. Although 30-nm fibres are consistent with low-angle X-ray diffraction and electron micrographs their structure and existence remains elusive in living cells (Tremethick, 2007).

Further compaction of chromatin in interphase nuclei and metaphase chromosomes is poorly understood. Chromatin loops possibly attach to the nuclear lamina and chromosome scaffolds, organizing chromosomal domains (Belmont, 2006). Nuclei are organized in compartments, including regions occupied by individual chromosomes (chromosome territories) and
Introduction

The interchromatin space harbouring functional activities like gene transcription, replication, and DNA repair (Meaburn and Misteli, 2007). In addition, chromosomal regions are packaged in chromatin with distinct structural features, which, based on early microscopic observations of stained nuclei, have been classified into heterochromatin and euchromatin. Heterochromatin constitutes a condensed structure and is associated with gene-poor regions including telomeric and pericentric chromosomal regions. Euchromatin is less condensed and contains more loci of transcriptionally active genes. Heterochromatin also exerts repression of “euchromatic genes” that were inserted into heterochromatic regions, a process referred to as transcriptional silencing. A characteristic feature of heterochromatin is its ability to propagate, and thereby influence gene expression in a region-specific, sequence-independent manner (Grewal and Jia, 2007).

Chromatin is heterogeneous and dynamic and its structure is regulated by a wide range of modifications including posttranslational modifications of histones, ATP-driven chromatin remodelling, and incorporation of histone variants into nucleosomes (Eberharter et al., 2005). Distinct histone variants create specialized nucleosomes functioning in chromosome segregation, transcription, and DNA repair (Sarma and Reinberg, 2005). Chromatin remodelling shifts nucleosomes or ejects histones from DNA templates, regulating the accessibility of DNA (Saha et al., 2006). Histone modifications can serve as signals for the binding of non-histone proteins, can regulate chromatin compaction and affect nucleosome stability (Mersfelder and Parthun, 2006; Nightingale et al., 2006). Combinations of specific histone modifications correlate with gene transcription or DNA repair (Jenuwein and Allis, 2001; Strahl and Allis, 2000).

Yeast is an excellent model organism for the study of histone function, since, in contrast to the multicopy histone genes of higher eukaryotes, the yeast genome carries only two copies each of the major core histone genes. The ease of genetic manipulation of budding yeast allows the introduction of histone mutations and their combination with mutations in chromatin associated factors. Much of the insight into the roles of the core histones and their posttranslational modifications on chromatin structure and function has been gained from investigations with S. cerevisiae (Mersfelder and Parthun, 2006).

1.2 The core histones

The core histones H2A, H2B, H3, and H4 are abundant, small, and highly basic proteins, ranging between 11 and 16 kDa (Malik and Henikoff, 2003). Core histones are among the most conserved proteins, also present in archaeabacteria (Starich et al., 1996), indicating that sequence, structure, and function are highly interdependent in these proteins (Sullivan and Landsman, 2003). The four core histones share a common structural motif, termed the histone fold (Arents et al., 1991; Arents and Moudrianakis, 1995). The histone fold consists of a long central α-helix, flanked on either side by a loop and a short α-helix. The helices and loops are denoted, from their N-
terminus to their C-terminus, as α1, L1, α2, L2, α3. Apart from the central histone fold region, histones H2A and H3 contain an additional α-helix in the N-terminal region (αN), whereas histone H2B contains an additional α-helix in the C-terminal region (αC) of the histone fold (Ramakrishnan, 1997). Interestingly, the histone fold motif is also found in some transcriptional coactivators and repressors (Burley et al., 1997). Beyond the histone fold, all core histones have a lysine rich N-terminal tail and H2A is the only core histone that also possesses a canonical C-terminal tail (Hansen, 2002).

H2A and H3 have several variants generally distinguished from the canonical histones by (i) their time of expression and incorporation into chromatin, (ii) the structure (primary structure, N- or C-terminal extensions or truncations), and (iii) their specific function in transcription, DNA repair, or chromosome segregation (Henikoff and Ahmad, 2005). The H2A variants H2A.Z and H2A.X have been found in most eukaryotes, whereas macroH2A and H2A.Bbd (bar-body deficient) seem to be vertebrate-specific (Chadwick and Willard, 2001; Pehrson and Fried, 1992). Yeast has no canonical histone H2A, its major H2A rather belongs to the H2A.X variant of higher eukaryotes (Mannironi et al., 1989). H2A.X variants do not differ substantially from H2A except for a C-terminal extension, that contains a serine four residues from the C terminus in an invariant SQ motif, which is important for DNA double-strand break repair (Redon et al., 2002; Stucki and Jackson, 2006). The variant H2A.Z is highly conserved from yeast to vertebrates (>90% sequence identity among different organisms), but shares only ~60% sequence identity with H2A in yeast (Jin et al., 2005; Raisner and Madhani, 2006; Redon et al., 2002). Higher eukaryotes have H3 isoforms that differ only in a few amino acids from each other (Hake and Allis, 2006). H3.3 is considered to be a variant, since it is constitutively expressed and incorporated into chromatin in a replication-independent manner (Henikoff and Ahmad, 2005). In S. cerevisiae the major H3 is a H3.3 version, though its expression is highly cell cycle regulated and it is incorporated into the genome in a replication-dependent manner (Malik and Henikoff, 2003). In addition all eukaryotes express a centromere-specific H3 variant, in S. cerevisiae Cse4p (for chromosome segregation) (Henikoff and Dalal, 2005; Smith, 2002). Centromere-specific H3 variants are evolutionarily well conserved in their globular core region, which is similar to that of canonical H3, but not in their extended N-terminal tails (Sarma and Reinberg, 2005).

H2B has testis-specific variants whose functions have not yet been determined (Churikov et al., 2004; Zalensky et al., 2002). For histone H4 no variants have been reported so far (Sarma and Reinberg, 2005).

1.2.1 The core histone genes

In higher eukaryotes, the major histones are encoded from clustered, multicopy genes, whose expression is tightly cell cycle regulated and coupled to DNA replication (Hentschel
and Birnstiel, 1981). The histone variants are mostly encoded as single copy genes and their expression is variably regulated (Pusarla and Bhargava, 2005). In contrast to higher eukaryotes, the \textit{S. cerevisiae} genome contains only four loci encoding the major core histones. Each locus consists of a set of two genes that are divergently transcribed from a central promoter (Osley, 1991). Two of these loci, \textit{HTA1-HTB1} and \textit{HTA2-HTB2}, carry genes for H2A (Histone H Two A) and H2B (Histone H Two B) (Smith and Andresson, 1983). The others, \textit{HHT1-HHF1} and \textit{HHT2-HHF2}, carry genes for H3 (Histone H Three) and H4 (Histone H Four) (Hereford et al., 1979). Identical proteins are encoded by the two gene pairs \textit{HHF-HHT} for H3 and H4, respectively (Cherry et al., 1997). In contrast, the two \textit{HTA-HTB} loci encode for slightly different H2A and H2B isoforms (Cherry et al., 1997).

Although genes encoding the major histones are essential for growth, deletion strains with either one gene set are viable (Clark-Adams et al., 1988; Cross and Smith, 1988; Kayne et al., 1988; Norris and Osley, 1987; Smith and Stirling, 1988). The \textit{(hta2-HTB2)}\textsc{Δ} mutant has been shown to grow normally, owing to increased transcription of the \textit{HTA1-HTB1} pair (Moran et al., 1990; Norris and Osley, 1987). In contrast, \textit{(hta1-HTB1)}\textsc{Δ} mutants have several mutant phenotypes, including defects in transcription and chromatin structure (Clark-Adams et al., 1988; Hirschhorn et al., 1992; Norris et al., 1988; Norris and Osley, 1987). In addition, they amplify the \textit{HTA2-HTB2} locus to dosage-compensate for H2A and H2B (Libuda and Winston, 2006). Haploid strains with deletions of either \textit{HHT-HHF} gene set show no obvious phenotypes (Smith and Stirling, 1988). Normally, the mRNAs from the \textit{HHT2-HHF2} represent about 80% of the total H3 and H4 transcripts. Remarkably, \textit{(hta2-HTB2)}\textsc{Δ} mutants seem not to increase \textit{HHT1-HHF1} transcription (Cross and Smith, 1988).

Deletion of the gene encoding the centromer-specific histone H3 variant, \textit{CSE4}, is lethal (Meluh et al., 1998; Stoler et al., 1995). Deletion of \textit{HTZ1}, the only H2A variant in yeast, allows viability but produces a slow-growth phenotype (Adam et al., 2001; Jackson et al., 1996; Jackson and Gorovsky, 2000). Htz1p and major H2A cannot substitute for each other, indicating that these histones have distinct important functions (Jackson and Gorovsky, 2000).

\subsection*{1.2.2 Core histone assembly into nucleosomes}

In \textit{S. cerevisiae} all histones are expressed during S phase of the cell cycle (Cho et al., 1998; Spellman et al., 1998). Whereas production of Htz1p is only partially restricted to this phase, the replication-dependent expression of the major histone is strictly controlled on multiple levels, including transcription, pre-mRNA processing, mRNA stability, and histone protein degradation (Gunjan et al., 2005; Gunjan et al., 2006). Histones and DNA tend to aggregate when they are mixed at physiological ionic strength \textit{in vitro} and a histone excess can be toxic (Gunjan et al., 2006; Gunjan and Verreault, 2003). In the cell histones occur in complexes with DNA or histone chaperones (Gunjan et al., 2005; Verreault, 2000). Newly synthesized histones

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are escorted by histone chaperones and delivered to sites of nucleosome formation (Loyola and Almouzni, 2004), where they are assembled into nucleosomes with the aid of chromatin assembly factors (Polo and Almouzni, 2006). Chromatin assembly factors and histone chaperons are not only important for de novo deposition of nucleosomes behind the replication fork, but also for nucleosome disassembly/reassembly during transcription, recombination, and DNA repair (Mello and Almouzni, 2001; Polo and Almouzni, 2006).

1.3 Structure of the nucleosome core particle

The nucleosome core particle (NCP) is a highly conserved nucleoprotein complex, consisting of ~146 bp of DNA and an octamer formed by two each of the histone proteins, H2A, H3B, H3 and H4 (Luger et al., 1997a). It has a mass of ~206 kDa that is equally distributed between the DNA and the histones (Richmond et al., 1984). A variety of X-ray crystal structures of the NCP have been obtained at high resolution (Chakravarthy et al., 2005b; Luger, 2003; Luger, 2006; Luger and Hansen, 2005). Most NCPs were reconstituted from a palindrome DNA sequence derived form a 73 bp unit of a human α-satellite sequence. The NCPs used for crystallization and structure determinations include nucleosomes reconstituted with recombinant histones from different species (Luger et al., 1997a; Tsunaka et al., 2005; White et al., 2001), recombinant histones containing mutations (sin mutant histones) (Muthurajan et al., 2004), recombinant histone H2A variants (Chakravarthy et al., 2005a; Suto et al., 2000), and histones purified from chicken erythrocyte nuclei (Chantalat et al., 2003; Harp et al., 2000). Other structures were obtained from NCP crystals with a different α-satellite DNA sequence (Davey et al., 2002), with a DNA containing a poly(dA·dT) element (Bao et al., 2006), and from NCP in a complex with a minor groove DNA-binding ligand (Suto et al., 2003). A common feature of these structure determinations is that the overall structure of the NCP is highly conserved, with changes in histone sequence resulting in subtle modulations of histone-histone and/or histone-DNA interactions (Luger, 2006). For instance, the yeast histones are among the most divergent from metazoans histones (Baxevanis and Landsman, 1998), with amino acid changes distributed throughout the proteins (White et al., 2001). Between X. laevis and S. cerevisiae H2A and H2B are more divergent (72 and 67% identity) than H3 and H4 (84 and 92% identity) (White et al., 2001). Despite of these histone sequence divergences, the overall architecture of the histone octamer, as well as all of the residues that are involved in direct protein-DNA interactions are essentially unchanged between S. cerevisiae and X. laevis NCPs (White et al., 2001). However, significant differences were observed which might be relevant for nucleosome stability as well as for nucleosome-nucleosome interactions in vivo (see below).

The crystal structures of NCPs generally have a pseudo-twofold rotational symmetry (Harp et al., 2000; Luger and Richmond, 1998a; Ramakrishnan, 1997). The octamer shows a tripartite architecture of a (H3-H4)₂ tetramer, which binds to the central 60 bp of DNA, and two H2A-H2B
dimers organizing 30 bp toward either end of nucleosomal DNA (Arents et al., 1991; Luger et al., 1997a). The histones have α-helical histone fold domains inside of the particle and disordered N-terminal tails that pass between and over the gyres of the DNA superhelix (Luger et al., 1997a). The 146-bp α-satellite DNA palindrome forms a left-handed superhelix of 1.65 turns around the histone octamer complex (Luger et al., 1997a). DNA binding to the octamer places a central base pair to the particle’s pseudodyad axis leaving two segments of unequal length on both halves of the particle (Luger et al., 1997a). The organization of the DNA in the NCP is defined relative to the central bp, where the major groove faces the octamer, as superhelix location 0 (SHL 0). For each successive helical turn the location number increases by an integer up to SHL +7 on one half and decreases to SHL –7 on the other half of the NCP (Luger et al., 1997a).

1.3.1 Structure of the histone octamer in the NCP

The principal structural unit for protein-protein and protein-DNA interactions in the NCP are the four histone heterodimers, two each of H2A·H2B and H3·H4, which are stabilized by the interaction of the histone folds (Arents and Moudrianakis, 1995). The three connected α-helices of the paired histones intertwine in a head-to-tail manner in a so-called handshake motif (Arents et al., 1991). Their antiparallel arrangement places the L1 loop of one histone in juxtaposition with the L2 loop of the other, a configuration designated as L1L2 (Arents and Moudrianakis, 1995). The C-terminal halves of the H3 histone folds interact together in the (H3·H4)2 tetramer through the formation of a four-helix bundle (Arents et al., 1991). The same motifs in H4 of the tetramer and in H2B of the H2A-H2B dimers associate to form the octamer (Arents and Moudrianakis, 1995). Additional stabilization of the dimer-tetramer interaction is provided by the α3 helix and C-terminal histone fold extension of H2A, referred to as the docking domain, which forms a short β-sheet with the H4 C terminus (Luger et al., 1997a). Sequence differences between the canonical H2A and the variant H2A.Z lead to changes in the docking domain, which seems to destabilize the dimer-tetramer interaction in H2A.Z containing nucleosomes (Abbott et al., 2001; Suto et al., 2000).

The two H2A-H2B dimers interact through a small interface comprising hydrogen bonds formed between the L1 loops of two adjacent H2A, suggesting a cooperativity in dimer binding (Luger et al., 1997a). Remarkably, NCPs containing H2A.Z or the histone domain of the H2A variant macroH2A, differ in this L1 loop-interaction interface (Chakravarthy et al., 2005a; Suto et al., 2000). This variation has been suggested to be important for the incorporation of two identical H2A molecules into a nucleosome (Chakravarthy et al., 2005b). Furthermore, in H2A.Z containing nucleosomes the L1 loop-interaction interface appears to stabilize the nucleosome (Suto et al., 2000), which is supported by fluorescence resonance energy transfer (FRET) and thermal mobilization experiments (Flaus et al., 2004; Park et al., 2004). However, these studies
disagree with a sedimentation analysis of NCPs under changing ionic strength, showing a substantial instability of the H2A.Z containing NCP (Abbott et al., 2001). In the budding yeast NCP no stabilizing interactions exist between the two H2A·H2B dimers in the interface formed by the L1 loops (White et al., 2001). These missing stabilizing L1 loop-interactions support the observation that yeast nucleosomes are less stable compared to metazoan nucleosomes (Lee et al., 1982; Pineiro et al., 1991).

1.3.2 DNA binding by the histone octamer

Each dimer has three distinct DNA binding sites formed by contacts of the individual histones in the dimer: two L1L2 sites at either end of the dimer and one α1α1 site which is formed by the two N-terminal α-helices of each histone fold (Arents and Moudrianakis, 1995; Luger et al., 1997a). In the octamer these 12 sites are responsible for binding 121 bp of DNA at 12 minor groove locations facing the octamer (SHL ±0.5, ±1.5…±6.5). The two remaining 13 bp segments where the DNA enters and leaves the NCP are bound by the αN extensions of the H3 histone fold (Luger and Richmond, 1998a). At the molecular level, more than 120 direct interactions are made between the histones and the 14 minor groove locations of the DNA (Davey et al., 2002).

The sin (switch-independent) mutations in the histones H3 and H4 affect residues in the L1L2 loops that bind the nucleosomal DNA at SHL ±0.5 near the pseudodyad axis (Luger, 2003). They were originally isolated from yeast in a genetic screen for H3 and H4 mutations that partially alleviate the transcription defects caused by the inactivation of the chromatin remodelling complex SWI/SNF (Kruger et al., 1995). Histone sin mutations disturb chromatin structure in vivo and in vitro (Horn et al., 2002; Kurumizaka and Wolffe, 1997; Wechser et al., 1997). Introducing the corresponding mutations into X. laevis NCPs only moderately affects the global NCP structure (Muthurajan et al., 2004). Though, only one of the mutated residues is involved in making direct contacts with the DNA, namely arginine 45 of H4, which is inserted into the minor groove (Muthurajan et al., 2004). The other affected residues seem to stabilize a specific arrangement of the L1L2 loop, leading to a local disruption of only very few selected histone-DNA interactions (2 to 6 of the ~120 direct contacts) (Muthurajan et al., 2004). Remarkably, loss of these few contacts significantly increases thermal mobility of nucleosome sliding and reduces nucleosome stability at high salt concentrations (Flaus et al., 2004; Muthurajan et al., 2004).

1.3.3 Histone tails

Some portions of the N- and C-terminal tail regions extending outside of the NCP are not
resolved in the crystal structure and are thus flexible (Luger et al., 1997a). The N-terminal tails do not contribute to the stability of the complex (Luger et al., 1997b), but are thought to be important in stabilisation of higher order chromatin structures (Hansen et al., 1998; Wolffe and Hayes, 1999). The N-terminal tails of H2B and H3 pass between the gyres of the DNA superhelix through channels formed by adjacent minor grooves (Luger et al., 1997a). The N-terminal tails of H2A and H4 extend from the octamer surface and the H2A N-terminal tails pass over the DNA to the outside of the superhelix (Luger et al., 1997a). The two H4 N-terminal tails have different structures within the same NCP (Luger and Richmond, 1998b). One tail makes extensive contacts with an acidic patch on the surface of the H2A-H2B dimer of an adjacent particle in the crystal lattice, while the other is not localized (Luger et al., 1997a). In the *S. cerevisiae* NCP the localized N-terminal tail of H4 is not interacting with the acidic patch, but instead is poised to interact with the DNA of the neighboring NCP in the crystal lattice, although the amino acid sequence within this region of H4 is almost completely conserved between *S. cerevisiae* and *X. laevis* (White et al., 2001). The C-terminal tails of H2A run antiparallel to the $\alpha$N of the H3 histones in direction of the entering and exiting DNA (Luger et al., 1997a; Luger and Richmond, 1998a). Again, differences in the traces of the C-terminal tails of H2A between *S. cerevisiae* and *X. laevis* NCPs were observed (White et al., 2001). Markedly however, the major H2A of yeast is rather a H2A.X variant than a canonical H2A, differing mainly in their C-terminal tails (Mannironi et al., 1989).

### 1.3.4 Nucleosomal DNA

The central 128-bp DNA segment within the NCP is tightly bent, whereas the two 9-bp terminal segments are relatively straight, contributing little to the curvature of the complete superhelix (Luger et al., 1997a). Two DNA segments of unequal length, 72 bp on one and 73 bp on the other halves of the particle related by the pseudo-twofold symmetry, are extending from the central base pair. The two halves have closely similar structure apart from a 12-bp segment, which is stretched by 1 bp in the 72 bp half between the two LIL2 sites of a H3-H4 dimer. A different 146-bp palindrome DNA sequence displays a similar distortion at another location, namely between the L1L2 sites of a H2A-H2B dimer. DNA stretching at different locations argues that it is rather not caused by octamer-DNA contacts, but by base stacking forces between terminal base pairs of adjacent particles in the crystal lattice (Davey and Richmond, 2002; Luger et al., 1997a). Compared to naked B-form DNA that has an average twist of 10.5 bp per turn, nucleosomal DNA is overwound with an average twist of about 10.2 bp per turn. Overwinding is mainly caused by DNA stretching (Richmond and Davey, 2003).

In comparison with the protein histone-fold regions, the DNA in the crystal structure of the NCP is more mobile or statically disordered (Luger et al., 1997a). But a significant improvement of the electron density of the DNA was achieved by extending the DNA from 146 to 147 bp (Davey...
and Richmond, 2002; Richmond and Davey, 2003). A 147-bp DNA has two segments of the same length on both halves of the particle, and thus reduces its distortion (Davey and Richmond, 2002). These NCP crystals diffracted to 1.9 Å resolution and the structure showed that the DNA is predominantly B-form, with an average twist of about 10.4 bp per turn (Richmond and Davey, 2003). However, the DNA has double the base pair step curvature necessary to produce the superhelix path, and some unusual conformational parameters (Richmond and Davey, 2003). The excess DNA curvature around the histone core is owing to anisotropic flexibility of the DNA, local structural features intrinsic to the DNA sequence, and irregularities dictated by the underlying histone octamer (Richmond and Davey, 2003).

1.4 Posttranslational histone modifications

The histones are subject to a wide variety of posttranslational modifications, at their flexible tails as well as at their structured globular domains (Mersfelder and Parthun, 2006; Nightingale et al., 2006). These modifications include the acetylation, ubiquitylation, and SUMOylation of lysines, the methylation of lysines and arginines, the phosphorylation of serines and threonines, and the ADP-ribosylation of glutamic acids. Lysine residues can accept one, two, or even three methyl groups, and arginines can be either mono- or di-methylated, and this in a symmetric or asymmetric manner (Margueron et al., 2005; Peterson and Laniel, 2004). In addition, as a non-covalent histone modification, cis-trans isomerization of proline residues in the N-terminal tail of histone H3 has been reported recently (Nelson et al., 2006).

So far, many studies focused on the histone modifications on the histone tails being that the primary method for discovering histone modifications, Edman degradation, favoured the analysis of the first 20–30 amino acids (Mersfelder and Parthun, 2006). More recently many histone modifications on the structured globular histone domains have been discovered by mass spectrometry (Cocklin and Wang, 2003; Freitas et al., 2004; Zhang et al., 2002a; Zhang et al., 2003). With the exceptions of acetylation of lysine 56 and the methylation of lysine 79 of H3, these modifications have not been examined in detail (Cosgrove et al., 2004; Cosgrove and Wolberger, 2005; Mersfelder and Parthun, 2006).

A long-standing model suggests that histone N-terminal tail modifications regulate the interaction between the highly basic histone tails and nucleosomal DNA or linker DNA (Angelov et al., 2001). Although some observations support this view, the primary mechanism by which tail modifications act, appears to be through altering the ability of non-histone proteins to interact with chromatin (Grewal and Moazed, 2003; Iizuka and Smith, 2003; Jenuwein and Allis, 2001; Martin and Zhang, 2005). These and other observations have led to the proposal of a histone “code” which might be read by various nuclear non-histone regulatory proteins (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Modifications of histones are set, removed, or can be recognized by specific proteins, some of which can be grouped into distinct families (Bottomley,
Introduction

The modifying enzymes and proteins recognizing the modifications often occur in multi-subunit protein complexes implicated in chromatin remodelling, transcription, silencing, DNA repair, and chromosome segregation (Bottomley, 2004; Khorasanizadeh, 2004; van Attikum and Gasser, 2005). The generation of histone modifications can be interdependent on each other, designated as histone modification cross-talk (Fischle et al., 2003). The modification cross-talk can act between different residues on the same or on different histone tails and can operate, either by generating complex interaction surfaces that modulate the binding of non-histone regulatory proteins, or through sequential patterns of modifications, in which defined modifications recruit histone modifying enzymes (Fischle et al., 2003; Turner, 2002).

1.5 Nucleosome dynamics

Nucleosomes are not static protein-DNA complexes but are rather intrinsic highly dynamic (Luger, 2006). The DNA in nucleosomes in solution can spontaneously unwrap and rewrap from the histone octamer (nucleosome breathing) (Hansen, 2002; Luger, 2003). The histone octamer can translational reposition along the DNA (nucleosome sliding) (Becker, 2002). In addition, the H2A·H2B dimers are less stably associated in the nucleosome than the (H3·H4)$_2$ tetramer, reflected by a sequential release of first H2A·H2B dimers and then the (H3·H4)$_2$ tetramer from the DNA, when salt concentration is raised (Hansen, 2002).

Using reconstituted mononucleosomes and stopped-flow FRET, it has been shown that the ends of nucleosomal DNA unwrap and rewrap rapidly (within 50–250 ms) from the histone surface (Li et al., 2005; Li and Widom, 2004; Tomschik et al., 2005). The brief excursions into an extended open state would create windows of opportunity for proteins to bind to or translocate along the DNA (Li et al., 2005). Nucleosomes assembled onto DNA fragments can be shifted to different positions following thermal incubation (Beard, 1978; Flaus et al., 1996; Meersseman et al., 1991; Meersseman et al., 1992). Compared to un- and rewrapping of nucleosomal DNA, spontaneous translational movements of nucleosomes under physiological conditions are very slow. However, chromatin remodelling enzymes that lower the energy barrier of nucleosome repositioning by coupling the disruption of histone-DNA contacts to ATP hydrolysis can enhance this mobility (Becker and Horz, 2002; Johnson et al., 2005; Saha et al., 2006; Tsukiyama, 2002).

Mechanisms for the catalyzed nucleosome translocation remain poorly understood and several different models have been suggested (Johnson et al., 2005; Langst and Becker, 2004; Saha et al., 2006). These models propose mechanisms which include twisting (twist diffusion) (Gavin et al., 2001; Havas et al., 2000), looping of DNA (bulge diffusion) (Saha et al., 2005; Zofall et al., 2006), or disassembly and reassembly of the octamer complex (histone eviction) (Gutierrez et al., 2007; Lorch et al., 1999; Phelan et al., 2000).
In addition to ATP-dependent chromatin remodelling, nucleosome mobility is assisted by histone chaperones and assembly factors (Luger and Hansen, 2005) and might be regulated by posttranslational histone modifications that lie at the histone-DNA interface (Cosgrove et al., 2004). The latter suggestion is supported by the coincidence of several modified residues with side chains in histone H3 and H4 that are sites of sin mutations (Cosgrove et al., 2004; Freitas et al., 2004). However, yet these modifications are poorly investigated (Mersfelder and Parthun, 2006).

1.6 The linker histone and its S. cerevisiae homologue Hho1p

In higher eukaryotes, linker histones (H1, H5) comprise a large family (up to 8 distinct members per organism) of chromatin associated proteins that are not structurally related to core histones (Hansen, 2002). Members of the canonical linker histone family possess a conserved globular winged-helix domain (Cerf et al., 1994; Clore et al., 1987; Ramakrishnan et al., 1993), flanked by a short N-terminal tail, and a long (~100 amino acids), highly basic C-terminal tail (Allan et al., 1980). The N- and C-terminal tails are variable in length and amino acid sequence among different variants and unstructured in solution (Allan et al., 1980; Wolffe, 1997). Linker histones protect ~20 bp linker DNA segments from MNase digestion of native as well as reconstituted chromatin, suggesting a binding to the DNA where it enters and exits the nucleosome (Meersseman et al., 1991; Noll and Kornberg, 1977; Simpson, 1978). In support of this observation, linker histones reduce mobility of reconstituted mononucleosomes (Pennings et al., 1994; Ura et al., 1995). The exact location of the linker histone on the nucleosome is not clear and several models have been proposed (Thomas, 1999; Travers, 1999). In an initial model the globular domain is centred directly on the nucleosomal pseudodyad axis and contacting both, entering and exiting DNA segments (Allan et al., 1980; Staynov and Crane-Robinson, 1988). The X-ray and NMR structures of the globular domains of H5 and H1 (GH5 and GH1) revealed two basic clusters on opposite sides of the domains (Cerf et al., 1994; Ramakrishnan et al., 1993). Mutagenesis of amino acids in these two putative DNA binding sites and a photoactivatable protein-DNA crosslinking approach, provided support for a bridging model, in which the globular domain forms a bridge between one terminus of chromatosomal DNA and the DNA in the vicinity of the pseudodyad axis of the nucleosome (Goytisolo et al., 1996; Zhou et al., 1998). Intriguingly, similar photocrosslinking experiments, though using a different DNA template, showed a biding of the globular domain 65 bp away from the pseudodyad axis, suggesting an off-axis model that located the globular domain inside the gyres of the DNA (An et al., 1998a; An et al., 1998b; Hayes et al., 1994; Hayes and Wolffe, 1993; Pruss et al., 1996). Using a computational docking approach it has recently been shown, that all three models might be correct, however in case of the off-axis model, the globular domain would be placed outside the gyres of DNA (Fan and Roberts, 2006). These results suggest that different binding
sites on the nucleosome might depend on different linker histone variants, modifications on the core histones, and/or linker histones (Fan and Roberts, 2006). 

*S. cerevisiae* has no classical linker histone, but a histone H1 homologue (Hho1p). In contrast to the tripartite domain structure of canonical linker histones, Hho1p has two globular winged-helix domains, GI and GII, separated by a basic segment of 42 amino acids, and a N-terminal extension (Ali et al., 2004; Landsman, 1996; Ono et al., 2003). The NMR structure of both globular domains, GI and GII, suggests two DNA binding sites on opposite surfaces, similar to GH5 and GH1 (Ali et al., 2004; Ono et al., 2003).

### 1.7 Chromatin higher order structure

Levels of chromatin folding beyond the linear array of nucleosomes are defined as chromatin higher order structures (Woodcock, 2006). The term chromatin higher order structure covers a wide range of hierarchical levels of chromatin folding from positioned nucleosomes up to the large-scale organization of interphase chromosomes. For convenience, a hierarchical system has been proposed for classifying chromatin structure (Woodcock and Dimitrov, 2001). In this system, primary chromatin structure is essentially the nucleosomal array, defined by nucleosome positions, nuclease hypersensitive sites and other prominent landmarks. Secondary structure is formed by the folding of primary structure and involves inter-nucleosomal contacts and interactions with linker histones and non-histone chromosomal proteins. This organizational level is often also referred to as the 30-nm chromatin fibre. The tertiary structure, formed by interactions between elements of the secondary structure, is made up of long-distance contacts or looped chromatin domains (Woodcock, 2006; Woodcock and Dimitrov, 2001). Though, chromatin structure is conformational dynamic and influenced by many factors, including posttranslational histone modifications, incorporation of histone variants into nucleosomes, nucleosome repeat length, and the presence of linker histones and non-histone chromosomal proteins. All these factors give rise to a high degree of heterogeneity and complexity in structure within all levels of chromatin folding.

Different strategies have been followed to investigate chromatin structure in vivo (Simpson, 1999). Conventional methods apply MNase or DNase I digestion of chromatin and indirect end-labelling to map positions of nucleosomes and nuclease hypersensitive sites along the DNA sequence of specific genomic loci (Gregory et al., 1998; Gregory and Horz, 1999; Livingstone-Zatchej and Thoma, 1999; Schmid et al., 2004).

