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Mechanism of chromatin reassembly at the yeast *PHO5* promoter upon repression

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I. Introduction

1. Chromatin organization

Due to the limited space inside the eukaryotic nucleus, the genetic material of the cell has to be massively compacted. This is achieved by forming a nucleoprotein complex between the negatively charged DNA molecules and positively charged structural proteins, the histones. In the context of this complex, 146 bp of DNA are wrapped in about two turns around a histone octamer, consisting of one histone H3/H4 tetramer and two histone H2A/H2B dimers (Luger et al., 1997; Richmond and Davey, 2003). This arrangement of histones and DNA is called a nucleosome core particle and constitutes the basic repeating unit of chromatin. Nucleosomes are regularly spaced on the DNA, with short stretches of histone-free linker DNA being interspersed between the nucleosomes. Electron microscopic imaging revealed that such nucleosomal arrays are arranged in a beads-on-a-string like fashion, generating a chromatin fiber of 10 nm in diameter (Horn and Peterson, 2002; Olins and Olins, 2003). A secondary level of chromatin compaction is accomplished by folding up the 10 nm fiber into a higher order structure, yielding a 30 nm fiber (Everid et al., 1970; Woodcock and Dimitrov, 2001). The formation of this fiber is thought to be mediated by intermolecular nucleosome-nucleosome interactions and is, in most eukaryotes, stabilized by the presence of linker histones, e.g. H1 or H5 (Hansen, 2002; Hayes and Hansen, 2001). In addition to the linker histones, other non-histone nucleosome-binding proteins, e.g. MeCP2 (methyl CpG binding protein 2) or PCGC (polycomb group protein complex), impose further condensation upon the nucleosomal arrays and thereby contribute to the generation of secondary and tertiary chromatin fiber structures (Luger and Hansen, 2005). During mitosis, when the genetic material is segregated to the two daughter cells, the chromosome fiber has to compact 200-500 fold. In this process, histone tail modifications, e.g. H1 phosphorylation, as well as the binding of additional factors to chromatin, e.g. topoisomerase II and the condensin complex, appear to play a vital role (Khorasanizadeh, 2004; Luger and Hansen, 2005).

The packaging of DNA into chromatin poses a central challenge to all eukaryotic organisms. Many cellular processes like DNA replication, transcription and DNA damage disrupt the integrity of the chromatin structure, leaving behind regions in the genome with free, damaged

or insufficiently assembled DNA, respectively. In response to these disruptions cells have developed mechanisms taking care of the rapid assembly and disassembly of chromatin at different physiological instances, e.g. during the process of repairing DNA lesions or in the wake of transcription and DNA replication (Gunjan et al., 2005; Henikoff et al., 2004; Schwabish and Struhl, 2004).

2. Chromatin assembly

Chromatin assembly is a complex cellular process, as it requires the coordination between the synthesis of histones and their subsequent incorporation into chromatin. Proper coordination becomes especially important in light of the fact that excess soluble histones may, under certain circumstances, be toxic to the cell and subject to rapid degradation (Gunjan et al., 2005). Cells solve the problem of transporting free histones by making use of factors that possess histone chaperone activity. These proteins contribute to the ordered assembly of histones into nucleosomes by binding to histone proteins and delivering them to sites of chromatin assembly (Mello and Almouzni, 2001; Tyler, 2002). The common theme to all histone chaperones is their acidic nature. By binding to the rather basic histones the chaperones manage to antagonize the non-specific aggregation between histones and DNA (Loyola and Almouzni, 2004).

Probably the most impressive examples for the potency of histone chaperones to buffer excess soluble histones are two very specialized histone chaperones, nucleoplasmin and N1. Their main function is to associate with the enormous amounts of free histones in the *Xenopus* oocyte, thereby preventing the formation of insoluble aggregates (Laskey et al., 1993). Fertilization of the oocyte initiates a myriad of cell divisions, during which massive DNA replication and nucleosome assembly have to take place. During this process, nucleoplasmin and N1 serve as a histone sink steadily delivering histones to the nucleosome assembly machinery (Akey and Luger, 2003; Loyola and Almouzni, 2004).

Many histone chaperones have been identified as factors that catalyze nucleosome assembly in an *in vitro* reaction. One of the first histone chaperones discovered in this way showed a clear preference for histones H2A and H2B. Because of its role in facilitating nucleosome assembly, it has been named nucleosome assembly protein 1 (NAP1) and it turned out to be

highly conserved from yeast to man (Ishimi and Kikuchi, 1991; Yoon et al., 1995; Ishimi et al., 1984). NAP1 also shuttles the transport of H2A/H2B from the cytoplasm to the nucleus (Mosammaparast et al., 2002) and serves as linker histone chaperone in *Xenopus* egg extracts (Shintomi et al., 2005).

It has been assumed for the longest time that, in addition to H2A/H2B histone chaperones, other chaperones exist that specifically associate with histones H3 and H4. Up to date, there are numerous examples for dedicated H3/H4 histone chaperones (e.g. Asf1p and the Cac proteins in *S. cerevisiae*), many of which I will describe in detail in the following sections.

Apart from the classification of histone chaperones according to their specificity (either H3/H4 or H2A/H2B) chaperones are further categorized depending on their operational modes. Generally, two different modes of chromatin assembly can be distinguished. One pathway operates in a manner that is tightly coupled to the replication of DNA, whereas the other pathway works independently of replication (Ahmad and Henikoff, 2002; Tagami et al., 2004). Both pathways employ sets of histone chaperones that are characteristic for the respective pathway.

2.1.Replication-dependent chromatin assembly

The so-called replication-dependent chromatin assembly pathway is responsible for the bulk of chromatin assembly that occurs during the S-phase of the cell cycle when the genome is duplicated. This pathway ensures that nucleosomes are reformed immediately after passage of the travelling replication fork. The key player of the replication-coupled assembly is the heterotrimeric protein CAF1 (chromatin assembly factor 1), which is evolutionarily conserved (Ridgway and Almouzni, 2000). First evidence for a role of CAF1 in nucleosome assembly stems from *in vitro* studies, where this complex derived from human cell extracts was found to promote nucleosome assembly onto plasmid DNA (Smith and Stillman, 1989; Stillman, 1986). Chromatin assembly on the plasmid template occurs in a stepwise manner, with a tetramer of acetylated histones H3 and H4 being deposited first, rapidly followed by the incorporation of two H2A/H2B dimers (Smith and Stillman, 1991). A more direct demonstration for a histone chaperone activity of CAF1 comes from a study by Verreault and coworkers. They show that CAF1 has the capacity to interact with and assemble histones H3

and H4. Interestingly, these H3 and H4 histones display an acetylation pattern reminiscent of the one newly synthesized H3 and H4 exhibit (Verreault et al., 1996).
Surprisingly, the CAF1 complex possesses a very distinct specificity in the chromatin assembly reaction. It preferentially uses newly synthesized DNA as a substrate, either during replication or nucleotide excision repair, and it incorporates newly synthesized H3 and H4 into nucleosomes (Smith and Stillman, 1991). The molecular basis accounting for this link between replication / repair and *de novo* nucleosome assembly turned out to be a physical interaction between the DNA polymerase processivity factor PCNA and the largest subunit of CAF1, p150 (Shibahara and Stillman, 1999; Moggs et al., 2000; Green and Almouzni, 2003). In agreement with this finding, CAF1 complex in HeLa cells colocalizes with DNA replication foci in S-phase and is recruited to sites of UV-induced DNA damage (Krude, 1995; Martini et al., 1998).
It is intuitive that the loss of factors involved in restoring proper chromatin structure in the aftermath of DNA replication or repair may generate severe phenotypes. In fact, depletion or inhibition of CAF1 in human cell lines leads to reduced nucleosome assembly activity during DNA synthesis, cell cycle arrest in S-phase and elicits a DNA damage response. These results highlight the importance of the complex in packaging nascent DNA into chromatin (Hoek and Stillman, 2003; Nabatiyan and Krude, 2004; Ye et al., 2003). Data from the mouse system provide evidence that CAF1 is also important for the reestablishment of heterochromatic states in the course of DNA replication. The large subunit of CAF1 is thought to sequester HP1 (heterochromatin protein 1) to heterochromatic sites, where the retention of HP1 molecules is mediated via an interaction with methylated H3-K9 and RNA (Quivy et al., 2004).
In the budding yeast *S. cerevisiae*, the *CAC1/ 2/ 3* (chromatin assembly complex 1/ 2/ 3) genes code for the respective subunits of the CAF1 complex. Strikingly, disruption of these genes does not bring about any apparent growth or cell cycle defect, as was previously observed in higher eukaryotes (Hoek and Stillman, 2003; Nabatiyan and Krude, 2004; Ye et al., 2003). The lack of severe phenotypes suggests the existence of redundant histone chaperones in yeast. However, deleting *CAC1-3* disturbs chromatin-mediated silencing at a series of genomic loci, i.e. the telomeres, the silent mating type loci as well as at the rDNA genes (Enomoto and Berman, 1998; Kaufman et al., 1997; Smith et al., 1999). Consistent with the suggested role for the CAF1 complex in chromatin assembly *in vivo*, the *cac1/ cac2*

mutations in yeast manifest themselves in a genome-wide under-assembly of chromatin (Adkins and Tyler, 2004) and in an increased sensitivity to double-strand DNA damaging agents and UV-irradiation (Kaufman et al., 1997; Linger and Tyler, 2005).

For yeast, it is speculated that networks of chaperones, rather than individual chaperones mediate the nucleosome assembly process. One of the chaperones acting in synergy with CAF1 is the antisilencing factor 1 (Asf1p). Asf1p was originally identified in two independent genetic screens in *S. cerevisiae* as a factor that depresses the silent mating type loci upon its overexpression (Le et al., 1997; Singer et al., 1998). In concert with CAF1, Asf1 promotes nucleosome assembly onto newly synthesized DNA in *Drosophila* embryo extracts (Tyler et al., 1999) and nucleosome assembly in human cell extracts during nucleotide excision repair (Mello et al., 2002). In the current model Asf1 serves as a histone donor in the assembly reaction by delivering H3-H4 tetramers to CAF1. The experimental data for Asf1 function strongly favour this idea. Asf1 physically binds histones H3 and H4, but not histones H2A and H2B (Umehara et al., 2002). The coordinated role for Asf1 and CAF1 in nucleosome assembly is emphasized by the fact that the two proteins in *Drosophila* interact *in vitro* and colocalize on polytene chromosomes (Tyler et al., 2001). Moreover, like CAF1, Asf1 interacts with a component of the replicational machinery, RF-C (replication factor C) (Franco et al., 2005). In Figure 1, the interactions between the key players of the replication-coupled nucleosome assembly pathway are summarized and a model for targeting the pathway to sites of DNA replication and repair is proposed.

Yeast cells lacking *ASF1* share some phenotypes with the *cac1/2* mutants, e.g. they also have silencing defects at the telomeres and the silent mating type loci (Le et al., 1997; Singer et al., 1998). Moreover, in *Drosophila* both mutations result in an accumulation of gross chromosomal rearrangements, reflecting the genomic instability of *ASF1* and *CAF1* mutant strains due to the activation of DNA damage and replication checkpoints during S-phase (Myung et al., 2003). On the other hand, inactivating *ASF1* leads to growth defects and renders cells more sensitive to a broad range of DNA damage inducing agents like hydroxyurea, phenotypes that are not characteristic for *CAC1* deletions (Le et al., 1997; Tyler et al., 1999). The genetic analyses indicate that the two factors are functionally distinct and might act in different chromatin assembly pathways.

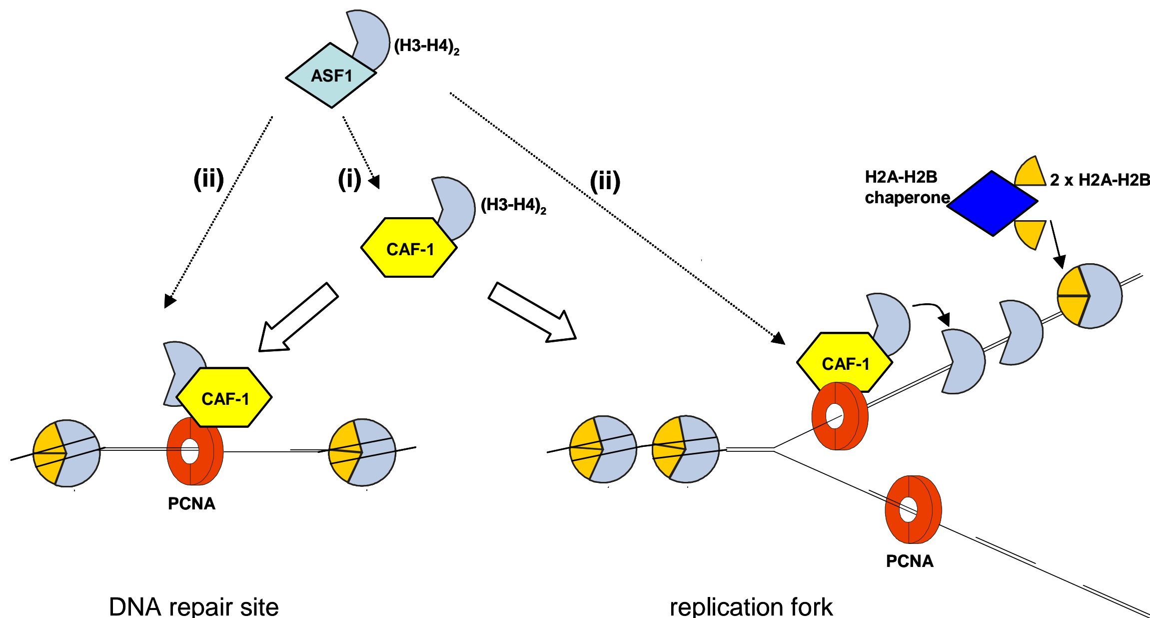


Figure 1. Model for targeting replication-dependent nucleosome assembly to sites of DNA replication and DNA repair.

The *de novo* assembly of chromatin at DNA lesions and at the replication fork requires the stepwise deposition of a H3/H4 tetramer and two H2A/H2B dimers. The assembly machinery is targeted to sites of chromatin assembly via a physical interaction between CAF-1 and the DNA polymerase processivity factor PCNA (ii). The histone chaperone Asf1 functions as a donor for histones H3-H4, either by shuttling the tetramer to CAF-1 prior to its interaction with PCNA (i), or by directly delivering H3-H4 to DNA replication and repair sites. Taken and modified from Mello and Almouzni, 2001.

2.2.Replication-independent chromatin assembly

Undoubtedly, the vast majority of histone incorporation into chromatin takes place during S-phase and is coupled to the DNA replication process. It has been, however, assumed for a while that in addition replication-independent mechanisms exist dealing with the replacement of nucleosomes, e.g. after transcription or during assembly of specific heterochromatic structures. This notion is strongly supported by the discovery of a whole set of so-called histone variants. These variant histones are related to the canonical histones and can be found in all kinds of species (Henikoff and Ahmad, 2005; Kamakaka and Biggins, 2005). Unlike expression of the canonical histones, which is restricted to S-phase, the expression of the variant histones occurs at low levels throughout the cell cycle. Based on this, their deposition

can also take place outside of S-phase. Histone variants have been shown to play important roles in the modulation of chromatin and epigenetic maintenance (for overview see Table 1).

Histone variant	Function
H3	canonical core histone
H3.3	transcriptional activation
CENPA	kinetochore assembly
H2A	canonical core histone
H2AX	DNA repair and recombination, major core histone in yeast
H2AZ	gene expression, chromosome segregation
macroH2A	X chromosome inactivation, transcriptional repression
H2ABBD	transcriptional activation
H4	canonical core histone
H2B	canonical core histone

Table 1. The functionality of histone variants.
Taken and modified from Sarma and Reinberg, 2005.

Examples of histone variants that impinge on specification and inheritance of chromatin domains are found e.g. at the centromers and the inactive X-chromosome in mammals, as well as at subtelomeric regions of the yeast genome. In the fission yeast *Saccharomyces pombe* the centromers are made up of two domains. A specialized histone variant, Cnp1p, resides in the so-called central domain of the centromer. This domain is embedded in silenced chromatin (Pidoux and Allshire, 2004). In higher eukaryotes like mammals, the histone variant CENP-A is the building block of the centromer in mammals whereas the vertebrate-specific variant macroH2A associates with the inactive X-chromosome (Palmer et al., 1991; Chadwick and Willard, 2002). Both variants contribute to the maintenance of the silenced state in these regions.

The opposite phenomenon, namely the specification of an active chromatin state, is observed for the histone variant H2AZ in yeast. H2AZ localizes to subtelomeric regions, where it protects euchromatin from spreading of silent heterochromatin beyond the telomeres (Meneghini et al., 2003; Raisner et al., 2005; Zhang et al., 2005).

Studies in *Drosophila* unraveled new insights into two variants of histone H3. The predominant variant is H3.1, whereas H3.3 serves as replacement variant. Strikingly, the two variants behave completely different regarding both, their mode of assembly as well as their sites of incorporation. Deposition of the major H3 occurs exclusively coupled to replication, whereas the replacement variant is specifically incorporated at transcriptionally active genes in a replication-independent fashion (Ahmad and Henikoff, 2002). These findings raised the question of which histone chaperones are involved in this replication-independent chromatin assembly of H3.3. A milestone in answering this question has been set by Almouzni and coworkers who elegantly demonstrated the capacity of *Xenopus* HIRA to promote nucleosome assembly specifically onto DNA independently of replication. Depleting the chaperone from *Xenopus* egg extracts led to severely impaired ability to assemble nucleosomes in a replication-independent manner, while replication-coupled assembly was not affected (Ray-Gallet et al., 2002). The observed effect of HIRA in nucleosome assembly was not due to a reduction of histone pools in the depleted egg extracts, since H3 levels were not significantly altered there. Old *in vitro* data indicating that HIRA interacts with histones in mammals further corroborates its suggested function as a histone chaperone (Lorain et al., 1998; Magnaghi et al., 1998).

Notably, a role for human HIRA in replication-independent chromatin assembly was discovered using a biochemical approach that aimed at clarifying how the histone variants H3.1 and H3.3 are incorporated into chromatin. Epitope tagged H3.1 and H3.3 from HeLa cells were affinity purified and components copurifying with the respective histone variants were subsequently analysed by mass spectroscopy. Thereby, HIRA was found to be specifically associated with the H3.3, but not with the H3.1 complex (Tagami et al., 2004). This connection between the HIRA complex and the H3.3 variant now offers a plausible explanation to the question of how a specific histone variant can be targeted to chromatin outside of S-phase.

In yeast, four HIRA homologues, Hir1p, Hir2p, Hir3p (histone regulation 1,2,3) and Hpc2p (histone periodic control 2) have been isolated. They have originally been discovered through genetic screens as mutants that abolish the repression of the histone genes outside of late G1 and S-phase (Osley and Lycan, 1987; Xu et al., 1992; Sherwood et al., 1993). Indications for the involvement of the yeast Hir proteins in chromatin assembly come from genetic and biochemical data. Firstly, *hir* deletion strains and *cac* strains display mild heterochromatic

silencing defects. Combining these deletions results in a synthetic phenotype, suggesting that the Hir proteins and the Cac proteins promote chromatin assembly through partially overlapping pathways (Kaufman et al., 1998; Sharp et al., 2002). In addition, two very recent studies provide evidence for the formation of a nucleosome assembly complex in yeast that comprises the four Hir proteins together with the histone donor Asf1p (Green et al., 2005; Prochasson et al., 2005). Analogous to the human and the *Drosophila* system, the Hir complex and Asf1p cooperate in replication-independent chromatin assembly also in yeast. A mutation in Asf1p that precludes its binding to the Hir complex impairs nucleosome assembly activity. The very same pathway is implicated in the replacement of histones in the wake of elongating RNA polymerases (Formosa et al., 2002).

2.3. DNAse I hypersensitive sites

In the early 1980ies, independent laboratories discovered multiple sites in the *Drosophila*, chicken and human genome that were hypersensitive to digestion with DNAse I (Forrester et al., 1986; Keene et al., 1981; Tuan et al., 1985; Wu, 1980). When the position of these sites was mapped, the nuclease susceptible region was found to colocalize with the 5`end of genes. Strikingly, the presence of such sites, e.g. in the human as well as the adult chicken beta-globin gene, correlated with transcriptional activity (McGhee et al., 1981; Tuan et al., 1985). Based on this it has been speculated that hypersensitive sites are used as recognition modules for elements of the transcriptional machinery. Because of the open, accessible chromatin structure there transcription factors possibly gain entry to promoter regions of genes and turn on their expression. Regarding the underlying structural basis of hypersensitive sites, however, little was known. Under DNAse I digestion regimes they behaved similar to naked DNA, which implied that they might be deficient of nucleosomes (Karpov et al., 1984; McGhee et al., 1981; Thomas and Elgin, 1988). 25 years after their discovery, hypersensitive sites are widely recognized as a hallmark of active genes and significant advances have been made in understanding their molecular nature.

The enzymatic activities contributing to the formation of hypersensitive sites turned out to be nucleosome remodeling machines. In an ATP-dependent manner they mobilize nucleosomes and thereby impose fluidity on chromatin (Eberharter et al., 2005). Nucleosome remodelers

may generate a hypersensitive site by one of the following mechanisms: In the first scenario remodeling loosens DNA-histone contacts and nucleosomes are removed from the underlying DNA by getting transfered to an acceptor, e.g. a histone chaperone. The second scenario involves the generation of so-called persistently altered nucleosomes, whereby a change in the conformational state of the nucleosomes renders them susceptible to nucleases. However, nucleosomes do not get lost from the underlying DNA. As a third possibility, remodeled nucleosomes may slide away from their original position, leaving behind a hypersensitive stretch of DNA. There is experimental evidence for both, the generation of a hypersensitive site with or without concomitant histone loss. At the activated *PHO5* promoter, histone displacement is the mechanism responsible for formation of the 600 bp long hypersensitive region (Boeger et al., 2003; Reinke and Hörz, 2003). An example for the formation of hypersensitive sites without concomitant histone loss is the MMTV promoter. There histones are retained upon activation by hormone induction even though a hypersensitive site is generated, presumably speaking for a stable remodeled state of a nucleosome (Nagaich and Hager, 2004; Richard Foy and Hager, 1987; Truss et al., 1995).
DNA accessibility in chromatin can be analyzed in various ways (reviewed in Reinke and Hörz, 2004). The most commonly used one is the digestion of chromatin with micrococcal nuclease (MNase). MNase preferentially cleaves in linker regions between nucleosomes, as linkers are more susceptible to nuclease digestion than DNA that is protected by nucleosomes. By directly hybridizing the MNase digested chromatin to a labeled probe corresponding to the region of interest, the abundance of canonical nucleosomes in that region can be detected. Another easy method to analyze the accessibility of DNA in chromatin is the digestion with sequence-specific restriction enzymes. After treatment with the respective restriction nuclease, the DNA fragment of interest is excised by secondary digestion and visualized by hybridization to an appropriate probe. Depending on the accessibility of the DNA at the restriction site, the DNA fragment will be cleaved to a greater or lesser extent and give rise to the appearance of a mixture of fragments: One long fragment in the case the restriction site has been resistant to cleavage and a mix of long and smaller fragments if the restriction site has been susceptible to the nuclease. A third method constitutes the digestion of chromatin with DNAse I. The protocol used for DNAse I digests is similar to the one used for restriction enzyme digestion and also involves secondary digestion. However, only partial digestion with DNAse I is desired in order to achieve a single cut in the region of interest. In this way a

mixture a fragments with different lenghts are obtained that yield a characteristic pattern reflecting nucleosome positioning in the region of interest. This method has an exclusive advantage over the other methods that I have described, i.e. it can be used to map quite precisely the positions nucleosomes adopt.

2.4. Nucleosome remodeling machines in chromatin assembly

Nucleosome remodeling is intimately linked to chromatin assembly. This is because histone chaperones, albeit being able to catalyze histone deposition onto DNA, lack the ability to produce regularly spaced nucleosomal arrays (Nakagawa et al., 2001). Hence, there is the demand for additional factors to impose physiological spacing onto irregularly spaced nucleosomal arrays, which are typically assembled by histone chaperones. Notably, the hydrolysis of ATP is needed to obtain the nucleosomal pattern that is characteristic for native chromatin in crude cell extracts (Almouzni and Mechali, 1988). The enzymatic activities being responsible for such a mobilization of nucleosomes resulting in the generation of native chromatin turned out to be ATP-dependent nucleosome remodeling machines (Becker and Hörz, 2002; Haushalter and Kadonaga, 2003; Owen-Hughes, 2003).

Generally, four classes of ATP-dependent nucleosome remodeling complexes can be distinguished, the CHD/Mi-2, the ISWI, the SWI/SNF and the INO80/SWR1 classes (Cairns, 2005; Eberharter and Becker, 2004). Common to them is their SNF2-type ATP-hydrolyzing subunit (Lusser and Kadonaga, 2003). There is a substantial amount of evidence that nucleosome remodelers do not act alone, but rather cooperate with histone chaperones in the chromatin assembly process. The nucleosome remodeler CHD1 is one of the examples where a remodeling machine works in synergy with a histone chaperone. *Drosophila* CHD1 transfers histones from the histone chaperone NAP1 onto DNA in an ATP-dependent fashion to generate regularly spaced nucleosomal arrays (Lusser et al., 2005). Yeast Chd1p is less well characterized than its counterpart in *Drosophila*. However, it has been shown to remodel chromatin in an *in vitro* reaction and catalyze nucleosome sliding in *cis* (Tran et al., 2000; Stockdale et al., 2006).

Similar findings of a synergistic interaction between nucleosome remodelers and histone chaperones have been reported for members of the ISWI (imitation switch) class. The ISWI-

containing remodeling complexes from *Drosophila* CHRAC (chromatin accessibility complex) and ACF (ATP-utilizing chromatin assembly and remodeling factor) can assemble periodic nucleosome arrays when mixed with purified histones, DNA, ATP and a histone chaperone as NAP1 (Fyodorov et al., 2004; Ito et al., 1997; Ito et al., 1999). Interestingly, an ACF-related complex from humans, RSF (remodeling and spacing factor), serves a similar function in chromatin assembly, but is able to deposit histones onto DNA even in the absence of a histone chaperone (Loyola et al., 2001). In the budding yeast *S. cerevisiae*, the ISWI class of remodelers is represented by two factors, Isw1p and Isw2p. Unlike their equivalents in higher organisms, Isw1p and Isw2p so far have not been found to act in concert with histone chaperones. Nonetheless, by virtue to catalyze nucleosome sliding they play an important role in gene regulation. In an *isw2* mutant a whole set of genes is derepressed (Goldmark et al., 2000). For the two Isw2p regulated genes *POT1* and *REC104*, the mechanism underlying transcriptional repression appears to be the repositioning of nucleosomes at critical promoter sites (Fazzio and Tsukiyama, 2003). It can be easily imagined that the positioning of nucleosomes at specific sites in promoter regions might assist repression by blocking the access of regulatory elements for components of the transcription machinery.