From such studies it became clear that active genes in chromatin are more quickly digested by the enzymes than inactive chromatin (Wolffe and Kurumizaka, 1998). Moreover, promoter and enhancer regions are DNase I-hypersensitive (Rando, 2006). Detailed studies of individual genes have identified many of the components and basic principles that control transcription (Saunders et al., 2006; Workman, 2006).
Genomic approaches to study chromatin structure have become available with the sequencing of whole genomes, the development of chromatin immunoprecipitation (ChIP), the generation of antibodies that recognize specific histone modification, and finally, advances in microarray technology (Loden and van Steensel, 2005; Orlando, 2000; Rando, 2006; van Steensel, 2005). These new methods allowed detailed genome-wide mappings of nucleosome positions (Yuan et al., 2005), distributions of non-histone chromosomal proteins (Kim et al., 2005; Ren et al., 2000; van Steensel, 2005), and histone modifications (Millar and Grunstein, 2006; Pokholok et al., 2005; Roh et al., 2005; Schubeler et al., 2004; Wiren et al., 2005). These studies revealed several parameters regulating chromatin structure, including DNA sequences favouring or impairing nucleosome positioning, histone modifications, histone variants, ATP-dependent chromatin remodelling, and the interaction of chromatin with other non-histone chromosomal proteins (Giresi et al., 2006; Polo and Almouzni, 2006; Raisner and Madhani, 2006; Rando, 2006; Varga-Weisz and Becker, 2006; Woodcock, 2006).

The development of imaging technology and fluorescent proteins allowed the investigation of dynamic properties of chromatin organization (Gasser, 2002), and the monitoring of the mobility of many chromosomal proteins including histones in living cells of higher eukaryotes (Kimura, 2005; Phair et al., 2004). Fluorescence recovery after photobleaching (FRAP) experiments revealed that (H3-H4)$_2$ tetramers remain bound to DNA once assembled during DNA replication. In contrast, H2A and H2B exhibit significant exchange (Kimura and Cook, 2001). While a small population of H2B exchanges rapidly (<10%), ~40% H2B exchanges slowly ($t_{1/2} \sim 130$ min), independent of DNA replication and transcription, and >50% remain bound stably ($t_{1/2} > 8.5$ h). On the other hand, photobleaching studies showed rapid exchange of proteins involved in transcription and DNA repair (Bustin et al., 2005; Hager et al., 2004; Mone et al., 2004; Phair et al., 2004). GFP-tagged H1 also rapidly exchanges in euchromatin as well as in heterochromatin and has an estimated residence time of a few minutes (Bustin et al., 2005; Lever et al., 2000; Misteli et al., 2000).

These molecular and cytological in vivo approaches to study chromatin structure do, however, have limitations (Horowitz-Scherer and Woodcock, 2006). They generate a highly detailed, but essentially one-dimensional description of the local and global primary structure of chromatin in terms of its molecular composition (Horowitz-Scherer and Woodcock, 2006; Woodcock, 2006). The experimental study of chromatin secondary structures has proven remarkably challenging and, despite an intense effort, many aspects of chromatin compaction remain poorly understood (Hansen, 2002; McBryant et al., 2006). Early investigations of chromatin fibre structure by electron microscopy utilized nuclease-digested endogenous chromatin fragments isolated from whole cells. These studies established the effect of different ionic strengths on chromatin folding and the stabilization of the folded structures by linker histones (Finch and Klug, 1976; Thoma and Koller, 1977; Thoma et al., 1979). In the condensed structures, induced by an increase of ionic strength and the presence of low concentration of divalent cations, individual nucleosomes are hardly visible and the trajectory of the DNA can not be resolved by electron microscopy,
making it very difficult to identify the arrangement of nucleosomes in secondary chromatin structures (Daban, 2003).

Chromatin fibres isolated from endogenous sources are quite heterogeneous in length, contain different histone isoforms, posttranslational modifications, associated proteins, and have varying lengths of linker DNA between adjacent nucleosomes. This structural and compositional heterogeneity made it difficult to rigorously interpret physical measurements. These considerations have led many investigators to turn to defined nucleosomal arrays reconstituted from recombinant histones and tandem repeats of DNA containing nucleosome-positioning sequences (Hansen, 2002; Woodcock, 2006).

1.7.1 Defined nucleosomal arrays

Much of our understanding of the behaviour of nucleosomal arrays has come from biophysical analyses of defined arrays reconstituted from tandem repeats of DNA containing nucleosome-positioning sequences and purified native histones or recombinant *X. laevis* histones (Hansen, 2002). By using 12 repeats of a 208-bp sea urchin 5S rDNA gene fragment (“208-12” array) (Simpson et al., 1985) or 12 tandem 177-bp repeats of the SELEX-derived 601 sequence (“12_177_601” array) (Lowary and Widom, 1998) it was possible to reconstitute quite homogeneous populations of model nucleosomal arrays suitable for sedimentation velocity and equilibrium studies (Hansen et al., 1989). In low-ionic-strength buffers, these model arrays sediment in the analytical ultracentrifuge in a fairly uniform distribution that can be modelled as a fully extended beads-on-a-string fibre (Bednar et al., 1995). In contrast, the addition of 1 to 2 mM divalent cations establishes a heterogeneous population of folded arrays, where the most condensed species can be modelled as a compact 30-nm fibre (Bednar et al., 1995; Hansen, 2002). Above 2 mM MgCl₂, the arrays also self-associate to high molecular weight oligomers, which form precipitates (Schwarz et al., 1996; Schwarz and Hansen, 1994). Oligomerization is cooperative, fully reversible upon removal of the divalent cations, and dependent on the presence of histone tails, and therefore inter-array interactions are believed to mimic fibre-fibre interactions of tertiary chromatin structures (Gordon et al., 2005; Schwarz et al., 1996).

By reconstitution of nucleosomal arrays with recombinant histones it has been shown that chromatin fibres can fully compact with any one of the histone tails deleted with the exception of that of histone H4 (Dorigo et al., 2003). In contrast, all four core histone N-terminal tails contribute to the salt-dependent oligomerization of chromatin fibres (Gordon et al., 2005). A chemical ligation strategy to generate histone H4 that was homogeneously acetylated at lysine 16, revealed that incorporation of this modified histone into nucleosomal arrays inhibits the formation of compact 30-nm–like fibres and impedes the ability of chromatin to form fibre-fibre interactions (Shogren-Knaak et al., 2006). Nucleosomal arrays containing the H2A variant H2A.Z fold into more compacted arrays that H2A containing arrays but show reduced fibre
oligomerization (Fan et al., 2002). The intra-array folding is promoted by the acidic patch on the histone octamer surface (Fan et al., 2004), which is extended on H2A.Z nucleosomes (Suto et al., 2000).

The analysis of sedimentation velocity of nucleosomal arrays reconstituted with a sin mutant histone, indicate that enhanced nucleosome mobility may disrupt chromatin folding (Flaus et al., 2004; Horn et al., 2002; Muthurajan et al., 2004). The sin mutation R45C of histone H4 has been demonstrated to eliminate Mg$^{2+}$-dependent, intra-array folding, although the mutant histone does not disrupt nucleosome positioning on the nucleosomal array (Horn et al., 2002). In contrast, this mutation of H4 shows normal fibre oligomerization, which indicates that fibre compaction is not a prerequisite for inter-array association (Horn et al., 2002; Pollard et al., 1999; Schwarz et al., 1996). Thus, the nucleosomal array data suggest that sin versions of histones may alleviate the need for SWI/SNF in vivo by disrupting higher order chromatin folding (Horn et al., 2002). Other histone H3 and H4 mutations, which impair transcriptional silencing and are called lrs (loss of rDNA silencing), have been speculated to disturb chromatin higher order structure (Park et al., 2002; Thompson et al., 2003). In contrast, the lrs mutation R83A of H3 does not disturb the formation of 30-nm-like fibres (Fry et al., 2006). Similar to arginine 45 of H4, arginine 83 of H3 is inserted into the minor groove of the DNA and forms a histone-DNA binding site. These studies suggest that each histone-DNA contact site is functionally distinct (Fry et al., 2006).

Although condensation is an intrinsic property of the nucleosomal array, the binding of linker histones stabilizes both intramolecular folding and fiber-fiber interactions (Carruthers et al., 1998; Lu and Hansen, 2004; McBryant et al., 2006). The removal of the histone tails abolishes condensation also in the presence of linker histone (Carruthers and Hansen, 2000). In addition, the C-terminal tail is essential for the stabilization of compact chromatin structures (Allan et al., 1986; Lu and Hansen, 2004).

### 1.7.2 Structural models for condensed nucleosomal arrays

Based on transmission electron microscopy, electron cryo-microscopy, atomic force microscopy, and X-ray diffraction of chromatin at various ionic strengths different structural models have been proposed for higher order chromatin structures (McBryant et al., 2006; Robinson and Rhodes, 2006; Woodcock, 2006). Most models have in common that an open zigzag arrangement of nucleosomes folds into a helical structure, ~30 nm in diameter. These models can be classified into the one-start and the two-start helix. In a one-start helix consecutive nucleosomes of an array coil up and follow each other immediately along the same helical path (Widom and Klug, 1985). The linker DNA between adjacent nucleosomes continues the curvature established in the nucleosome and thus is bent. In a two-start helix consecutive nucleosomes of an array arrange in a zigzag course in the helix and are connected by straight linker DNA segments (Woodcock
et al., 1984). The most prominent representative of the one-start class is the solenoid, where the nucleosomes coil around a central cavity with linker DNA in the interior of the fibre and with six to eight nucleosomes per turn (Finch and Klug, 1976; McGhee et al., 1983; Thoma et al., 1979). The two-start class is essentially divided between two main models named the helical ribbon model and the crossed-linker model. In the helical ribbon model, nucleosomes arranged in a zigzag, form a ribbon that is coiled into a helix (Woodcock et al., 1984; Worcel et al., 1981). The diameter of this fibre is not dependent upon the nucleosome repeat length, because the linker DNA is arranged more or less along the fibre axis. The crossed-linker model is based on linker DNA extended in the fibre interior and connecting nucleosomes on opposite sides of the fibre (Williams et al., 1986). Linker DNA segments of adjacent pairs of nucleosomes cross in the fibre interior and is arranged more or less horizontally to the fibre axis. Thus, the diameter of such a fibre is related to the nucleosome repeat length.

Recent progress with the objective to determine the structure of condensed chromatin fibres has included the reconstitution of fully defined nucleosomal arrays (Robinson and Rhodes, 2006; Woodcock, 2006). Compacted nucleosomal arrays, that were stabilized by introduction of disulfide cross-links between neighbouring NCPs, and analysed by restriction digestion, revealed a two-start organization of the chromatin fibre (Dorigo et al., 2004). A crystal structure of a compact tetranucleosome, consisting of four core particles incorporating 147-bp segments of the “601” sequence and three 20-bp segments of linker DNA, was determined at a resolution of 9 Å (Schalch et al., 2005). The structure was solved by molecular replacement using the high resolution NCP structure and revealed that the NCPs are arranged in two stacks forming a truncated two-start helix (Schalch et al., 2005). Modelling continuous fibres by successively stacking tetranucleosomes most closely fit with the crossed-linker model (Schalch et al., 2005). However, these models have short nucleosomal repeat lengths of 167 bp, close to that found in *S. cerevisiae*, and result in fibre diameters of only 24–25 nm. Longer linker DNA segments of 30–90 bp, typical of many higher eukaryotic cells, could be included by increasing fibre diameter. Though, questions of how longer or non-uniform linker DNA lengths are accommodated are not conclusively answered by these models. Large and abrupt changes in linker DNA lengths would cause a structural perturbation in the chromatin fibre (Schalch et al., 2005).

Chromatin fibre models derived from the tetranucleosome structure contrast recent measurements of dimensions of long, compact nucleosomal arrays visualized by electron microscopy (Robinson et al., 2006; Robinson and Rhodes, 2006). Reconstituted nucleosomal arrays containing between 50 and 70 nucleosomes, the linker histone, and different nucleosomal repeat lengths (10 to 70 bp of linker DNA), revealed that the dimensions of these fully compacted chromatin fibres do not increase linearly with increasing linker DNA length (Robinson et al., 2006). Instead, two distinct classes of fibre dimensions were found, both with remarkably high nucleosome density: Arrays with 10 to 40 bp of linker DNA produce fibres with a diameter of 33 nm and a nucleosome packing ratio of 11 nucleosomes per 11 nm fibre lengths. Longer repeat lengths with 50 to 70 bp of linker DNA, form thicker fibres with a diameter of 44 nm and 15 nucleosomes per 11 nm
fibre lengths (Robinson et al., 2006). Constraints imposed by these measurements can only be satisfied by a one-start helical structure (Robinson et al., 2006; Robinson and Rhodes, 2006). The proposal of two strikingly different models for the secondary structure of chromatin may indicate that, depending on the presence or absence of linker histones, chromatin might indeed condense into different higher order structures. Certainly, the controversy of how nucleosomes are organized in condensed 30-nm fibres appears to continue (Robinson and Rhodes, 2006).

1.7.3 Nucleosome positioning in *S. cerevisiae* chromatin

Nucleosomes can be positioned along the DNA sequence (translational and rotational positioning), with variable precision (Simpson, 1991; Thoma, 1992), though most nucleosome are well positioned in the yeast genome (Rando, 2006; Yuan et al., 2005). High-resolution nucleosome footprinting studies revealed that nucleosomes can occupy multiple and overlapping positions, which suggests that nucleosomes are mobile and a dynamic equilibrium between several positions may exist in living cells (Buttinelli et al., 1993; Fragoso et al., 1995; Tanaka et al., 1996). Nucleosome positioning is influenced by numerous factors, including the DNA sequence, chromatin remodelling, binding of non-histone proteins to DNA, and chromatin folding (Giresi et al., 2006; Ivanovska and Orr-Weaver, 2006; Kiyama and Trifonov, 2002; Segal et al., 2006; Thoma, 1992; Whitehouse and Tsukiyama, 2006).

DNA sequences as positioning signals are relatively weak and difficult to detect in any individual nucleosomal sequence (Giresi et al., 2006; Kiyama and Trifonov, 2002). However, recent reports indicate that the DNA sequence itself may significantly regulate nucleosome stability and positioning at loci across the genome (Ioshikhes et al., 2006; Segal et al., 2006). Microarray-based studies combined with ChIP and/or nuclease-protection assays of genome-wide nucleosome occupancy in yeast indicate generally, that coding sequences are more stably associated with nucleosomes than are non-coding sequences (Giresi et al., 2006; Lee et al., 2004; Pokholok et al., 2005; Sekinger et al., 2005; Yuan et al., 2005). A stereotyped chromatin organization at promoters of RNA polymerase II transcribed genes consists of a nucleosome-free region, which is enriched in poly(dA·dT) elements, flanked on both sides by positioned nucleosomes (Yuan et al., 2005). The nucleosome-free regions generally overlap with DNase I-hypersensitive sites found in the promoter region of nearly every gene across a ~45 kb region of chromosome III (Ercan and Simpson, 2004). The nucleosome-free regions at promoters of both active and inactive genes are typically flanked by two positioned nucleosomes containing the H2A variant Htz1p (Raisner et al., 2005).

Positioned nucleosomes can be mobilized to alternative positions along the DNA by chromatin remodelling complexes (Saha et al., 2006; Tsukiyama, 2002). They regulate the accessibility of DNA to nuclear factors also by nucleosome assembly/disassembly and the exchange of histones with variants of different properties (Saha et al., 2006; Varga-Weisz and Becker, 2006). Most
studies of chromatin remodelling complexes have focused on their roles in transcriptional control, although they are also involved in replication, repair, and recombination (Chai et al., 2005; Gaillard et al., 2003; Gong et al., 2006; Hara and Sancar, 2002; Martens and Winston, 2003; Saha et al., 2006; Tsukiyama, 2002; van Attikum and Gasser, 2005). Similar to in vitro observations, SWI/SNF remodelling complexes primarily disorder nucleosomal arrays, forming several alternative overlapping nucleosome positions also in vivo (Kim and Clark, 2002; Kim et al., 2006; Saha et al., 2006; Shen et al., 2001). ISWI plays a more subtle role in determining ordered nucleosome positions, which are different from those preferred by SWI/SNF (Kim et al., 2006; Saha et al., 2006; Whitehouse and Tsukiyama, 2006). Moreover, it has been suggested that Isw2p targets contain DNA sequences that are inhibitory to nucleosome formation and that these sequences facilitate the formation of nuclease-accessible open chromatin in the absence of Isw2p, and therefore Isw2p functions to position nucleosomes onto unfavourable DNA segments (Fazzio et al., 2001; Goldmark et al., 2000; Tsukiyama, 2002; Whitehouse and Tsukiyama, 2006).

1.7.4 Histone modifications and chromatin structure of *S. cerevisiae*

Modifying histones is another mechanism used by cells to control the accessibility to DNA in chromatin (Millar and Grunstein, 2006; Nightingale et al., 2006). Histone modifications function either by disrupting intra-nucleosome and inter-nucleosome contacts or by affecting the interaction of chromatin with non-histone proteins (Kouzarides, 2007). Depending on the type of modification, localized changes in charge can occur. Thus acetylation and phosphorylation of histone tails have a potential to alter DNA-histone or nucleosome-nucleosome contacts, influencing chromatin structure through electrostatic mechanisms (Mersfelder and Parthun, 2006). The most prominent candidate modification directly modulating chromatin compaction is acetylation of lysine 16 of histone H4 (Shia et al., 2006b; Shogren-Knaak et al., 2006). In *S. cerevisiae* this is the favoured acetylated site, with 80% of all H4 molecules acetylated at K16 (Clarke et al., 1993; Smith et al., 2003). Consistently, most of the *S. cerevisiae* genome exists in a decondensed state, with a minor heterochromatin fraction compared to higher eukaryotes (Lohr et al., 1977; Shia et al., 2006b).

Recent ChIP experiments have described the relative genome-wide distribution of several histone modifications in *S. cerevisiae* (Millar and Grunstein, 2006; Rando, 2006). These studies have shown that certain histone modifications occur together, that some are correlated with transcription or DNA repair, and that similar patterns of modified and unmodified sites are found on biologically related genes (Millar and Grunstein, 2006; Rando, 2006). Histone modification patterns can be created by (i) targeting of modifying enzymes to specific genomic locations (e.g. promoters, coding regions), by (ii) recruitment of modifying enzymes to relatively large, but restricted regions (e.g. subtelomeric regions), and by (iii) global histone modification activities
According to specific modification patterns, the traditionally distinguished domains euchromatin and heterochromatin can be subdivided into chromosomal domains with characteristic combinations of histone modification patterns (Kurdistani et al., 2004; Liu et al., 2005; Pokholok et al., 2005; Robyr et al., 2002; Xu et al., 2005b).

Most of the genome-wide characterized histone modifications are localized on the N-terminal histone tails (Millar and Grunstein, 2006). Mutations that alter sites of histone tail modifications have been shown to affect processes such as transcription, transcriptional silencing, and DNA repair (Hirschhorn et al., 1995; Kelly et al., 2000; Ma et al., 1998; Megee et al., 1990; Megee et al., 1995; Park and Szostak, 1990; Qin and Parthun, 2002; Thompson et al., 1994). However, the effects in many cases are minor (Mersfelder and Parthun, 2006). Moreover, the individual histone N-terminal tails are dispensable for viability (Lenfant et al., 1996; Recht and Osley, 1999). But the H2A and H2B or H3 and H4 tails, respectively, cannot be removed simultaneously without loss of viability, although they can be interchanged between H2A/H2B or H3/H4 pairs (Grunstein, 1990; Kayne et al., 1988; Ling et al., 1996; Morgan et al., 1991; Schuster et al., 1986). Deletions at the N-terminal tails affects chromatin structure (Fisher-Adams and Grunstein, 1995; Kayne et al., 1988; Recht and Osley, 1999; Roth et al., 1992; Sabet et al., 2003; Zhang et al., 1998). However, the histone H3 and H4 N-terminal tails as well as specific lysine residues of H4 are important for nucleosome assembly (Ling et al., 1996; Ma et al., 1998), and CAF-1, HIR, and asf1 mutations similarly affect chromatin structure (Adkins and Tyler, 2004; Sharp et al., 2002).

Single amino acid substitutions of modifiable residues within the histone fold domains have been shown to dramatically affect transcription, DNA repair, chromatin structure, chromatin assembly and heterochromatic gene silencing (Driscoll et al., 2007; Han et al., 2007; Hyland et al., 2005; Masumoto et al., 2005; Ng et al., 2002a; Ozdemir et al., 2005; van Leeuwen et al., 2002; Xu et al., 2005a; Xu et al., 2005b; Ye et al., 2005). The solvent accessible face of the nucleosome provides a large surface on which interactions may occur, which impact the regulation of chromatin structure (Luger, 2006). Therefore, modifications to this surface may function in a similar way as N-terminal tail modifications (Mersfelder and Parthun, 2006): On one hand they may control the ability of non-histone proteins to bind to the nucleosome (Fry et al., 2006; Huyen et al., 2004; Park et al., 2002; Thompson et al., 2003). On the other hand some of these modifications may directly influence nucleosome-nucleosome contacts (Mersfelder and Parthun, 2006; Park et al., 2002; Thompson et al., 2003).

Modifications on the lateral surface of the nucleosome are likely to influence chromatin structure through the modulation of histone-DNA interactions (Cosgrove et al., 2004). The sin alleles of H3 and H4, which partially alleviate the requirement for SWI/SNF for the transcriptional activation of some genes (Kruger et al., 1995), are indications for the capacity of such modifications to affect chromatin (Cosgrove et al., 2004; Luger, 2003; Mersfelder and Parthun, 2006). Some sin versions of H4 lead to an increased accessibility of chromatin to MNase and Dam methyltransferase and impair the ability of nucleosomes to supercoil the
2 μ plasmid (Wechser et al., 1997). Similarly, acetylation of lysine 56 of H3 may modulate histone-DNA interactions, where the DNA enters and exits the nucleosome (Masumoto et al., 2005; Mersfelder and Parthun, 2006). Acetylation of lysine 56 is important for transcription of a subset of SWI/SNF-regulated genes (Xu et al., 2005b). In addition, mutation of lysine 56 leads to an increased chromatin sensitivity to MNase digestion and a decrease in 2 μ plasmid supercoiling (Masumoto et al., 2005).

There are a number of modifiable residues that are within regions of the histone proteins that are involved in intra-nucleosomal interactions (Cosgrove and Wolberger, 2005; Mersfelder and Parthun, 2006). Modifications located at contact sites between histone proteins may alter chromatin structure by destabilization of the histone octamer (Mersfelder and Parthun, 2006). Mutations in histone H2B that show a Sin– phenotype, occur among others in residues involved in H2A·H2B dimer formation and dimer-tetramer association (Recht and Osley, 1999). However, these mutations do not affect the distribution of 2 μ topoisomers, and thus seem not to alter nucleosome assembly or stability (Recht and Osley, 1999). Acetylation of lysine 91 of histone H4 is the best characterized example of a modification that affects the interface between the (H3·H4)2 tetramer and H2A·H2B dimers (Ye et al., 2005). Mutation of this residue confers phenotypes consistent with defects in chromatin assembly such as sensitivity to DNA damaging agents and derepression of silent heterochromatin (Ye et al., 2005). In addition, this mutation destabilizes the histone octamer, leading to defects in chromatin structure (Ye et al., 2005).

1.7.5 Silenced chromatin in *S. cerevisiae*

In the yeast *S. cerevisiae* only a few discrete portions of the genome, such as the chromosome ends within 5–10 kb of the telomeres and the silent mating-type loci *HML* and *HMR*, are transcriptionally silent (Chen and Widom, 2004; Fox and McConnell, 2005; Grewal and Moazed, 2003; Talbert and Henikoff, 2006). Silencing differs from targeted gene-specific repression, in that the repression of RNA polymerase II transcribed genes depends on its chromosomal location within silenced regions and is a long range effect, occurring over distances larger than a single gene (Rusche et al., 2003). Another characteristic of silencing is a stable epigenetic inheritance of the transcriptional state during cell division, indicating a duplication of the specific chromatin pattern during DNA replication (Rusche et al., 2003). In addition to silencing at subtelomeric regions and the *HM* loci, marker genes inserted into the rDNA array are also expressed at a lower level than they are at their normal chromosomal loci (Bryk et al., 1997; Smith and Boeke, 1997). However, silencing at the rDNA array differs markedly from the other two classes of silencing in that it requires a special DNA-associated protein complex termed regulator of nucleolar silencing and telophase (RENT) (Straight et al., 1999). Sir2p is the only silencing factor known to be shared among the three classes of silencing (see below) (Rusche et al., 2003). The mechanism of rDNA silencing remains largely unresolved (Rusche et al., 2003),
Introduction

and therefore this section focuses on subtelomeric and HM silencing.
Heterochromatin was originally defined as regions of the genome in which the structure of chromatin is highly condensed throughout the cell cycle, which would be repressive for gene expression (Grewal and Jia, 2007; Grewal and Moazed, 2003). The discovery that the N-terminal tail of histone H4 is required for silencing revealed that silencing in S. cerevisiae also involves a specialized structure of chromatin (Kayne et al., 1988; Thompson et al., 1994). Silenced chromatin regions are less accessible to restriction nucleases (Loo and Rine, 1994), DNA methyltransferases (Gottschling, 1992; Singh and Klar, 1992), and photolyase (Bucceri et al., 2006; Livingstone-Zatchej et al., 2003). In addition, silenced genomic regions display ordered, regularly spaced nucleosomes (Ravindra et al., 1999; Weiss and Simpson, 1998), have an altered topology (Bi and Broach, 1997; Cheng et al., 1998), and the N-terminal tails of the histones H3 and H4 are hypoacetylated (Braunstein et al., 1993; Suka et al., 2001). Moreover silent chromosomal domains contain a continuous distribution of the silent information regulator (Sir) proteins (Hecht et al., 1996; Lieb et al., 2001; Rusche et al., 2002; Strahl-Bolsinger et al., 1997; Zhang et al., 2002b).

The formation of silent chromatin is thought to occur by nucleation at specific DNA elements, the silencer, and spreading of silent chromatin, which involves a sequential propagation of histone deacetylation and binding of Sir proteins (Talbert and Henikoff, 2006). The silencers are the telomeric (TG$_{1–3+n}$) repeats or small specialized sequences, known as the E and I silencers, which flank the HM loci (Rusche et al., 2003). The DNA binding proteins Rap1p, Abf1p, and ORC (origin recognition complex) nucleate silent chromatin by recruitment of Sir1p to telomeres and to the silencers at the silent mating-type loci (Bose et al., 2004; Hou et al., 2005; Hsu et al., 2005; Huang, 2002). The Sir2p-Sir4p complex is recruited to the nucleation sites through interactions with Sir1p and Rap1p (Rusche et al., 2003). Sir2p is an NAD$^+$-dependent histone deacetylase and silencing requires this enzymatic activity (Blander and Guarente, 2004; Imai et al., 2000; Moazed, 2001). The Sir2p-Sir4p complex deacetylates lysine 16 of H4, allowing Sir3p and additional Sir2-Sir4p to bind to histone H3 and H4 tails (Carmen et al., 2002; Hecht et al., 1995; Hoppe et al., 2002; Rudner et al., 2005). Periodic rounds of deacetylation and subsequent binding of Sir proteins to deacetylated histone tails propagate silencing along the chromosome (Rusche et al., 2003; Talbert and Henikoff, 2006). Oligomerization of Sir3p is thought to induce a conformational change that could contribute to the assembly process (Liou et al., 2005).

Several mechanisms have been proposed for preventing inappropriate spreading of silent chromatin into neighbouring euchromatin (Bi and Broach, 2001). Specific DNA elements have been suggested to function as silencing barriers either by excluding nucleosomes, or by recruiting histone modification enzymes to alter the underlying chromatin structure (Bi and Broach, 2001; Oki et al., 2004). Silencing at subtelomeric regions and the HM loci can be counteracted by the histone acetyltransferase complex SAS (something about silencing), (Kimura et al., 2002; Osada et al., 2001; Suka et al., 2002), which specifically acetylates lysine 16 of histone H4 (Shia et al.,
2005; Sutton et al., 2003). The histone H2A variant Htz1p can limit the spread of Sir proteins into nearby euchromatic regions (Kobor et al., 2004; Ladurner et al., 2003; Meneghini et al., 2003; Mizuguchi et al., 2004; Zhang et al., 2004), and SAS-mediated acetylation of histone H4 lysine 16 is required for Htz1p deposition at subtelomeric regions (Shia et al., 2006a). In addition, NuA4-dependent acetylation of Htz1p seems to be important for the boundary function of Htz1p (Babiarz et al., 2006).

The mechanism by which silent chromatin represses gene expression is not well understood (Chen and Widom, 2004). A model proposes that silencing inhibits the ability of site-specific activator proteins or RNA polymerases to access their DNA target sites simply through steric hindrance from a specialized compacted or sequestered chromatin structure (Rusche et al., 2003). This is supported by the reduced accessibility of silent chromatin to DNA metabolizing enzymes (Bucceri et al., 2006; Gottschling, 1992; Livingstone-Zatchej et al., 2003; Loo and Rine, 1994; Singh and Klar, 1992). In contrast, the promoter region of the α1 and α2 genes at the (silenced) *HML* is nucleosome free and more nuclease sensitive than the equivalent regions at the (active) *MAT* locus (Weiss and Simpson, 1998). Moreover, the *HMRα1* promoter is occupied by the TATA-box binding protein and RNA polymerase II (Sekinger and Gross, 2001). This findings support a model that suggests that upstream activators and RNA polymerase II have unhindered access and that transcription is instead blocked downstream of the transcription preinitiation complex formation (Sekinger and Gross, 2001). However, another report show, that silent chromatin acts primarily by reducing the probability that RNA polymerase II will occupy promoters at *HML* and *HMR* (Chen and Widom, 2005).

### 1.8 Chromatin and DNA repair

DNA is continuously exposed to endogenous as well as exogenous damaging agents, including reactive oxygen species, collapsed replication forks, environmental chemicals, and ionizing and ultraviolet (UV) radiation (Essers et al., 2006; Fillingham et al., 2006). These agents can induce modifications of bases, and/or generate abasic sites, DNA single-stand and double-stand breaks, which are removed or repaired by a variety of DNA repair mechanisms (Essers et al., 2006; Hoeijmakers, 2001). In response to DNA damage, cell-cycle checkpoints are activated, blocking cell cycle progression in G1 or G2 or slowing the S phase (Nyberg et al., 2002). The DNA damage checkpoint is a signal transduction pathway conducted by a cascade of phosphorylations and consisting of signal sensor, adaptor/mediator, and effector proteins (Melo and Toczyski, 2002; Nyberg et al., 2002). Central to DNA damage induced checkpoint responses are the phosphatidylinositol-3-OH kinase related kinases (PIKKs) ATM (ataxia telangiectasia mutated, in *S. cerevisiae* Tel1p) and ATR (ATM-and Rad3-related, in *S. cerevisiae* Mec1p) (Jeggo and Lorbich, 2006; Melo and Toczyski, 2002). These kinases act as “sensor kinases” at the top of the DNA damage checkpoint signalling pathway and propagate the signal to the effector kinases...
Chk1p and Rad53p (Chk2p in mammals) via the adaptor/mediator proteins Rad9p (BRCA1, 53BP1, NBS1, MDC1 in mammals) and Mrc1p (claspin in mammals) (McGowan and Russell, 2004; Melo and Toczyski, 2002).

Chromatin structure impacts the DNA damage response in all DNA repair pathways (Altal et al., 2007; Groth et al., 2007; Lydall and Whitehall, 2005; Osley and Shen, 2006). Due to technical reasons the role of chromatin structure in DNA repair has been characterized best for DNA double-strand break (DSB) repair and nucleotide excision repair (NER) of UV-induced lesions. In both pathways chromatin is altered by histone modifications, ATP-dependent chromatin remodelling, nucleosome disruption and reassembly (Driscoll et al., 2007; Gong et al., 2006; Groth et al., 2007; Han et al., 2007; Peterson and Cote, 2004; van Attikum and Gasser, 2005; Wurtele and Verreault, 2006).

1.8.1 Repair of DNA double strand breaks

Studies exploring the connection of DSB repair and chromatin remodelling in *S. cerevisiae* took advantage of yeast strains created by Haber and coworkers, in which a site-specific DSB at the *MAT* locus is generated by conditional expression of the homothallic switching (HO) endonuclease (Haber, 2006; Osley and Shen, 2006). DSBs are repaired by the two distinct but interconnected pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR) (Daley et al., 2005; Krogh and Symington, 2004). During NHEJ, the DNA ends are rejoined by ligation after little or no processing of the ends, which is often an error-prone process (Daley et al., 2005). This pathway depends on Ku70, Ku80, and the Lig4p-Lif1p (ligase IV-XRCC4 in mammals) DNA ligase (Dudasova et al., 2004). In contrast to NHEJ, repair by HR requires extensive 5’ resection to generate single-stranded DNA ends (Krogh and Symington, 2004). The single-stranded ends can subsequently invade a homologous template and prime DNA synthesis. Completion of repair requires strand resolution and ligation of the broken ends. Since this process involves sequence homology, this pathway is considered as error free. Central proteins of this pathway belong to the *RAD52* epistasis group and include the Mre11p-Rad50p-Xrs2 (MRE11-RAD50-NBS1 in mammals) complex (Krogh and Symington, 2004).

Upon formation of a DSB, histone H2A is rapidly and extensively phosphorylated at serine 129 and this occurs over a domain of ~50 kb (Foster and Downs, 2005; Shroff et al., 2004). In mammals, the histone H2A.X variant gets phosphorylated at serine 139 in a domain of over a megabase around the DSB and its phosphorylation is required for the stable accumulation of numerous DNA repair and checkpoint factors into ionizing radiation-induced nuclear foci (IRIF) (Celeste et al., 2003; Rogakou et al., 1999; Stucki and Jackson, 2006). Phosphorylation of the C-terminal H2A(X) serine residue depends on the checkpoint kinases Tel1p and Mec1p (ATM and ATR in mammals) (Downs et al., 2000; Rogakou et al., 1998). In yeast, ChIP experiments revealed an accumulation of the ATP-dependent chromatin remodelling factors SWI/SNF,
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1. Introduction

RSC, INO80, the histone acetyltransferases NuA4, and cohesion at the DSB in a phospho-
H2A dependent manner (Osley and Shen, 2006; Shim et al., 2007; Strom et al., 2004; Unal et
al., 2004; van Attikum and Gasser, 2005). Recruitment of remodelling factors to the DSB is
accompanied by acetylation of histones (Bird et al., 2002; Downs et al., 2004; Tamburini and
Tyler, 2005), INO80 dependent histone loss (Tsukuda et al., 2005), and SWR1-C dependent
exchange of H2A with Htz1p (Papamichos-Chronakis et al., 2006). Following displacement
from chromatin, soluble phospho-H2A is dephosphorylated by the HTP-C phosphatase complex,
which is necessary for efficient recovery from the DNA damage checkpoint in yeast (Keogh et
al., 2006; Papamichos-Chronakis et al., 2006).

1.8.2 Repair of UV lesions

Chromatin structures play an important role in NER and photoreactivation (Ataian and Krebs,
2006; Gong et al., 2005; Reed, 2005; Thoma, 2005).

The NER pathway is responsible to remove DNA lesions that distort the DNA double helix,
such as UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4)-pyrimidone
photoproducts (6-4PPs) (Ataian and Krebs, 2006; Thoma, 1999). NER is a multistep pathway
which involves more than thirty proteins and is divided in two subpathways, global genome
repair (GGR) and transcription coupled repair (TCR) (Thoma, 1999). NER involves damage
recognition, binding of a multi-protein complex, dual incision of the damaged strand several
nucleotides away from the lesion, excision of the damage containing oligonucleotide, DNA
repair synthesis, and ligation (Prakash and Prakash, 2000; Reardon and Sancar, 2005). The
two NER subpathways are modulated by chromatin structure and transcription (Ataian and
Krebs, 2006). ATP-dependent chromatin remodelling enhances DNA repair efficiency by NER
in vitro and in vivo (Gong et al., 2006; Hara and Sancar, 2002; Ura et al., 2001), and subunits
of SWI/SNF interact with the NER damage-recognition heterodimer Rad4p-Rad23p in a UV
irradiation dependent manner (Gong et al., 2006). Moreover, the N-terminal tails of histone
H3 get hyperacetylated at the repressed MFA2 promoter after UV irradiation, which depends
on Gcn5p, and this is accompanied by enhanced promoter accessibility to restriction enzymes
(Yu et al., 2005). In mammals, UV irradiation induces H2A.X phosphorylation as a diffuse,
even, pannuclear staining, in contrast to IRIF (Marti et al., 2006; O’Driscoll et al., 2003). H2A.
X phosphorylation upon UV irradiation depends on NER factors (XPA, XPC, and XPG) and
possibly on the generation of DNA single strand gaps (Marti et al., 2006; Matsumoto et al.,
2007).

In many organisms, but not in humans, CPDs and/or 6-4PPs can also be repaired by light-
dependent single enzyme pathways, called photoreactivation (PR). PR is performed by
photolyases, which flip the damaged bases out of the double helix into an active site and restore
the bases by light-induced electron transfer using the cofactor $\text{FADH}_2$ (1,5-dihydroflavin ade

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1. Introduction

...dinucleotide) and either the MTHF (pterin 5,10-methenyltetrahydrofolate) or the 8-HDF (8-hydroxy 5-deazariboflavin) chromophore (Sancar, 2003). In *S. cerevisiae* the photolyase Phr1p, which reverses CPDs, is tightly modulated by chromatin structure (Thoma, 1999; Thoma, 2005). *In vivo* CPD repair is fast in linker DNA, nuclease-sensitive regions and slow in nucleosomes and silenced regions (Bucceri et al., 2006; Livingstone-Zatchej et al., 2003; Suter et al., 1997). Moreover, photoreactivation is transiently inhibited by RNA polymerases II and III stalled at DNA lesions on the transcribed strand of active genes (Aboussekhra and Thoma, 1998; Livingstone-Zatchej et al., 1997). *In vitro*, chromatin remodelling factors SWI/SNF and ISW2 can facilitate nucleosome repair by photolyase (Gaillard et al., 2003).

1.9 The aim of this study

The aim of this study was to test selected histone mutants with respect to nucleosome stability and formation of chromatin higher order structures in the yeast *S. cerevisiae*. First, it has been suggested that the SQ motif in the C-terminal tail of histone H2A, which is important for DSB repair, has a role in determining higher order chromatin structure (Downs et al., 2000; Paull et al., 2000). The C-terminal tail of H2A has been shown to contact the linker DNA region of nucleosomal DNA and rearranges when the linker DNA is removed or upon linker histone binding (Guschin et al., 1998; Lee and Hayes, 1998; Usachenko et al., 1994). Second, a nucleosome surface formed by the α1 helix and L1 loop of H3 and adjacent amino acid residues of H4, the LRS domain, is crucial for transcriptional silencing (Park et al., 2002; Thompson et al., 2003). Therefore this nucleosome surface has been proposed to function in higher order chromatin structure, either by affecting nucleosome-nucleosome contacts or Sir protein binding (Fry et al., 2006; Park et al., 2002; Thompson et al., 2003).

We investigated the contribution of the C-terminal tail of H2A, the putative yeast linker histone, Hho1p, and the LRS domain to chromatin structure and DNA accessibility in yeast. Chromatin was investigated by using nuclease digestion, supercoiling assays, and repair approaches. In contrast to our expectation and to published results, we found only subtle effects on chromatin structure. Only in the case of the subtelomeric *URA3* gene we observed that silencing and loss of silencing correlates with a change in the position of a promoter nucleosome. We conclude that the analysed mutations act in recruitment of repair or silencing proteins, rather than in destabilization of nucleosomes. A part of this work was published in: M. Fink, D. Imholz, and F. Thoma (2007) Contribution of the Serine 129 of Histone H2A to Chromatin Structure. *Mol. Cell. Biol.*, 27: 3589–3600.
2 Contributions of the Serine 129 of Histone H2A and the H1 Homologue Hho1p to Chromatin Structure


2.1 Introduction

The histones have flexible and lysine rich N-terminal tails that protrude from the nucleosome core particle (Luger et al., 1997a). The tails are thought to interact with the DNA or histones of adjacent nucleosomes thereby contributing to higher order structural organization of chromatin (Luger and Richmond, 1998b). The histone H2A is the only core histone that also possesses a C-terminal tail with similar types of residues (rich in serine and lysine) as the N-terminal tails (McBryant et al., 2006). The C-terminal tail of H2A is located close to the site where the DNA enters and exits the nucleosome and, in crystal structures obtained with yeast histones, it contacts H2A in the neighbouring nucleosome (White et al., 2001). Crosslinking data from chicken erythrocyte nucleosomes showed that the H2A C-terminal tail binds to linker DNA, but rearranges to bind the central part of nucleosome core DNA when the linker is removed (Usachenko et al., 1994). Moreover, the extended C-terminal tail of the wheat histone H2A protects additional base pairs of linker DNA of reconstituted nucleosomes against MNase digestion (Lindsey et al., 1991). Thus, it is conceivable that the C-terminal tail and its modifications impact the stability of individual nucleosomes, nucleosome positioning as well as nucleosome-nucleosome contacts in higher order structures.

Nucleosomes control the accessibility of DNA to proteins involved in transcription, replication, recombination, and DNA repair. Occluded sites may become exposed and accessible to proteins by dynamic transitions involving transient dissociation of histones, unwrapping of DNA, or changing the position of histone octamers on the DNA sequence (nucleosome mobility). These intrinsic properties may be modulated by chromatin remodelling activities that chemically modify the histones, exchange histone variants, and/or alter the structure and position of nucleosomes. Posttranslational modifications of histones include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and poly (ADP)-ribosylation (Felsenfeld and Groudine, 2003; Peterson and Laniel, 2004; Varga-Weisz and Becker, 2006). Histone modifications can act as binding interfaces for the recruitment of chromatin associated proteins (Jenuwein and Allis, 2001; Strahl and Allis, 2000). On the other hand, modifications that alter the charge of histones such as histone acetylation and phosphorylation may alter the stability of nucleosomes and higher order structures (Hansen et al., 1998; Shogren-Knaak et al., 2006; Wolffe and Hayes, 1999).
Increasing evidence supports a role of chromatin remodelling in DNA-repair pathways. In nucleotide excision repair (NER) of UV-lesions, remodelling includes rearrangement and/or reassembly of nucleosomes after DNA-repair synthesis, acetylation of histones after UV-damage induction, as well as monoubiquitination of histone H2A (Gong et al., 2005; Gontijo et al., 2003; Kapetanaki et al., 2006; Yu et al., 2005). Double-strand break repair involves phosphorylation of H2A.X, acetylation of histones in the vicinity of the lesion as well as a local chromatin expansion immediately after DNA damage induction (Kruhlak et al., 2006; Tsukuda et al., 2005; van Attikum and Gasser, 2005). The C-terminal region of the H2A variant H2A.X of mammals and the bulk H2A (Hta1 and Hta2) of S. cerevisiae contain a serine four residues from the carboxyl terminus in an invariant SQ motif. This serine residue is phosphorylated in response to DSBs by the checkpoint kinases ATM/ATR (Tel1 and Mec1 in S. cerevisiae) (Downs et al., 2000; Rogakou et al., 1998). In mammals, phosphorylated H2A.X (γ-H2A.X) spreads over megabase chromatin domains and is required for the stable accumulation of repair proteins into nuclear foci (Celeste et al., 2003; Paull et al., 2000; Rogakou et al., 1999). The major mediator of γ-H2A.X recognition is likely MDC1 that binds γ-H2A.X through its tandem BRCT domain (Stucki et al., 2005). Similarly in S. cerevisiae γ-H2A spreads ~50 kb in either direction from a defined DSB, which is accompanied by the recruitment of repair factors to the break (Mre11 and Rad51) (Shroff et al., 2004). In addition, several chromatin remodelling complexes, including INO80 (Morrison et al., 2004; van Attikum et al., 2004), NuA4 and SWR1 (Downs et al., 2004), as well as cohesins (Strom et al., 2004; Unal et al., 2004) assemble on chromatin in a γ-H2A-dependent manner. But, phosphorylation of H2A and nucleosome loss at the break seems to occur independently (Tsukuda et al., 2005). After completion of DSB repair, γ-H2A(X) becomes dephosphorylated that is necessary for efficient recovery from the DNA damage checkpoint (Chowdhury et al., 2005; Keogh et al., 2006).

Downs et al. showed that mutation of serine 129 in the SQ motif of H2A to alanine (hta1-S129A) results in sensitivity to MMS to a similar degree as when the last four C-terminal amino acids (SQEL) are missing. In contrast, if serine 129 of histone H2A is replaced by glutamic acid (hta1-S129E), yeast cells survive in the presence of MMS almost as well as the H2A (HTA1) wild-type strain indicating that glutamic acid 129, at least in part, mimics constitutive phosphorylation. Since hta1-S129E showed enhanced nuclease sensitivity of chromatin and changes in supercoiling of a 2 µ plasmid, it was suggested that the C-terminal motif of H2A has a role in higher order chromatin structure and that phosphorylation of the SQ motif may modulate chromatin structure in the vicinity of a DSB (Downs et al., 2000).

The SQ motif of H2A is assumed to be located near to proposed binding sites for the linker histone to the nucleosome. In the most accepted models the linker histone is placed in the vicinity of the pseudodyad axis of the nucleosome, binds the entering and/or exiting linker DNA segments (Thomas, 1999; Travers, 1999), and thereby stabilizes higher order chromatin structure (Thoma et al., 1979). It has been shown that in presence of linker histones the C-terminal tail of H2A slightly alters contacts with DNA, indicating that the H2A C-terminal tail...
is repositioned upon linker histone binding (Guschin et al., 1998; Lee and Hayes, 1998). Thus, the C-terminal tail of H2A may influence linker histone binding to the nucleosome. The H1 homologue (Hho1p) of *S. cerevisiae* is a candidate linker histone, but with a domain structure different from the canonical linker histones (Woodcock et al., 2006). Canonical linker histones have a conserved globular winged-helix domain, a long and highly basic C-terminal, and a short N-terminal tail (Allan et al., 1980). The X-ray and NMR structures of the globular domains of the linker histones H5 and H1 revealed two basic clusters on opposite sides of the domains, suggesting the binding of two DNA strands (Cerf et al., 1994; Ramakrishnan et al., 1993), and providing a structural rationale for “bridging” a linker DNA segment with the DNA in the vicinity of the pseudodyad axis of the nucleosome (Zhou et al., 1998). Hho1p has two globular winged-helix domains, separated by a basic segment, and an N-terminal extension (Ali et al., 2004; Landsman, 1996; Ono et al., 2003). Similar to canonical linker histones, Hho1p reconstituted with H1-stripped chromatin confers a MNase kinetic pause at ~168 bp (Ali and Thomas, 2004; Patterton et al., 1998). Moreover, Hho1p forms a stable ternary complex with a reconstituted core di-nucleosome (Freidkin and Katcoff, 2001; Patterton et al., 1998). Based on *in vitro* experiments, showing each globular domain of Hho1p can interact independently with two different four-way junction DNA molecules, Hho1p has been suggested to bind two serially adjacent nucleosomes (Schäfer et al., 2005). But, whereas depletion of H1 variants in mice subtly reduced nucleosome repeat length (Fan et al., 2005), *hho1Δ* yeast cells have no detectable changes in nucleosome repeat length of bulk chromatin nor in nucleosome positioning on specific genomic loci (Freidkin and Katcoff, 2001; Patterton et al., 1998; Puig et al., 1999). In addition, *HHO1* deletion causes no detectable phenotype on yeast cell viability, growth, and sporulation (Patterton et al., 1998; Ushinsky et al., 1997). Gene expression is only modestly affected in the *hho1Δ* strain (Hellauer et al., 2001; Veron et al., 2006) and the only significant phenotype detected so far is an increase in homologous recombination (Downs et al., 2003). However, in contrast to higher eukaryotes, yeast chromatin is generally active, relatively highly acetylated (Waterborg, 2000), and has a short nucleosome repeat length (Lohr et al., 1977; Thomas and Furber, 1976). Consistently, Hho1p abundance in yeast cells is relatively low compared to linker histones in cells of higher eukaryotes (Downs et al., 2003; Freidkin and Katcoff, 2001). In additional support that Hho1p might be the putative yeast linker histone, *HHO1* transcription is tightly coordinated with transcription of the core histones (Spellman et al., 1998); and Hho1p dissociates in concert with the core histones from repressed promoters upon heat shock (Zanton and Pugh, 2006). Here, we used the *hta1-S129E, hta1-S129A, hta1-S129*/hta2-S129*, *hho1Δ*, and *hta1-S129*/hta2-S129* *hho1Δ* mutants to investigate the influence of the H2A-C-terminal tail and Hho1p on chromatin organization and stability in detail. Minichromosomes and genomic loci were analysed with respect to nucleosome positioning, nucleosome-nucleosome contacts and nucleosome stability. The accessibility of DNA was tested by micrococcal nuclease digestion of nuclei and, as an alternative *in vivo* approach, by DNA-repair of UV-lesions. We found
that in hta1-S129E strains chromatin stability is not compromised and indistinguishable from chromatin of HTA1 wild-type cells and the hta1-S129A mutant. The data support a role of S129 phosphorylation in recruitment of repair proteins rather than in destabilizing chromatin. Moreover, we show that neither deletion of the C-terminal tail nor deletion of HHO1 have any obvious effect on nucleosome stability and positioning.

2.2 Results

2.2.1 Mutations of the phosphorylation site S129 of H2A do not affect nucleosome positioning and stability on minichromosomes

To test whether the mutations S129A and/or S129E of histone H2A affect the stability of nucleosomes, nucleosome positioning and nucleosome-nucleosome contacts, we used two minichromosomes with well characterized nucleosome positions (YRpFT35 and YRpFT68; Figure 1 A) (Thoma and Zatchej, 1988). YRpFT35 shows nuclease sensitive regions at the TRP1-promoter (EcoRI site) and at the origin of replication (ARS1), four nucleosomes on TRP1, and nine nucleosomes in a region between EcoRI and ARS1 that contains a tandem repeat of Umid sequences (UmidA and UmidB; Umid corresponds to 3.5 nucleosomes of the URA3 coding region). Four of these nucleosomes are in close contact forming a nuclease resistant “tetranucleosome”. In YRpFT68 that was generated by insertion of the URA3 gene into YRpFT35, most of the structural features of YRpFT35 were maintained except the linker DNA in the tetranucleosome became accessible to micrococcal nuclease (MNase). The different structures on UmidA and UmidB demonstrate that the DNA-sequence does not determine nucleosome positioning in these regions (Thoma and Zatchej, 1988). The presence of positioned nucleosomes, tightly packed nucleosomes and nuclease sensitive regions makes those minichromosomes suitable substrates to test nucleosome positioning, stability and nucleosome-nucleosome contacts.

The minichromosomes were transformed into yeast strains in which both genomic loci coding for histones H2A and H2B (HTA1-HTB1 and HTA2-HTB2) were disrupted and which carry a wild-type or a mutant allele on a centromeric plasmid (Downs et al., 2000; Hirschhorn et al., 1995) (Table 1). Figure 1 B shows chromatin analysis by MNase of several strains, that contained the wild-type HTA1 (MFY37) or the mutant alleles hta1-S129A (MFY13) or hta1-S129E (MFY14). Both, the DNA patterns after MNase digestion of nuclei (nucleosomal repeats) as well as the digestion kinetics were indistinguishable in those strains, indicating that the mutations had no obvious effect on the general stability and organization of bulk genomic chromatin.

Figures 1 C and D reveal details of the chromatin structures of the minichromosomes with respect to nucleosome positioning and stability. Cleavage sites in chromatin are compared with those in naked DNA. Regions that are protected from cleavage in chromatin and encompass
Table 1. Histone H2A C-terminal and HHO1 mutant S. cerevisiae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>2µ</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY406</td>
<td>MATa his3Δ200 leu2Δ1 lys2-1285 trp1Δ63 ura3-52 GAL2' (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 YCpSAB6 [pRS316-HTA1-HTB1]</td>
<td>cir⁺</td>
<td>Hirschhorn et al., 1995</td>
</tr>
<tr>
<td>MFY1</td>
<td>as JHY2, but (hta2-htb2)Δ::trp1::URA3</td>
<td>n. a.</td>
<td>this study</td>
</tr>
<tr>
<td>MFY2</td>
<td>as JHY3, but (hta2-htb2)Δ::trp1::URA3</td>
<td>n. a.</td>
<td>this study</td>
</tr>
<tr>
<td>MFY3</td>
<td>as JHY8, but (hta2-htb2)Δ::trp1::URA3</td>
<td>n. a.</td>
<td>this study</td>
</tr>
<tr>
<td>MFY5</td>
<td>as MFY2, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>MFY6</td>
<td>as MFY3, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>MFY36</td>
<td>as MFY1, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>MFY13</td>
<td>as JHY3, plus YRpFT68 [URA3]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>MFY14</td>
<td>as JHY8, plus YRpFT68 [URA3]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>MFY37</td>
<td>as JHY2, plus YRpFT68 [URA3]</td>
<td>cir⁺</td>
<td>this study</td>
</tr>
<tr>
<td>W303a-JD</td>
<td>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100</td>
<td>n. a.</td>
<td>J.A. Downs</td>
</tr>
<tr>
<td>JDY22</td>
<td>as W303a-JD, but hta1-S129* hta2-S129*</td>
<td>n. a.</td>
<td>J.A. Downs</td>
</tr>
<tr>
<td>JDY41</td>
<td>as W303a-JD, but hho1Δ::HIS3</td>
<td>n. a.</td>
<td>J.A. Downs</td>
</tr>
<tr>
<td>JDY42</td>
<td>as W303a-JD, but hta1-S129* hta2-S129* hho1Δ::HIS3</td>
<td>n. a.</td>
<td>J.A. Downs</td>
</tr>
<tr>
<td>RHY1</td>
<td>as W303a-JD, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>R. Hulsker</td>
</tr>
<tr>
<td>RHY2</td>
<td>as JDY22, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>R. Hulsker</td>
</tr>
<tr>
<td>RHY3</td>
<td>as JDY41, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>R. Hulsker</td>
</tr>
<tr>
<td>RHY4</td>
<td>as JDY42, plus YRpFT35 [TRP1]</td>
<td>cir⁺</td>
<td>R. Hulsker</td>
</tr>
<tr>
<td>RHY5</td>
<td>as JDY42, plus YRpFT35 [TRP1]</td>
<td>n. a.</td>
<td>R. Hulsker</td>
</tr>
<tr>
<td>RHY6</td>
<td>as JDY22, plus YRpFT68 [URA3]</td>
<td>n. a.</td>
<td>R. Hulsker</td>
</tr>
</tbody>
</table>

140–160 bp are interpreted as positioned nucleosomes (Thoma et al., 1984). Most clearly, the cutting patterns were similar in all strains, both in YRpFT35 (Figure 1 C) and YRpFT68 (Figure 1 D). Nucleosome footprints (boxes in Figure 1 C, D) are readily identified as well as a long footprint characteristic for the tetranucleosome (dark box in UmidA) and the nuclease sensitive regions around the EcoRI site (arrow heads).

In order to better resolve the nuclease sensitive regions on YRpFT35 and the coding and terminator region of the URA3 gene on YRpFT68 the membranes were stripped and rehybridized with a probe on the other side of the XbaI site (Figure 1 A). YRpFT35 showed an extended nuclease sensitive region at the ARS1 and between the nucleosomes I and R1 (Figure 1 E, arrow heads). YRpFT68 revealed the characteristic nucleosome footprint of the URA3 gene and the nuclease sensitive terminator (Figure 1 F). These structures were maintained in the hta1-S129E and hta1-S129A mutants demonstrating that the S129 mutations did neither affect positions of spaced nucleosomes nor tightly packed nucleosomes. Moreover, the strong footprints indicate that nucleosomes were not destabilized. In addition, all nuclease sensitive regions were maintained in the different genetic background indicating that the mutations in the H2A-C-terminal tail
Figure 1. Chromatin structures of minichromosomes in hta1-S129 mutants. (A) Schematic representation of the minichromosomes YRpFT35 and YRpFT68. YRpFT35 contains the TRP1 gene and the autonomously replicating sequence ARS1. UmidA and UmidB are fragments of the URA3 gene inserted in the TRP1ARS1 circle. YRpFT68 was generated by insertion of URA3 in TRP1 of YRpFT35. Indicated are the EcoRI-XbaI and XbaI-EcoRV fragments used as probes for indirect end-labeling and nucleosome positions (circles) determined by MNase digestion. Four nucleosomes are tightly packed in YRpFT35 forming a tetranucleosome (dark circles).
In YRpFT68, the linker in the tetranucleosome is accessible (Thoma and Zatchej, 1988). (B) Analysis of bulk chromatin by MNase digestion. Nuclei from HTA1 (MFY37), hta1-S129A (MFY13), and hta1-S129E (MFY14) were digested with increasing amounts of MNase (0, 1, 2, 5, 10 and 15 units/ml). Purified DNA was separated on a 1% agarose gel containing ethidium bromide. M is a 2 log DNA ladder (New England BioLabs). (C) Nucleosome footprints on YRpFT35 obtained by MNase digestion and hybridization with the EcoRI-XbaI fragment. Chromatin (C) and DNA (D) isolated from yeast strains expressing the wildtype HTA1 (MFY36) or the mutant alleles hta1-S129A (MFY5) or hta1-S129E (MFY6) were analyzed by limited MNase digestion and indirect end-labeling. Wedges on top of the lanes denote increasing MNase concentrations. Rectangles on the left of the blot mark the positions of nucleosomes on the sequences UmidA and B and flanking sequences of the minichromosome. The arrow denotes the direction of TRP1 transcription. X indicates the XbaI restriction site. Arrow heads indicate open, non-nucleosomal regions. (D) Nucleosome footprints on YRpFT68 obtained by hybridization with the EcoRI-XbaI fragment. Chromatin and DNA isolated from HTA1 (MFY37), hta1-S129A (MFY13), and hta1-S129E (MFY14) strains was analyzed as in (C). 10 × 5S is a molecular size marker indicating multiples of 256 bp (Thoma et al., 1984). Dots indicate cleavage between nucleosomes at elevated levels of digestion. (E) Nucleosome footprints on YRpFT35 obtained by MNase digestion and hybridization with the XbaI-EcoRV fragment, analysed as in (C). (F) Nucleosome footprints on YRpFT68 obtained by hybridization with the XbaI-EcoRV fragment, analysed as in (D).

does not affect the origin of replication ARS1 and the TRP1 promoter regions.

Based on our previous observations, we expected enhanced cleavage between nucleosomes in UmidA of YRpFT68. Instead, the four nucleosomes remained quite resistant and enhanced cutting was evident only at higher levels of digestion (Figure 1 D, dots). This is not an effect of the S129 mutants, since it is also observed in MFY37 containing a wildtype HTA1 allele. The different accessibility of the linker DNA in the tetranucleosome is possibly related to the partial purification of minichromosomes used in the earlier study (see discussion).

Taken together these data suggested that neither of the hta1-mutants destabilized nucleosomes in minichromosomes nor in bulk chromatin, but maintained the structural and dynamic characteristics of wildtype chromatin.

2.2.2 H2A-S129E maintains chromatin stability

Since digestion kinetics with MNase are sensitive to variations in nuclear preparations, we performed co-digestion experiments with hta1-S129E and HTA1 wild-type cells. We prepared in parallel nuclei from HTA1 cells containing YRpFT68 (MFY37) and nuclei from hta1-S129E cells containing YRpFT35 (MFY6) from cultures of equal cell densities. The nuclei suspensions were split into two equal fractions. One fraction of MFY37 nuclei was mixed with an equal fraction of MFY6 nuclei for co-digestion with MNase. The remaining nuclei fractions were digested separately. Visualized by ethidium bromide staining, bulk chromatin from both HTA1 and hta1-S129E cells had similar accessibilities to MNase and indistinguishable nucleosomal repeat patterns (Figure 2 A). Scans of the lanes with clearly visible nucleosomal repeats (Figure 2 A, lanes 16 and 17) revealed no obvious difference in global nucleosomal organization.
Figure 2. Comparative MNase analyses of Hta1- and hta1-S129E chromatin. Nuclei isolated from HTA1 cells containing the minichromosomes YRpFT68 (MFY37) and nuclei from hta1-S129E cells containing the minichromosome YRpFT35 (MFY6) were separately digested or mixed and co-digested with increasing amounts of MNase (0–60 units/ml). (A) Analysis of bulk chromatin of separately digested nuclei. Purified genomic DNA was analyzed on a 1% agarose gel containing ethidium bromide. S (serine) and E (glutamic acid) indicate the residue at position 129 of histone H2A that was present in the digested nuclei. M (lanes 1 and 18) is a 2 log DNA ladder (New England BioLabs). (B) Scans of the lanes corresponding to 60 units MNase/ml shown in (A). The values were normalized in respect to the maximal band intensities (trinucleosomes). (C) Nucleosome footprints on YRpFT35 and YRpFT68. The DNA shown in (A) was digested with XbaI (X), separated on 1% agarose gels and transferred to Zeta-Probe GT membranes. The membranes were probed with an EcoRI-XbaI fragment of the TRP1 gene. Dots indicate cleavage between nucleosomes on YRpFT68 at elevated levels of digestion. (D) Equal amounts of nuclei of both strains were mixed and co-digested. DNA was analyzed as in (A). (E) Nucleosome footprints of co-digested YRpFT35 and YRpFT68. The DNA shown in (D) was analysed as in (C). 10 X 5S is a molecular size marker indicating multiples of 256 bp. (F) PhosphorImager scans of the two top bands of lanes 6–12 of the blot shown in (E). The values were normalized in respect to the band intensities of lane 6.

between HTA1 and hta1-S129E cells (Figure 2 B). The co-digestion of mixed nuclei also
showed a similar kinetic and the distinct bands reflecting the nucleosomal repeat manifested no difference in nucleosomal repeat length of *HTA1* and *hta1*-S129E chromatin (Figure 2 D). Indirect endlabelling confirmed the arrangement of nucleosomes in YRpFT35 and YRpFT68 in the individual digests (Figure 2 C). Probing the co-digested samples with a *TRP1* fragment detects both minichromosomes and allowed to assess digestion kinetics quantitatively (Figure 2 E, F). The signal corresponding to the full length linearized YRpFT68 and YRpFT35 (top bands) decreased similarly with increasing amounts of MNase. From scanning the top bands of the minichromosomes it became evident that *hta1*-S129E chromatin (YRpFT35) was not more susceptible to degradation by MNase than *HTA1* chromatin (YRpFT68) (Figure 2 F). Thus, both genomic chromatin and minichromosomes maintain stable nucleosomes and nucleosome positions in the *hta1*-S129E mutant.