The third group of remodeling machines, the SWI/SNF class, comprises the nucleosome remodelers RSC and SWI/SNF. The SWI/SNF complex was one of the first nucleosome remodeling complexes that has been purified from yeast (Logie and Peterson, 1999). Since then extensive studies have focused on this complex and diverse roles of the complex ranging from chromatin remodeling to gene regulation have been documented so far (Cairns et al., 1999; Mohrmann and Verrijzer, 2005; Sudarsanam et al., 2000). One characteristic feature of this class of nucleosome remodelers is the ability to bring about nucleosome movements in *trans*. The SWI/SNF complex promotes the removal and exchange of H2A-H2B dimers (Bruno et al., 2003). In addition, both, the SWI/SNF as well as the RSC complex catalyze the transfer of histone octamers onto acceptor DNA *in vitro* even in the absence of histone chaperones (Lorch et al., 1999b; Phelan et al., 2000). Whether these mechanisms are physiologically relevant also *in vivo* remains unclear. Conceivably, histone chaperones might also be involved in the chromatin assembly reaction *in vivo*. One interesting aspect of the interplay between remodeling complexes and chaperones is the finding that in *S. cerevisiae*, the HIR complex has an inhibitory effect on the remodeling activity of SWI/SNF (Prochasson et al., 2005).

Intriguingly, remodelers belonging to the INO80/SWR1 class have been associated with distinct histone variants. In some cases they promote the deposition of these variant histones. An example for that is the Ino80 complex that is directed to DNA double-strand breaks by the specific damage-induced histone variant phospho-H2AX. The Ino80 complex is believed to facilitate the removal of nucleosomes at the damage site and contribute to the efficient recruitment of homologous recombination proteins (Morrison et al., 2004; Tsukuda et al., 2005; van Attikum et al., 2004). The *Drosophila* Tip60 complex acetylates phospho-H2Av, the *Drosophila* homologue of H2AX, at DNA lesions and substitutes it with unmodified H2Av (Kusch et al., 2004). The same exchange reaction can also be performed by the Domino/p400 complex, which is homologous to the SWR complex in yeast.
Other studies from three independent laboratories identified the yeast SWR complex as the assembly machinery taking charge of replacing H2A by H2AZ (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). Based on its physical association with the SWR complex and its ability to exchange H2A and H2B, the histone chaperone NAP1 is proposed to shuttle the H2AZ/H2B dimer to the SWR complex (Park et al., 2005).
Two recent microarray studies analyzed H2AZ binding targets in yeast. Consistent with previous results that provided evidence for a role of H2AZ in specifying an active chromatin state (Meneghini et al., 2003), the bulk of H2AZ was found at gene promoters in euchromatin (Raisner et al., 2005; Zhang et al., 2005). The authors postulated a model, in which H2AZ helps to maintain the transcription initiation site of promoters in a euchromatic state by marking the 5´ end of inactive and active genes. The localization of H2AZ was found to be independent of ongoing transcription, but instead it was dependent on the SWR1 complex component Bdf1, indicating a role for the SWR1 complex in H2AZ deposition on a global level. These examples clearly illustrate the role ATP-dependent nucleosome remodelers play in differentiating chromatin domains by incorporation of variant histones there.

2.5. Transcription and chromatin remodeling

Transcription in eukaryotic cells requires the displacement of nucleosomes from the DNA template and the restoration of chromatin structure after RNA polymerase passage. The priniciple of removing and replacing nucleosomes is thereby reminiscent of the chromatin

remodeling process as it is carried out by ATP-dependent remodeling machines. However, the important difference between the two is that the remodeling process primarily takes place in the promoter regions of genes whereas transcription, at least for the bulk part, is restricted to open reading frames.

In the course of transcriptional elongation by RNA polymerase II, histones H2A/H2B are transiently evicted (Kireeva et al., 2002). Nucleosomes have to be reloaded in the wake of transcription (Studitsky et al., 2004). These may be either canonical histones or histone variants, as was described for replication-independent chromatin assembly catalyzed by HIRA (Ahmad and Henikoff, 2002). In an attempt to purify accessory factors for productive transcription by Pol II, the so-called FACT complex was discovered (Orphanides et al., 1998). It was shown to facilitate transcription by assisting the unravelling of nucleosomes during transcript elongation, i.e. it constitutes a disassembly machinery. In addition, FACT also took part in the opposite process, namely the regeneration of chromatin in the wake of the RNA polymerase (Belotserkovskaya et al., 2003; Schwabish and Struhl, 2004). As most of the yeast genome is actively transcribed, a large portion of the histones H2A and H2B get exchanged even outside of S-phase by such a transcription-dependent mechanism.

Another elongation factor serving a similar function as FACT is Spt6. Spt6 also helps restoring chromatin structure after RNA polymerase passage (Kaplan et al., 2003). Lack of Spt6 leads to the initiation of transcription from cryptic promoters, probably because chromatin structure in an *spt6* mutant is more permissive to the binding of the transcriptional machinery at sites that are normally inaccessible. Both, FACT and Spt6 are examples for factors implicated in transcription-dependent chromatin assembly, where not variant, but canonical histones are replaced.

In summary, Figure 2 shows how histone variants can become incorporated into chromatin in the course of DNA replication, transcription and in histone exchange reactions.

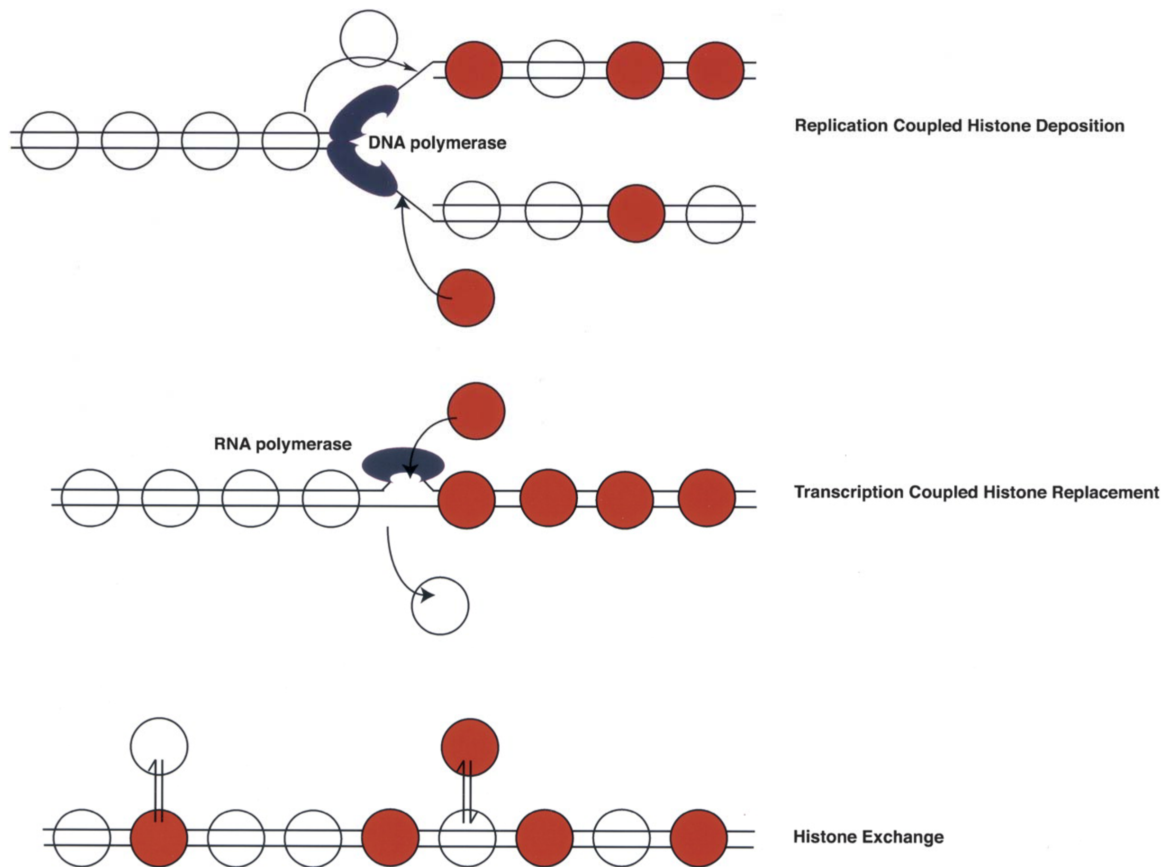


Figure 2. Schematic depicting by which mechanisms histone variants can become incorporated into chromatin. Red circles indicate variant histones, white circles indicate canonical histones. Taken from Kamakaka and Biggins, 2005.

3. The yeast *PHO* system

The *PHO* system in *S. cerevisiae* comprises a set of genes that is regulated in response to adverse growth conditions under which inorganic phosphate in the medium becomes scarce. Starving yeast cells for phosphate brings about the initiation of the *PHO* signal transduction cascade. Transmission of the phosphate starvation signal occurs by a yet unknown mechanism and culminates in binding of the transactivator Pho4p to UASp elements in the promoter regions of specific *PHO* genes, concomitantly inducing their gene expression. *PHO* responsive genes typically encode phosphatases (e.g. *PHO5* and *PHO8*) or phosphate transporters (e.g. *PHO84*). Their gene products counteract nutrient limitation by either optimizing the uptake of phosphate from the medium (Pho84p) or cleaving phosphate-

containing compounds from the environment or the vacuole (Pho5p, Pho8p). In that way inorganic phosphate is made readily available for the cell (Gregory et al., 2000).

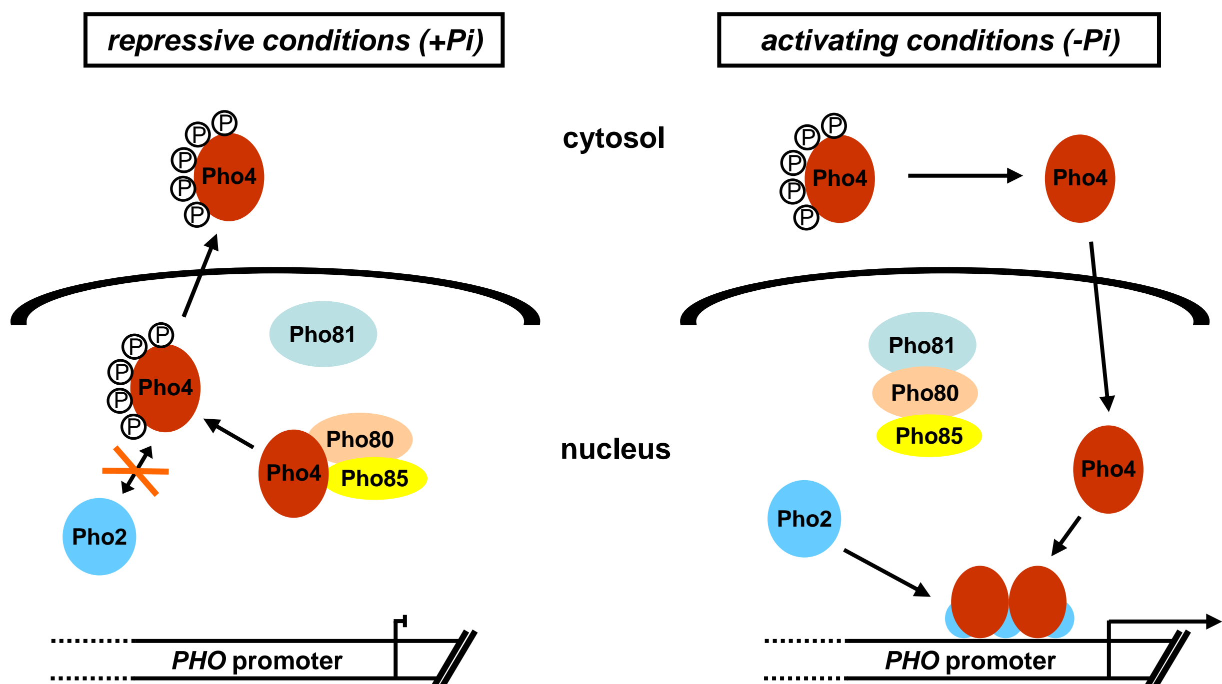


Figure 3. The *PHO* signal transduction pathway.
Adapted from H. Feldmann.