### 2.2.3 Deletion of the C-terminal SQEL motif of H2A does not destabilize chromatin

The C-terminal SQEL motif of H2A might stabilize nucleosomes or higher order structures irrespective of the presence or absence of a negative charge at position 129. We therefore examined the chromatin structure of YRpFT68 in the strain RHY6, in which serine 129 of both genomic copies of H2A was mutated to a stop codon (*hta1*-S129*/hta2-S129*) (Downs et al., 2000). Chromatin from RHY6 showed a similar accessibility to MNase compared to chromatin isolated from an isogenic wild-type strain (RHY5) (Figure 3 A and B). The *hta1*-S129*/hta2-S129* mutant maintained the nucleosome positions in YRpFT68 (Figure 3 C) demonstrating
1, 8, and 15) is a 2 log DNA ladder (New England BioLabs). (B) Scans of the lanes corresponding to 30 units MNase/ml shown in (A). The values were normalized in respect to the maximal band intensities (dimucleosomes). (C) Nucleosome footprints on YRpFT68. The DNA shown in (A) was analysed as described in Figure 1. Solid dots indicate cleavage between nucleosomes on YRpFT68 at elevated levels of digestion. White dots represent double bands possibly originating from alternative nucleosome positions. In the W303 genetic background the EcoRI-XbaI fragment cross-hybridizes with the genomic TRP1 locus (asterix). 10 × 5S is a molecular size marker indicating multiples of 256 bp.

that the loss of the last four C-terminal amino acids of H2A does not destabilize chromatin. However, compared to strains in a different background we observed a subtle difference in positioning of nucleosomes R1, R2, R4 (see discussion).

MNase footprinting was also performed in a hta1-S129*/hta2-S129* strain (RHY2) containing the minichromosome YRpFT35 (Figure 4). However, this experiment did not produce a clear nucleosomal repeat (data not shown), presumably due to a significant fraction of unlysed cells in the nuclei extract. Incomplete accessibility of chromatin to MNase is reflected by the strong top band of YRpFT35 in Figure 4, indicating that a large part of the DNA has not been digested. Nonetheless, the cutting pattern in YRpFT35 was similar to that observed in previous experiments (Figure 1 C), except of subtle differences in nucleosome positioning on Umid B, as observed in YRpFT68 isolated from RHY5 and RHY6 (see discussion).

2.2.4 The putative yeast linker histone Hho1p does not control nucleosome positioning

In the crystal structure of the nucleosome the C-terminal tail of H2A is located close to the proposed binding site of the linker histone (Luger et al., 1997a; Thomas, 1999; Travers, 1999; White et al., 2001). Therefore the C-terminal tail of H2A may control linker histone association with chromatin (Paull et al., 2000). Above all, at least in higher eukaryotes, linker histones themselves affect chromatin structure in vitro and in vivo (Woodcock et al., 2006). To test if Hho1p might affect nucleosome positioning and nucleosome-nucleosome contacts, we tested
Chromatin structure of a hho1Δ strain containing the minichromosome YRpFT35 (RHY3). Chromatin isolated from a hho1Δ strain (RHY3) had a nucleosomal repeat (Figure 5 A and B) comparable to the HHO1 strains RHY5 and RHY6 (Figure 3 A). Moreover, YRpFT35 showed a similar nucleosome footprint in RHY3 (Figure 5 C) as in other strains (Figure 1 C). Thus, lack of Hho1p does not affect nucleosome positioning, which is consistent with previous observations (Freidkin and Katcoff, 2001; Patterton et al., 1998; Puig et al., 1999).

2.2.5 The H2A-S129E mutation maintains nucleosome positions in chromosomal loci

To test whether maintaining nucleosome positioning in hta1-S129E mutant cells was a unique property of minichromosomes, chromatin structures of the chromosomal GAL1-10 and PHO3-5 loci were analysed (Figure 6). Under repressing conditions intergenic regions between YBR094W and PHO5, PHO5 and PHO3 as well as between GAL1 and GAL10 contain the histone variant H2AZ (Htz1) (Raisner and Madhani, 2006), but HA-tagged Hta1 was relatively uniformly distributed across these loci (Santisteban et al., 2000). Cells were grown under conditions where the GAL1, GAL10 and PHO5 were not transcribed. Nucleosome footprinting by MNase revealed the characteristic chromatin structure in the GAL1-10 region (Cavalli and Thoma, 1993), namely positioned nucleosomes in GAL10, three nucleosomes in the promoter region flanking the UASG, as well as less precisely and partially overlapping nucleosome positions in GAL1 (Figure 6 B). Similarly, the PHO3-5 locus showed the characteristic chromatin structure, in particular the positioned nucleosomes in the PHO5 gene and the PHO5 promoter (Figure 6 C) (Almer and Horz, 1986). The chromatin structures in both loci were indistinguishable in HTA1 and hta1-S129E cells.

Figure 5. Chromatin of a HHO1 deletion strain. Nuclei isolated from hho1Δ cells containing the minichromosomes YRpFT35 (RHY3) was digested with increasing amounts of MNase (0, 1.5, 3, 5, 10, and 20 units/ml). (A) Purified genomic DNA was analyzed on a 1% agarose gel containing ethidium bromide. M (lane 7) is a 2 log DNA ladder (New England BioLabs). (B) Scan of the lane corresponding to 20 units MNase/ml shown in (A). The values were normalized in respect to the maximal band intensity (dinucleosomes). (C) Nucleosome footprint on YRpFT35 was analysed as described in Figures 1 and 3.
2.2.6 H2A-S129 mutations an HHO1 deletion have no effect on superhelical density of minichromosomes and the 2 \( \mu \) plasmid

Studying the superhelical density of circular minichromosomes can be applied to characterize chromatin (Simpson et al., 1985). Histone depletion (Kim et al., 1988) as well as histone mutations (including \( hta1 \)-S129E) were reported to affect supercoiling of the yeast 2 \( \mu \) plasmid (Downs et al., 2000; Masumoto et al., 2005; Wechsler et al., 1997). Since the MNase assay did not detect substantial chromatin changes in the \( hta1 \) mutants, we tested first supercoiling of the minichromosomes YRpFT35 and YRpFT68. The H2A mutants and the \( hho1 \Delta \) strain were grown in SD minimal medium and DNA was purified in parallel under conditions that prevent changes in plasmid DNA topology during cell lysis (Johnston and Williamson, 1978).
Figure 7. Superhelical densities of the minichromosomes and the 2 µ plasmids isolated from H2A mutant or HHO1 deletion strains. (A) Topoisomerase distribution of YRpFT35 and YRpFT68. The DNA isolated from the indicated strains was electrophoresed on 1% agarose gels containing 1 µg/ml chloroquine and transferred to a Zeta-Probe GT membrane. YRpFT35 and YRpFT68 superhelical density was analyzed by hybridization with a radiolabeled EcoRI-XbaI fragment from the TRP1 gene. A (alanine), E (glutamic acid), and S (serine) indicate the residue at position 129 of histone H2A. (B) Topoisomerase distribution of 2 µ plasmid. Indicated yeast strains were grown either in YPD (Y) or SD (S) minimal media. The DNA isolated from these strains was separated on a 0.75% agarose gel containing 1 µg/ml chloroquine and transferred to a Zeta-Probe GT membrane. The 2 µ plasmid superhelical density was analyzed by hybridization with a radiolabeled SnaBI-XbaI fragment from the 2 µ origin of replication. (C) Topoisomerase distribution of 2 µ plasmid in two dimensions. The same DNA as shown in (B) was separated on a 0.8% agarose gel in the first dimension in presence of 10 µg/ml chloroquine, rotated 90°, and in the second dimension in presence of 20 µg/ml chloroquine. The 2 µ plasmid superhelical density was analyzed as described in (B).

Plasmid supercoils were resolved in chloroquine-agarose gels and analyzed by Southern blotting. Topoisomer distributions for YRpFT35 and YRpFT68 were indistinguishable in all tested strains. Subtle differences in YRpFT35 supercoiling observed in RHY2 and RHY4 compared with the other strains (Figure 7) were not reproduced in other experiments (data not shown). Thus, the mutations did not obviously affect the average number of nucleosomes or their stability which is consistent with the MNase digestion results. Since the hta1-S129E mutation was reported to change supercoiling in the endogenous 2 µ plasmid (Downs et al., 2000) and since the culture medium might influence transcription and thereby supercoiling, we analysed 2 µ DNA of cells grown in YPD and SD minimal medium. No obvious variations in
supercoiling of the 2 µ plasmid was in the different strains, neither in SD minimal medium nor in YPD (Figure 7 B). Moreover, similar supercoil distributions in 2 µ plasmids were observed using two dimensional gels (Figure 7 C). Thus, the supercoiling assays confirm that neither hta1-S129A nor hta1-S129E destabilized nucleosomes.

2.2.7 Repair of UV lesions is not affected by H2A-S129E

As an alternative approach to study chromatin structure and accessibility of DNA to proteins in living cells we measured repair of UV-lesions by photolyase and NER. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) are two major classes of DNA lesions generated by UV-light (Friedberg et al., 1995). Both CPDs and 6-4PPs are repaired by NER, a multistep pathway that includes damage recognition, excision of an oligonucleotide containing the DNA-lesion, DNA-repair synthesis, and chromatin remodelling to reestablish chromatin structures (Gong et al., 2005; Thoma, 2005). Alternatively, many organisms, including yeast, have a CPD-photolyase, which selectively binds CPDs and reverses the damage in a light-dependent reaction (photoreactivation, PR) (Friedberg, 2003). NER and PR are modulated in chromatin by protein-DNA interactions, positioned nucleosomes, and heterochromatin (Thoma, 2005).

Budding yeast hta1-S129A mutants are sensitive to methyl methanesulphonate (MMS) and camptothecin (CPT) but insensitive to UV (Downs et al., 2000; Redon et al., 2003), indicating that H2A-S129 phosphorylation is not important for the repair of UV lesions. We confirmed that the hta1-S129A mutation renders cells more sensitive to MMS than wild-type or the hta1-S129E mutation (Figure 8 A). Furthermore, none of the hta1-S129 mutant strains was hypersensitive to UV-irradiation, but we noticed a very weak UV-sensitivity of hta1-S129A and hta1-S129E compared to HTA1 cells at a relative high dose used for repair experiments (150 J/m²) (Figure 8 B). Interestingly, a wild-type strain with the W303 genetic background showed an increased sensitivity towards UV light compared to the strains FY406, MFY1, and MFY36, in the S288C genetic background, but expressing the HTA1 allele from a centromeric plasmid. The different UV sensitivity might be attributed to RAD5, which is involved in postreplication repair of UV-damaged DNA (Torres-Ramos et al., 2002), and is mutated in the original W303 strain (rad5-535) (Fan et al., 1996).

DNA repair of UV-lesions by photolyase (photoreactivation) and NER was investigated in MFY36 (HTA1, YRpFT35) and MFY6 (hta1-S129E, YRpFT35). Cells were UV-irradiated with 150 J/m² to generate about 0.3 CPDs/kb, and either exposed to light for photoreactivation or incubated in the dark for NER. To analyze the distribution of CPDs in YRpFT35 and GAL1-10, the DNA was purified, cut at CPDs with T4-endonuclease V and digested with XbaI or EcoRV, respectively. The DNA fragments were separated on alkaline gels, transferred to nylon membranes, and hybridized to short strand-specific probes.
DNA of non-irradiated cells showed the intact restriction fragment (top band) irrespective of T4-endonuclease V treatment (Figure 9 A and B, lanes 1 and 2). DNA of irradiated cells showed the intact restriction fragment in the mock-treated lanes (lanes 12), but a specific band pattern and a weaker top band when cut at CPDs with T4-endonuclease V (lanes 11). The bands represent the yields and distribution of CPDs along the DNA fragment. Top and bottom strands revealed different patterns demonstrating strand specificity. The CPD patterns disappeared when cells were exposed to photoreactivating light (PR + NER, lanes 3 to 9) or incubated in the dark (NER, lanes 13 to 19). Repair of CPDs was quantified as a time-dependent increase of the intact restriction fragment (Figure 9 C and D). The CPDs were slowly removed by NER alone (about 50-70% in 4 hours for YRpFT35 and GAL1-10). However, CPDs were efficiently removed by PR and NER on GAL1-10 and YRpFT35 (> 80% in one hour) indicating that under those conditions photolyase was the predominant pathway to repair CPDs. Most clearly, there was no dramatic difference between repair curves of MFY36 (HTA1) and MFY6 (hta1-S129E). Thus, the accessibility and repair of UV-lesions is largely independent of the hta1-S129E mutation, which confirms the results of the MNase digestion and supercoiling. We noticed, however, that CPDs in the hta1-S129E strain seemed to be repaired more slowly, which is consistent with the
Figure 9. Repair of cyclobutane pyrimidine dimers (CPDs) by photolyase and NER. *HTA1* (MFY36) and *hta1-S129E* (MFY6) cells containing the minichromosome YRpFT35 were irradiated with 150 J/m² of UV light (lanes 3–20), exposed to photoreactivating light (PR + NER) for 30 to 120 minutes (lanes 3–10) or kept in the dark for 0 to 240 minutes for NER (lanes 11–20). The DNA was purified and cut at CPDs with T4-endonuclease V (T4).

(A) To analyse CPD repair on the minichromosome YRpFT35 the DNA was digested with XbaI (X) separated
on 1.5% alkaline agarose gels, transferred to Zeta-Probe GT membranes and hybridized with strand specific end-label probes generated by primer extension using the RIX DNA fragment as template (Figure 1). PhosphorImager scenes are shown for top and bottom strand. (B) To analyse CPD repair on the GAL1-10 locus the DNA was digested with EcoRV (V), and further processed as in (A). The GAL1-10 locus was hybridized with strand specific end-label probes generated by primer extension using the GAL1-SS1 DNA fragment as template (Figure 3). (C, D) Quantitative analysis of CPD repair on GAL1-10 and YRpFT35. The data are means and standard deviations of five gels from three experiments. (These experiments have been carried out mostly by Daniela Imholz.)

subtle UV hypersensitivity of the hta1-S129E mutant. However, whether this very subtle effect reflects a less accessible and, hence, a more stable chromatin structure in context with repair or a lower repair capacity in the mutant remains speculative.

### 2.2.8 Serine 129 of Hta1p is phosphorylated after UV irradiation

In mammalian cells serine 139 of H2A.X is phosphorylated in response to UV irradiation independent on the presence of DSBs, but dependent on NER factors (Marti et al., 2006; Matsumoto et al., 2007; O’Driscoll et al., 2003). UV irradiation mainly induces H2A.X phosphorylation as a diffuse, even, pannuclear staining in contrast to ionizing radiation-induced nuclear γ-H2A.X foci (Marti et al., 2006). Phosphorylation of serine 129 of H2A after UV irradiation may bias our study of chromatin structure by measuring accessibility of DNA lesions by repair enzymes in MFY36 (HTA1) cells. Therefore, preliminary experiments were carried out to test by Western blot if S129 of Hta1p is phosphorylated after UV irradiation of yeast cells. First, to test the specificity of the antibody against phosphorylated H2A, histones were extracted from cells, that were either untreated or treated with camptothecin, which has been reported to result in H2A phosphorylation (Redon et al., 2003). The phosphor-specific antibody recognized H2A isolated from MFY36 (HTA1) cells (Figure 10 A), but fails to recognize H2A that was extracted from MFY5 (hta1-S129A) cells (Figure 10 A, lanes 1 and 2). Since H2A in MFY5 can not be phosphorylated at position 129, these results indicate that the antibody was specific for γ-H2A. Treatment of MFY36 cultures with camptothecin for 15 or 30 minutes induced a time-dependent increase in antibody signal (Figure 10 A, lanes 3–6). Figure 10 B reveals that H2A is phosphorylated also after treatment of MFY36 (HTA1) with UV light. Accumulation of γ-H2A was observed already within 15 minutes incubation of the yeast cultures at 30°C after UV irradiation. Treatment of MFY36 with camptothecin, leading to DSB during replication and subsequent H2A-S129 phosphorylation (Redon et al., 2003), showed a similar γ-H2A accumulation only after incubation for 30 minutes at 30°C (Figure 10 A and B). This suggests that upon UV irradiation, H2A phosphorylation specifically depends on UV damages rather than on DSBs formed by processing of UV lesions. Regrettably, γ-H2A levels from these Western blots can not be quantified, because of lacking loading controls (an anti-H3 antibody did not work on Western blots).
Figure 10. Phosphorylation of serine 129 of H2A after UV irradiation. Western blot analysis of crude histone extracts from yeast cells with anti-phospho-H2A antibody. (A) Specificity test of the antibody. Indicated yeast strains were grown in SD media supplemented with amino acids and nucleotides to an $A_{\text{600}}$ of about 1 at 30°C. Camptothecin (10 mg/ml in DMSO) was added to a final concentration of 20 µM to two aliquots of MFY36 and they were incubated at 30°C for 15 and 30 minutes, respectively. The cells from 5 ml samples were lysed using glass beads in presence of 0.2 M H$_2$SO$_4$ and the extracted proteins were precipitated with TCA. The proteins were resolved in SDS-PAGE sample buffer, separated on 15% SDS polyacrylamide gels, and transferred in a Towbin buffer to nitrocellulose membranes. The membranes were incubated with an anti-phospho-H2A antibody diluted 1/1000 in 3% BSA in TBS-Tween. Wedges on top of the lanes denote increasing sample amount loaded on the gel (5 and 10 µl). (B) Monitoring Hta1p-S129A phosphorylation after UV irradiation. MFY36 was grown to an $A_{\text{600}}$ of about 0.5 at 30°C and two aliquots were treated as in (A) with camptothecin. The remaining culture was centrifuged, the cells were resuspended in SD, and aliquots were distributed to small Petri dishes for UV irradiation (50–600 J/m²). Amino acids and nucleotides were added and the samples were incubated for 15 minutes at 30°C before histones were extracted and analysed by Western blotting as in (A).

2.3 Discussion

2.3.1 Serine 129 of histone H2A and chromatin structure

Driven by the hypothesis that phosphorylation of yeast H2A might disturb chromatin structures to facilitate repair of strand breaks, we tested chromatin structures in an hta1-S129E mutant mimicking phosphorylation and the corresponding wild-type strain ($HTA1$). In contrast to a previous observation (Downs et al., 2000), we did not observe a difference between these two strains with respect to nuclease digestion nor by supercoiling assays. In addition, we show that the hta1-S129E mutation does neither affect stability nor positioning of nucleosomes. Finally, repair of UV lesions, as an alternative approach to assess chromatin accessibility in vivo, revealed similar repair rates in both strains. So far, we can not explain the different observations made by us and Downs et al. (Downs et al., 2000). However, we strongly emphasize that the discrepancy on chromatin accessibility does not affect the major conclusions on DNA repair by Downs et al.

So far we can only speculate that the different observations reflect subtle variations in the nuclease digestion and supercoiling assays. Indeed, since supercoiling depends not only on the number and stability of nucleosomes, but also on the transcriptional activity, temperature and
topoisomerase activity during isolation, variations in growth or preparation conditions might impact supercoiling (e.g. (Giaever and Wang, 1988; Kim et al., 1988; Saavedra and Huberman, 1986; Simpson et al., 1985)). We therefore investigated two different artificial minichromosomes with one active gene each (TRP1 and URA3, respectively) as well as the natural 2 μ plasmid and we used YPD as well as SD minimal medium. Moreover, we checked the strains with respect to UV-sensitivity, MMS sensitivity, and verified the hta1-mutations in our strains by sequencing (not shown). Thus, we are confident that under our conditions, there is no substantial difference in supercoiling in the different histone mutants. It should, however, be mentioned that other histone mutants were reported to impair the ability of nucleosomes to constrain supercoils in vivo: a histone H3 residue (hht1-K56Q) that makes water mediated DNA contacts at the entry and exit site of the nucleosome core (Masumoto et al., 2005) and sin mutations in histone H4, that define a discrete domain on the nucleosome surface (Wechser et al., 1997).

There is also variability in chromatin analysis by MNase digestion. First, it must be considered that MNase cuts DNA and RNA (Anfinsen et al., 1971). Hence, variations in the RNA content of nuclear preparations might affect digestion kinetics. Second, MNase digestions might be compromised by variations in nuclear and chromatin preparations that affect composition and stability of chromatin. Different chromatin preparations (e.g. partial purification of minichromosomes versus crude nuclear preparations) might explain different extent of cleavage of linker in the tetranucleosome region of YRpFT68. Third, disruption of either histone locus, HTA1-HTB1 or HTA2-HTB2, was shown to result in different phenotypes (Clark-Adams et al., 1988; Norris and Osley, 1987), including alterations of chromatin structure (Norris et al., 1988). (hta1-htb1)Δ strains can compensate histone levels by amplification of the HTA2-HTB2 gene pair (Libuda and Winston, 2006), whereas (hta2-htb2)Δ strains increase transcription of HTA1-HTB1 (Moran et al., 1990; Norris and Osley, 1987). Thus, it is conceivable that, depending on the genetic background, the strains might have different histone levels which could affect chromatin structure.

Here, we compared chromatin results using the same chromatin preparation technique and found no major differences between hta-mutants and wild-type strains of the same genetic background. Interestingly, we observed subtle differences in nucleosome positions R1, R2, and R4 of YRpFT68, depending on the genetic background of the stains. Strains expressing H2A and H2B from endogenous alleles showed two double bands of similar intensity (Figure 3 C, white dots). However, in stains which express the HTA1-HTB1 allele from a centromeric plasmid and miss the HTA2-HTB2 allele, the upper bands of those double bands were more pronounced (Figures 1 and 2). It is possible that the differences in nucleosomes positions R1, R2, and R4 are related to the different genetic background expressing all histone loci and the strains expressing only one subtype or mutation from a centromeric plasmid.

Contacts of the C-terminal tail of H2A with the N-terminal tail of H2A of a neighbouring particle in crystallized yeast nucleosome core particles (White et al., 2001), crosslinking of the C-terminal tail to linker DNA (Usachenko et al., 1994), as well as additional protection of linker
DNA against MNase digestion in nucleosomes containing H2A with an extended C-terminal tail (Lindsey et al., 1991) indicated that this tail might affect both the stability of nucleosomes and potential interactions with flanking nucleosomes and linker DNA in higher order structures. Phosphorylation at H2A-S129, as it occurs in DNA-repair, as well as the hta1-S129E mutation alters the charge of an amino acid residue and thereby might mediate changes in chromatin by disruption of histone-DNA contacts or nucleosome-nucleosome interactions. Our experiments in yeast do not support that hypothesis. Nucleosomes remained stable with respect to nuclease sensitivity and positioning in all regions analysed. Moreover no effect was obvious in the region of tightly packed nucleosomes. In addition, we show that photoreactivation and NER, two different DNA repair pathways which are known to be modulated by chromatin structures (Thoma, 2005), did not reveal major effects of the hta1-S129E mutant on chromatin structure and stability. Thus, we take this as an indication that a negative charge at S129 of the C-terminal tail has no direct role in the organization and stability of nucleosomes and nucleosome-nucleosome contacts in yeast.

2.3.2 Phosphorylation of Serine 129 of histone H2A and DNA repair

Preliminary data suggest that Hta1p becomes phosphorylated in response to UV irradiation (Figure 10). Although these results are not quantitative, they indicate that H2A gets phosphorylated already at a relatively low UV dose of 50 J/m². Moreover, compared to camptothecin treatment, H2A phosphorylation after UV irradiation occurs quite fast, with significant phosphorylation after 15 minutes incubation of the culture at 30°C. In human fibroblasts, H2A.X phosphorylation after UV treatment has been reported to depend on NER and not on DSBs (Marti et al., 2006; Matsumoto et al., 2007). In fibroblasts, UV irradiation displays a diffuse γ-H2A.X staining of the whole nucleus, in contrast to ionizing radiation induced foci. γ-H2A.X staining is more intense in S phase of the cell cycle than in G1 (Marti et al., 2006; Matsumoto et al., 2007). Phosphorylation of H2A.X in response to UV irradiation and ionizing irradiation seems not to be performed by the same kinase (Marti et al., 2006; O’Driscoll et al., 2003). Moreover, γ-H2A.X levels after UV irradiation are reduced in cells defective for XPA, XPC, or XPG within G1 and S phase (Marti et al., 2006; Matsumoto et al., 2007). Therefore it would be interesting to investigate the cause of H2A-S129 phosphorylation in response to UV irradiation in yeast. On one hand, H2A phosphorylation should be investigated within different phases of the cells cycle. On the other hand, UV induced γ-H2A levels should be compared in different NER defective strains, including rad14Δ (XPA homologue), rad4Δ (XPC homologue). However, H2A phosphorylation seems not to play an important role in repair of UV lesions, since hta1-S129A stains do not show a strong hypersensitivity after UV-irradiation, as observed with MMS or camptothecin (Downs et al., 2000; Redon et al., 2003). Nevertheless, it might be interesting to test if hta1-S129A stains show a reduced NER capacity.
γ-H2A.X might act in DSB repair by disruption of chromatin and/or by recruitment of chromatin remodelling activities and repair components. Tsukuda et al. showed that no histone loss occurred in the first 30 min after induction of a DSB, although γ-H2A accumulated rapidly and extensively on either side of the break. Furthermore, an H2A mutant lacking the SQ motif lost histones at the break to the same extent as a wild-type strain (Tsukuda et al., 2005). While those experiments do not address the stability of nucleosomes, they indicate that nucleosome displacement at the break depends on the recruitment of remodelling factors. These data are consistent with our observation that the negative charge at H2A-S129E does not destabilize chromatin structure.

Studies with mammalian cells revealed that presence of H2A.X is not prerequisite for chromatin decondensation around a DSB. Decondensation rather depends on ATP, indicating the action of remodelling factors near the break, also in the absence of H2A.X (Kruhlak et al., 2006). Phosphorylated H2A.X appears to regulate cellular responses to DSB by binding effector proteins, as shown for MDC1 (Stucki et al., 2005). In yeast, phosphorylated H2A interacts with Nhp10, a subunit of the INO80 remodelling complex (Morrison et al., 2004) and the NuA4 subunit Arp4p binds phospho-H2A peptides (Downs et al., 2004; Morrison et al., 2004). Thus, phosphorylation of H2A(X) may rather act as a binding interface for chromatin-associated proteins involved in DSB repair than directly alter the level of chromatin compaction. In support of this suggestion, recent data showing that loss of MDC1-γ-H2A.X interaction or ablation of H2A.X has quite the same phenotype in mammalian cells and in mice as the H2A.X deletion (Lou et al., 2006; Stucki et al., 2005), thus supporting the idea that in mammals, the main function of γ-H2A.X is to interact with MDC1.

2.3.3 Contributions of the C-terminal tail of histone H2A and the histone H1 homologue Hho1p to chromatin structure

The carboxyl terminus of H2A makes contacts with the linker DNA and, if linker DNA is missing, repositions to contact the DNA at the pseudodyad axis of the nucleosome (Usachenko et al., 1994). Therefore, a truncation of the C-terminal tail of H2A by four amino acids might destabilize chromatin structure. On the other hand, the location of the globular domain of canonical liker histones on the vicinity of the pseudodyad axis might be influenced by the carboxyl terminus of H2A (Guschin et al., 1998; Lee and Hayes, 1998). Thus, H2A truncated at its C-terminal tail could affect binding of the linker histone to the nucleosome, and thereby disturb chromatin higher order structure. Hho1p in S. cerevisiae contains two regions of sequence homology to the central globular domain of the canonical histone H1 and, therefore is a candidate linker histone, albeit with unusual domain organisation. An H1 homologue might be needed to stabilise nucleosome-nucleosome contacts in yeast.

Our results showed that chromatin isolated from hta1-S129*/hta2-S129* mutant strains
had normal nuclease susceptibility and nucleosome positions on YRpFT35 and YRpFT68 (Figure 3 and 4). Moreover, an *hta1-S129*/hta2-S129* mutant did not affect supercoiling of a minichromosome (Figure 7). These data indicate that the loss of the last four amino acids of the H2A C-terminal tail did not markedly affect chromatin structure. Second chromatin studies with a *hho1Δ* strain revealed that the loss of Hho1p neither affected nucleosome repeat length, nor nucleosome positioning (Figure 5), or minichromosome supercoiling (Figure 7). Third, we showed that the combination of the C-terminal truncation in H2A with the *HHO1* deletion did not have an obvious effect on the superhelical density of a minichromosome (Figure 7).

The observations that deletion of the last four amino acids of the C-terminal tail of H2A did neither affect chromatin stability nor higher order chromatin structure are consistent with the finding that mutations at position 129 of H2A had no influence on chromatin structure. Thus, this investigation strongly suggests that the C-terminal tail of H2A is not directly involved in regulating chromatin compaction.

Nucleosome positioning on the genomic loci *STE6*, *YIL161w-POT1*, and in the centromeric region of chromosome III has been reported to be independent of Hho1p (Patterton et al., 1998; Puig et al., 1999). Moreover, previous findings revealed that deletion of *HHO1* has no detectable effect on the nucleosomal repeat (Freidkin and Katcoff, 2001; Patterton et al., 1998). These observations are in agreement with our data, that loss of Hho1p did not affect chromatin structure. Abundance of Hho1p in yeast is lower than linker histones in many higher eukaryotic cells. The stoichiometry of Hho1p and nucleosomes has been variously reported as 1 Hho1p per 4 nucleosomes (Downs et al., 2003), 1 Hho1p per 10 nucleosomes (Ghaemmaghami et al., 2003), and 1 Hho1p per 37 nucleosomes (Freidkin and Katcoff, 2001). Therefore it is possible that Hho1p is not associated with YRpFT35, but might play a role in chromatin organization at specialized genomic loci. Genomic localization of Hho1p have been reported variously as being widely associated with a variety of genomic locations (*MAT* locus, *HIS3*, an intergenic region on chromosome X, *CEN4*, a subtelomeric region on chromosome VI, and the rDNA repeat) (Downs et al., 2003) or being preferentially bound to the rDNA locus (Freidkin and Katcoff, 2001). Interestingly, in partially purified minichromosomes the linker DNA in the tetranucleosome of YRpFT68 showed a better accessibility to MNase than in crude nuclei extracts (Thoma and Zatchej, 1988) (Figure 1 D and 2 C). It can be speculated that during minichromosome purification certain chromatin bound proteins, which stabilized this structure in crude nuclei extracts, were detached from the tetranucleosome. Hho1p could be a candidate, and therefore it would be interesting to test nucleosome positioning on YRpFT68 in crude nuclei extracts isolated from *hho1Δ* cells.
3 Contributions of Histone H3 Nucleosome Core Surface Mutations to Chromatin Structure

3.1 Introduction

The nucleosome core particle shows a distinct, fissured and irregular disk surface of the histone octamer with a very distinctive charge distribution (Arents et al., 1991; Luger et al., 1997). This surface was implicated in nucleosome-nucleosome contacts to promote chromatin higher order structure formation (Luger, 2006). A surface located at a H3-H4 handshake motif flanks a large protuberance made up of several residues, including lysine 79, from the L1 loop of histone H3. Between this surface and the adjacent surface of histone H2B lies a rather deep groove (Figure 11 D). Based on manual docking experiments with a histone H3 N-terminal tail peptide, this groove is wide enough to accommodate a peptide, possibly an N-terminal histone tail (Park et al., 2002). Another surface near the centre of the octamer disc is an intensely negatively charged region formed by the H2A-H2B dimer, the so called acidic patch, which interacts with the N-terminal tail of H4 of an adjacent nucleosome core in the crystal structure (Luger et al., 1997). The N-terminal tail of histone H4 is required for condensation of nucleosomal arrays in vitro (Dorigo et al., 2003) and acetylation of lysine 16 of H4 prevents fibre folding (Shogren-Knaak et al., 2006), presumably by inhibiting internucleosomal interaction between the N-terminal tail of H4 and the acidic patch. Similarly the groove between H2B and the H3-H4 handshake motif and the adjacent surfaces might also be involved in nucleosome-nucleosome contacts.