The yeast *PHO* system constitutes a very well established model system for studying eukaryotic gene regulation. Key components of the pathway and their interactions are depicted in Figure 3. Central to the *PHO* pathway is the transactivator helix-loop-helix protein Pho4p that induces the *PHO* genes under phosphate starvation conditions (-Pi) (Ogawa and Oshima, 1990). Pho4p is regulated on multiple levels. Firstly, a cyclin/cyclin-dependent kinase (CDK) complex of Pho80p and Pho85p phosphorylates Pho4p at five specific serine residues under repressive conditions (+Pi) (Kaffman et al., 1994). These modifications lead to an active export of Pho4p from the nucleus into the cytoplasm and at the same time prevent reimport of Pho4p back into the nucleus (Kaffman et al., 1998a; Kaffman et al., 1998b). Secondly, phosphorylated Pho4p cannot interact anymore with the homeodomain protein Pho2p, that assists Pho4p in the activation of some of the *PHO* genes, e.g. *PHO5* (Komeili

and O'Shea, 1999). As a consequence, the *PHO* genes are transcriptionally silent under repressive conditions.
In the situation of phosphate starvation the CDK inhibitor Pho81p inhibits the activity of the CDK complex. This will result in Pho4p being in an unphosphorylated state, which then localizes to the nucleus. There Pho4p binds to the promoter regions of phosphate-responsive genes, thereby switching on their gene expression (Lenburg and O´Shea, 1996).
Of all the genes participating in phosphate regulation, the *PHO5* gene has been investigated most thoroughly in our laboratory. *PHO5* encodes a secreted acid phosphatase, whose expression is switched on by the cooperative binding of Pho4p and Pho2p to intrinsic UASp elements in the promoter region (Barbaric et al., 1998). The chromatin structure of the *PHO5* promoter under repressive and activating conditions has been mapped in detail by DNaseI and restriction nuclease digestion. Under repressive conditions (+Pi), the *PHO5* promoter is packaged into an array of four positioned nucleosomes. Subjecting yeast cells to phosphate starvation (-Pi) renders a 600 bp stretch of promoter DNA highly susceptible to DNaseI and gives rise to a hypersensitive site in this region (Fig. 4)(Almer and Hörz, 1986). It is intuitive that due to the open, accessible chromatin structure, components of the transcriptional machinery can gain access to promoter regions and turn on gene expression. Indeed, hypersensitive sites have been tightly associated with active genes and were discovered in all kinds of species (Keene et al., 1981; McGhee et al., 1981). Regarding the molecular nature of hypersensitive promoter sites, a significant leap forward has been made in our own as well as the Kornberg laboratory. In an attempt to investigate the histone acetylation status of the *PHO5* promoter upon activation, Reinke and Hörz discovered that phosphate starvation does not only lead to transient hyperacetylation of the *PHO5* promoter, but also to a complete loss of histones from the promoter in the course of chromatin remodelling (Boeger et al., 2003; Reinke and Hörz, 2003). Later on, this phenomenon of histone displacement turned out to be not a feature unique to *PHO5*, but rather was observed throughout the entire yeast genome. Microarray data demonstrated that nucleosome occupancy in yeast is especially low at promoters, but also at some coding regions of actively transcribed genes (Bernstein et al., 2004; Lee et al., 2004).

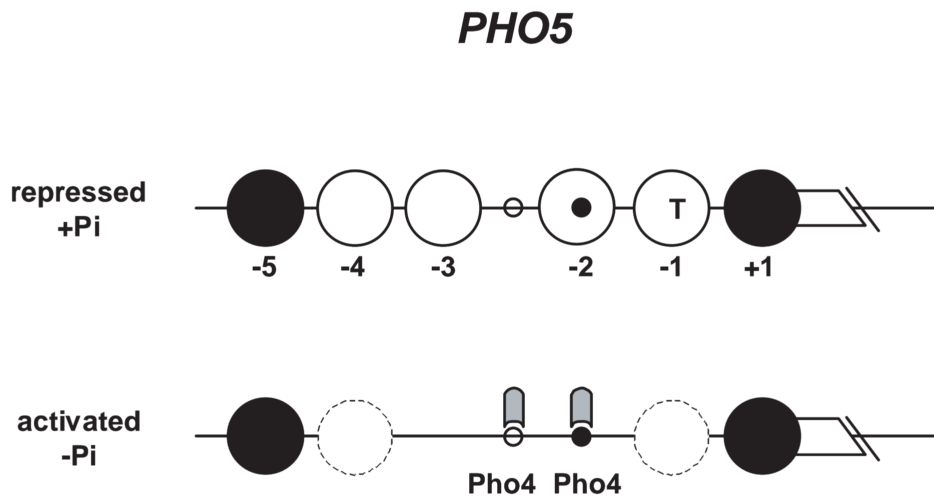


Figure 4. Schematic of the chromatin structure at the *PHO5* promoter region under repressive and activating conditions.
Black circles denote nucleosomes neighbouring the *PHO5* promoter, whereas white circles denote *PHO5* promoter nucleosomes. Upon activation, promoter nucleosomes get displaced giving rise to a hypersensitive site. Not all of the four promoter nucleosomes get lost from the promoter upon the shift to activating conditions. On average, about 2 nucleosomes are present at the open *PHO5* promoter. Stippled circles thereby indicate the nucleosomes that become remodeled less often. Small circles represent the binding sites for the *trans* activator Pho4p. Under repressive conditions, one of the binding sites lies within a hypersensitive stretch (small white circle), whereas the other binding site is covered by nucleosome -2 (small black circle).

What could be the possible mechanism accounting for histone loss? Concerning the mobilization of nucleosomes, two possibilities can be distinguished. Octamers could slide away in *cis* with respect to the underlying DNA, leaving behind a histone-free DNA stretch. The only *in vivo* example for this *cis*-mechanism comes from the Tsukiyama laboratory. They find that the yeast remodelling factor Isw2 regulates expression of the *POT1* and *REC104* genes by inducing the sliding of nucleosomes towards the promoter regions of these genes upon repression (Fazzio et al., 2001; Fazzio and Tsukiyama, 2003). A second mechanism involves the complete disassembly of nucleosomes, leading to histone removal from the DNA in *trans*. This latter scenario has been observed at the *PHO5* promoter during activation (Boeger et al., 2004; Korber et al., 2004).

4. Objectives

The important finding that nucleosomes are cleared from the *PHO5* promoter in the course of activation raises the question of how the opposite process, namely the reassembly of chromatin at the *PHO5* promoter during repression is accomplished. To date, little is known about how nucleosomes are reassembled at promoter regions. At the activated *PHO5* promoter, the shift from phosphate starvation to repressive conditions results in a chromatin transition back to the inactive state, as was determined by carrying out restriction enzyme accessibility two hours after this shift (Schmid et al., 1992). This suggests that within this time window of two hours nucleosomes are incorporated in order to restore *PHO5* promoter chromatin upon repression. However, the mechanism by which this reassembly of the *PHO5* promoter occurs remains unknown.

The goal of this study was to elucidate mechanisms of chromatin reassembly at the *PHO5* promoter during repression. In particular, I wanted to investigate whether the histones for promoter reassmbly originate from a source in *trans* or a source in *cis*.

Generally, the same distinction between movement of histones in *cis* and in *trans* does not only hold true for the eviction of histones in the course of gene activation, but can also be made for nucleosome reassembly in the course of gene repression. Even though histone eviction upon activation was shown to occur by a *trans*-mechanism, this does not mean that histones for reassembly necessarily have to arise from a source in *trans* as well.

II Materials and Methods

1. *Saccharomyces cerevisiae* strains

Strain	Genotype	Reference
WZY42	*hht1-hhf1*; *hht2-hhf2*;	S. Roth Dent
	Ycp50 [*HHT1-HHF1; CEN; URA3*]	(Zhang et al., 1998)
USY6	*HHF2-MYC-HHT2*	this study
	GAL1/10-FLAG-HHT1-HHF1	
W303a	*wt*	A. Verreault
W303 *asf1*	*asf1*	A. Verreault
W303 *hir1*	*hir1*	A. Verreault
W303 *asf1 hir1*	*asf1 hir1*	M. A. Osley
W303 *hir2*	*hir2*	M. A. Osley
YAV119	*cac1*	A. Verreault
CY407	*snf2*	C. Peterson
CY407 *asf1*	*asf1 snf2*	this study
YTT227	*isw1 isw2 chd1*	T. Tsukiyama
BY4341	*wt*	Euroscarf
BY4341 *nap1*	*nap1*	Euroscarf
YAG116	*hht1-hhf1, rad5*	A. Verreault
YAG107	*hht2-hhf2, rad5*	A. Verreault

2. Plasmids and yeast transformations

Plasmid	Reference
pNOY 439 [*HHF2-MYC-HHT2; CEN; TRP1*]	M. Nomura

YIplac211pGAL1/10 *HHF1-FLAG-HHT1* A. Verreault
[*GAL1/10-FLAG-HHT1-HHF1, CEN; URA3*]

To create the histone double-tag strain USY6, strain WZY42 was transformed with plasmid pNOY 439, driving the expression of a MYC-tagged version of the histone H3 gene (*MYC-HHT2*) and a nontagged version of the H4 gene (*HHF2*) from their endogenous promoter. The plasmid Ycp50, which harbours nontagged versions of the H3 (*HHT1*) and the H4 (*HHF1*) gene was subsequently lost by selection on 5-FOA. Transformation with ApaI-linearised plasmid YIplac211pGAL1/10 *HHF1-FLAG-HHT1* resulted in integration of the histone H4 (*HHF2*) and FLAG-tagged histone H3 (*FLAG-HHT2*) genes at the *URA3* locus. Expression of these versions of nontagged H4 and FLAG-H3 is regulated by the GAL1/10 promoter (see also Fig. 11A in the result section).
To generate the strain CY407 *asf1*, CY 407 was transformed with an *asf1::kan* disruption fragement. This fragment was PCR-amplified using genomic DNA of the *asf1* disrupted strain BY4741 *asf1* (Euroscarf) as template and the following primers:

ASF1 fwd. 5`GGTGGCGTCTTTTGCTG 3`,
ASF1 rev. 5`GGAGAGGTCTCCGGTTC 3`.

Yeast transformations were performed as described in (Gietz et al., 1992).

3. *Saccharomyces cerevisiae* media

3.1. YPDA complete medium

1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 100 mg/l adenine

3.2. Phosphate-free minimal medium

2 g/l L-asparagine, 500 mg/l $MgSO_4$ x $7H_2O$, 100 mg/l NaCl, 100 mg/l $CaCl_2$ x $2H_2O$, 2 mg/l inositol, 500 µg/l H_3BO_3, 40 µg/l $CuSO_4$ x $5H_2O$, 100 mg/l KJ, 200 µg/l Fe(III)Cl_3 x $6H_2O$,

400 mg/l $MnSO_4$ x H_2O, 200 µg/l $(NH_4)_6MO_7O_{24}$ x $4H_2O$, 200 mg/l $ZnSO_4$ x $7H_2O$, 200 µg/l riboflavin, 200 µg/l para-aminobenzoic acid, 2 µg/l biotin, 2 µg/l folic acid; 400 µg/l nicotinic acid, 400 µg/l pyridoxin-HCl, 400 µg/l thyamine chloride, 13.4 mM KCl, 50 mM sodium citrate pH 5.0, 2% (w/v) glucose, 1.6 g/l amino acid dropout mix (2 g adenine, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartate, 2 g cysteine, 2 g glutamine, 2 g glutamate, 2 g glycine, 2 g meso-inositol, 2 g isoleucine, 2 g lysine, 2 g methionine, 0.2 g para-aminobenzoic acid, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g valine, 2 g histidine, 2 g uracil, 2 g leucine).
For experiments with the histone double-tag strain USY6, the medium contained 2% raffinose instead of glucose. Expression of FLAG-H3 was induced by addition of galactose to a final concentration of 2% to the medium.

3.3. High phosphate minimal medium

As phosphate-free minimal medium, but with 1g/l KH_2PO_4.

4. Induction of the *PHO* genes

In order to activate the *PHO* genes, logarithmically growing yeast cells were incubated in phosphate-free medium for the indicated times (Almer et al., 1986). Reassembly of the *PHO5*, *PHO8* and *PHO84* promoters was induced by the addition of phosphate (KH_2PO_4) to a final concentration of 1g/l.

5. Oligonucleotide sequences for ChIP experiments

*PHO*5-adjacent ORF C: 5´-GATCAAACGGTTCATTAGACAATAGGT-3´;
*PHO*5-adjacent ORF D: 5´-TGAGTGGATATTAATCGATGGAACTC-3´;
*PHO*5-adjacent ORF probe: 5´-CAGCCCGATATTTGCGCACGATG-3´;
PHO5 UASp2 C: 5´-GAATAGGCAATCTCTAAATGAATCGA-3´;
PHO5 UASp2 D: 5´-GAAAACAGGGACCAGAATCATAAATT-3´;
PHO5 UASp2 probe: 5´-ACCTTGGCACTCACACGTGGGACTAGC-3´;

TEL A: 5´TCCGAACGCTATTCCAGAAAGT 3´,
TEL B: 5`CCATAATGCCTCCTATATTTAGCCTTT 3´,
TEL probe: 5´TCCAGCCGCTTGTTAACTCTCCGACA 3´,
ACT1 coding A: 5´TGGATTCCGGTGATGGTGTT 3´,
ACT1 coding B: 5´TCAAAATGGCGTGAGGTAGAGA 3´,
ACT1 coding probe: 5´CTCACGTCGTTCCAATTTACGCTGGTTT 3´,
ATF2 promoter A: 5´CGCCACAATCTCAGGCTACAT 3´,
ATF2 promoter B: 5´GAAACTCGTTGAATTCGTTTACTCATT 3´,
ATF2 promoter probe: 5´AACTCTGTAGGCCACCGATAAATATTGCGG 3´,
PHO84 promoter C: 5´GAAAAACACCCGTTCCTCTCACT 3´,
PHO84 promoter D: 5´CCCACGTGCTGGAAATAACAC 3´,
PHO84 promoter probe: 5´ CCCGATGCCAATTTAATAGTTCCACGTG 3´,
PHO8 promoter C: 5´ TGC GCC TAT TGT TGC TAG CA 5´,
PHO8 promoter D: 5´ AGT CGG CAA AAG GGT CAT CTA C 5´,
PHO8 promoter probe: 5´ ATC GCT GCA CGT GGC CCG A 3´,

6. Antibodies for ChIP and Western blot analysis

Antibody	Reference
anti-FLAG	M2 beads, order no: A1205, Sigma
	monoclonal anti-FLAG, order no: F1804, Sigma
anti-MYC 9E11	K. Nasmyth
anti-H3 C-terminal	Abcam, order no: ab 1791
anti-H4 C-terminal	A. Verreault

7. ChIP analysis

7.1. Crosslinking yeast cell cultures with formaldehyde and fragmentation of chromatin

50 ml yeast cultures were grown to an OD of 0.5-2 (≡0.5-2 x 10^7 cells/ml). Crosslinking proteins to DNA was achieved by adding 37% formaldehyde to a final concentration of 1%

for 15 minutes at room temperature (RT). The crosslinking reaction was quenched by adding 2.5 M glycine to a final concentration of 125 mM for 5 minutes at RT. The crosslinked yeast cells were then harvested by centrifugation for 5 minutes at 4000 rpm, 4°. Cell pellets corresponding to 25-100 OD were subsequently washed 2 x with icecold H_2O, and either processed immediately or frozen at -20°.

Cell pellets were resuspended in 15 µl / 10^7 cells FA150 buffer (50 mM Hepes/KOH pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X 100, 0.1 % sodium deoxycholate, 0.1% SDS) plus protease inhibitors (EDTA-free, Roche). Yeast cells were broken by shaking 300 µl of the cell suspension with an equal volume of glass beads on an Eppendorf shaker, maximum setting, for 1 hour at 4°. Chromatin was fragmented by sonifying the lysate in a BIORUPTOR waterbath (Diagenode) 3 x 30", setting "HIGH", interrupted by two 45" cooling periods on ice. Shearing the chromatin in this way yielded DNA fragments of 500 bp on average.

For the experiment in Fig. 17, where we probed for the abundance of canonical nucleosomes in the *PHO5* promoter region, chromatin was not fragmented by sonication, but instead by MNase digestion. Therefore, the crosslinked cell pellet was washed twice with icecold H_2O and 1 x with MNase buffer (15 mM Tris pH8.0, 50 mM NaCl, 1.4 mM CaCl2, 0.2 EGTA, 0.2 mM EDTA). Cells were resuspended in 400 µl MNase buffer and broken with an equal volume of glass beads for 1 hour on an Eppendorf shaker at 4°. For the preparation of lysates with different fragment sizes, chromatin was treated with varying amounts of micrococcal nuclease (25-200 U/ml lysate). The digest was stopped by adding EDTA and SDS to a final concentration of 200 mM and 10% respectively. The lysate was centrifuged and the supernatant was taken and diluted 1:2 with Adjust IP buffer (75 mM Hepes pH 7.5, 200 mM NaCl, 1.5% Triton X 100, 0.15% sodium deoxycholate) for the IP.

7.2. Immunoprecipitation

The lysate was centrifuged for 10 minutes at 20000 rpm, 4°. 50 µl aliquots of the supernatant were mixed with 50 µl FA150 buffer and taken for chromatin immunoprecipitation. For anti-H3, anti-H4 and anti-MYC IPs, the supernatant was incubated overnight at 4° with 15 µl of Protein G beads (50% slurry in FA150 buffer) and either 1.5. µl anti-H3, 1.5 µl anti-H4 or 10

µl anti-MYC 9E11 respectively. For anti-FLAG IPs the supernatant was incubated with 10 µl M2 anti-FLAG beads overnight at 4°. Subsequently, IPs were washed 2 x with 200 µl FA 150 (10 minutes and 5 minutes at RT), 2 x with FA 500 (as FA 150, but with 500 mM NaCl instead of 150 mM NaCl) (10 minutes and 5 minutes at RT), and 1 x with deoxycholate buffer (10 mM Tris pH 8.0, 0.25 M LiCl, 0.5% NP40, 0.5% sodium deoxycholate, 1 mM EDTA) for 5 minutes at RT. The DNA was eluted from the beads with 100 µl elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) at 65° for 20 minutes. The elution step was repeated once more and crosslinks were reversed by incubating the eluted material overnight at 65°.

7.3. Purification of the immunoprecipitated DNA

Having reversed the crosslinks, 40 µg protease K and 20 µg glycogene were added to the supernatant and incubated for 3 hours at 56°. The DNA was subsequently extracted with 1 volume of phenol: chloroform: isoamylic alcohol (25:24:1). The upper phase was taken and 25 µl 3 M sodium acetate pH 7 and 750 µl 100% icecold ethanol were added and mixed. After 30 minutes of centrifugation (20000 rpm, 4°), the precipitated DNA pellet was washed with 70% ethanol and air-dried. IP DNA was resuspended in 50 µl TE pH 8.0 and diluted 1:10 in water for quantitative real-time PCR.

7.4. Quantification of immunoprecipitated DNA using Taqman quantitative real-time PCR

For quantification 6 µl of the 1:10 DNA solution in TE were mixed with:

2.25 µl of each oligonucleotide (10 µM)
1.25 µl fluorescent probe (MWG Biotech)
12.5 µl 2x Taqman Mastermix (Applied Biosystems)
0.75 µl H_2O
25 µl reaction

Each PCR reaction was performed in duplicates or triplicates. For DNA amplification and quantification, the ABI PRISM 7000 Sequence Detection System and the corresponding software were used.

Quantitative real-time PCR (qRT-PCR) is based on the fact that there is a quantitative relationship between the amount of starting DNA and the amount of PCR product in the exponential phase of the PCR reaction. By using fluorescently labeled probes, the accumulation of fluorescence signal and thereby PCR product is measured in the course of the PCR reaction. The point when the fluorescent signal is becoming detectable is called threshold (see Figure 5). Depending on the amount of starting DNA in the reaction, the threshold will be reached at an earlier or later timepoint during the PCR reaction. The PCR cycle number at which the threshold is crossed is called the Ct value (cycle-threshold value). Accordingly, a high amount of starting DNA will manifest itself in a low Ct value, whereas a low amount of starting DNA will give rise to a high Ct value.

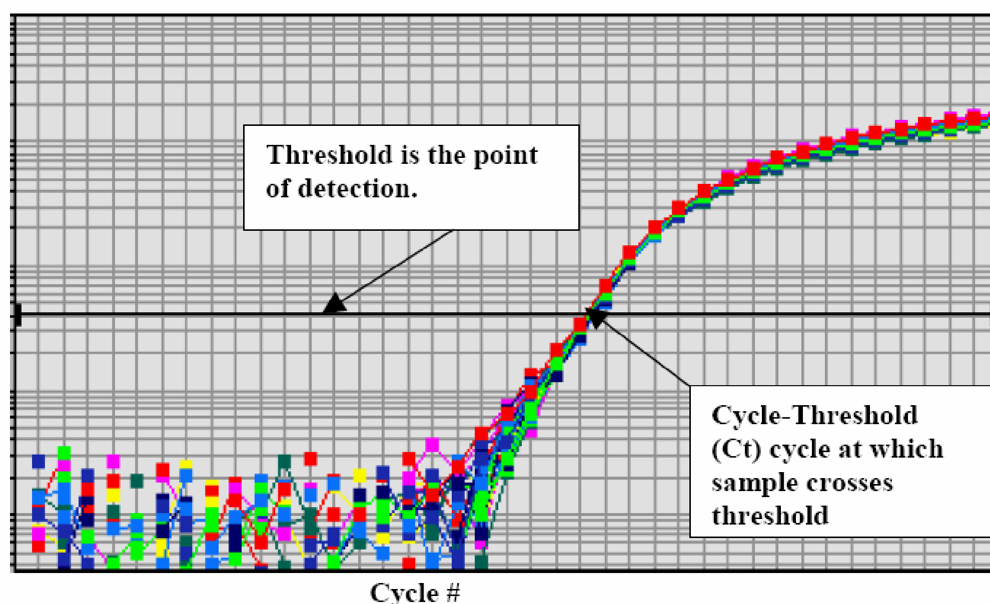


Figure 5. Example for a characteristic amplification curve.
The PCR cycle number is plotted against the intensity of fluorescence signal. The Ct value indicates the PCR cycle number at which the fluorescence signal of the sample crosses the so-called threshold. Taken from a tutorial of Applied Biosystems.

To ensure that quantification of the accumulating PCR product was in the exponential range and to control for primer efficiency, standard curves were pipetted for each amplicon using input DNA (purified DNA from chromatin extracts before immunoprecipitation). To obtain

standard curves, the input DNA was diluted 1:10, 1:100, 1:1000, 1:10000 and 1:100000 respectively. The absolut DNA amount of these dilutions was determined by qRT-PCR (an example standard curve is shown in Figure 6). Each dilution yielded a distinct Ct value that is reflecting the absolute amount of input DNA. The concentrations of the input DNA dilutions were plotted against the corresponding Ct values of the input DNA dilutions. For data analysis of immunoprecipitated DNA, the intercept with the Y-axis as well as the slope of the generated standard curve were taken into consideration. Primer efficiency was controlled for by normalizing the signals obtained for immunoprecipitated DNA to signals obtained for input DNA.

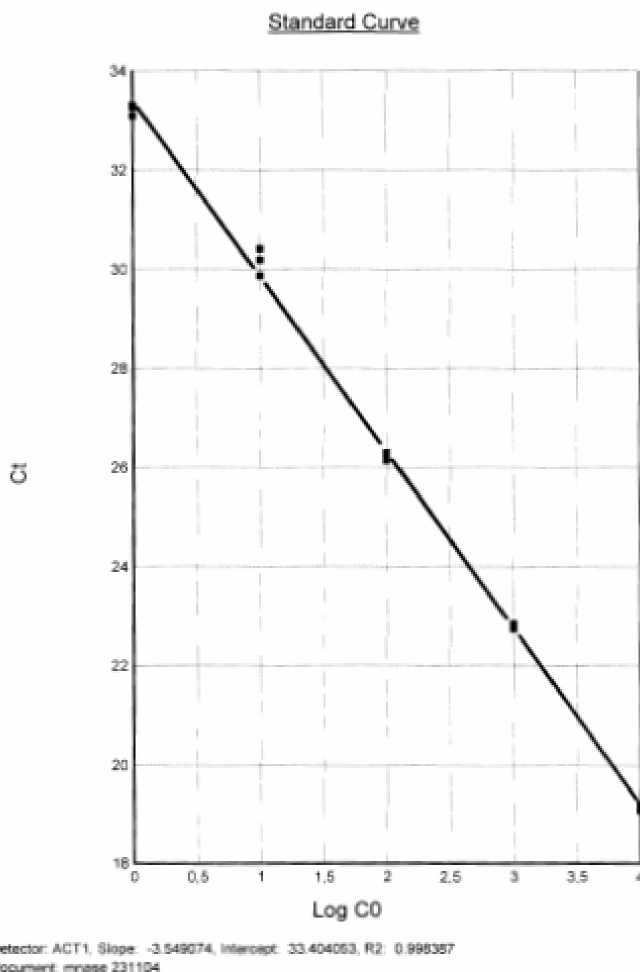


Figure 6. Example for a standard curve.

Five different dilutions of input DNA were subjected to qRT-PCR analysis. Each dilution was pipetted in triplicates. The concentrations of these input DNA dilutions were plotted against the Ct values of the corresponding input DNA dilutions. The resulting standard curve was used for quantification of the immunoprecipitated DNA. For this analysis, the intercept with the Y axis and the slope of the standard curve were taken into consideration.

8. Preparation of whole cell extracts and Western blot analysis

50 ml yeast cultures were grown to an OD of 1-2, harvested and washed with icecold water. The cell pellet was resuspended in 500 µl spheroblasting solution (24 mM Tris pH 7.5, 0.6 M sorbitol) and centrifuged for 5 minutes at 4000 rpm. The pellet was weighed and resuspended in 5 ml spheroblasting solution per 1g wet weight. In order to prepare spheroblasts, 10 µl zymolyase (ICN Biomedicals, 20 mg/ml) were added per 0.1 g of pellet wet weight and incubated for 30 minutes at 28°. Spheroblasts were lysed by addition of Triton X 100 to a final concentration of 3.33%. Whole cell extract from approximately 2 x 10^7 cells per lane was loaded on 15% SDS acrylamide gels. FLAG-tagged histone H3 was detected using monoclonal anti-FLAG antibody (Sigma).

9. Determination of *PHO5* mRNA levels

Yeast cells were grown in phosphate starvation medium for the indicated times and then shifted to phosphate containing medium. Total RNA was prepared by hot-phenol extraction as described (Schmitt et al., 1990). Reverse transcriptase PCR was carried out in 10 µl 1 x RT buffer (Promega), 0.5 mM dNTPs each, 1U/µl RNasin, 0.1 µg/µl 9mers, 1 µl of a total RNA dilution, and 10U/µl M-MLV reverse transcriptase (Promega) at 23° for 10 minutes, 42° for 30 minutes and 95° for 5 minutes. The complementary DNA was quantified using the ABI PRISM 7000 Sequence Detection System. *PHO5* mRNA levels were normalized to levels of the housekeeping gene *ACT1*. The following primers and probes were used:

PHO5 coding C: 5´ TGC AGA CTG TCA GTG AAG CTG AA 3´,
PHO5 coding D: 5´ CCC AAG CAG GAC ATG AGT TAC A 3´,
PHO5 coding probe: 5´ CGC TGG TGC CAA CAC TTT GAG TGC 3´,
ACT1 coding A: 5´TGGATTCCGGTGATGGTGTT 3´,
ACT1 coding B: 5´TCAAAATGGCGTGAGGTAGAGA 3´,
ACT1 coding probe: 5´CTCACGTCGTTCCAATTTACGCTGGTTT 3´.

III. Results

1. Chromatin reassembly at the *PHO5* promoter during repression

1.1. Transcriptional repression of *PHO5* occurs rapidly

Phosphate starved *S. cerevisiae* cells exhibit the characteristic open *PHO5* promoter chromatin structure, the molecular basis of this structure being an extended stretch of histone-free promoter DNA (Reinke and Hörz, 2003). Shifting a phosphate starved cell culture to medium containing phosphate brings about transcriptional repression of the *PHO5* gene and is manifested in the regeneration of the inactive promoter chromatin pattern (Schmid et al., 1992).