In a genetic screen for histone H3 and H4 mutations that impair rDNA or telomeric silencing (Park et al., 2002; Thompson et al., 2003), mutated amino acid residues were identified that cluster in a discrete region on the surface of the (H3-H4)4 tetramer around lysine 79 of H3. According to their “loss of rDNA silencing” (Lrs-) phenotype, the mutant H3 and H4 alleles were called lrs (Park et al., 2002). Their phenotypes are similar to those of yeast sir2 mutants, which are defective in silencing of rDNA, HM loci, and subtelomeric regions. Some of the altered residues are located in the L1L2 motif of H3-H4 that bind the DNA at the superhelical location (SHL) ±2.5 (Figure 11 A and B). They resemble mutations that partially alleviate the transcription defects caused by the inactivation of the chromatin remodelling complex SWI/SNF, thus called switch-independent (sin) (Fry et al., 2006; Kruger et al., 1995). The lrs mutations in the L1L2 motif which contacts the DNA at SHL±0.5 can be structurally superimposed with the sin mutations (Fry et al., 2006) and thus could destabilize histone-DNA interactions similar to sin mutations (Flaus et al., 2004; Muthurajan et al., 2004; Park et al., 2002; Thompson et al., 2003). However, other mutations mapped distant from DNA binding sites on the nucleosome core surface near the groove between the H3-H4 handshake and H2B, and may not simply destabilize the nucleosome, but may rather disrupt an interface for silencing protein binding or internucleosomal interactions in higher order chromatin structure (Park et al., 2002; Thompson...
Lysine 79 of H3 is methylated by the conserved histone methyltransferase Dot1p (disruptor of telomeric silencing) (Ng et al., 2002a; Singer et al., 1998; van Leeuwen et al., 2002), and this occurs only when H3 is assembled into nucleosomes (van Leeuwen et al., 2002). Methylation of lysine 79 in transcriptionally active chromatin has been shown to be important for gene silencing at telomeres, HM loci, and rDNA, presumably by preventing the spreading of Sir proteins throughout the genome (Ng et al., 2003; van Leeuwen et al., 2002). On the other hand, the presence of Sir proteins at subtelomeric regions and the HM loci seems to limit Dot1p to methylate lysine 79 of H3, supporting an indirect effect of Dot1p on gene repression in silenced chromatin (Katan-Khaykovich and Struhl, 2005; Ng et al., 2003). For efficient methylation of lysine 79 (as well as lysine 4) of histone H3, ubiquitylation of histone H2B at lysine 123 by Rad6p is required (Briggs et al., 2002; Ng et al., 2002b; Sun and Allis, 2002). However, H2B ubiquitylation is not essential for Dot1p recruitment and monomethylation of lysine 79, but is necessary for di- and trimethylation (Shahbazian et al., 2005), which seems to be important for gene silencing (Emre et al., 2005).

In mammalian cells methylated lysine 79 of H3 serves as a binding site for the conserved checkpoint protein 53BP1 (p53 binding protein 1) near to DSBs (Huyen et al., 2004). 53BP1 interacts through Tudor domains with high affinity with a H3 peptide dimethylated at lysine 79 and with lower affinity with a monomethylated peptide (Huyen et al., 2004). In S. cerevisiae the DNA damage-dependent checkpoint protein Rad9p shares homology with 53BP1 and contains a globular domain, which is structurally similar to the Tudor domains of 53BP1 (Alpha-Bazin et al., 2005). In yeast Dot1p-dependent methylation of lysine 79 is required for DNA damage checkpoint function of Rad9p in response to DNA damage (Game et al., 2005; Giannattasio et al., 2005; San-Segundo and Roeder, 2000; Wysocki et al., 2005). However, after ionizing irradiation methylation of lysine 79 is not enhanced, like phosphorylation of serine 129 of H2A (Huyen et al., 2004). Therefore it was suggested that increased exposure of pre-existing methylated lysine 79 by changes in higher order chromatin structure induced for instance by a DSB would account for the recruitment of 53BP1 (Huyen et al., 2004). A recent report showed that in yeast the chromatin assembly factor CAF1 interacts with H3 methylated at lysine 79 in the processes of silencing and DNA repair (Zhou et al., 2006).

To test chromatin structure in yeast, we have chosen lrs mutations in histone H3 (HHT2) that are located on the α1L1 histone fold motif (Figure 11 C), which has been proposed to represent a distinct functional domain playing a key role in silencing (Thompson et al., 2003). The residues of leucine 70 (L70) and glutamic acid 73 (E73) map to the N-terminal end of the α1-helix of H3. Lysine 79 (K79) and threonine 80 (T80) are in the L1 loop adjacent to α1 of H3 (Figure 11 C). The mutations H3-L70S (hht2-L70S) and H3-E73D (hht2-E73D) were reported to reduce telomeric silencing of a URA3 reporter gene and to be defective in basal repression of a GAL1-URA3 construct (Thompson et al., 2003). These observations suggested that these particular mutations affect general properties of chromatin. In addition, whereas H3-E73D shows a strong
Figure 11. Mapping of lrs mutations to the surface of the nucleosome core particle. (A) Front view of one half NCP structure down the DNA superhelix axis. 73 bp of two superimposed DNA sequences (light blue and pale yellow) and associated histone protein main chains (histone folds of H2A, H2B, H3, and H4 in yellow, red, blue, and green, respectively, tails and extensions in white,) are shown as ribbon traces. The pseudo-twofold axis is aligned vertically with the central base pair of the DNA at the top. The superhelical locations (SHLs) are indicated, as are the location of the sin and the lrs mutations on H3 and H4, as well as the α1-helix and the L1-loop of H3. (B) Side view of the NCP structure obtained by a 90° rotation of an overall structure shown in (A) around the vertical pseudo-twofold axis. Parts of the DNA are omitted to reveal the location of the α1-helix and L1-loop of histone H3. (C) Enlarged ribbon diagram view of the α1-L1 region of histone H3. The α1-helix comprising residues 64–78 and the adjacent L1-loop comprising residues 79–85 are in dark grey and parts of H4 are in light grey. The DNA is shown in stick representation in the background. Positions of the lrs H3 mutations are indicated as dots, in red those used in this study and others in white. The N-terminal tail and the L2-loop of H4 are denoted. (Adapted and modified from (Thompson et al., 2003)). (D) Enlarged view along a groove between the lrs region (in dark grey on the right) and histone H2B (in light grey on the left) on the NCP. Histone moieties are displayed as isosurface and the DNA as stick representation in the front. The red asterisk denotes the position of the ε-nitrogen of the lysine 79 residue of histone H3 that is methylated by Dot1. The arrow indicates the location of the groove. 2.5 refer to the superhelical location. ((A) and (B) are reproduced and modified from (Luger, 2003), (C) from (Thompson et al., 2003), and (D) from (Park et al., 2002)).

reduction of silencing at HML, the H3-L70S mutant causes only a minimal effect at HML, but is lethal at 37°C. In contrast, the mutations H3-K79E (hht2-K79E) and H3-T80A (hht2-T80A) appear to affect silencing in a more specific manner. These mutations have very pronounced
effects on telomeric *URA3* and *HML* silencing, but have no obvious effect on basal repression and do not exhibit any apparent growth defects. Furthermore, the mating deficiencies caused by E73D and K79E are restored to near-wild-type levels by *sir3* suppressor alleles (Thompson et al., 2003). The exposure of the side chains of those mutated residues on the nucleosome surface, suggested that they may either be involved in silencing protein binding or internucleosomal interactions (Park et al., 2002; Thompson et al., 2003).

Here we analysed nucleosome positioning, stability, and nucleosome-nucleosome contacts in a minichromosome and in subtelomeric *URA3* gene in the *hht2*-L70S, *hht2*-E73D, *hht2*-K79E, and *hht2*-T80A mutants. Surprisingly, none of the mutations showed any severe effect on nucleosome contacts, stability, and positioning. However, loss of silencing of the subtelomeric *URA3* gene seemed to be caused by a shift of a nucleosome at the promoter. Since a similar effect was observed in a *sir3Δ* strain, we speculate that the impact of silencing in these mutants is rather due to an altered recruitment and impaired spreading of silencing factors than a direct histone-induced change in nucleosome positions.

### 3.2 Results

#### 3.2.1 Histone H3 *lrs* mutations largely maintain chromatin stability

Strains in which both genomic loci coding for histones H3 and H4 (*HHT1*-*HHF1* and *HHT2*-*HHF2*) were disrupted and which carry a wild-type or a mutant allele on a centromeric plasmid (Thompson et al., 1994; Thompson et al., 2003) were transformed with the minichromosome YRpFT35 (Table 2). To test whether the mutations of histone H3 affect the stability, positioning of nucleosomes and internucleosomal contacts, nuclei were prepared from the strains MFY15 (*HHT2*), MFY16 (*hht2*-T80A), MFY17 (*hht2*-K79E), MFY18 (*hht2*-L70S), and MFY19 (*hht2*-E73D) and digested with MNase. Purified DNA was separated on a 1% agarose gel, stained with ethidium bromide. Bulk chromatin from all tested strains displayed clear nucleosomal ladders and a similar accessibility to MNase (Figure 12 A and B). These results suggest that the mutations *hht2*-T80A, *hht2*-K79E, *hht2*-L70S, and *hht2*-E73D do not affect global chromatin organization.

Indirect end-labelling from the XbaI site of YRpFT35 revealed the arrangement of the nucleosomes in this minichromosome. Cleavage sites in chromatin are compared with those in naked DNA. Regions that are protected from cleavage in chromatin and encompass 140–160 bp are interpreted as positioned nucleosomes (Thoma et al., 1984). The cutting patterns in YRpFT35 were similar in all tested strains (Figure 12 C). Nucleosome footprints (boxes in Figure 12 C) are readily identified as well as a long footprint characteristic for the tetranucleosome (dark box in UmidA) and the nuclease sensitive regions around the EcoRI site and *ARS1* (arrow heads). In the area of the nucleosomes R1, R2, and R4, we observed subtle differences between the strains.
expressing the divergent HHT2 alleles. The hht2-K79E (MFY17), hht2-L70S (MFY18), and hht2-E73D (MFY19) strains showed two double bands of similar intensity (Figure 12C, white dots), while in the HHT2 (MFY15) and the hht2-T80A (MFY16) strain the upper bands of the double bands were more pronounced. These differences are similar to those observed between strains differing in their genetic background in respect of the HTA2-HTB2 allele, discussed in Part I. Though, in these cases the differences seem to depend on the specific lrs mutation in the HHT2 allele.

Table 2. S. cerevisiae strains carrying histone H3 lrs mutations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>2 µ</th>
<th>Reference/Source</th>
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<tr>
<td>JTY34U</td>
<td>MATα ade2-101(och) his3Δ200 LEU2 or leu2-3,112 lys2-801 (amb) trp1Δ901 ura3-52 adh4Δ::URA3-TEL (hhf1-hht1)Δ::LEU2 (hhf2-hht2)Δ::HIS3 YCpJT34 [pRS317-HHF2-HHT2]</td>
<td>n. a.</td>
<td>J. S. Thompson</td>
</tr>
<tr>
<td>MFY15</td>
<td>as JTY34U, plus YRpFT35 [TRP1]</td>
<td>cir⁻</td>
<td>this study</td>
</tr>
<tr>
<td>MFY16</td>
<td>as JTY307U, plus YRpFT35 [TRP1]</td>
<td>cir⁻</td>
<td>this study</td>
</tr>
<tr>
<td>MFY17</td>
<td>as JTY308U, plus YRpFT35 [TRP1]</td>
<td>cir⁻</td>
<td>this study</td>
</tr>
<tr>
<td>MFY18</td>
<td>as JTY309U, plus YRpFT35 [TRP1]</td>
<td>cir⁻</td>
<td>this study</td>
</tr>
<tr>
<td>MFY19</td>
<td>as JTY319U, plus YRpFT35 [TRP1]</td>
<td>cir⁻</td>
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Chromatin Structure of lrs H3 Mutants

Figure 12. Chromatin structures of minichromosomes in lrs H3 mutants. (A) Analysis of bulk chromatin by MNase digestion. Nuclei from HHT2 (MFY15), hht2-T80A (MFY16), hht2-K79E (MFY17), hht2-L70S (MFY18), and hht2-E73D (MFY19) were digested with increasing amounts of MNase (1.5, 3, 5, 10 and 20 units/ml for MFY15, MFY18, and MFY19 and 1, 5, 10, 20, and 45 units/ml for MFY16, MFY17). Purified DNA was separated on a 1% agarose gel containing ethidium bromide. M is a 2 log DNA ladder (New England BioLabs). Ethidium bromide staining below 0.1 kb for MFY16 and MFY17 samples (lanes 8–16) represents RNA that was incompletely digested during purification of DNA from crude nuclei extracts. (B) Scans of the lanes corresponding to 20 units MNase/ml shown in (A). The values were normalized in respect to the maximal band intensities (mononucleosomes). (C) Nucleosome footprints on YRpFT35 obtained by MNase digestion and hybridization with the EcoRI-XbaI fragment. Chromatin (C) and DNA (D) isolated from yeast strains expressing the wildtype HHT2 (MFY15) or the mutant alleles hht2-T80A (MFY16), hht2-K79E (MFY17), hht2-L70S (MFY18) or hht2-E73D (MFY19) were analyzed by limited MNase digestion and indirect end-labeling. Wedges on top of the lanes denote increasing MNase concentrations. Rectangles on the left of the blot mark the positions of nucleosomes on the sequences UmidA and B and flanking sequences of the minichromosome. The arrow denotes the direction of TRP1 transcription. X indicates the XbaI restriction site. Arrow heads indicate open, non-nucleosomal regions. White dots represent double bands possibly originating from alternative nucleosome positions.

In general, the characteristic structures of YRpFT35 were maintained in the hht2 mutants demonstrating that these mutations did not markedly affect positions of spaced nucleosomes nor tightly packed nucleosomes. Moreover, the strong footprints indicate that nucleosomes were not destabilized. In addition, the nuclease sensitive regions were maintained in all lrs H3 mutant strains, suggesting that these mutations do not affect the origin of replication ARS1 and the TRP1 promoter regions.

Taken together, the hht2-T80A, hht2-K79E, hht2-L70S, and hht2-E73D mutants do not obviously change the accessibility of bulk chromatin to MNase. These mutations also largely maintain characteristic features of chromatin in a minichromosome.

3.2.2 H3 lrs mutations maintain nucleosome positioning in the coding region of a subtelomeric URA3 gene but shift a nucleosome in the promoter region

The lrs histone H3 mutants used in this study were originally identified in a screen for derepression of a URA3 reporter gene inserted at the telomere on the left arm of chromosome VII (Thompson et al., 1994; Thompson et al., 2003). Assayed by sensitivity to 5-fluoroorotic acid (5-FOA), the URA3 gene was strongly repressed in a strain expressing wild-type HHF2 from a centromeric plasmid. The mutations hht2-T80A, hht2-K79E, hht2-L70S, and hht2-E73D strongly increased sensitivity of yeast cells to 5-FOA, indicating a loss of repression of the subtelomeric URA3 gene (Thompson et al., 2003). To test whether these mutations change nucleosome positioning on URA3, chromatin structure adjacent to the telomere on the left arm of chromosome VII (Tel VII L) was analysed (Figure 13). Nuclease footprinting by MNase revealed the characteristic chromatin structure of the URA3 gene as observed in the normal locus on chromosome V (Tanaka et al., 1996), the subtelomeric regions on chromosomes V (Livingstone-Zatchej et al., 2003),
as well as in the minichromosomes YRpFT68 (see Part I), YRpTRURAP, and YRpTRARUP (Thoma, 1986). The footprint showed five positioned nucleosomes in the coding region and one at the 5’ end next to the nuclease sensitive promoter. Characteristically, a protected region in the centre of *URA3* of approximately 460 to 480 bp, which is hardly cut by MNase, represents three nucleosomes that are relatively tightly packed. The nucleosome at the 5’ end may occupy two extreme positions (gray and white circles), defined by double bands at the 5’ end of the gene (Livingstone-Zatchej et al., 2003). Deproteinized DNA exhibit a strong cutting site in the promoter of *URA3*, resulting from enhanced cutting of MNase in the TATA-box, due to some sequence preference by cutting pA and pT faster than pC or pG (Bellard et al., 1989).

The arrangement of the nucleosomes is similar in all tested strains therefore these *Irs* mutants do not disrupt chromatin structure near the telomere, which confirms the results obtained with the minichromosome. However, in *HHF2* wild-type cells (MFY15) the nucleosome in the 5’ region partially protects the strong cutting site at the TATA box and the lower band of the double bands

**Figure 13.** Nucleosome positions in the subtelomeric region on the left arm of chromosome VII in the *Irs H3* mutants. (A) Schematic representation of the *URA3* gene integrated at the HindIII site of *ADH4* locus adjacent to the telomere on the left arm of chromosome VII (Gottschling et al., 1990). Shown is the HhaI restriction fragment including the 3’-end of *ADH4* (adh4a) the *URA3* gene and promoter containing the TATA box. (TG<sub>T</sub>, TG<sub>G</sub>) refers to the telomeric repeat sequence. The black rectangle indicates the end-label probe used for the Southern blots. (B) Nucleosome footprint on the left arm of chromosome VII. DNA (shown in Figure 11 A) was digested with HhaI and separated on a 1% agarose gel. The Southern blot probed with an end-label fragment near the HhaI site shows positioned nucleosomes (circles). Tow circles next to the lanes indicate major (bigger circle) and minor (smaller circle) cutting site at the 5’-end of *URA3*. (C) Enlarged section of the *URA3* promoter and 5’ coding region shown in (B).
3.2.3 Superhelical density of a minichromosome and the 2 µm plasmid is essentially similar in the lrs strains

To further investigate the stability of nucleosomes on minichromosomes and on the endogenous 2 µm plasmid we tested the superhelical density of plasmid DNA. DNA was purified from wild-type and lrs mutant cells that were grown in YPD or SD minimal medium. Plasmid topoisomers
were separated in chloroquine-agarose gels and analyzed by Southern blotting. As shown in Figure 14 topoisomer distributions for YRpFT35 and the 2 µ plasmid were similar in all tested strains. Unfortunately MFY15 (HHT2) contained no 2 µ plasmid (Figure 14 B lanes 1 and 2). Notably, YRpFT35 as well as the 2 µ plasmid isolated form hht2-L70S (MFY18) strains appear to exhibit a subtly broader distribution of supercoils (Figure 14 A to D). Possibly, hht2-L70S mildly shifts the 2 µ supercoil distribution towards the band representing the nicked circles (top band).

Figure 14. Superhelical densities of the minichromosomes and the 2 µ plasmids isolated from lrs H3 mutant strains. (A) Topoisomer distribution of YRpFT35. The DNA isolated from the indicated strains was electrophoresed on 1% agarose gels containing 1 µg/ml chloroquine and transferred to a Zeta-Probe GT membrane. YRpFT35 superhelical density was analyzed by hybridization with a radiolabelled EcoRI-XbaI fragment from the TRP1 gene. (B) Topoisomer distributions of YRpFT35 were quantified from the Southern blot shown in (A) by densitometric tracing. (C) Topoisomer distribution of 2 µ plasmid. Indicated yeast strains were grown either in YPD (Y) or SD (S) minimal media. The DNA isolated from these strains was separated on a 0.75% agarose gel containing 1 µg/ml chloroquine and transferred to a Zeta-Probe GT membrane. The 2 µ plasmid superhelical density was analyzed by hybridization with a radiolabelled SnaBI-XbaI fragment from the 2 µ origin of replication. (D) Topoisomer distributions of the 2 µ plasmids isolated from cells grown in YPD were quantified from the Southern blot shown in (C) by densitometric tracing. (E) Topoisomer distribution of YRpFT35 at different chloroquine concentrations. DNA isolated from the indicated strains was separated on 1% agarose gels containing 1, 5, 10, or 15 µg/ml chloroquine and analysed as in (A)
To verify a putative effect of the hht2-L70S mutation on YRpFT35 topoisomer distribution DNA was purified from yeast cultures using a standard protocol in which spheroplasts were lysed with SDS (Sherman et al., 1986). Topoisomers were separated on agarose gels containing 1, 5, 10, or 15 µg/ml chloroquine to test topological differences at various chloroquine concentrations (Figure 14 C). An increase of chloroquine concentration from 1 to 5 µg/ml resulted in a shift of topoisomers in direction of the nicked circles. This observation manifested that at 1 µg/ml chloroquine concentration closed circular DNA was largely negatively supercoiled and became relaxed by enhanced chloroquine intercalation at 5 µg/ml chloroquine. A further increase to 10 and 15 µg/ml chloroquine induced positive supercoils. These results did not reveal pronounced variations in YRpFT35 supercoil distribution between strains expressing the different HHT2 alleles. At a chloroquine concentration of 5 µg/ml the minichromosome isolated from the hht2-L70S mutant showed subtly enhanced bands below the nicked circle. Thus, superhelical density of a minichromosome and the 2 µ plasmid confirms that none of the lrs mutation substantially destabilized nucleosomes.

### 3.2.4 UV-sensitivity of lrs H3 mutants and repair of UV lesions

As an alternative approach to study chromatin structure and accessibility of DNA to proteins in living cells we intend to measure repair of UV-lesions by photolyase and NER. Deletion of DOT1 results in a weak hypersensitivity towards UV irradiation (Game et al., 2005; Zhou et al., 2006) and methylation of lysine 79 of H3 was suggested to be required for effective repair of ionizing radiation induced damages (Game et al., 2006). To test if the lrs mutants might play a role in repair of UV lesions we monitored UV sensitivity of these strains (Figure 15). The hht2-T80A (MFY16), hht2-K79E (MFY17), and hht2-L70S (MFY18) mutants showed a similar sensitivity to UV irradiation as the HHT2 (MFY15) strain and W303α (RHY1). Though, the hht2-E73D (MFY19) mutant was slightly UV hypersensitive. This indicates that the lrs mutants are unlikely to affect UV damage recognition or repair.

![Figure 15. UV sensitivity of lrs H3 mutant strains. Survival after exposure to different UV-doses. W303 and strains expressing the indicated H3 allele from centromeric plasmids were grown in SD minimal media and plated in serial 10-fold dilutions (10^5 to 10 cells) on YPD media. The plates were UV-irradiated and incubated for 3 days](image-url)
3.3 Discussion

The “loss of rDNA silencing” (lrs) mutant alleles of histone H3 and H4 genes were identified in screens for specific phenotypes of impaired silencing (Park et al., 2002; Thompson et al., 2003). The mutations alter a distinct cluster of amino acid residues of a discrete surface on the histone octamer. Mutations on the lateral octamer surface may affect DNA-histone contacts at SHL ±2.5 and thus could destabilize nucleosomes. However, mutations on the octamer surface, including hht2-T80A, hht2-K79E, hht2-L70S, and hht2-E73D which were investigated in this study, are located distant from DNA binding sites. It has been speculated, that these lrs mutations may not destabilize the nucleosome, because the affected residues are exposed on the nucleosome surface (Park et al., 2002; Thompson et al., 2003). These residues have been suggested to represent a distinct functional domain on the nucleosome surface, playing a specific role in silencing (Park et al., 2002; Thompson et al., 2003). This surface may either represent an interface for silencing protein binding and/or internucleosomal interactions in higher order chromatin structure. Hence it was of interest to investigate chromatin structure in these lrs H3 mutant strains.

Our results indicate that chromatin isolated from hht2-T80A, hht2-K79E, hht2-L70S, and hht2-E73D mutant cells have a normal nucleosomal repeat and maintain nucleosome positions, and nucleosome stability. Moreover, the lrs mutants do not destabilize nucleosomes on a subtelomeric region (Figure 13). Except, a nucleosome in the 5’ region of a subtelomeric URA3 reporter gene was shifted compared to the position in the corresponding wild-type strain (HHT2), which most probably accounts for the loss of URA3 silencing in the mutants (see below).

The rearrangement of the nucleosome on the TATA-box of the subtelomeric URA3 gene in lrs mutant cells is likely not caused by defects in nucleosome-nucleosome interactions but rather by inability of these mutant nucleosomes to associate with Sir proteins. The nucleosome rearrangement in the URA3 promoter depends on the distance of this gene from the telomere and the expression level of Sir3p (Aparicio et al., 1991; Gottschling et al., 1990; Renauld et al., 1993). In strains used in this study, the URA3 gene is placed immediately adjacent to the telomere with its promoter ~1.3 kb from the chromosome end (Gottschling et al., 1990). In stains used in another study (Livingstone-Zatchej et al., 2003) the promoter of URA3 is either 2 kb or 6 kb distant from the end of chromosome V. In the HHT2 wild-type cells, the structure of the URA3 promoter looks similar to that observed in URA3 2 kb from the telomere in cells overexpressing SIR3 (Livingstone-Zatchej et al., 2003). On the other hand, the promoter chromatin in the lrs mutants resembles that of the 2 kb subtelomeric promoter region in sir3Δ cells. In addition, this URA3 promoter structure in the hht2 mutants is also similar to that of URA3 placed 6 kb away from the telomere in wild-type cells or at its natural locus (Livingstone-Zatchej et al., 2003; Tanaka et al., 1996). Transcriptional silencing is enhanced or abolished when SIR3 is
overexpressed or deleted, respectively (Renauld et al., 1993), and the position of the nucleosome in the \textit{URA3} promoter correlates with transcriptional activity of \textit{URA3}. ChIP experiments provided evidence for a spreading of the Sir proteins from the telomere into adjacent, telomere-proximal chromatin regions (de Bruin et al., 2000; Hecht et al., 1996). Therefore repositioning of the nucleosome in the \textit{URA3} promoter most likely depends on spreading of Sir proteins, which might be defective in the \textit{hht2} mutants.

This interpretation is consistent with the report, that \textit{hht2}-K79E and \textit{hht2}-E73D cells (\textit{hht2}-T80A and \textit{hht2}-L70S were not tested) have reduced Sir2p and Sir4p binding near the right telomere of chromosome VI, although cellular levels of these proteins were not affected (Fry et al., 2006). Rap1p, which binds to the (TG\textsubscript{1-3}\textsubscript{n}) repeat and represses subtelomeric transcription through its interaction with Sir proteins (Moretti et al., 1994; Shore, 1997), is normally recruited to telomeres in \textit{lrs} mutant strains (Fry et al., 2006). Therefore our results together with the observations from Fry et al. suggest that the \textit{lrs} mutations may prevent spreading of the silencing complex from the telomere along the subtelomeric regions by disrupting an interaction of Sir proteins with the nucleosome core surface (Fry et al., 2006). It was proposed, that hypomethylated lysine 79 of H3 is a potential binding site for Sir proteins (Ng et al., 2003; Varga-Weisz and Dalgaard, 2002), which could be disrupted by hypermethylation of lysine 79 in euchromatic regions. The mutations neighbouring lysine 79 may reflect additional residues involved in Sir protein interactions or may reflect residues required for recognition by the Dot1p methyltransferase. Indeed, methylation of lysine 79 is almost completely abolished in cells expressing \textit{hht2}-E73D, although recombinant Dot1p is able to partially methylate chromatin isolated form \textit{hht2}-E73D cells (Fry et al., 2006). Thus, mutations neighbouring lysine 79 may indirectly disrupt silencing by preventing binding of Dot1p and thereby reducing methylation of lysine 79. On the other hand, the \textit{hht2}-E73D mutation has a much stronger effect on silencing at \textit{HML} than do any of the other \textit{lrs} mutations, indicating that E73 must play another role in silencing in addition to methylation of lysine 79 (Thompson et al., 2003). Therefore it might be interesting to test if also \textit{hht2}-T80A and \textit{hht2}-L70S reduce methylation of lysine 79. Given the previously established interactions between the H3 and H4 N termini and Sir3p and Sir4p (Hecht et al., 1995; Johnson et al., 1990), along with the close proximity between the H4 N terminus and the H3 α1-L1 domain (Figure 11 C), it is also reasonable to speculate that the Sir proteins interact with a histone-binding site encompassing the H3 and H4 N termini and the H3 α1-L1 domain (Thompson et al., 2003).

Histone modifications on the octamer face surface, including methylation of lysine 79 of H3 have been speculated to potentially modulate inter-nucleosome interactions involved in the formation of higher order chromatin structures (Mersfelder and Parthun, 2006). However, nucleosome-nucleosome contacts in chromatin secondary structures such as the 30-nm fibre are difficult to identify \textit{in vivo} (Horowitz-Scherer and Woodcock, 2006). Conventional MNase mappings of nucleosome positions may miss alterations in nucleosome-nucleosome contacts that do not affect nucleosome positioning. Though, measuring DNA accessibility by photolyase may detect
changes in local chromatin compaction affected by interactions between nucleosomes. But in the same way also chromatin bound non-histone proteins such as Sir proteins may determine photolyase accessibility (Bucceri et al., 2006; Livingstone-Zatchej et al., 2003). Therefore our experiments can not exclude that the \textit{hht2}-T80A, \textit{hht2}-K79E, \textit{hht2}-L70S, or \textit{hht2}-E73D mutation may affect chromatin compaction mediated by inter-nucleosomal interactions. Conclusive results on the \textit{lrs} mutations affecting the nucleosome face surface may be provided by sedimentation analysis of reconstituted nucleosomal arrays. One \textit{lrs} mutation, H3-R83A, that affects an octamer lateral surface amino acid residue, sediment like wild-type chromatin in the analytical ultracentrifuge and does not impede fibre-fibre interactions (Fry et al., 2006). On the other hand a structurally related \textit{sin} mutation (H4-R43C) eliminates Mg\textsuperscript{2+}-dependent condensation of nucleosomal arrays \textit{in vitro} (Horn et al., 2002). The observation that \textit{sin} mutations destabilize nucleosomes and enhance nucleosome sliding, suggested that an increased propensity of nucleosome mobility inhibits the formation of higher order chromatin structure (Flaus et al., 2004; Muthurajan et al., 2004). Strikingly, \textit{hht2}-R83A has a similar silencing phenotype as \textit{hht2}-K79E, which is different to the silencing phenotype of \textit{sin} mutations (Fry et al., 2006; Park et al., 2002). Therefore \textit{hht2}-R83A may act in a similar manner as the here tested \textit{lrs} mutations in abrogating transcriptional silencing by reducing Sir2p and Sir4p binding to silenced chromatin (Fry et al., 2006).
4 Concluding Remarks

4.1 The C-terminal tail of histone H2A

While the effect of deletions and mutations at the N-terminal histone tails on chromatin structure has been addressed in many respects in vivo and in vitro, less knowledge is available on the role of the C-terminal tail of histone H2A. The C-terminal tail of H2A contains similar types of amino acid residues as the N-terminal histone tails, which include modifiable serine, threonine, and lysine residues (McBryant et al., 2006). It has been suggested that the replacement of serine 129 of H2A with glutamic acid renders nucleosomes more susceptible to degradation by MNase (Downs et al., 2000). Thus, the C terminus of H2A has been postulated to influence higher order chromatin structure and phosphorylation of serine 129 in response to DSB to decrease chromatin compaction, allowing better access of repair factors to the DNA (Downs et al., 2000).

Our results show that neither the C-terminal tail of H2A itself nor mimicking constitutive phosphorylation at serine 129 define DNA accessibility by determining nucleosome positioning. This suggests that the C-terminal H2A tail and its phosphorylation rather functions to interact with effector proteins, as shown for the MDC1-γ-H2A.X interaction in mammalian cells (Lou et al., 2006; Stucki et al., 2005). MDC1 binds directly with its tandem BRCT (BRCA1 carboxyl-terminal) domain to a phosphor-peptide of the C-terminus of H2A.X (Stucki et al., 2005). Yeast does apparently not possess a MDC1 homologue (Stucki and Jackson, 2006), but a potential candidate for a phoso-H2A binding protein might be the DNA damage checkpoint adaptor/mediator protein Rad9p, which contains an BRCT domain (Javaheri et al., 2006; Lydall and Whitehall, 2005). A mammalian orthologue of Rad9p, 53BP1, has been shown to bind to methylated lysine 79 of histone H3 through its tudor domains (Huyen et al., 2004). 53BP1 also possesses a tandem BRCT domain, which has been suggested to directly interact with γ-H2A.X (Lydall and Whitehall, 2005; Ward et al., 2003). Other potential interaction partners for phospho-H2A in yeast are Nhp10 and Arp4p (Downs et al., 2004; Morrison et al., 2004).

Another H2A serine residue, serine 122, is important for survival in the presence of DNA damaging agents (Harvey et al., 2005; Moore et al., 2006). Phosphorylation of serine 122 and in addition threonine 126 was observed in response to some damage conditions (Moore et al., 2006). Therefore, the C-terminal tail of H2A may represent an interaction interface for different DNA repair factors. Interestingly, a lysine residue, lysine 127, of H2A has been identified as a site for SUMOylation (Nathan et al., 2006). The function of H2A SUMOylation in S. cerevisiae is not known (Nathan et al., 2006), and it might be interesting to test, if it plays a role in DNA damage response.
4.2 The putative *S. cerevisiae* linker histone Hho1p

The yeast Hho1p, contains two globular domains, which have a structure very similar to the single globular domain of the linker histone H1 (Ali et al., 2004; Ono et al., 2003). Recombinant Hho1p shows a similar behaviour as canonical linker histones when reconstituted with chromatin fibres (Ali and Thomas, 2004; Patterton et al., 1998). Canonical linker histones have an established role in stabilizing native as well as reconstituted 30-nm chromatin fibres (Robinson and Rhodes, 2006; Woodcock et al., 2006). Yeast chromatin may also condense into 30-nm fibres that resemble those characteristic of chromatin isolated from higher eukaryotic cells (Lowary and Widom, 1989), and Hho1p may therefore stabilize these fibres.

We and others showed that deletion of *HHO1* results in no detectable changes in chromatin structure *in vivo* (Freidkin and Katcoff, 2001; Patterton et al., 1998; Puig et al., 1999). Depletion of H1 subtypes in mammalian cells has been reported to reduce nucleosome spacing *in vivo* (Fan et al., 2003; Fan et al., 2005). The lack of a detectable effect on the nucleosomal repeat upon deletion of *HHO1* in yeast might be due to a relatively low abundance of Hho1p in wild-type cells (Downs et al., 2003; Freidkin and Katcoff, 2001). However, though expression of H1 in yeast is lethal (Linder and Thoma, 1994; Miloshev et al., 1994), its overexpression does not affect nucleosome spacing in yeast chromatin (Linder and Thoma, 1994). Therefore, the role of linker histones in yeast chromatin organization remains elusive.

4.3 Histone H3 lrs mutations and higher order chromatin structure

The surface of the nucleosome is highly contoured, has an uneven distribution of charged amino acid residues and has been speculated to potentially modulate nucleosome-nucleosome contacts involved in the formation of higher order chromatin structures (Luger, 2006; Mersfelder and Parthun, 2006). Particularly the interaction of the N-terminal tail of H4 with the acidic patch formed by the H2A-H2B dimer of an adjacent nucleosome favour this hypothesis (Dorigo et al., 2003; Dorigo et al., 2004; Fan et al., 2004; Shogren-Knaak et al., 2006). On the same lines the nucleosome face may act as docking surface for the binding of non-histone proteins (Mersfelder and Parthun, 2006; Tremethick, 2007). This ability has been shown recently with a viral protein that interacts with the acidic patch on H2A-H2B (Barbera et al., 2006).

We demonstrate that lrs mutant alleles of H3 affecting residues on the nucleosome core surface clustering around lysine 79 do not generally alter chromatin structure. In a subtelomeric region chromatin is rather specifically modulated at the promoter region of a reporter gene. Others showed that residues changed by lrs mutations are required for the accumulation of Sir proteins to subtelomeric and silent mating type loci (Fry et al., 2006). In addition, a mutation resulting in substitution of lysine 79 to arginine in histone H3 weakens silencing of rDNA but enhances subtelomeric silencing (Park et al., 2002). If lysine 79 would be important for
Concluding Remarks

chromatin condensation by inter-nucleosomal interactions, a similar impact on both forms of silencing could be expected in an *hht2*-K79R strain. Thus, it has been suggested that lysine 79 may represent an interaction site for the binding of silencing proteins to nucleosomes and that distinct silencing complexes could interact differently with an arginine residue than with a lysine residue at position 79 (Park et al., 2002). Together these results favour the hypothesis that the nucleosome surface around lysine 79 contributes to Sir protein binding and not to nucleosome-nucleosome interactions. Interestingly, lysine 79 of H3 may also facilitate the binding of checkpoint proteins to chromatin containing damaged DNA (Lydall and Whitehall, 2005). As mentioned above, 53BP1 interacts with H3 methylated at lysine 79 upon induction of DSBs in mammalian cells (Huyen et al., 2004). Because methylation of lysine 79 is unaltered in response to DNA damage, it was proposed that 53BP1 senses DSBs indirectly through changes in higher order chromatin structure that expose the 53BP1 binding site (Huyen et al., 2004).
5 Materials and Methods

5.1 Chemicals

Chemicals used for buffers, media, and solutions are listed in Table 3.

Table 3. General chemicals

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### Chemicals

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## Materials and Methods

### Chemicals

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</tbody>
</table>

Oligonucleotides used for sequencing, generation of DNA fragments for indirect end-labeling, and generation of radiolabeled strand-specific probes were all purchased from Microsynth and are listed in Table 4. They were obtained in amounts of 30–90 nmol lyophilized DNA and were resolved in TE (10 mM Tris, pH 8.0; 1 mM EDTA) to a final concentration of 100 µM.

### Table 4. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>No</th>
<th>Sequencea</th>
<th>ntb</th>
<th>SGD Coordinatesc</th>
<th>T [°C]d</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP1 RIX 160.L</td>
<td>1036</td>
<td>5'-GCA AGC CGC AAA CTT TCA CCA ATG GAC CAG-3'</td>
<td>30</td>
<td>IV 461896C</td>
<td>58</td>
</tr>
<tr>
<td>TRP1 RIX 25.U</td>
<td>1037</td>
<td>5'-GAG GGC CAA GAG GGA GGG CAT TGG TGA C-3'</td>
<td>28</td>
<td>IV 461761W</td>
<td>58</td>
</tr>
<tr>
<td>3'-ChrVII-TelURA</td>
<td>1132</td>
<td>5'-CAT GTG AAT GAC ACA CGA AAG-3'</td>
<td>21</td>
<td>VII 16861C</td>
<td>60</td>
</tr>
<tr>
<td>5'-ChrVII-TelURA</td>
<td>1133</td>
<td>5'-GAC ATA CTG TTG GCT TTG AAC-3'</td>
<td>21</td>
<td>VII 16566W</td>
<td>60</td>
</tr>
<tr>
<td>GAL1-668W</td>
<td>1164</td>
<td>5'-GCG GTG AGG ACA ATG ATG C-3'</td>
<td>19</td>
<td>II 279687W</td>
<td>60</td>
</tr>
<tr>
<td>GAL1-908C</td>
<td>1165</td>
<td>5'-GAT TAC CTT TAT TCG TGC TCG-3'</td>
<td>21</td>
<td>II 279948C</td>
<td>60</td>
</tr>
<tr>
<td>3'-HTA1-215d</td>
<td>1190</td>
<td>5'-AGC AGC ATT ACC AGC TAA TTC-3'</td>
<td>21</td>
<td>IV 91767C</td>
<td>60</td>
</tr>
<tr>
<td>3'-HTA1-607d</td>
<td>1191</td>
<td>5'-TGA ACC TAA ACA GAG CAA CAG-3'</td>
<td>21</td>
<td>IV 92159C</td>
<td>60</td>
</tr>
<tr>
<td>5'-HTA1-82d</td>
<td>1192</td>
<td>5'-CAG TCG GTA GAG TGC ACA G-3'</td>
<td>19</td>
<td>IV 91634W</td>
<td>60</td>
</tr>
<tr>
<td>5'-HTA1-251u</td>
<td>1193</td>
<td>5'-CCT TTC TCA CCC CCT TCC C-3'</td>
<td>19</td>
<td>IV 91301C</td>
<td>60</td>
</tr>
</tbody>
</table>

*a* All sequences were retrieved from the *Saccharomyces* Genome Database (SGD) [http://www.yeastgenome.org](http://www.yeastgenome.org) (Cherry et al., 1997).

*b* Length of the oligonucleotide indicated in nucleotides (nt).

*c* SGD coordinates indicate the chromosome number in roman numerals and the position of the 5' end of the oligonucleotide sequence of the Watson (W) or Crick (C) strand.

*d* T corresponds to the annealing temperature in °C used in the PCR, primer extension, or sequencing reactions.

DNA size markers used for agarose gel electrophoresis are listed in Table 5. Commercially available markers (all, but 10 × 5S) were diluted to 100 µg/ml in 1 × TBE Sample Buffer (90 mM Tris; 90 mM boric acid; 20 mM EDTA; pH 8.0; 0.01% (w/v) bromophenol blue; 0.01% (w/v) xylene cyanol FF; 6% (v/v) glycerol) before used for agarose gel electrophoresis.
Table 5. DNA size markers

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Origin/Supplier</th>
<th>Reference/Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 kb DNA Ladder</td>
<td>500 µg/ml</td>
<td>New England Biolabs</td>
<td>N3232L</td>
</tr>
<tr>
<td>100 bp DNA Ladder</td>
<td>500 µg/ml</td>
<td>New England Biolabs</td>
<td>N3231L</td>
</tr>
<tr>
<td>λ DNA/HindIII Markers</td>
<td>500 µg/ml</td>
<td>Promega</td>
<td>G171A</td>
</tr>
<tr>
<td>φX174 RF DNA/Hae III Fragments</td>
<td>500 µg/ml</td>
<td>Gibco BRL</td>
<td>15611-015</td>
</tr>
<tr>
<td>10 × 5S</td>
<td>1 ng/ml</td>
<td>A. Meier</td>
<td>Thoma et al., 1984</td>
</tr>
</tbody>
</table>

5.2 Enzymes and antibodies

Enzyme used for spheroplasting of yeast cells, DNA isolation, digestion of nuclei, cloning, and CPD analysis are listed in Table 6.

Table 6. General enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (Units)</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyticase</td>
<td>518 U/mg</td>
<td>Fluka</td>
<td>62982</td>
</tr>
<tr>
<td>Nuclease S7 (MNase)</td>
<td>15 000 U/mg</td>
<td>Roche Diagnostics</td>
<td>107 921</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>≥30 U/mg</td>
<td>Roche Diagnostics</td>
<td>3 115 852</td>
</tr>
<tr>
<td>Ribonuclease A (RNase A)</td>
<td>33 U/mg</td>
<td>Sigma</td>
<td>R-5503</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 U/µl</td>
<td>Roche Diagnostics</td>
<td>481 220</td>
</tr>
<tr>
<td>T4 Endonuclease V</td>
<td>20 U/µl</td>
<td>Epicentre</td>
<td>TE665K</td>
</tr>
<tr>
<td>Zymolyase-100T</td>
<td>100 U/mg</td>
<td>Seikagaku Kogyo Co.</td>
<td>120493</td>
</tr>
</tbody>
</table>

Restriction enzymes used for cloning and DNA analysis are listed in Table 7.

Table 7. Restriction enzymes and buffers

<table>
<thead>
<tr>
<th>Enzyme/Buffer</th>
<th>Activity/Composition</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BglII</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>567 639</td>
</tr>
<tr>
<td>EcoRI</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>1 175 084</td>
</tr>
<tr>
<td>EcoRI, conc.</td>
<td>40 U/µl</td>
<td>Roche Diagnostics</td>
<td>606 189</td>
</tr>
<tr>
<td>EcoRV</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>667 145</td>
</tr>
<tr>
<td>EcoRV, conc.</td>
<td>40 U/µl</td>
<td>Roche Diagnostics</td>
<td>1 040 197</td>
</tr>
<tr>
<td>HhaI</td>
<td>20 U/µl</td>
<td>New England Biolabs</td>
<td>R0139L</td>
</tr>
<tr>
<td>SalI</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>10 567 663 001</td>
</tr>
<tr>
<td>SnaBI</td>
<td>20 U/µl</td>
<td>New England Biolabs</td>
<td>R0130L</td>
</tr>
<tr>
<td>Stul</td>
<td>100 U/µl</td>
<td>New England Biolabs</td>
<td>R0187M</td>
</tr>
<tr>
<td>Xbal</td>
<td>5 U/µl</td>
<td>New England Biolabs</td>
<td>R0145S</td>
</tr>
<tr>
<td>Xbal, conc.</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>674 273</td>
</tr>
<tr>
<td>XhoI</td>
<td>10 U/µl</td>
<td>Roche Diagnostics</td>
<td>703 770</td>
</tr>
<tr>
<td>10 × SuRE/Cut Buffer B</td>
<td>10 mM Tris, pH 8.0 (at 37°C); 100 mM NaCl; 5 mM MgCl₂; 1 mM 2-mercaptoethanol</td>
<td>Roche Diagnostics</td>
<td>1 417 967</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Enzyme/Buffer</th>
<th>Activity/Composition</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 × SuRE/Cut</td>
<td>50 mM Tris, pH 7.5 (at 37°C); 100 mM NaCl; 10 mM MgCl₂; 1 mM DTT</td>
<td>Roche Diagnostics</td>
<td>1 417 991</td>
</tr>
<tr>
<td>Buffer H</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 × SuRE/Cut</td>
<td>10 mM Tris, pH 7.5 (at 37°C); 50 mM NaCl; 10 mM MgCl₂; 1 mM DTT</td>
<td>Roche Diagnostics</td>
<td>1 417 983</td>
</tr>
<tr>
<td>Buffer M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 × NEBuffer 2</td>
<td>10 mM Tris, pH 7.5 (at 25°C); 50 mM NaCl; 10 mM MgCl₂; 1 mM DTT</td>
<td>New England Biolabs</td>
<td>B7002S</td>
</tr>
<tr>
<td>10 × NEBuffer 4</td>
<td>20 mM Tris, pH 7.9 (at 25°C); 50 mM KOAc; 10 mM Mg(OAc)₂; 1 mM DTT</td>
<td>New England Biolabs</td>
<td>B7004S</td>
</tr>
<tr>
<td>100 × BSA</td>
<td>10 mg/ml</td>
<td>New England Biolabs</td>
<td>B9001S</td>
</tr>
</tbody>
</table>

Antibodies used for western blotting are listed in Table 8.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration</th>
<th>Origin/Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-H3 IgG</td>
<td>500 µg/ml</td>
<td>Abcam</td>
<td>ab 1791</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-phosphoH2A IgG</td>
<td>500 µg/ml</td>
<td>Abcam</td>
<td>ab 15083</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-HRP conjugate</td>
<td>400 µg/ml</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-2054</td>
</tr>
</tbody>
</table>

5.3 Apparatus

General apparatus used in this study are listed in Table 9.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Supplier</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI Prism 310 Genetic Analyser</td>
<td>PE Applied Biosystems</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>Balances, AE 100, PM600, PM4000</td>
<td>Mettler</td>
<td>Weight measurements</td>
</tr>
<tr>
<td>BioPhotometer 6131</td>
<td>Eppendorf</td>
<td>A₅₀₀ of yeast and E. coli cultures</td>
</tr>
<tr>
<td>DNA Thermal Cycler</td>
<td>Perkin Elmer</td>
<td>Primer extension reactions</td>
</tr>
<tr>
<td>Electro Cell Manipulator 600</td>
<td>BTX Harvard Apparatus</td>
<td>Electroformation of E. coli cells</td>
</tr>
<tr>
<td>Gel Documentation System</td>
<td>UVItc</td>
<td>Analysis of agarose gels</td>
</tr>
<tr>
<td>Gel Dryer, Model 583</td>
<td>Bio-Rad</td>
<td>Drying polyacrylamide gels</td>
</tr>
<tr>
<td>GeneAmp PCR System 9600</td>
<td>Perkin Elmer</td>
<td>PCR for DNA sequencing</td>
</tr>
<tr>
<td>Heating Block, Thermostat 2100-D A</td>
<td>Liebisch Labortechnik</td>
<td>Heating samples to 95°C</td>
</tr>
<tr>
<td>Horizontal Gel Electrophoresis System, Mini, Midi, Maxi</td>
<td>homemade</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Horizontal Gel Electrophoresis System, Horizon 20-25</td>
<td>Gibco BRL</td>
<td>Agarose maxi gel electrophoresis</td>
</tr>
<tr>
<td>Incubation Shaker, 37°C</td>
<td>Infors AG</td>
<td>Growth of E. coli liquid cultures</td>
</tr>
<tr>
<td>Incubator, 37°C</td>
<td>Heraeus</td>
<td>Growth of E. coli liquid cultures</td>
</tr>
<tr>
<td>Irradiation box containing 6 Type G15 T8 germicidal lamps (254 nm)</td>
<td>Homemade Sylvania (lamps)</td>
<td>UV irradiation of yeast</td>
</tr>
<tr>
<td>Irradiation box containing 6 Type F15 T8 BLB lamps (366 nm)</td>
<td>Homemade Sylvania (lamps)</td>
<td>Photoreactivation</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Supplier</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid Scintillation Analyzer, 1500 TRI-CARB</td>
<td>Packard</td>
<td>Determination of radioactivity after labeling reaction</td>
</tr>
<tr>
<td>MAXI dry lyo</td>
<td>Kleiner AG</td>
<td>Lyophilization of DNA</td>
</tr>
<tr>
<td>Milli-Q Synthesis</td>
<td>Millipore</td>
<td>Water purification</td>
</tr>
<tr>
<td>Mini-PROTEAN 3 Electrophoresis System</td>
<td>Bio-Rad</td>
<td>SDS-PAGE</td>
</tr>
<tr>
<td>Mini Trans-Blot cell</td>
<td>Bio-Rad</td>
<td>Western blotting</td>
</tr>
<tr>
<td>Oven, 80°C</td>
<td>WTC Binder</td>
<td>Baking Southern blots</td>
</tr>
<tr>
<td>Oven, 65°C</td>
<td>Memmert</td>
<td>Washing Southern blots</td>
</tr>
<tr>
<td>Peristaltic Tubing Pump, Midi Vario</td>
<td>Ismatec SA</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>pH Meter, Toledo 320</td>
<td>Mettler</td>
<td>pH measurements</td>
</tr>
<tr>
<td>PhosphorImager Scanner, Storm 820</td>
<td>Amersham Biosciences</td>
<td>Scanning PhosphorImager screens</td>
</tr>
<tr>
<td>Photocells UVX-25 and UVX-36</td>
<td>UVP Inc.</td>
<td>Measuring flux at 254, 366 nm</td>
</tr>
<tr>
<td>Power Supply, Power Pac 300</td>
<td>Bio-Rad</td>
<td>Electrophoresis</td>
</tr>
<tr>
<td>RoboCycler Gradient 96</td>
<td>Stratagene</td>
<td>PCR</td>
</tr>
<tr>
<td>Rocker platform, Rockomat</td>
<td>Tecnomara AG</td>
<td>Washing membranes</td>
</tr>
<tr>
<td>Rotary Oven, 65°C</td>
<td>Bachofer</td>
<td>Hybridization of Southern blots</td>
</tr>
<tr>
<td>Shakers</td>
<td>Ismatec SA</td>
<td>Incubation of yeast cultures</td>
</tr>
<tr>
<td>Stirrer, Combinimag Ret</td>
<td>IKA Labortechnik</td>
<td>Stirring buffers, Electrophoresis</td>
</tr>
<tr>
<td>Stirrer, RCT basic</td>
<td>IKA Labortechnik</td>
<td>Stirring buffers, Electrophoresis</td>
</tr>
<tr>
<td>Vortex, MS2 Minishaker</td>
<td>IKA Labortechnik</td>
<td>Vortexing</td>
</tr>
<tr>
<td>Vortex, VIBRAX-VXR</td>
<td>IKA Labortechnik</td>
<td>Glass bead lysis of yeast cells</td>
</tr>
<tr>
<td>Waterbath, C 20</td>
<td>MGW Lauda</td>
<td>Enzymatic reactions</td>
</tr>
<tr>
<td>Waterbath, PTR Regler R20K, K4R Electronic</td>
<td>MGW Lauda</td>
<td>Temperature control during photoreactivation</td>
</tr>
</tbody>
</table>

Centrifuges used are listed in Table 10. The relative centrifugal force (RCF), given in multiples of the earth gravity g (9.81 m/s²), depends on the centrifugal speed in revolution per minutes (rpm) and the radius \( r \) [mm] of the centrifugation rotor and was calculated using the following equation:

\[
RCF = 1.118 \left( \frac{rpm}{1000} \right)^2 r
\]

For Sorvall centrifuges RCF were converted to centrifugal speed using the Centrifugation Calculator: [http://researchlink.labvelocity.com/tools/](http://researchlink.labvelocity.com/tools/)

| Centrifuge, Manufacturer,Rotor, Maximal Speed, Maximal Radius |
|---------------------------------------------------------------|-------------------------------------|
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall SS-34 (fixed 34° angel) | 16 000 rpm, 107 mm |
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall SA-600 (fixed 34° angel) | 16 000 rpm, 129.6 mm |
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall GSA (fixed 25° angel) | 13000 rpm, 145.6 mm |
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall GS-3 (fixed 20° angel) | 9000 rpm, 151.3 mm |
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall HB-4 (Swinging bucket) | 13 000 rpm, 146.8 mm |
| Sorvall RC 5B, Sorvall RC 5C plus, Sorvall HS-4 (Swinging bucket) | 7000 rpm, 172.3 mm |
| Megafuge 1.0 R, Heraeus Instruments Swinging bucket | 4000 rpm, 187 mm |
5 Materials and Methods

<table>
<thead>
<tr>
<th>Centrifuge, Manufacturer</th>
<th>Rotor</th>
<th>Maximal Speed</th>
<th>Maximal Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>4236 Centrifuge, Kontron Instruments</td>
<td>Swinging bucket</td>
<td>3800 rpm</td>
<td>115 mm</td>
</tr>
<tr>
<td>Biofuge pico, Heraeus Instruments</td>
<td>Fixed angel</td>
<td>13 000 rpm</td>
<td>85 mm</td>
</tr>
<tr>
<td>Biofuge 15, Heraeus Sepatech</td>
<td>Fixed angel</td>
<td>14 000 rpm</td>
<td>85 mm</td>
</tr>
</tbody>
</table>

5.4 Materials

General materials used in this study are listed in Table 11.

Table 11. General materials

<table>
<thead>
<tr>
<th>General Materials</th>
<th>Supplier (Cat. No.)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottle Top Filter, Steritop, 0.22 µm pores</td>
<td>Millipore (SCGPT02RE)</td>
<td>Sterile filtration</td>
</tr>
<tr>
<td>Film, RX-100</td>
<td>Fujifilm</td>
<td>Exposure of membranes</td>
</tr>
<tr>
<td>Gene Pulser Cuvettes, 1 mm gap</td>
<td>Bio-Rad (165-2089)</td>
<td>Electroporation of E. coli</td>
</tr>
<tr>
<td>Glass beads, 425–600 µm diameter</td>
<td>Sigma (G-8772)</td>
<td>Disruption of yeast cells</td>
</tr>
<tr>
<td>Glass Douncer homogenizer and piston</td>
<td>B. Braun Melsungen AG</td>
<td>Spheroplast homogenization</td>
</tr>
<tr>
<td>Haemocytometer</td>
<td>Neubauer</td>
<td>Counting yeast cells</td>
</tr>
<tr>
<td>Hybridisation Cylinders, 4 cm diameter</td>
<td>homemade</td>
<td>Southern blot hybridisation</td>
</tr>
<tr>
<td>Petri Dish, 35 mm diameter, polyethylene</td>
<td>Greiner Bio-one</td>
<td>UV-irrad. of cells, 5 ml</td>
</tr>
<tr>
<td>Plastic trays, 219 × 312 mm</td>
<td>--</td>
<td>UV-irrad. of cells, 250 ml</td>
</tr>
<tr>
<td>PhosphorImager Screens</td>
<td>Molecular Dynamics</td>
<td>Exposure of Southern blots</td>
</tr>
<tr>
<td>Safety Light, Type F36W T8 Yellow Fluorescent</td>
<td>Sylvania</td>
<td>Prevention of undesired photoreactivation</td>
</tr>
</tbody>
</table>

Kits used for DNA purification, DNA sequencing, DNA labelling, cloning, and Western blot detection are listed in Table 12.

Table 12. Kits

<table>
<thead>
<tr>
<th>Kits</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BigDye Terminator Cycle Sequencing Kit</td>
<td>Applied Biosystems</td>
<td>4303149</td>
</tr>
<tr>
<td>HexaLabel DNA Labeling Kit</td>
<td>Fermentas</td>
<td>K0612</td>
</tr>
<tr>
<td>Immun-Star HRP Substrate Kit</td>
<td>Bio-Rad</td>
<td>170-5041</td>
</tr>
<tr>
<td>QIAfilter Plasmid Maxi Kit</td>
<td>QIAGEN</td>
<td>12263</td>
</tr>
<tr>
<td>QIAGEN Genomic-tip 100/G</td>
<td>QIAGEN</td>
<td>13343</td>
</tr>
<tr>
<td>QIAGEN Genomic-tip 500/G</td>
<td>QIAGEN</td>
<td>13362</td>
</tr>
<tr>
<td>QIAprep Spin Miniprep Kit</td>
<td>QIAGEN</td>
<td>27104</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>QIAGEN</td>
<td>28704</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>QIAGEN</td>
<td>28104</td>
</tr>
<tr>
<td>Quick Spin Columns G-50 Sephadex</td>
<td>Roche Diagnostics</td>
<td>11 273 973 001</td>
</tr>
<tr>
<td>Taq DNA Polymerase Kit</td>
<td>Fermentas</td>
<td>EP0402</td>
</tr>
</tbody>
</table>
Membranes and Whatman paper used for Southern and Western blotting are listed in Table 13.

<table>
<thead>
<tr>
<th>Materials for Blotting</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocellulose Membrane (0.45 µm pore size)</td>
<td>Schleicher &amp; Schull</td>
<td>401196</td>
</tr>
<tr>
<td>Nylon Membrane, Zeta-Probe GT</td>
<td>Bio-Rad</td>
<td>162-0196</td>
</tr>
<tr>
<td>Whatman Chromatography Paper, 3MM CHR</td>
<td>Whatman</td>
<td>3030-931</td>
</tr>
</tbody>
</table>

### 5.5 Media

#### 5.5.1 Yeast media

The media listed in Table 14 were used for growth of *S. cerevisiae*. Media and ingredient solutions (amino acids, adenine, and uracil, listed in Table 15) were prepared essentially as described in (Rose et al., 1990). They were sterilized in an autoclave for 15 min at 121°C, except $100 \times$ Trp, as well as the $20 \times$ Dropout Solution, were filter-sterilized using a Steritop filter (Millipore). SD (agar) was supplemented after sterilization with the appropriate $100 \times$ concentrated ingredient solutions (Table 15) to obtain synthetic minimal media (SMM) for growth of yeast strains bearing minichromosomes under selective conditions.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPD (Yeast Extract Peptone Dextrose)</td>
<td>1% Bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>2% Bacto Peptone</td>
</tr>
<tr>
<td></td>
<td>2% D-(+)-Glucose (Dextrose)</td>
</tr>
<tr>
<td></td>
<td>2% Agar (only for plates)</td>
</tr>
<tr>
<td>SD (Synthetic Dextrose)</td>
<td>0.67% YNB without amino acids</td>
</tr>
<tr>
<td></td>
<td>2% D-(+)-Glucose (Dextrose)</td>
</tr>
<tr>
<td></td>
<td>2% Agar (only for plates)</td>
</tr>
</tbody>
</table>

The yeast strains MFY15, MFY16, MFY17, MFY18, and MFY19 did not grow in SMM. In order to grow them under selective conditions, synthetic complete dropout (SC-DO) media had to be used. To prepare SC-DO media, SMM were additionally supplemented with a $20 \times$ concentrated Dropout Solution (Table 15).
Table 15. Amino acid, adenine, and uracil supplements and Dropout Solution

<table>
<thead>
<tr>
<th>Nutrition Supplement</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 × Ade</td>
<td>2 mg/ml Adenine sulfate</td>
</tr>
<tr>
<td>100 × Ura</td>
<td>2 mg/ml Uracil</td>
</tr>
<tr>
<td>100 × His</td>
<td>2 mg/ml L-Histidine HCl</td>
</tr>
<tr>
<td>100 × Leu</td>
<td>3 mg/ml L-Leucine</td>
</tr>
<tr>
<td>100 × Lys</td>
<td>3 mg/ml L-Lysine HCl</td>
</tr>
<tr>
<td>100 × Trp</td>
<td>2 mg/ml L-Tryptophan</td>
</tr>
<tr>
<td>20 × Dropout Solution</td>
<td>0.4 mg/ml L-Arginine HCl</td>
</tr>
<tr>
<td></td>
<td>2 mg/ml L-Glutamic acid</td>
</tr>
<tr>
<td></td>
<td>0.6 mg/ml L-Isoleucine</td>
</tr>
<tr>
<td></td>
<td>1 mg/ml L-Phenylalanine</td>
</tr>
<tr>
<td></td>
<td>8 mg/ml L-Serine</td>
</tr>
<tr>
<td></td>
<td>0.6 mg/ml L-Tyrosine</td>
</tr>
<tr>
<td></td>
<td>4 mg/ml L-Threonine</td>
</tr>
<tr>
<td></td>
<td>3 mg/ml L-Valine</td>
</tr>
</tbody>
</table>

5.5.2 E. coli media

The media listed in Table 16 were used for growth of E. coli. They were sterilized in an autoclave for 15 min at 121°C. If required, ampicillin or kanamycin was added to the media at a concentration of 100 µg/ml or 50 µg/ml, respectively.

Table 16. E. coli media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB (Luria Bertani)</td>
<td>1% Bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>0.5% Bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>0.5% NaCl</td>
</tr>
<tr>
<td></td>
<td>2% Agar (only for plates)</td>
</tr>
<tr>
<td>2 × YT (Yeast extract Trpytone)</td>
<td>1.6% Bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>1% Bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>0.5% NaCl</td>
</tr>
</tbody>
</table>

5.6 Strains

5.6.1 Yeast strains

Histone H2A C-terminal and HHO1 mutant strains are listed in Table 1 (chapter 2). The following S. cerevisiae strains were kindly provided by J. A. Downs: FY406 ((hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 YCpSAB6 [pRS316-HTA1-HTB1]), JHY2 ((hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 YCpJH2 [pRS313-HTA1-HTB1]), JHY3 ((hta1-htb1)Δ::LEU2 (hta2-
Materials and Methods

htb2)\(\Delta::TRP1\) YCpJH3 [pRS313-htal-S129A-HTBI]), and JHY8 ((htal-htb1)\(\Delta::LEU2\) hta2-htb2::TRP1 YCpJH8 [pRS313-htal-S129E-HTBI]) are all isogenic to S288C (MATa his3\(\Delta200\) leu2\(\Delta1\) lys2-128\(\delta\) trp1\(\Delta63\) ura3-52), except that all strains are GAL2+ (Hirschhorn et al., 1995; Winston et al., 1995). JDY22 (hta1-S129*/hta2-S129*), JDY41 (hho1\(\Delta::HIS3\)), and JDY42 (hta1-S129*/hta2-S129* hho1\(\Delta::HIS3\)) are in the W303\(\alpha\) wild-type background (MATa ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-52) (Downs et al., 2000).