To get a more detailed view of how fast *PHO5* transcription is shut off after the shift to high phosphate conditions, *PHO5* mRNA levels were measured in a time course of repression. For that a wildtype yeast strain was induced by phosphate starvation and subsequently transfered to high phosphate medium in order to initiate *PHO5* repression. *PHO5* mRNA levels were determined using reverse transcriptase PCR (Fig. 7).

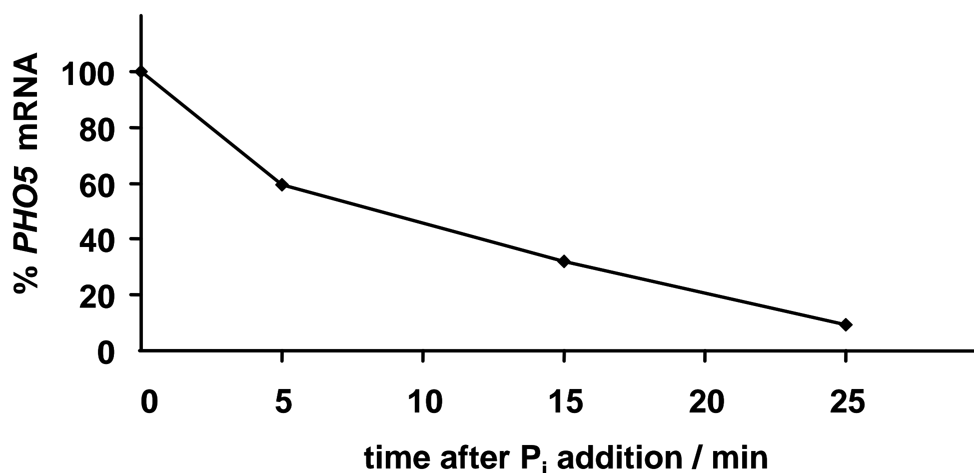


Figure 7. Transcriptional repression of the *PHO5* gene occurs very rapidly.
Wildtype yeast strain W303 was induced for 3 hours in phosphate-free medium and then shifted to phosphate-containing medium. Total RNA was prepared at the indicated time points and analyzed for *PHO5* mRNA by reverse transcriptase PCR and quantitative real-time PCR. *PHO5* mRNA levels were normalized to levels of *ACT1* mRNA. The normalized *PHO5* mRNA level at 0 min after phosphate addition was set as 100%.

Unlike activation, *PHO5* repression occurs very rapidly. Nearly full repression is achieved after 25 minutes. This finding indicates that *PHO5* mRNA has a very short half-life with about 90% of the mRNA being degraded within this time period.

1.2. Principle of chromatin immunoprecipitation (ChIP)

As chromatin immunoprecipitation is the central technique in this study it should be briefly introduced. What makes ChIP such a powerful method is the fact that it allows the detection of protein binding to chromatin *in vivo*. The underlying principle is simple (illustrated in Figure 8). Proteins that are associated with chromatin are crosslinked to the DNA by treating the yeast cells *in vivo* with a crosslinking agent, most commonly formaldehyde. The cells are lysed by vortexing them with glass beads and subsequently the chromatin is fragmented by sonication. The resulting lysate is incubated with an antibody directed against the protein one wishes to detect. After immunoprecipitation, the crosslinks are reversed and the IP DNA is purified and subjected to quantitative PCR. Thereby, primers and probes are employed corresponding to the genomic region where protein binding should be investigated. The relative enrichment of a particular DNA fragment (compared to input DNA) reflects the binding of the protein of interest in that region. If e.g. ChIP is performed with an antibody against RNA polymerase and the DNA fragment corresponding to the *PHO5* promoter region is enriched compared to input DNA, then this result would imply that the RNA polymerase is associated with the *PHO5* promoter region. To properly judge whether a particular PCR fragment is enriched, normalization procedures are of crucial importance. As in our case we investigate the incorporation of histones at the *PHO* promoters upon changes in phosphate levels, an ideal control region should not respond to changes in phosphate levels. Good control regions are therefore often transcriptionally inert, e.g. heterochromatic regions like centromers and telomers. Moreover, it is important that protein binding to the control region is constant, no matter which mutant strains are compared.
In this study, the bulk of normalization was done with a telomeric region, 500 bp away from the end of the right arm of chromosome 6 (Suka et al., 2001). This has previously served as control in ChIP experiments in the Grunstein laboratory. The underlying principle is that histone density at the telomere should be constant, since telomeres are packaged in heterochromatin and the turnover of histones there should be low.

In our laboratory the ChIP technique has been previously established by Hans Reinke. All the amplicons I used in this study for qRT-PCR have been tested by him and shown to be specific for the desired PCR fragment.

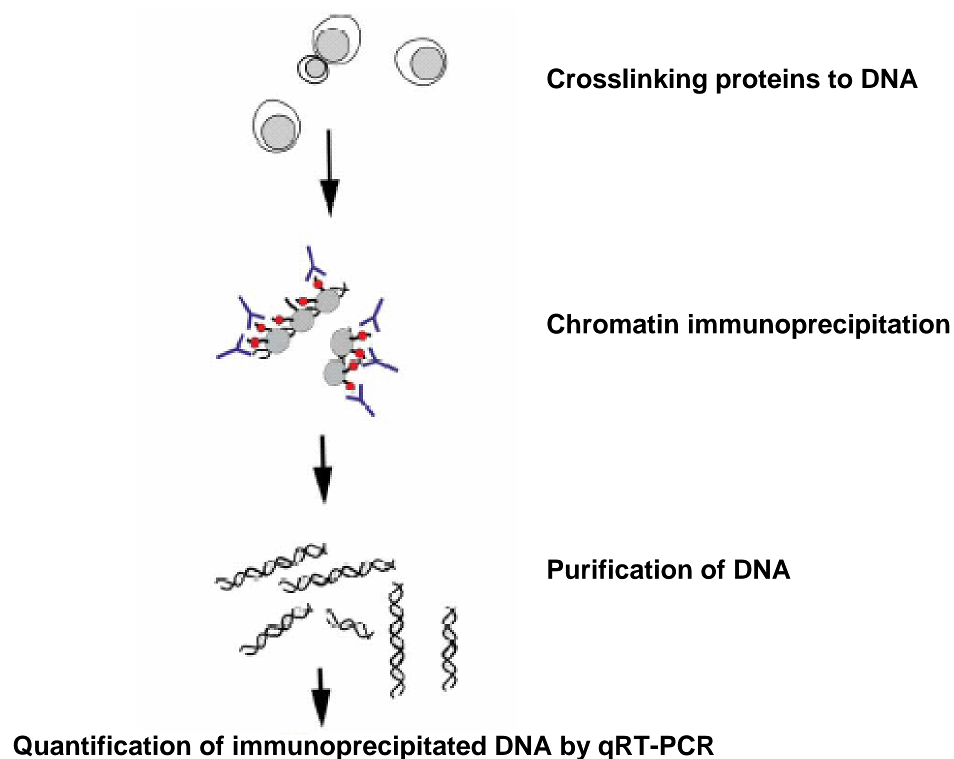


Figure 8. The ChIP technique.
Proteins are crosslinked to DNA by treating yeast cells with formaldehyde *in vivo*. The cells are then lysed and chromatin is sheared by sonication. Proteins that are associated with chromatin are pulled down by using an appropriate antibody. After immunoprecipitation the crosslinks are reversed and the IP DNA is purified and subjected to qRT-PCR. Modified from (Kurdistani and Grunstein, 2003).

1.3. Transcriptional repression of *PHO5* is accompanied by the deposition of histones at the closing promoter

It is conceivable that the restoration of the inactive *PHO5* promoter chromatin structure will require the reassembly of nucleosomes in that region. Therefore, it was investigated whether the kinetics of transcriptional repression correlate with the incorporation of histones at the

closing *PHO5* promoter. Yeast cells were induced by phosphate starvation prior to the addition of phosphate to the culture at time point t0. Histone levels were measured by ChIP and real-time PCR at increasing times after re-repression of *PHO5*. Antibodies directed against the C-terminus of histone H4 were employed for ChIP and an amplicon corresponding to the -2 nucleosome of the promoter region (*PHO5* UASp2) was chosen for real-time PCR analysis (Fig. 9).

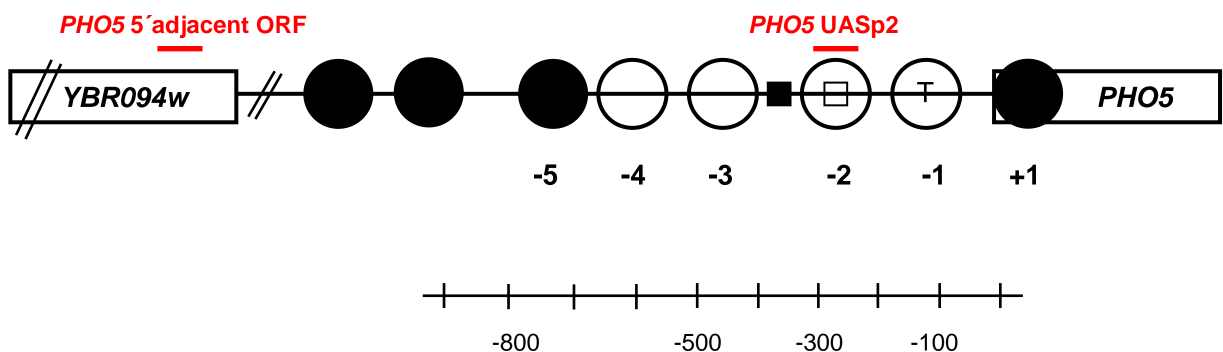


Figure 9. Schematic of the *PHO5* promoter and the upstream ORF *YBR094w*.
Regions that were analyzed by quantitative real-time PCR with the amplicons "*PHO5* 5´adjacent ORF" and "*PHO5* UASp2" are indicated in grey. The scale indicates the distance from the *PHO5* transcriptional start site in bp.

In Figure 10, the deposition of histone H4 at the *PHO5* promoter upon repression is shown. The H4-ChIP signal increases about 4-fold (relative to telomere) after the shift to repressive conditions and reaches a plateau after 20 minutes. When subjecting the very same cell lysate to ChIP analysis using an anti-H3 antibody, an almost identical result was obtained (data not shown).

The data illustrate how transcriptional repression of *PHO5* is accompanied by the deposition of histones at the closing promoter. Remarkably, the kinetics of histone incorporation match the kinetics of transcriptional repression very well.

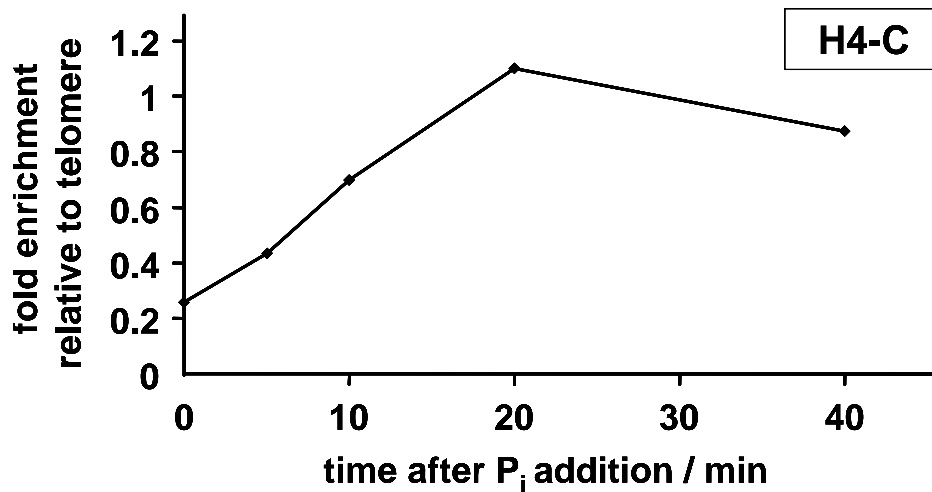


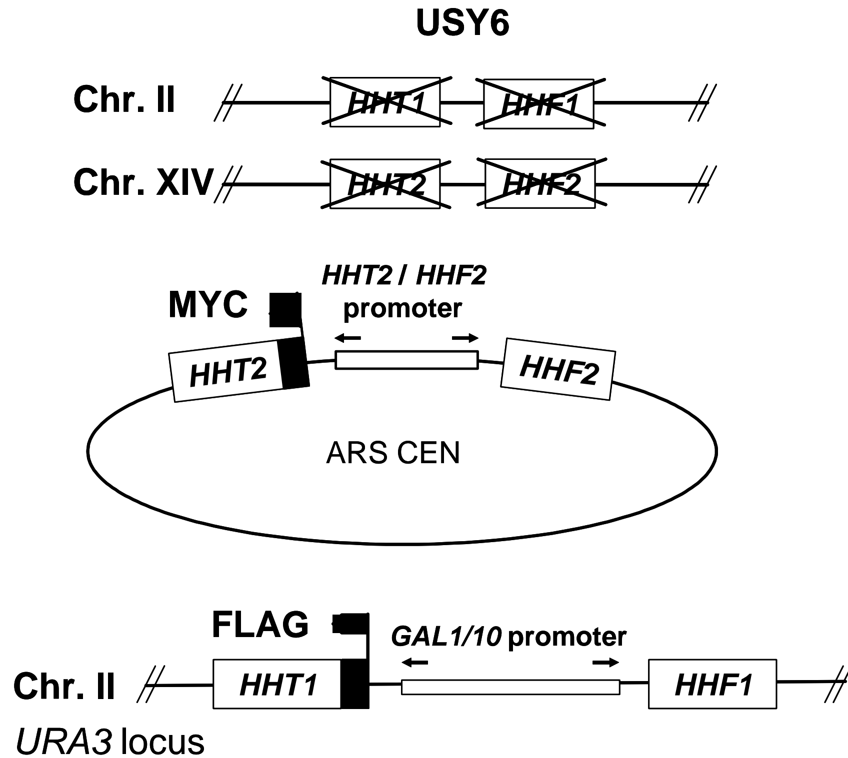
Figure 10. Histone H4 is deposited at the UASp2 site of the *PHO5* promoter during reassembly.
Wildtype yeast strain W303 was induced for 5 hours in phosphate-free medium and then repressed by addition of phosphate. Aliquots were taken at the indicated time points and analyzed using ChIP against the C-terminal part of histone H4. ChIP data were normalized to input DNA and to an amplicon in the telomere region.

1.4. Histones are incorporated in *trans* during reassembly of the *PHO5* promoter

Our initial experiments have shown that in the course of *PHO5* repression, transcription of the corresponding gene is shut off rapidly and concomitantly chromatin is reassembled over the *PHO5* promoter region. This result raises a central question, namely where the histones for this reassembly reaction come from. Two main mechanisms of histone deposition could be envisioned. Nucleosomes could either slide onto the promoter from neighbouring regions, thereby filling up the hypersensitive site with nucleosomes from a source in *cis*. Alternatively, histones could be incorporated at the promoter from a soluble pool of histones, which would constitute a *trans*-deposition mechanism. The general problem encountered when performing ChIP with antibodies against the C-terminal part of histones (e.g. in Fig. 10) was that this method was not conclusive regarding the source of the histones. In order to solve this ambiguity we needed to find a way to distinguish between the two modes of incorporation, *cis* and *trans*. I addressed the question by means of differentially tagging and differentially regulating the two different histone fractions. For the following series of experiments the yeast strain USY6 was constructed that does not harbour endogenous copies of H3 and H4 anymore (see Fig. 11A for details). Instead, a MYC-tagged version of the histone H3 gene and an untagged version of the histone H4 gene controlled by the wildtype H3-H4 promoter

are supplied on an episomal plasmid. In addition, the strain carries a galactose-inducible version of a FLAG-tagged H3 gene integrated at the chromosomal *URA3* locus (Fig.11A).

A.



B.

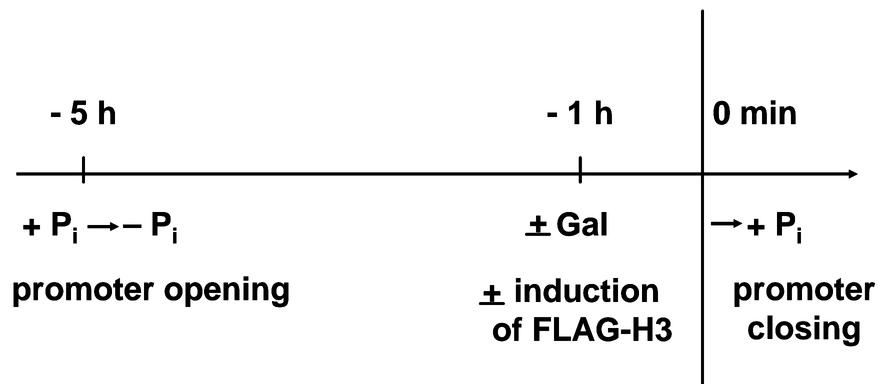


Figure 11. The histone double-tag strategy and experimental design.

A. In strain USY6 both endogenous copies of the H3 and H4 genes are deleted. Instead, a MYC-tagged version of H3 and an untagged version of H4 under the control of the wildtype H3-H4 promoter are expressed from an episomal ARS CEN plasmid. In addition, a FLAG-tagged version of H3 and an untagged version of H4 are integrated at the chromosomal *URA3* site. Both are regulated by the inducible *GAL1/10* promoter.

B. Strain USY6 was induced for 5 hours in phosphate-free medium leading to the opening of the *PHO5* promoter. In the last hour of the phosphate starvation period, expression of FLAG-H3 can be turned on by addition of galactose to the medium. At time point t0, promoter closure was initiated by adding phosphate to the culture.

In Fig. 11B, a schematic of the experimental setup is depicted. USY6 cells are grown in phosphate-free medium for 5 hours leading to the opening of the *PHO5* promoter. In the last hour of phosphate starvation, the expression of FLAG-H3 can be turned on by adding galactose to the medium. At time point 0 min, phosphate is added back to the culture, initiating promoter reassembly.

We observed that due to the phosphate starvation conditions, the yeast cells cease to replicate during the five hour incubation in phosphate-free medium (data not shown). Therefore replication will be largely absent at the time point of FLAG-H3 expression and FLAG-histones that are produced during the last hour of phosphate starvation will not become significantly incorporated into chromatin via replication. For that reason, FLAG-H3 should be solely present in the soluble histone pool and represents a histone source in *trans*. In contrast, the chromatin fraction contains almost exclusively MYC-histones, as this histone variant is constitutively expressed and assembled into chromatin. MYC-histones may not only be part of the chromatin fraction, but also part of the soluble histone pool since some of them probably remain in the soluble fraction as a left-over from the last round of replication. Accordingly, MYC-H3 corresponds to a source in *cis* or in *trans*.

In a first experiment with the double-tag strain USY6, galactose induction of FLAG-H3 was omitted, leaving MYC-H3 as the only histone H3 variant present. In the absence of FLAG-H3 expression, MYC-H3 should become incorporated at the UASp2 site of the *PHO5* promoter upon re-repression, analogous to the H4 incorporation previously observed (compare to Fig.10).

When monitoring MYC-H3 occupancy by ChIP using the 9E11 anti-MYC antibody, MYC-H3 levels were found to be low at the UASp2 site at time points t-60 and t0 (Fig. 12, solid line). However, from the onset of repression on, MYC-H3 levels at the closing *PHO5* promoter increase rapidly about 10-fold, while MYC-H3 occupancy at control regions (*ACT1* and *PHO5* adjacent ORF) remains unaffected by changes in phosphate levels. As the ChIP data were normalized to a telomere region, ChIP values lower than one were observed for the time points t-60 and t0, since the heterochromatic telomere region is tightly packaged into MYC-H3 nucleosomes, whereas the active *PHO5* promoter is relatively histone depleted.

This result demonstrates that MYC-tagged histones and endogenous H4 histones are deposited in a very similar manner at the *PHO5* promoter during closure. However, MYC-ChIP is not suitable to distinguish whether the source of the histones for reassembly of the

PHO5 promoter lies in *cis* or in *trans*. Therefore, I resorted to anti-FLAG ChIP analysis by inducing the expression of FLAG-histones through addition of galactose to the medium. Promoter closure was initiated as in the previous experiment. FLAG-H3 levels in the course of rerepression were followed by ChIP using an anti-FLAG antibody.

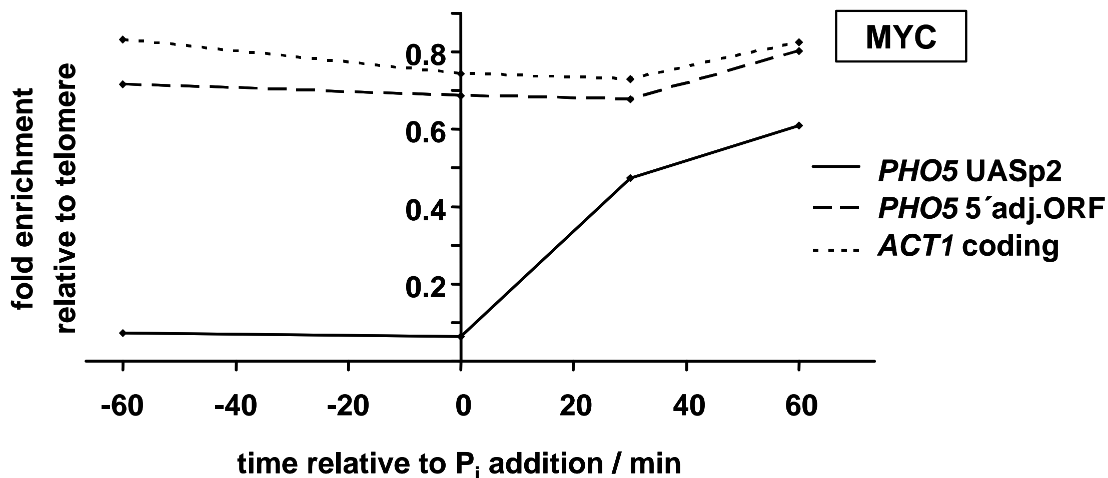


Figure 12. MYC-H3 is deposited at the *PHO5* promoter upon repression.
Strain USY6 was induced for 5 hours in phosphate-free medium leading to the opening of the *PHO5* promoter. Galactose induction was omitted. At time point t0, promoter closure was initiated by adding phosphate to the culture. MYC-H3 levels were followed at the indicated time points by anti-MYC ChIP using amplicons corresponding to either nucleosome -2 of the *PHO5* promoter (*PHO5* UASp2), the *ACT1* coding region (*ACT1* coding) or an ORF upstream of the *PHO5* gene (*PHO5* 5´ adj. ORF). ChIP data were normalized to input DNA and to an amplicon in the telomere region.

In Fig. 13, the ChIP kinetics of FLAG-H3 incorporation at the *PHO5* promoter as well as at other coding and promoter regions, are shown. Under phosphate starvation conditions, FLAG-H3 is not significantly deposited at the *PHO5* hypersensitive site (t-60 and t0). Upon phosphate addition, I observed an enrichment of FLAG-H3 at the *PHO5* UASp2 site (Fig.13, solid line). This enrichment of FLAG-histones occurs specifically in that particular region, as control regions that are not regulated by phosphate levels do not exhibit any changes in FLAG-histone ChIP signal (*ACT1* coding and *ATF2* promoter). The peak of FLAG-H3 incorporation is reached after 20 min in high phosphate, which again correlates well with the kinetics of transcriptional shutdown of *PHO5* (see also Figs. 7 and 10). The fast kinetics argue for a replication-independent mechanism contributing to *PHO5* repression and are consistent with previous results from our laboratory showing that the restoration of repressive

PHO5 promoter chromatin is rapid and not dependent on replication (Schmid et al., 1992). The maximum FLAG-ChIP signal enrichment in this experiment is 7-fold relative to the telomere region. This is as expected since histones are presumably not rapidly exchanged at the telomere and the FLAG-histone density there is low and independent of changes in phosphate levels. In contrast to this, the *PHO5* promoter region gets subject to FLAG-histone incorporation upon repression, which explains the relative FLAG-enrichment of maximally 7-fold over telomere at this site.

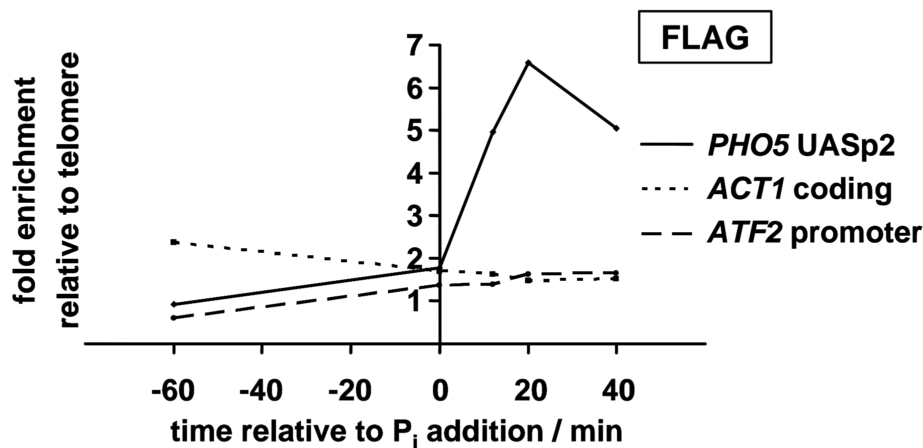


Figure 13. FLAG-H3 is specifically incorporated at the *PHO5* promoter upon repression.
Strain USY6 was induced for 5 hours in phosphate-free medium leading to the opening of the *PHO5* promoter. This time FLAG-H3 expression was turned on in the last hour of phosphate starvation. At time point t0, promoter closure was initiated by adding phosphate to the culture. FLAG-H3 levels were followed at the indicated time points by anti-FLAG ChIP using amplicons corresponding to either nucleosome -2 of the *PHO5* promoter (*PHO5* UASp2), the *ACT1* coding region (*ACT1* coding) or the promoter region of the *ATF2* gene (*ATF2* promoter). ChIP data were normalized to input DNA and to an amplicon in the telomere region.

The experimental approach we established to distinguish between *cis* and *trans* deposition is crucially dependent on a fast pulse of FLAG-H3 synthesis and would be biased if there was leaky expression prior to galactose induction. I therefore tested whether the GAL1/10 promoter exerts a sufficiently tight control on FLAG-H3 expression and performed two control experiments, ChIP and Western blot analysis.

Anti-FLAG ChIP analysis in the absence of galactose induction confirmed that the *PHO5* promoter specific increase in FLAG-H3 ChIP signal could only be seen when FLAG-H3 is

synthesized (Fig. 14A). In agreement with this, FLAG-H3 was hardly detectable by Western blot analysis prior to galactose induction (Fig. 14B).

In summary, the data provide strong evidence that chromatin reassembly at the *PHO5* promoter occurs by using a histone source in *trans*.