MFY1, MFY2, and MFY3 were constructed by disruption of TRP1 in JHY2, JHY3, and JHY8 ((hta2-htb2)\(\Delta::trp1::URA3\)) using an EcoRI-XhoI disruption fragment of pTU10 (Cross, 1997) as described below. MFY5, MFY6, MFY36, RHY1, RHY2, RHY3, and RHY4 were derived from MFY2, MFY3, MFY1, W303\(\alpha\), JDY22, JDY41, and JDY42 respectively, by transformation with the minichromosome YRpFT35 [TRP1 ARS1 UmidA UmidB] as described in section 5.6.1.2. MFY13, MFY14, MFY37, RHY5, and RHY6, were derived from JHY3, JHY8, JHY2, W303\(\alpha\), and JDY22, respectively, by transformation with the minichromosome YRpFT68 [trpl::URA3 ARS1 UmidA UmidB] as described below. All transformations were verified by Southern blot hybridization.

Histone H3 lrs mutant strains are listed in Table 2 (chapter 3). The following S. cerevisiae strains were kindly provided by J. S. Thompson: JTY34U (YCpJT34 [pRS317-HHF2-HHT2]), JTY307U (YCpJT307 [pRS317-HHF2-hht2-T80A]), JTY308U (YCpJT308 [pRS317-HHF2-hht2-K79E]), JTY309U (YCpJT309 [pRS317-HHF2-hht2-L70S]), and JTY319U (YCpJT319 [pRS317-HHF2-hht2-E73D]) are all MATa ade2-101(och) his3\(\Delta200\) LEU2 or leu2-3,112 lys2-801 (amb) trpl\(\Delta901\) ura3-52 adh4\(\Delta::URA3-TEL\) (hhf1-hht1)\(\Delta::LEU2\) (hhf2-hht2)\(\Delta::HIS3\) (Thompson et al., 1994; Thompson et al., 2003).

MFY15, MFY16, MFY17, MFY18, and MFY19 were derived from JTY34U, JTY307U, JTY308U, JTY309U, and JTY319U, respectively, by transformation with the minichromosome YRpFT35 [TRP1 ARS1 UmidA UmidB] as described in section below.

Construction of the strains MFY1, MFY2, and MFY3. The TRP1 nutritional marker gene in the (hta2-htb2)\(\Delta::TRP1\) locus of the strains JHY2, JHY3, and JHY8 had to be replaced in order to introduce the minichromosome YRpFT35. A “marker swap” plasmid, pTU10, developed by F. R. Cross was used to interconvert the TRP1 marker gene to an URA3 marker gene (Cross, 1997). An EcoRI-XhoI disruption fragment containing URA3-kan\(^8\) in the EcoRV site of TRP1 (trpl::URA3, URA3 in the opposite orientation to trpl) was excised from pTU10 and transformed into JHY2, JHY3, and JHY8.

The “marker swap” plasmid pTU10 (6.6 kb) was isolated from the E. coli strain FT136 as described in section 5.8.2. About 6 \(\mu\)g of pTU10 was digested with EcoRI (10 U/\(\mu\)l) and XhoI (10 U/\(\mu\)l), each 40 U, in a total volume of 60 \(\mu\)l 1 \(\times\) SuRE/Cut Buffer H at 37°C for 2 h. A 3.4 kb fragment containing the vector backbone and a 3.2 kb disruption fragment (trpl::URA3) was obtained. 5 \(\mu\)l of this digestion mixture (about 250 ng disruption fragment) were transformed without purification into JHY2, JHY3, and JHY8 as described in section 5.7.1.
Some colonies were tested for growth on SD agar supplemented with adenine, histidine, leucine, lysine, and tryptophane and on SD agar supplemented with adenine, uracil, histidine, leucine, and lysine. Two colonies, of each transformation, growing on media lacking uracil but not on media lacking tryptophane were frozen in 15% glycerol and named MFY1, MFY2, and MFY3, respectively. One clone of each of these strains was further characterized by Southern blot. The DNA fragments containing the \textit{trp1::URA3} insertion showed the expected sizes when digested either with EcoRI or with BglIII (Figure 16). These clones were used for subsequent strain constructions.

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**Figure 16. Characterization of hta1-S129 mutant strains by Southern blot.** (A) Maps of the genomic loci \textit{HTA2-HTB2} on chromosome II and \textit{TRP1} on chromosome IV in the W303 genetic background. Shown are the BglIII and EcoRI restriction sites used for strain characterization and the corresponding fragments of \textit{TRP1} analysed in (D) by hybridization with the RIX probe (EcoRI-Xbal fragmen). Indicated is the restriction site EcoRV in \textit{TRP1}, that was used in a plasmid borne version of this gene to insert the \textit{URA3-kanR} cassette for pTU10 construction (Cross, 1997). (B) Map of the \textit{(hta2-htb2)}\textsubscript{Δ}::\textit{TRP1} locus on chromosome II in the strains FY406, JHY2, JHY3, and JHY8 with the indicated restriction sites and fragments used for characterization in (D). (C) Map of the \textit{(hta2-htb2)}\textsubscript{Δ}::\textit{trp1::URA3} locus on chromosome II in the strains MFY1, MFY2, and MFY3 as in (B). These strains were obtained from JHY2, JHY3, and JHY8, respectively, by transformation with the \textit{trp1::URA3} disruption fragment released form pTU10 by EcoRI/Xhol digestion. (D) Southern blot of genomic DNA isolated from the indicated strains and digested either with EcoRI or with BglIII. The membrane was hybridized with the RIX fragment. This fragment hybridizes to the endogenous \textit{trp1-1} locus in W303, but not to the \textit{trp1Δ63} allele in the genetic background (S288C) of the FY406 derived strains. M is a 1 kb DNA ladder (New England BioLabs) hybridizing to random DNA sequences.
**Construction of yeast strains carrying minichromosomes.** The minichromosomes YRpFT35 and YRpFT68 were released from pBT2UmidR-YRpFT35 as a 2527 pb fragment and from pFT54-YRpFT68 as a 3693 bp fragment, respectively, by EcoRI digestion and circularized by ligation prior to transformation.

The plasmids pBT2UmidR-YRpFT35 and pFT54-YRpFT68 were isolated from the *E.coli* strains FT016 and FT054, respectively, as described in section 5.8.2. About 50 µg of plasmid DNA was digested with 240 U EcoRI (40 U/µl), in a total volume of 100 µl 1× SuRE/Cut Buffer H at 37°C for 2 h. A 3 kb fragment containing a pBR322 derivative vector backbone (Thoma and Simpson, 1985) and a 2.5 kb (YRpFT35), respectively 3.7 kb (YRpFT68) minichromosome fragment were obtained. To circularize the minichromosomes the digestion mixture was diluted to 3 ml in 1× T4 DNA Ligase Buffer (66 mM Tris, pH 7.5; 5 mM MgCl$_2$; 1 mM DTE; 1 mM ATP) supplemented with 7 U T4 DNA Ligase (1 U/µl) and incubated overnight at 4°C. To precipitate the DNA, the ligation mixture was transferred to a 30 ml Corex tube, 300 µl 3 M NaOAc, pH 4.8, and 9 ml cold EtOH abs. were added, mixed and left for 1 h at –20°C. The precipitate was centrifuged for 30 min at 16 500×g (10 000 rpm, Sorvall, HB-4 rotor) and 4°C. The pellet was washed with 5 ml cold 70% EtOH, air-dried, and resuspended in 50 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA). 5 µl of ligated DNA (about 2.5 µg ligated minichromosome) were used for yeast transformation (section 5.7.1). Some colonies were tested by Southern blot for bearing a minichromosome of the expected size and two of each strain was frozen in 15% (v/v) glycerol at –80°C.

**5.6.2  *E. coli* strains and plasmids**

All *E.coli* strains used for plasmid DNA isolation were in the DH5α genetic background, F$^{-}$ supE44 Δ(lacZYA-argF)U169 φ80lacZΔM15 hsdR17(r$^-$ m$^-x$) recA1 endA1 gyrA96 thi-1 relA1 deoR phoA λ$^-$, and are listed in Table 17.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers <em>a</em></th>
<th>bp $^b$</th>
<th>Usage/Purpose</th>
<th>Stain</th>
<th>Reference/Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>p425GPD</td>
<td>amp$^R$ LEU2</td>
<td>7760</td>
<td>SX-2micron fragment</td>
<td>AB6</td>
<td>ATTC No. 87357</td>
</tr>
<tr>
<td>pBT2UmidR- YRpFT35</td>
<td>amp$^R$ TRP1</td>
<td>5844</td>
<td>YRpFT35 construction</td>
<td>FT16</td>
<td>Thoma and Zatchej, 1988</td>
</tr>
<tr>
<td>FT54- YRpFT68</td>
<td>amp$^R$ URA3</td>
<td>7010</td>
<td>YRpFT68 construction</td>
<td>FT54</td>
<td>Thoma and Zatchej, 1988</td>
</tr>
<tr>
<td>pTU10</td>
<td>cap$^R$ kan$^R$ URA3</td>
<td>6600</td>
<td>marker swap</td>
<td>FT136</td>
<td>Cross, 1997</td>
</tr>
<tr>
<td>pUC18RIX</td>
<td>amp$^R$</td>
<td>2833</td>
<td>RIX fragment</td>
<td>FT63</td>
<td>M. Zatchej</td>
</tr>
<tr>
<td>pUC18XRV</td>
<td>amp$^R$</td>
<td>2879</td>
<td>XRV fragment</td>
<td>FT64</td>
<td>F. Thoma</td>
</tr>
<tr>
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<td>amp$^R$</td>
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<td>SS1</td>
<td>S. Schalbetter</td>
</tr>
<tr>
<td>pSS2</td>
<td>amp$^R$</td>
<td>3221</td>
<td>PHO5-SS2 fragment</td>
<td>SS2</td>
<td>S. Schalbetter</td>
</tr>
<tr>
<td>YCpJH2</td>
<td>amp$^R$ HIS3</td>
<td>7537</td>
<td>Sequencing of HTA1</td>
<td>MF3</td>
<td>this study</td>
</tr>
</tbody>
</table>
5.7 Transformation methods

5.7.1 Yeast transformation

All plasmids and DNA fragments were transformed into yeast cells using the lithium acetate method as described in (Gietz and Woods, 2002) with modifications. Yeast cells were grown in 2 ml YPD overnight at 200–250 rpm and 30°C. The cells were counted using a haemocytometer (Neubauer) and 5 ml YPD were inoculated to a cell density of about $5 \times 10^6$ cell/ml. The culture was incubated at 200–250 rpm and 30°C until a cell density of about $2 \times 10^7$ cell/ml was reached (3–6 h, depending on the strain). Per transformation $10^8$ cells were harvested by centrifugation for 5 min at $1150 \times g$ (3000 rpm, 4236 Centrifuge) and RT, washed with 2.5 ml sterile H$_2$O, resuspended in 100 µl 1 M LiOAc and transferred to an Eppendorf tube. The cells were centrifuged for 30 s at $16 000 \times g$ (13 000 rpm, Biofuge pico) and RT and the pellet was resuspended in 40 µl 100 mM LiOAc. 240 µl 50% polyethylene glycol 3350, 36 µl 1 M LiOAc, 50 µl 2 mg/ml single stranded DNA from herring sperm (boiled for 5 min and chilled on ice for 2 min before used), and 34 µl diluted DNA (0.2 up to 2.5 µg) was added to the cells. This transformation mixture was vortexed vigorously for about 1 min, incubated for 30 min at 30°C, and heat shocked for 15 min at 42°C in a water bath. The cells were pelleted as described above, the supernatant was removed by aspiration and the yeast pellet was resuspended in 1 ml sterile H$_2$O by short vortexing and pipetting up and down. 200 µl of the suspension was plated on SD agar supplemented with the appropriate ingredients. The plates were incubated for 2 to 4 days at 30°C.

5.7.2 E. coli transformation by electroporation

To recover yeast shuttle vectors for sequencing, genomic DNA isolated from yeast (section 5.8.4) was used to transform E. coli DH5α by electroporation, according to a method described in (Dower et al., 1988).
1 µl (200 ng) yeast genomic DNA (containing roughly 100 pg plasmid DNA) was mixed with 35 µl electrocompetent DH5α cells (section 5.7.3) in a Gene Pulser Cuvette (0.1 cm gap, Bio-Rad). Using an Electro Cell Manipulator 600 (BTX Harvard Apparatus) set to a voltage of 1.5 kV, a capacitance of 25 µF, and an impedance of 129 Ω, E. coli were electroporated by a pulse with a time constants of 4.5–4.8 ms. The cells were immediately transferred to a 1.5 ml Eppendorf tube by rinsing the cuvette with 1 ml LB broth, and shaken for 60 min at 37°C and 250 rpm. The E. coli cells were collected by a short spin at 16 000 × g (13 000 rpm, Biofuge pico) and RT, resuspended in 100 µl sterile H₂O, spread on LB agar supplemented with 100 µl/ml ampicillin, and incubated for 1 day at 37°C. 30 up to 450 colonies were obtained per transformation.

5.7.3 Preparation of electrocompetent E. coli DH5α cells

E. coli DH5α cells were prepared for electroporation using a procedure adapted from (Dower et al., 1988), but modified. DH5α E. coli cells grown on a LB agar plate were used to inoculate 10 ml LB broth. E. coli were grown overnight at 37°C and 250 rpm. 5 ml of this starter culture was used to inoculate into 1 12 × YT medium that then was shaken at 200 rpm and 37°C. Every hour the A600 was measured until an A600 of 0.45–0.55 (BioPhotometer 6131) was reached. The culture was chilled on ice-water for 5 min, centrifuged for 5 min at 6000 × g (6000 rpm, Sorvall, GS-3 rotor) and 4°C. The total pellet was washed, first with 800 ml, then with 400 ml ice-cold sterile H₂O, and finally with 80 ml ice-cold 10% glycerol. The bacterial cells were resuspended in 2 ml ice-cold 10% glycerol, distributed to 70 µl aliquots in Eppendorf tubes, flash-frozen in liquid nitrogen and stored at –80°C.

5.8 DNA isolation methods

5.8.1 Small scale alkaline lysis plasmid DNA isolation from E. coli

For sequencing the HTA1-HTB1 or hta1-HTB1 loci on the plasmids YCpJH2, YCpJH3, and YCpJH8, respectively (section 5.12), these plasmids were purified from E.coli MF3, MF24, and MF25 using a QIAprep Spin Miniprep Kit (QIAGEN) as described in QIAprep Miniprep Handbook (2002).

From an LB agar plate containing 100 µg/ml ampicillin E. coli were used to inoculate 2 ml LB broth supplemented with 100 µg/ml ampicillin. The culture was grown overnight at 37°C and 250 rpm. 1.5 ml of the culture was transferred to an Eppendorf tube and the bacterial cells were harvest by short spinning at 16 000 × g (13 000 rpm, Biofuge pico) and RT. The cell pellet was resuspended in 250 µl of Buffer P1 (50 mM Tris, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A),
250 µl of Buffer P2 (200 mM NaOH; 1% (w/v) SDS) was added, and mix by inverting the tube. Then 350 µl of Buffer N3 (QIAGEN) was add, mixed by inverting the tube, and centrifuged for 10 min at 16 000 × g (13 000 rpm, Biofuge pico) and RT. The supernatant was pipetted to a QIAprep spin column and the column was centrifuged for 1 min as above. The flow-through was discarded and the column was first washed with 500 µl of Buffer PB (QIAGEN) and then with 750 µl of Buffer PE (QIAGEN, mixed with 4 volumes EtOH abs. before used). After the flow-through was discarded the column was centrifuged for an additional 1 min as above. The column was placed in a clean 1.5 ml Eppendorf tube, 50 µl sterile H₂O was added, and the column was let stand for 1 min. To elute the DNA the column was centrifuged as above. Plasmid yield was about 5 µg DNA, estimated from agarose gel electrophoresis (section 5.13.1).

5.8.2 Large scale alkaline lysis plasmid DNA isolation from *E. coli*

Plasmid DNA isolation from *E. coli* strains for preparation of minichromosomes, the *trp1::URA3-kan*<sub>8</sub> disruption fragment, and DNA fragments for labeling has been carried out as described in QIAGEN Plasmid Purification Handbook (2003), using the QIAfilter Plasmid Maxi Kit (QIAGEN).

From a selective LB agar plate *E. coli* were used to inoculate a starter culture of 2 ml LB broth containing 100 µg/ml ampicillin or 50 µg/ml kanamycin. The starter culture was incubated for about 8 h at 37°C on a shaker at 250 rpm and then (depending on the copy number of the vector) diluted 1/500 into 250 ml (low-copy plasmids pBT2UmidR-YRpFT35 and pFT54-YRpFT68) or 1/1000 into 100 ml (high-copy plasmids) selective LB broth in an Erlenmeyer flask. This culture was incubated overnight at 37°C and 180 rpm. The bacterial cells were harvested by centrifugation for 15 min at 6000 × g (6000 rpm, Sorvall, GSA rotor) and resuspended in 10 ml Buffer P1 (50 mM Tris, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). The cells were lysed by adding 10 ml Buffer P2 (200 mM NaOH; 1% (w/v) SDS), gently inverting the centrifugation bottle, and incubation at RT for 5 min. 10 ml of chilled Buffer P3 (3 M KOAc, pH 5.5) was added, mixed by inverting the bottle, and the suspension was poured to a QIAfilter Cartridge. The Cartridge was incubated for 10 min at RT while a QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) 2-propanol). The cap from the QIAfilter outlet was removed and the cell lysate was filtered into the equilibrated QIAGEN-tip by inserting a plunger into the QIAfilter. After the filtered lysate flew through the QIAGEN-tip, the tip was washed twice with 30 ml Buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) 2-propanol). The DNA was then eluted into a 30 ml Corex tube with 15 Buffer QF (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) 2-propanol), 10.5 ml 2-propanol (RT) was added, and mixed to precipitate the DNA. The precipitate was centrifuged for 30 min at 16 500 × g (10 000 rpm, Sorvall, HB-4 rotor) and 4°C, the pellet washed with 5 ml cold 70% EtOH, air-dried and resuspended in 100 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA).
5.8.3 Small scale isolation of yeast genomic DNA

Genomic DNA for characterization of yeast strains was isolated essentially as described in (Sherman et al., 1986).

Cells were grown in 12–20 ml YPD or SD supplemented with the appropriate ingredients to an $A_{600}$ of 0.8–1.5 (BioPhotometer 6131) (about $1.5 \times 10^8$ cell) and harvested by centrifugation for 5 min at 1150 × g (3000 rpm, 4236 Centrifuge) and RT. The cells were resuspended in 500 µl Buffer Y1 (1 M Sorbitol; 100 mM EDTA, pH 7.5; 1 µl/ml 2-mercaptoethanol), transferred to an Eppendorf tube, 50 µl 10 mg/ml Lyticase were added, and the cell suspension was incubated at 30°C for about 30 min (until ~90% of the cells were lysed in 1% (w/v) SDS as checked by microscopy). The spheroplasts were pelleted by centrifugation for 1 min at 16 000 × g (13 000 rpm, Biofuge pico) and RT. The pellet was resuspended in 500 µl Lysis Buffer (50 mM Tris; 20 mM EDTA, pH 7.4; 1% (v/v) SDS) and the suspension was incubated for 30 min at 65°C. 200 µl 3 M KOAc, pH 5.5 was added, mixed, and the tube was placed on ice for 60 min. The white precipitate was pelleted by centrifugation for 10 min at 16 000 × g (13 000 rpm, Biofuge pico) and RT. The supernatant was transferred to a fresh Eppendorf tube, 700 µl 2-propanol (RT) was added and the tube was left at RT for 5 min. The DNA was centrifuged for 15 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The pellet was lyophilized (MAXI dry lyo), resuspended in 300 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA), 3 µl 10 mg/ml RNase A (in 10 mM Tris, pH 7.5; 15 mM NaCl) was added, and incubated for 1 h at 37°C. To precipitate the DNA, 30 µl 3 M NaOAc, pH 4.8 and 200 µl 2-propanol was added, mixed and left for 5 min at RT. The precipitate was centrifuged for 15 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C, the pellet washed with 500 µl 70% EtOH, lyophilized and resuspended in 30–50 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA).

5.8.4 Isolation of yeast genomic DNA using QIAGEN Genomic-tips

In order to isolate genomic DNA form lysed yeast cells for CPD mapping, plasmid DNA topology analysis, or from crude nuclei extracts, Genomic-tips 100/G or 500/G (QIAGEN) were used essentially as described in QIAGEN Genomic DNA Handbook (2001). Genomic-tips 100/G and 500/G have binding capacities of 100 and 500 µg genomic DNA, respectively. The genome of a haploid *S. cerevisiae* cell, in size of about 12.5 Mbp, has a mass of $8.25 \times 10^9$ Da/genome (provided an average of 660 Da/bp), corresponding to about 0.0137 pg DNA per cell. Consequently, to isolate DNA from up to $7 \times 10^9$ cells Genomic-tips 100/G and from up to $3.5 \times 10^{10}$ cells Genomic-tips 500/G were used. Buffer volumes for Genomic-tips 100/G and in brackets for Genomic-tips 500/G are denoted.

For DNA isolation form lysates or crude nuclei extracts in Buffer G2 (sections 5.8.5, 5.9.2, or 5.10.3), the samples were centrifuged for 10 min at 3000 × g (4000 rpm, Megafuge 1.0R)
and 4°C. The supernatant was transferred to a 15 ml (50 ml) Falcon tube, vortexed for 10 s
and applied to a Genomic-tip 100/G (500/G), which before was equilibrated with 4 ml (10 ml)
Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) 2-propanol; 0.15% (v/v) Triton
X-100). The Genomic-tip was washed twice with 7.5 ml (15 ml) Buffer QC (1 M NaCl; 50 mM
MOPS, pH 7.0; 15% (v/v) 2-propanol) and the DNA was eluted with 5 ml (15 ml) Buffer QF
(1.25 M NaCl; 50 mM Tris, pH 8.5; 15% (v/v) 2-propanol) prewarmed to 50°C. To precipitate
the DNA 3.5 ml (10.5 ml) 2-propanol was added to the eluate and the samples were centrifuged
for 30 min at 9500 × g (7000 rpm, Sorvall, HS-4 rotor) and 4°C. The DNA pellet was washed
with 2 ml (4 ml) cold 70% EtOH and centrifuged for 20 min as above. The pellet was air-dried
for 10 min and resuspended in 200 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA).

5.8.5 Lysis of yeast cells for plasmid DNA topology analysis

Yeast cells were grown at 30°C in about 20 ml SD, supplemented with appropriate ingredients
to an A_{600} of about 1–1.5 (BioPhotometer 6131) or in YPD to an A_{600} of about 2.5–3.0, mixed
with an equal volume of buffered ethanol/toluene (20 mM Tris, pH 8.0; 95% (v/v) ethanol; 3%
(v/v) toluene) prechilled to −20°C followed immediately by addition of 0.5 M EDTA, pH 8.0
to a final concentration of 10 mM (Johnston and Williamson, 1978). Yeast cells were harvested
from this mixture by centrifugation for 5 min at 1150 × g (3000 rpm, 4236 Centrifuge) and
RT, washed with 4 ml TE (10 mM Tris, pH 8.0; 1 mM EDTA), resuspended in 4 ml Buffer Y1
(1 M sorbitol; 100 mM EDTA, pH 8.0; 14 mM 2-mercaptoethanol), and digested with 0.3 mg
of Zymolyase-100T in a waterbath at 30°C for about 30 min (until the A_{600} in 1% (w/v) SDS
was less than 10% of the starting value). The spheroplasts thus obtained were recovered by
centrifugation for 5 min at 3000 × g (4000 rpm, Megafuge 1.0R) and 4°C, resuspended in 5 ml
Buffer G2 (800 mM guanidine HCl; 30 mM Tris; 30 mM EDTA; pH 8.0; 5% (v/v) Tween 20;
0.5% (v/v) Triton X-100; 0.1 mg/ml RNase A; 0.1 mg/ml Proteinase K), and incubated for 2 h
at 50°C. The DNA was isolated using Genomic-tips 100/G (QIAGEN) as described in section
5.8.4.

5.9 Preparation and digestion of chromatin from yeast

5.9.1 Preparation of crude nuclei extracts

Crude nuclei extracts for micrococcal nuclease (MNase) mapping of nucleosome organization
or UV irradiation were prepared essentially as described in (Livingstone-Zatchej and Thoma,
1999).

Yeast cells were grown at 30°C in SD supplemented with appropriate ingredients to an A_{600}
of about 0.8–1.2 (BioPhotometer 6131). Cells from 6 l yeast culture \(5 \times 10^{10}\) cells) were harvested by centrifugation for 6 min at 6000 × g (6000 rpm, Sorvall, GS-3 rotor) and washed in 80 ml water. The volume of the yeast pellet was estimated (8–16 ml) and the pellet was resuspended in a double volume of Pre-incubation Solution \((2.8 \text{ mM EDTA, pH 8.0; 0.7 M 2-mercaptoethanol})\). The cell suspension was shaken for 30 min at 100 rpm and 30°C, the cells were centrifuged for 5 min at 1150 × g (3000 rpm, 4236 Centrifuge) and RT, and washed in 20 ml 1 M sorbitol. The cells were resuspended in 5 ml Spheroplasting Buffer \((1 \text{ M sorbitol; 5 mM 2-mercaptoethanol; 0.5 mM PMSF; 0.5 µg/ml pepstatin A; 0.5 µg/ml leupeptin})\) per 1 g of cells and digested with 10 mg of Zymolyase-100T in a waterbath at 30°C for about 30 min (until the \(A_{660}\) in 1% (w/v) SDS was less than 10% of the starting value). The spheroplasts thus obtained were recovered by centrifugation for 5 min at 3000 × g (4000 rpm, Megafuge 1.0R) and 4°C, washed in 50 ml ice-cold 1 M sorbitol, resuspended in 7 ml ice-cold Ficoll solution \((18\% \text{ (w/v) Ficoll 400; 20 mM KH}_{2}\text{PO}_{4}, \text{pH 6.8; 1 mM MgCl}_{2}; 0.25 \text{ mM K}_{2}\text{EDTA}; 0.25 \text{ mM EGTA; 1 mM PMSF; 1 µg/ml pepstatin A; 1 µg/ml leupeptin})\) per 1 g cells (wet weight) and lysed with a Douncer homogenizer. The lysate was centrifuged for 30 min at 27 000 × g (15 000 rpm, Sorvall, SS-34 rotor) and 4°C and the white fatty layer covering the sample was removed by aspiration. The nuclei containing pellet was resuspended in the same volume of ice-cold Buffer A \((20 \text{ mM Tris, pH 8.0; 150 mM NaCl; 5 mM KCl; 1 mM EDTA; 1 mM PMSF; 1 µg/ml pepstatin A; 1 µg/ml leupeptin})\) as the spheroplasts were resuspended in Ficoll solution.

5.9.2 Digestion of chromatin with micrococcal nuclease

Aliquots of 2 ml of the crude nuclei extract (section 5.9.1) were transferred to 14 ml polypropylene tubes on ice and CaCl\(_2\) was added to a final concentration of 5 mM. The aliquots were prewarmed (37°C) for 5 min in a waterbath, MNase \((1–60 \text{ U/ml})\) was added, and the samples were incubated for 5 min at 37°C. The reactions were terminated by addition of 3 ml 2.5 × Buffer G2 \((2 \text{ M guanidine HCl; 75 mM Tris; 75 mM EDTA; pH 8.0; 12.5% (v/v) Tween 20; 1.25% (v/v) Triton X-100; 200 µg/ml RNase A; 300 µg/ml Proteinase K})\) and incubation for 2 h at 50°C. To the remaining crude nuclei extract 1.5 volumes of 2.5 × Buffer G2 were added and it was incubated for 2 h at 50°C. Genomic-tips 100/G and 500/G (QIAGEN) were used as described in section 5.8.4 to isolate genomic DNA from MNase digested samples and from the remaining crude nuclei extract, respectively. The DNA pellets of MNase digested samples were resuspended in 200 µl TE \((10 \text{ mM Tris, pH 8.0; 1 mM EDTA})\) to a concentration of about 200 ng/µl.
5.10 UV-Irradiation and DNA Repair by NER and Photolyase

5.10.1 UV-Irradiation of Yeast in Suspension

UV irradiation of yeast cultures was done essentially as described in (Livingstone-Zatchej et al., 1999). Yeast cells were grown at 30°C in SD, supplemented with appropriate ingredients, to an $A_{600}$ of about 0.8–1.2 (BioPhotometer 6131). Cells from 6 l yeast culture ($5–10 \times 10^{10}$ cells) were harvested by centrifugation for 6 min at 6000 × g (6000 rpm, Sorvall, GS-3 rotor) and resuspended in SD to about $2.5–3.5 \times 10^{7}$ cells/ml. 250 ml aliquots were transferred to a plastic tray ($22 \times 31$ cm) and irradiated at RT at an average flux of 4.7 J/m$^2$·s (UVX-25 radiometer) with 150 J/m$^2$ of UV light (predominantly 254 nm) generated by type G15 T8 germicidal lamps (Sylvania).

5.10.2 DNA repair by NER and photolyase

After irradiation (section 5.10.1), the cells were pooled and the medium was supplemented with the appropriate ingredients. For photoreactivation and NER, $2 \times 500$ ml of the irradiated cell suspension were exposed to photoreactivating light (peak emission at 375 nm) generated by type F15 T8/BLB lamps (Sylvania) at an average flux of $\sim 13$ J/m$^2$·s (UVX-36 radiometer) in plastic trays ($22 \times 31$ cm) on a metal cooling plate (cooled to 20°C) in an irradiation box. After 15, 30, 60, and 120 min 250 ml aliquots were removed and chilled on ice. For NER alone 250 ml aliquots of the irradiated cells were incubated at RT (25°C to 27 ºC) for 30, 60, 120 and 240 min and then chilled on ice until they were prepared for DNA isolation (section 5.10.4).

5.10.3 Preparation of UV-irradiated and repair samples for DNA isolation

The samples (sections 5.10.2) were centrifuged for 8 min at 6000 × g (6000 rpm, Sorvall, GS-3 rotor), resuspended in 4 ml TE (10 mM Tris, pH 8.0; 1 mM EDTA) and transferred to 50 ml Falcon tubes. The cells were collected by centrifugation for 2 min at 3000 × g (4000 rpm, Megafuge 1.0R) and 4°C, resuspended in 4 ml Buffer Y1 (1 M sorbitol; 100 mM EDTA; 14 mM 2-mercaptoethanol), and digested with 2.5 mg of Zymolyase-100T in a waterbath at 30°C for about 30 min (until the $A_{600}$ in 1% (w/v) SDS was less than 10% of the starting value). The spheroplasts thus obtained were recovered by centrifugation for 5 min at 3000 × g (4000 rpm, Megafuge 1.0R) and 4°C, resuspended in 5 ml Buffer G2 (800 mM guanidine HCl; 30 mM Tris; 30 mM EDTA; pH 8.0; 5% (v/v) Tween 20; 0.5% (v/v) Triton X-100), 200 µl 10 mg/ml RNase A (in 10 mM Tris, pH 7.5; 15 mM NaCl) were added, and incubated at 30°C for 20 min. All steps until lysis of cells were done in yellow safety light using type F36W T8 lamps (Sylvania).
to prevent undesired photoreactivation. 200 µl 10 mg/ml Proteinase K (in 50 mM Tris, pH 8.0) were added and the samples were incubated for 2 h at 50°C. The DNA was isolated as described in section 5.8.4.

5.11 Digestion of yeast genomic DNA

5.11.1 MNase digestion of genomic DNA

To compare MNase cutting sites of chromatin with MNase cutting sites of deproteinized DNA, genomic DNA isolated from crude nuclei extracts (section 5.9.2) was digested with increasing amounts of MNase. About 1 µg genomic DNA was incubated at 37°C in 200 µl Buffer A without protease inhibitors (20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM KCl; 1 mM EDTA) and 5 mM CaCl$_2$ with 0.015, 0.03, 0.06, or 0.15 U MNase for 5 min. The DNA was immediately precipitated by adding 20 µl 3 M NaOAc, pH 4.8, 600 µl cold EtOH abs., quick vortexing, and incubation for 1 h at –20°C. The precipitate was spun down for 30 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The pellet was washed with cold 70% EtOH, centrifuged for 20 min as above, and lyophilized for 5–10 min. The extent of MNase digestion was checked by separation on a 1% agarose gel (section 5.13.1) before restriction digestion for indirect end-labeling (section 5.11.3).

5.11.2 Restriction digestion of genomic DNA for strain characterization

To characterize yeast transformants by Southern blotting, genomic DNA, isolated as described in section 5.8.3, was used for restriction digestion. To check if the trp1::URA3 disruption fragment form pTU10 inserted into the (hta2-htb2)Δ::TRP1 locus to obtain (hta2-htb2)Δ::trp1::URA3 (section 5.6.1.1), about 100–200 ng genomic DNA was digested with 10 U EcoRI (10 U/µl) in a total volume of 10 µl 1 × SuRE/Cut Buffer H or 10 U BglII (10 U/µl) in a total volume of 10 µl 1 × SuRE/Cut Buffer M for 3 h at 37°C. To characterize strains carrying minichromosomes, 100–200 ng genomic DNA was digested with 10 U XbaI (10 U/µl) in a total volume of 20 µl 1 × SuRE/Cut Buffer H for 2–4 h at 37°C.

Without further purification the digested samples were used for agarose gel electrophoresis (section 5.13.1) and subsequent Southern blot analysis.
5.11.3 Restriction digestion of genomic DNA for indirect end labelling

To map MNase cutting sites and CPDs by indirect end-labeling genomic DNA, purified as described in section 5.8.4, was used for restriction digestion. The minichromosomes YRpFT35 and YRpFT68 were analysed by digestion with XbaI (40 U/µl) in 1 × SuRE/Cut Buffer H. *GALI-10* was analysed by digestion with EcoRV (40 U/µl) in 1 × SuRE/Cut Buffer B. *PHO3-5* was analysed by digestion with StuI (100 U/µl) in 1 × NEBuffer 2. *adh4::URA3* was analysed by digestion with HhaI (20 U/µl) in 1 × NEBuffer 4, supplemented with BSA to an final concentration of 100 ng/µl. All restriction digestion reactions were carried out at 37°C for 2–4 h in a waterbath.

For mapping MNase cutting sites on minichromosomes or genomic loci about 2 or 5 µg DNA, respectively, were digested in a total volume of 100 µl with 60–120 U restriction enzyme. For mapping CPDs on minichromosomes or genomic loci about 8 or 20 µg DNA, respectively, were digested in a total volume of 250 µl with 120–240 U restriction enzyme. To precipitate the DNA after restriction digestion 0.1 volumes 3 M NaOAc, pH 4.8 and 3 volumes cold EtOH abs. were added, mixed by inverting the tube, and incubated at –20°C for at least 1 h or overnight. The precipitate was centrifuged for 30 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The pellet was washed with cold 70% EtOH, centrifuged for 20 min as above, and lyophilized for 5–10 min. DNA samples for mapping MNase cutting sites were separated on 1% agarose maxi gels as described in section 5.13.2 and DNA samples for mapping CPDs were resuspended in 45 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA) and followed up as described in section 5.11.4.

5.11.4 T4 endonuclease V digestion of genomic DNA

To nick genomic DNA at CPDs, 4–10 µg (about 20 µl) of restriction digested DNA (section 5.11.3) was incubated in 40 µl T4 Endo V Reaction Buffer (50 mM Tris, pH 7.5; 5 mM EDTA) with 14 U T4 Endonuclease V (20 U/µl) for 2 h at 37°C. In parallel mock treated samples were incubated at the same conditions without T4 Endonuclease V. Before loading on an alkaline gel (section 5.13.3), the DNA was denatured for 30 min at RT by adding 10 µl freshly prepared 5 × Alkaline Loading Buffer (12.5% (w/v) Ficoll 400; 5 mM EDTA; 250 mM NaOH; 0.125% (w/v) bromocresol green).

5.12 DNA sequencing

Sequencing of DNA samples was carried out according the dideoxy chain termination method described by F. Sanger (Sanger et al., 1977) using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyser (Applied Biosystems).
The kit consists of a Termination RR (ready reaction) Mix (containing dNTPs, differentially labeled ddNTPs, and an AmpliTaq DNA Polymerase, FS) and a Template Suppression Reagent. Basically a primer extension reaction from a DNA template to be sequenced is randomly terminated by incorporation of a fluorescently labeled ddNTP. The termination products are separated by capillary electrophoresis and analyzed by laser-induced fluorescence spectroscopy in the Genetic Analyser.

For the primer extension reaction 1 µl of plasmid DNA (100 ng/µl, section 5.8.1) was mixed with 2 µl Termination RR Mix and 3 µl primer solution (1 µM, Table 4, No. 1190 to 1193), diluted to 20 µl in 2 mM MgCl₂; 80 mM Tris, pH 9.0 in a 200 µl PCR tube and overlaid with 2 drops of mineral oil (Sigma). The sequencing reaction was carried out on a GeneAmp PCR System 9600 (Perkin Elmer) in 25 cycles of 30 s at 96°C, 15 s at 54°C, and 4 min at 60°C. To precipitate the DNA the sequencing reaction mix was transferred to a 1.5 ml Eppendorf tube, 2 µl 3 M NaOAc, pH 4.8 and 50 µl cold EtOH abs. was added, mixed by inverting the tube and immediately centrifuged for 30 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The DNA pellet was washed with 250 µl cold 70% EtOH and centrifuged for 5 min as above. The pellet was lyophilized for 5 min (MAXI dry lyo) and resuspended in 25 µl Template Suppression Reagent. The sequencing sample was then heated for 2 min at 95°C, left on ice for 2 min, transferred to a 500 µl PCR tube with cut lid and applied to the ABI Prism 310 Genetic Analyser, that was run according to the ABI Prism 310 Genetic Analyzer User’s Manual (1998). Eventually the sequence was analysed using the software EditView1.0.1.

5.13 Agarose gel electrophoresis

5.13.1 Neutral mini and midi agarose gel electrophoresis

DNA fragments and plasmid DNA from 100 up to 10 000 bp were separated and analysed on 1–1.5% agarose mini (6 × 10 cm, 40 ml) or midi (14.6 × 10 cm, 80 ml) gels in 1 × TBE (90 mM Tris; 90 mM boric acid; 20 mM EDTA; pH 8.0) and 0.5 µg/ml EtBr using horizontal gel electrophoresis systems.

Before loading on the gel, DNA samples were mixed with 0.25 volumes of 5 × TBE Sample Buffer (0.05% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol FF; 30% (v/v) glycerol, 5 × TBE). The gels were run at 100–150 V and RT for 30 min to 1 h and analysed using a Gel Documentation System (UVItect). For Southern blotting, the gels were treated as described in section 5.14.
5.13.2 Neutral maxi agarose gel electrophoresis

To analyse MNase digested chromatin samples the DNA was separated on 1% agarose maxi gels (20 × 25 cm, 300 ml) in 1 × TBE (90 mM Tris; 90 mM boric acid; 20 mM EDTA; pH 8.0) and 0.5 μg/ml EtBr using a horizontal gel electrophoresis system (Horizon 20-25, Gibco BRL).

After restriction digestion and DNA precipitation (section 5.11.3) the DNA was resuspended in 10 μl 1 × TBE Sample Buffer (0.01% (w/v) bromophenol blue; 0.01% (w/v) xylene cyanol FF; 6% (v/v) glycerol, 1 × TBE), separate on the gels at 50 V and RT for 15 h, and transferred to nylon membranes as described in section 5.14.

5.13.3 Alkaline agarose gel electrophoresis

To separate single stranded DNA after T4 Endonuclease V digestion (section 5.11.4), alkaline agarose gels (20 × 25 cm, 300 ml) were prepared according to (Sambrook et al., 1989), with modifications, and run in a horizontal gel electrophoresis system (Horizon 20-25, Gibco BRL).

1.5 % agarose was melted in 300 ml 50 mM NaCl; 1 mM EDTA, pH 8.0 and after the gel has solidified, it was soaked twice for 2 h in Alkaline Electrophoresis Buffer (50 mM NaOH; 1 mM EDTA). Per slot 24 μl (2–5 μg) of denatured DNA sample (section 5.11.4) was loaded and the gel was run in 2 l Alkaline Electrophoresis Buffer at 4°C, first at 56 V (about 300 mA) for 20 min, and then at a constant current of 250 mA (voltage increased from 40–44 V at the beginning to 70–80 V at the end of electrophoresis) with continuous buffer circulation using a peristaltic tubing pump (Ismatec) and magnetic stirrers. Electrophoresis was carried out for 15–18 h until the bromocresol green dye has migrated about 14–15 cm.

5.13.4 Chloroquine agarose gel electrophoresis

One-dimensional topology analysis. Plasmid DNA topoisomers were resolved on 0.75% (2 μ plasmid) or 1% (YRpFT35 and YRpFT68) midi (14.6 × 10 cm, 80 ml) or maxi (19.5 × 25 cm, 300 ml) agarose gels in 1 × TBE (90 mM Tris; 90 mM boric acid; 20 mM EDTA; pH 8.0) containing chloroquine using horizontal gel electrophoresis systems as described in (Wechser et al., 1997).

About 1 μg DNA, isolated as described in section 5.8.5, was mixed with 0.25 volumes of 5 × TBE Sample Buffer (0.05% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol FF; 30% (v/v) glycerol, 5 × TBE) and electrophoresed on 0.75 or 1% maxi agarose gels containing 1 μg/ml chloroquine. DNA was allowed to enter the gel at 100 V for 20 min. Hereafter 0.75% gels were
run for 40–47 h and 1% gels for 25–26 h at 35 V in the dark and the buffer was circulated using a peristaltic tubing pump (Ismatec) and magnetic stirrers. The DNA was transferred to nylon membranes as described in section 5.14.

**Two-dimensional topology analysis.** Two-dimensional chloroquine gels were done as described in (Kim and Clark, 2002). The DNA was isolated as described in section 5.8.5, about 1 µg DNA was mixed with 0.2 volumes of 4 × TPE Sample Buffer (0.04% (w/v) bromophenol blue; 0.04% (w/v) xylene cyanol FF; 40% (v/v) glycerol; 4 × TPE (see below); 40 µg/ml chloroquine) and loaded on 0.8% agarose maxi gels (19.5 × 25 cm, 300 ml) in 1 × TPE (40 mM Tris; 30 mM NaH$_2$PO$_4$; 1 mM EDTA; pH 8.2) and 10 µg/ml chloroquine. The first dimension was run at 55 V for 15 h in the dark and the buffer was circulated using a peristaltic tubing pump (Ismatec) and magnetic stirrers. The gel was soaked three times for 2 h in 1 × TPE containing 20 µg/ml chloroquine in the dark. About 5.5 cm of the top of the gel was cut, using a razor blade, and the gel was rotated 90° relative to the first dimension. The second dimension was run in 1 × TPE and 20 µg/ml chloroquine as described above, but at 35 V. The DNA was transferred to nylon membranes as described in section 5.14.

### 5.14 Southern blotting

In order to analyse DNA by hybridization with short $^{32}$P-labeled DNA fragments, the DNA was transferred from an agarose gel to a nylon membrane according to an alkaline blotting method described in (Reed and Mann, 1985). The membrane was hybridized with a short radiolabeled DNA fragment (200–350 bp) according to a standard protocol described in the Zeta-Probe GT Blotting Membranes Instruction Manual (Bio-Rad). The labeled membrane was then analysed by exposure to a PhosphorImager Screen (Molecular Dynamics) that was scanned on a Storm 820 scanner (Amersham Biosciences).

Following agarose gel electrophoresis (sections 5.13), the DNA was denatured by placing the agarose gel for 15 min in 0.4 M NaOH (except for alkaline gels). The agarose gel was laid upside down on two 3MM Whatman chromatography papers (pre-soaked in 0.4 M NaOH). A Zeta-Probe GT nylon membrane (Bio-Rad), pre-soaked in 0.4 M NaOH, was laid on the gel and covered with six pre-wetted 3MM Whatman papers. A stack of paper towels was put on the blot and covered with a gel tray. The DNA was allowed to transfer overnight. The membrane was neutralized twice for 20 min in 2 × SSC (0.3 M NaCl; 30 mM Na$_3$ citrate, pH 7.0) and baked for 1 h in an oven (WTC Binder) at 80°C between two 3MM Whatman papers.

In a hybridisation cylinder rotating in a rotary oven (Bachofer) at 65°C, the membrane was pre-hybridised for 1 h with 15 ml of pre-warmed (65°C) Hybridisation Solution (0.25 M Na$_2$HPO$_4$, pH 7.2; 7% (w/v) SDS), supplemented with 50 µg/ml tRNA, and hybridised overnight with 20 ml Hybridisation Solution containing 100 µl $^{32}$P-labeled DNA probe (sections 5.15.3 and
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5.15.4). The membrane was transferred to a plastic tray and washed three times for 20 min with pre-warmed (65°C) Wash Solution I (40 mM Na$_2$HPO$_4$, pH 7.2; 5% (w/v) SDS) and three times for 20 min with pre-warmed (65°C) Wash Solution II (40 mM Na$_2$HPO$_4$, pH 7.2; 1% (w/v) SDS) in an oven (Memmert) at 65°C. The membrane was dried between 3MM Whatman papers, wrapped in Saran Wrap and exposed on a RX-100 autoradiography film (Fujifilm) at –80°C or on a PhosphorImager (Molecular Dynamics) screen at RT. The PhosphorImager screen was scanned on a Storm 820 Scanner (Amersham Biosciences) and analysed and quantified using the ImageQuant software (Molecular Dynamics).

5.15 DNA fragments and radioactive labeling of DNA probes

DNA fragments used to generate radioactive DNA probes are listed in Table 18. The DNA fragments were generated by excision from a plasmid (Table 17), as described in section 5.15.1, or by polymerase chain reaction (PCR) amplification, using primers listed in Table 4, as described in section 5.15.2.

Table 18. DNA fragments for indirect end-labeling

<table>
<thead>
<tr>
<th>Name</th>
<th>Fragment Description</th>
<th>Detection</th>
<th>SGD coordinates*</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIX</td>
<td>EcoRI-XbaI fragment of TRP1</td>
<td>YRpFT35/YRpFT68</td>
<td>IV 461736-461922</td>
<td>186</td>
</tr>
<tr>
<td>XRV</td>
<td>XbaI-EcoRV fragment of TRP1</td>
<td>YRpFT35/YRpFT68</td>
<td>IV 461922-462121</td>
<td>199</td>
</tr>
<tr>
<td>GAL1-SS1</td>
<td>EcoRI fragment of pSS1</td>
<td>GAL1-10 EcoRV</td>
<td>II 279687-279948</td>
<td>261</td>
</tr>
<tr>
<td>PHO5-SS2</td>
<td>EcoRI fragment of pSS2</td>
<td>PHO3-5 StuI</td>
<td>II 433761-433964</td>
<td>203</td>
</tr>
<tr>
<td>URA3-HhaI</td>
<td>PCR with primers 1132, 1133</td>
<td>Adh4Δ::URA3 HhaI</td>
<td>VII 16566-16861</td>
<td>295</td>
</tr>
<tr>
<td>SX-2micron</td>
<td>SnaBI-XbaI fragment of p425GPD</td>
<td>2 µ plasmid ori</td>
<td>2 µ 3606-3945</td>
<td>339</td>
</tr>
</tbody>
</table>

* SGD coordinates were retrieved from Saccharomyces Genome Database (SGD) [http://www.yeastgenome.org/](http://www.yeastgenome.org/) (Cherry et al., 1997). The chromosome number in roman numerals and the position of fragment sequence on the corresponding chromosome is indicated. In case of the SX-2micron fragment, its position is indicated with regard to the 2 µ plasmid sequence, which was retrieved from the National Centre for Biotechnology Information Database (NCBI, with the accession number NC_001398) [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (Hartley and Donelson, 1980). The length of the DNA fragments is denoted in bp.

5.15.1 Generation of DNA fragments by excision from plasmid DNA

The DNA fragment RIX (Table 18) was excised from the plasmid pUC18RIX (Table 17), by digestion with EcoRI and XbaI and purified from an agarose gel by gel extraction.

About 50 µg pUC18RIX plasmid DNA, isolated from E. coli FT63 as described in section 5.8.2, was digested with EcoRI (10 U/µl) and XbaI (10 U/µl), each 100 U, in a total volume of 400 µl 1 × SuRE/Cut Buffer H (5 mM Tris, pH 7.5 (at 37°C); 10 mM NaCl; 1 mM MgCl$_2$;
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0.1 mM DTT) for 2 h at 37°C. The DNA was precipitated by adding 0.1 volume 3 M NaOAc, pH 4.8, and 2.5 volumes of cold EtOH abs., mixing, and incubation for 1 h at −20°C. The precipitate was pelleted by centrifugation for 30 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C, the pellet was air-dried for 15 min, and resuspended in 50 µl TE (10 mM Tris, pH 8.0; 1 mM EDTA). The DNA fragments were separated on a 1.5% agarose gel (section 5.13.1) and a gel slice containing a band of approximately 200 bp was used for gel extraction using a QIAquick Gel Extraction Kit (QIAGEN). The RIX fragment was eluted at a concentration of approximately 20 ng/µl in 10 mM Tris, pH 8.5.

The DNA fragment XRV (Table 18), excised from the plasmid pUC18XRV (Table 17), by digestion with BamHI (for the XbaI site) and HindIII (for the EcoRV site), and extracted from low melting agarose gel, was obtained from M. Livingstone-Zatchej and was about 25 ng/µl in TE (10 mM Tris, pH 8.0; 1 mM EDTA).

The DNA fragment SX-2micron (Table 18) was excised from the plasmid p425GPD (Table 17) (ATCC No. 87357), by digestion with SnaBI and XbaI and purified from an agarose gel by gel extraction.

About 5 µg p425GPD plasmid DNA, a gift from A. Bucceri, was digested with SnaBI (20 U/µl) and XbaI (5 U/µl), each 30 U, in a total volume of 40 µl 1 × NEBuffer 4 (2 mM Tris, pH 7.9 (at 25°C); 5 mM KOAc; 1 mM Mg(OAc)₂; 0.1 mM DTT), supplemented with BSA to an final concentration of 100 ng/µl, for 3 h at 37°C. The DNA fragments were separated on a 1% agarose gel (section 5.13.1) and a gel slice containing a band of approximately 340 bp was used for gel extraction using a QIAquick Gel Extraction Kit (QIAGEN). The SX-2micron fragment was eluted at a concentration of approximately 25 ng/µl in 10 mM Tris, pH 8.5.

The DNA fragments GAL1-SS1 and PHO5-SS2 (Table 18), excised from the plasmids pSS1 and pSS2 (Table 17), respectively, by digestion with EcoRI, and extracted from agarose gels, were obtained from S. Schalbetter and were about 12 and 25 ng/µl, respectively, in 10 mM Tris, pH 8.5.

5.15.2 Generation of DNA fragments by PCR

The DNA fragment URA3-HhaI (Table 18) was generated by PCR amplification from a genomic DNA template (Saiki et al., 1985), isolated from W303α-JD (section 5.8.4), using the primers 3'-ChrVII-TelURA (No. 1132) and 5'-ChrVII-TelURA (No. 1133) (Table 4) and a Taq DNA Polymerase Kit (QIAGEN).

About 500 ng genomic DNA was mixed with 10 µl 5 × Q Solution (QIAGEN); 1 µl 25 mM MgCl₂; dATP, dCTP, dGTP, and dTTP, each at a final concentration of 0.2 mM; primers, each at a final concentration of 1 µM; 1 U Taq DNA Polymerase (QIAGEN) in a total volume of 50 µl 1 × PCR Buffer (QIAGEN) in a 200 µl PCR tube. The PCR was performed in a RoboCycler Gradient 96 (Stratagene) PCR machine. The first stage was a denaturation phase at 94°C for 3
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min, followed by 30 cycles of a denaturation step at 94°C for 1 min, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min. The reaction was terminated by an extension step at 68°C for 10 min and subsequent cooling to 6°C.

A specific fragment of about 300 bp was obtained that was purified from the PCR mixture by gel extraction using a QIAquick Gel Extraction Kit (QIAGEN). The URA3-HhaI fragment was eluted at a concentration of approximately 20 ng/µl in 10 mM Tris, pH 8.5.

5.15.3 Labeling DNA fragments by random priming

DNA fragments were labelled with [α-32P]-dCTP by random oligonucleotide-primed synthesis (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) using a HexaLabel DNA Labeling Kit (Fermentas).

About 25 ng DNA fragment (Table 18) diluted to 33 µl in H2O was mixed with 10 µl 5 × Reaction Buffer (250 mM Tris, pH 8.0; 25 mM MgCl₂; 5 mM DTT) containing hexanucleotides, boiled for 5 min and cooled on ice for 2 min. 3 µl Mix C (dATP; dGTP; dTTP; each 0.33 mM), 1 µl Klenow Fragment, exo− (5 U/µl), and 3 µl [α-32P]-dCTP (3000 Ci/mmol; 10 µCi/µl) was added and incubated for 30 min at 37°C. The reaction was stopped by adding 2 µl 0.5 M EDTA, pH 8.0 and the mixture was diluted to 100 µl with TE (10 mM Tris, pH 8.0; 1 mM EDTA). Unincorporated nucleotides were removed using a Quick Spin Column G-50 Sephadex (in 10 mM Tris, pH 8.0; 1 mM EDTA; 100 mM NaCl) and the probe was denatured by boiling for 5 min and chilling on ice for 2 min before hybridisation (section 5.14).

A typical labeling reaction resulted in a probe activity of 1.5–4 × 10⁷ cpm (determined by scintillation counting of 1 µl of the probe using a Cerenkov protocol and a Liquid Scintillation Analyzer, 1500 TRI-CARB).

5.15.4 Labeling strand-specific DNA probes by primer extension

For strand-specific probes, single-stranded DNA was labelled with [α-32P]-dCTP by primer extension in a linear amplification reaction (Finckh et al., 1991) using a Taq DNA Polymerase Kit (Fermentas).

About 5 ng of template DNA fragment (Table 18) was mixed with MgCl₂ at 3 mM; dATP, dGTP, and dTTP, each at 25 µM; [α-32P]-dCTP (3000 Ci/mmol; 10 µCi/µl) at 0.25 µM; a primer (Table 4) at 0.25 µM final concentration; 1 U Taq DNA Polymerase (Fermentas); in 40 µl 1 × Taq PCR Buffer (75 mM Tris, pH 8.8; 20 mM (NH₄)₂SO₄; 0.01% Tween 20) in a 500 µl PCR tube and overlaid with 2 drops of mineral oil (Sigma). The primer extension reaction was cycled in a DNA Thermal Cycler (Perkin Elmer), in 20 cycles of a denaturation step at 94°C for 45 s, an annealing step at 58 or 60°C, depending on the primer (Table 4), for 5 min, and an extension
step at 72°C for 3 min. Thereafter the volume of the reaction mixture was adjusted to 100 µl with TE (10 mM Tris, pH 8.0; 1 mM EDTA), unincorporated nucleotides were removed using a Quick Spin Column G-50 Sephadex (in 10 mM Tris, pH 8.0; 1 mM EDTA; 100 mM NaCl), and the probe was denatured by boiling for 5 min and chilling on ice for 2 min before hybridisation (section 5.14).

A typical labeling reaction resulted in a probe activity of 1.5–3 × 10^7 cpm (determined by scintillation counting of 1 µl of the probe using a Cerenkov protocol and a Liquid Scintillation Analyzer, 1500 TRI-CARB). Strand specificity of the probes was checked with a mock primer extension reaction where the template, but no primer was added. After purification, less than 10% of the signal of the probe was detected in the mock reaction.

5.16 Protein analysis

5.16.1 Crude histone extraction for Western blotting

To monitor H2A phosphorylation by Western blotting, histone proteins were acid extracted from cell lysates essentially as described in (Redon et al., 2003). About 2 × 10^7 cells were spun for about 10 s at 16 000 × g (13 000 rpm, Biofuge pico) in 1.5 ml Eppendorf tubes containing 450 mg glass beads (425–600 µm diameter). The medium was removed by aspiration, 400 µl 0.2 M H₂SO₄ was added, and the tubes were vortexed (VIBRAX-VXR, at the highest setting) 5 times for 1 min with samples being placed on ice during 1-min intervals between vortexing. The cellular debris and the glass beads were pelleted by centrifugation for 1 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The supernatant was transferred to a new tube and the extracted proteins were mixed with 0.2 volumes of 100% TCA, kept on ice for 30 min, and centrifuged for 15 min at 16 000 × g (13 000 rpm, Biofuge 15) and 4°C. The pellet was washed twice with 500 µl cold acetone and centrifuged for 10 min as above. The pellet was air-dried for 10–15 min and resuspended in 20 µl SDS-PAGE Sample Buffer (62.5 mM Tris, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 2.5% (v/v) 2-mercaptoethanol; 0.01% (w/v) bromophenol blue). 10 µl (~0.5 µg) of these samples was loaded on a SDS polyacrylamide gel (section 5.16.2) for subsequent Western blotting.

5.16.2 SDS-PAGE of proteins

Protein samples were analysed on denaturing polyacrylamide gels (8.3 × 7.3 cm, 0.75 mm thick) in a continuous buffer system using a vertical Mini-PROTEAN 3 Electrophoresis System (Bio-Rad) as described by U. K. Laemmli (Laemmli, 1970), except that the running gel and the Electrode Buffer concentrations were doubled.
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To separate proteins of 10–45 kDa, resolving gels (~3.5 ml) were 15% (w/v) polyacrylamide (acrylamide/N,N’-methylene-bis-acrylamide 29:1) in 750 mM Tris, pH 8.8; 0.1% (w/v) SDS, overlaid with a 4% (w/v) polyacrylamide stacking gel (~1 ml) in 125 mM Tris, pH 6.8. TCA precipitated protein samples (section 5.16.1) in SDS-PAGE Sample Buffer (62.5 mM Tris, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 2,5% (v/v) 2-mercaptoethanol; 0.01% (w/v) bromophenol blue) were heated for 5–10 min at 95°C, spun for 20 s at 16 000 \( \times \) g (13 000 rpm, Biofuge pico) before loading ~0.5 µg protein on the gel. The gel was run in 2 \( \times \) Electrode Buffer (248 mM Tris; 1.92 M glycine; pH 8.3; 0.1% (w/v) SDS) at 25 mA/per gel for approximately 90 min.

The gel was either used for Western blotting (section 5.16.3) or stained for 45 min in 0.05% (w/v) Coomassie Brilliant Blue R 250; 50% (v/v) methanol; 10% (v/v) acetic acid and destained for at least 2–3 h or overnight in 45% (v/v) methanol; 10% (v/v) acetic acid. The gel was kept in 5% (v/v) acetic acid until it was dried for 2 h at 80°C on a 3MM Whatman paper in a Gel Dryer (Bio-Rad).

5.16.3 Western blotting

Transfer of proteins from SDS polyacrylamide gels to nitrocellulose membranes was performed in a Mini Trans-Blot cell (Bio-Rad) using a wet electroblotting Towbin buffer (2.5 mM Tris; 19.2 mM glycine; pH 8.3; 0.02% SDS) system (Towbin et al., 1979). Following SDS-PAGE (section 5.16.2) the polyacrylamide gel was equilibrated for 15 min in Towbin buffer. The membrane was wetted in \( \text{H}_2\text{O} \) then soaked for 5 min in Transfer Buffer. The blot was assembled between two stacks of three in Transfer Buffer pre-wetted 3MM Whatman chromatography papers sandwiched between two wet sponges in the transfer cassette. The transfer cassette was placed into the buffer tank in such a way as to enable the proteins to be transferred between gel and membrane from the cathode to the anode. The tank was filled with Transfer Buffer and a stir bar was added. Proteins were transferred for 2 h at 400 mA and 4°C begin stirring.

5.16.4 Western blot detection

Following protein transfer, the nitrocellulose membrane was blocked in 2–5% (w/v) dry milk in TBS-Tween (20 mM Tris, pH 7.4; 150 NaCl; 0.1% (v/v) Tween 20) (for detection with primary anti-H3 antibody) or in 3% (w/v) BSA in TBS-Tween (for detection with primary anti-phospho H2A antibody) on a rocker platform for 1 h at RT. The membrane was incubated with the primary antibody diluted in blocking solution (2–5% (w/v) dry milk in TBS-Tween or 3% (w/v) BSA in TBS-Tween) on a rocker platform for 1 h at RT or overnight at 4°C, washed 3 times for
10 min in TBS-Tween, and incubated with the secondary antibody HRP-conjugate in 5% (w/v) dry milk in TBS-Tween as with the primary antibody. The membrane was washed again 3 times for 10 min in TBS-Tween, rinsed with H₂O, and analysed by chemiluminescence detection using an Immun-Star HRP Substrate Kit (Bio-Rad) and exposure on a RX-100 autoradiography film (Fujifilm).

5.17 MMS and UV sensitivity assays

Yeast strains were grown at 30°C and 250 rpm in 2 ml SD media, supplemented with the appropriate ingredients, to an $A_{600}$ of about 1–1.5 (BioPhotometer 6131) or in 2 ml YPD media to an $A_{600}$ of about 1.5–2. The cells were counted using a haemocytometer (Neubauer) and diluted in sterile H₂O to a cell density of $10^7$ cells/ml. 10-fold serial dilutions ($10^3$ to $10^6$ cells/ml) were prepared in 1 ml sterile H₂O.

To assess MMS sensitivity, 10 µl of these 10-fold serial dilutions (10 to $10^5$ cells) were spotted on YPD plates with or without 0.02% (v/v) MMS (Sigma). The plates were incubated for 2 days at 30°C.

To monitor UV sensitivity, drop spots on YPD plates, as described above, were not irradiated or irradiated with UV-light (predominantly 254 nm) by use of type G15 T8 germicidal lamps (Sylvania) at 75, 100 or 150 J/m². The plates were incubated in the dark, to prevent photoreactivation, for 2 days at 30°C.
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