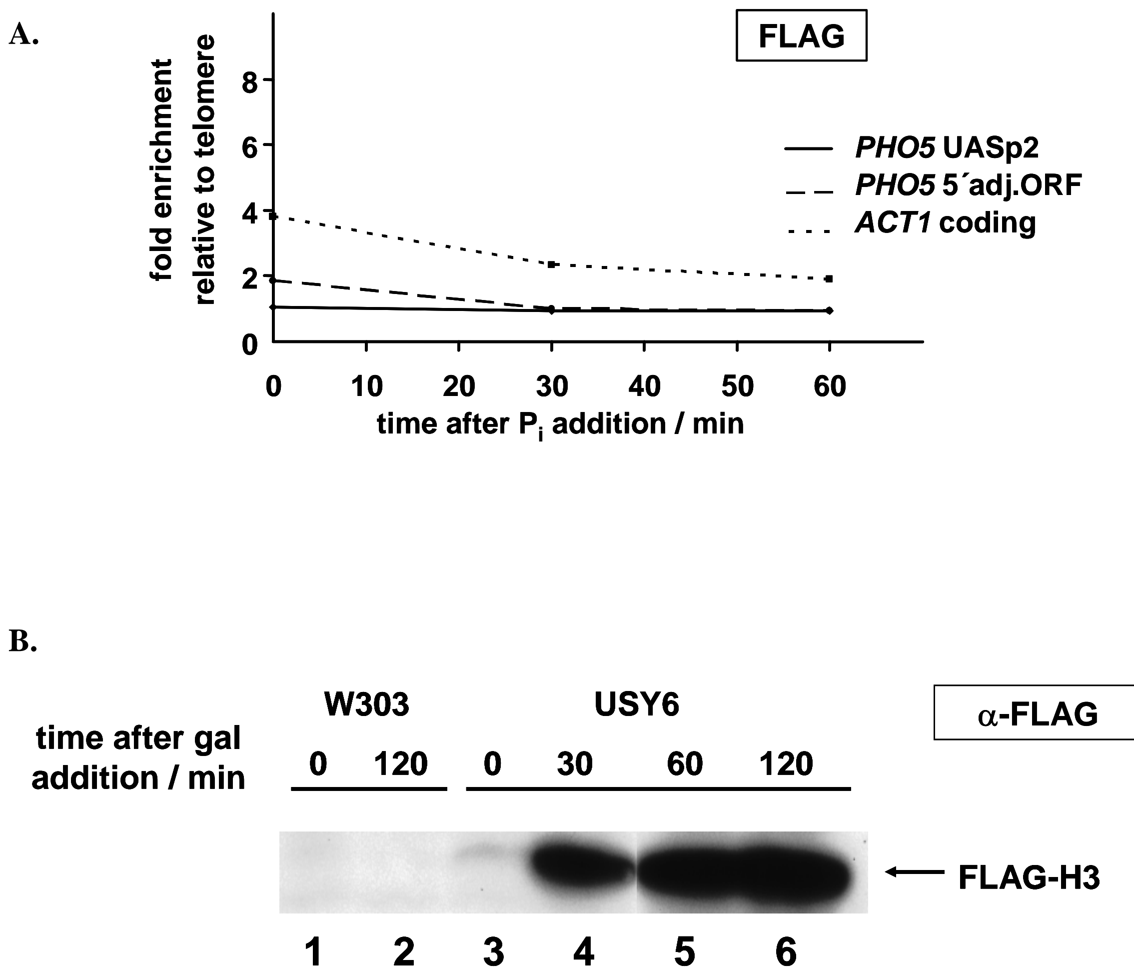


Figure 14. FLAG-H3 incorporation and expression is dependent on galactose induction.

A. Strain USY6 was starved for phosphate for 5 hours. Galactose induction was omitted. At time point 0 min, phosphate was added to initiate closing of the *PHO5* promoter. FLAG-H3 levels were followed by anti-FLAG ChIP using amplicons corresponding to the indicated regions. ChIP data were normalized to input DNA and to an amplicon in the telomere region.

B. Expression of FLAG-tagged histone H3 was monitored by Western blot using anti-FLAG-antibodies. Whole cell extracts were prepared from the strains USY6 and W303 (wildtype) after shift to medium containing 2% galactose for the indicated times.

1.5. FLAG-H3 is deposited to a significant extent at the closing *PHO5* promoter

The anti-FLAG ChIP experiment (Fig. 13) shows that chromatin reassembly at the closing *PHO5* promoter is, at least in part, mediated by a *trans*-acting mechanism. However, it is still unclear whether the observed FLAG-H3 incorporation at the UASp2 site corresponds to a minor or major fraction of the total amount of newly assembled histones at the closing promoter. Theoretically it could be that only a minor portion of the histones used for the reassembly reaction is supplied from a source in *trans*, whereas the majority of the histones is translocated to the promoter by nucleosome sliding from adjacent regions. If this was the case and only a negliable amount of histones would originate from a soluble histone pool, then FLAG-histones should not be able to compete significantly with MYC-histones during promoter reassembly. To rule out this possibility, I determined to which extent the two H3 variants (FLAG and MYC) contribute to the total amount of newly assembled histones at the *PHO5* promoter during repression. This problem was addressed by comparing MYC-H3 incorporation into the closing *PHO5* promoter in the presence and in the absence of FLAG-H3 expression. It has been already demonstrated previously that the *PHO5* promoter is filled up with MYC-histones when FLAG-H3 expression is abolished (Fig. 12). Thereby, a maximum anti-MYC ChIP value of 0.6 was repeatedly measured at the fully inactive promoter, e.g. under repressive high phosphate conditions (Fig. 16, time point -240) or after re-repression (Fig. 12, time point 60 min). This value represents a full complement of MYC-H3 histones at the inactive *PHO5* promoter in the absence of FLAG-H3 synthesis. Competition of the two histone H3 variants should therefore lead to a decrease in the maximum MYC-H3 value of 0.6. Indeed, anti-MYC ChIP kinetics conclusively demonstrate a 50% reduction in the final extent of MYC-H3 incorporation in the presence of FLAG-H3 expression, now reaching a maximum value of only 0.3 (Fig. 15).

The observed 1:1 ratio of FLAG-H3 : MYC-H3 deposition at the closing *PHO5* promoter leads us to conclude that at least half of the newly assembled histones at that site originates from a source in *trans*. No clear-cut conclusions can be derived about the other half, since MYC-H3 histones may originate from a source in *trans* as well as from a source in *cis*.

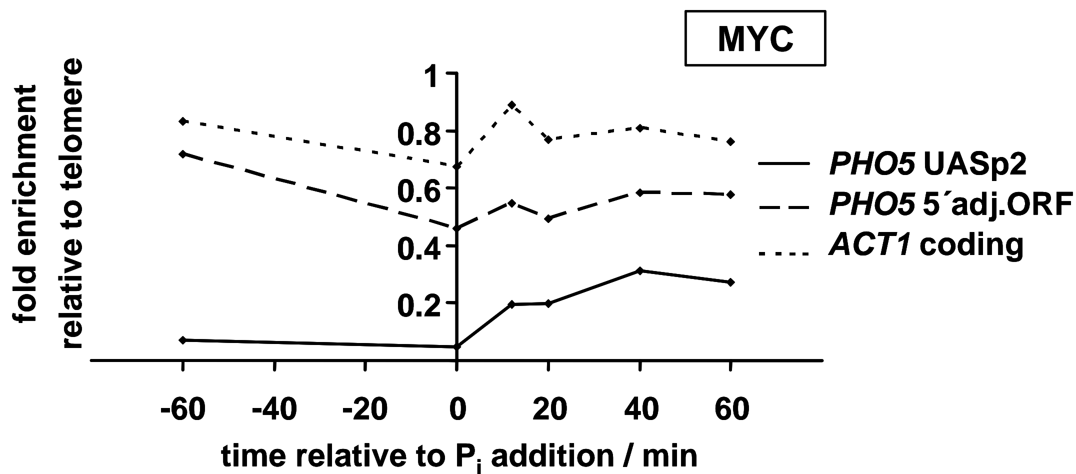


Figure 15. FLAG-H3 competes significantly with MYC-H3 for incorporation during *PHO5* promoter reassembly. USY 6 was induced by phosphate starvation for 5 hours and expression of FLAG-H3 was turned on in the last hour of induction. At time point t 0 min phosphate was added to initiate promoter closure. MYC-H3 levels were determined by anti-MYC ChIP analysis at the indicated time points using the indicated amplicons. Data were normalized to input DNA and an amplicon in the telomere region. Theoretically a region that has the same histone density than the telomere region would yield a MYC-ChIP signal value of 1. The reason for obtaining values lower than 1 (e.g. *PHO5* adj. ORF) may be due to transcriptional activity. In the course of transcription histones get displaced from the DNA. Accordingly, the histone density of transcribed regions may be lower than 1.

1.6. The kinetics of galactose induction determine the extent of MYC-H3 deposition at the closing *PHO5* promoter

Since the extent of MYC-H3 incorporation during *PHO5* promoter reassembly is dependent on how much FLAG-H3 is synthesized and FLAG-H3 expression in turn is dependent on the length of the galactose pulse, the idea arose whether varying the galactose induction times would allow to shift the observed 1:1 ratio of MYC-H3 : FLAG-H3 incorporation towards a higher MYC-H3 percentage. To test this hypothesis, I started out with a yeast culture that had been grown in phosphate-free medium for 4 hours. The culture was then split into 4 aliquots, which were subjected to galactose induction for varying time periods (0, 15, 30, 60 min), monitoring the promoter closure kinetics and anti-MYC ChIP analysis (Fig. 16).

When comparing the different galactose induction periods for strain USY6, I found that MYC-H3 deposition at the UASp2 site is indeed controlled by the length of the galactose pulse. Induction times shorter than 60 min lead to an increase in maximum MYC-ChIP signal from 0.3 towards 0.6 (Fig. 16), arguing that the extent of FLAG-H3 synthesis directly determines the extent of MYC-H3 incorporation into the closing *PHO5* promoter. In addition,

a dependency of FLAG-H3 deposition on galactose induction time using anti-FLAG-ChIP was observed (data not shown).

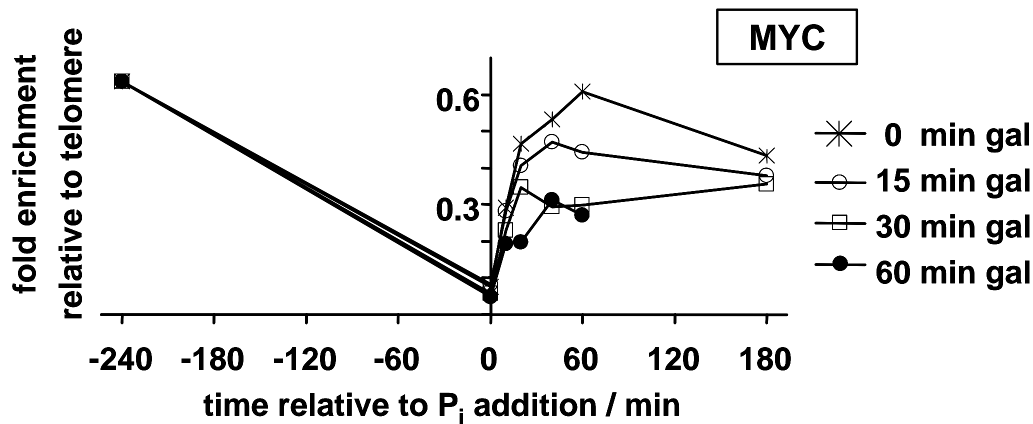


Figure 16. The kinetics of galactose induction control the extent of MYC-H3 deposition during *PHO5* promoter reassembly.

USY 6 was starved for phosphate for 4 hours. The culture was then split into 4 aliquots, which were induced for varying times with galactose (0, 15, 30, 60 min gal). At time point t0 phosphate was added to initiate promoter closure. MYC-H3 levels were determined by anti-MYC ChIP analysis at the indicated time points for the *PHO5* UASp2 site. Data were normalized to input DNA and an amplicon in the telomere region.

1.7. During reassembly FLAG-H3 is deposited to give rise to a canonical -2 nucleosome in the *PHO5* promoter region

Due to their basic nature, histone proteins tend to bind to negatively charged DNA in a non-specific manner. For that reason, the enrichment in FLAG-ChIP signal I found at the closing *PHO5* promoter could simply be the outcome of a non-nucleosomal interaction between the histone-free promoter DNA and the positively charged FLAG-histones. To verify that FLAG-H3 is really incorporated into a canonical nucleosome, promoter closure kinetics and ChIP analysis were performed as previously described. Importantly however, the chromatin was not fragmented by sonification. Instead nuclease digestion was performed. Micrococcal nuclease is widely used to screen for the abundance of canonical nucleosomes and by employing stringent digestion conditions, all chromosomal DNA can be converted to mononucleosomes. This strategy was used to break all chromatin in the cross-linked USY6 cell lysate down to mononucleosomes (Fig. 17, M).

A.

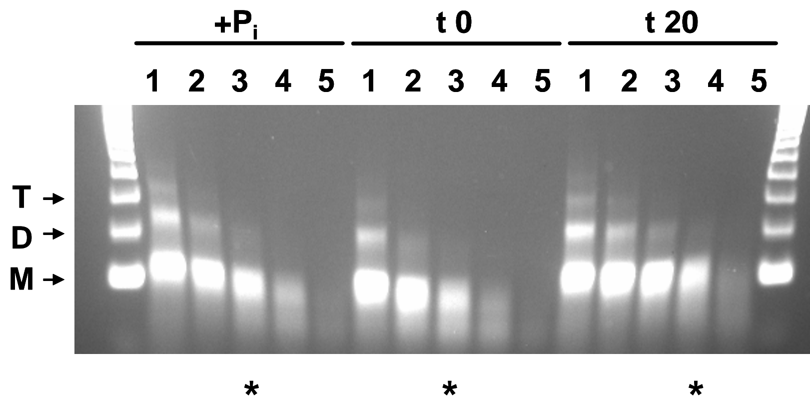
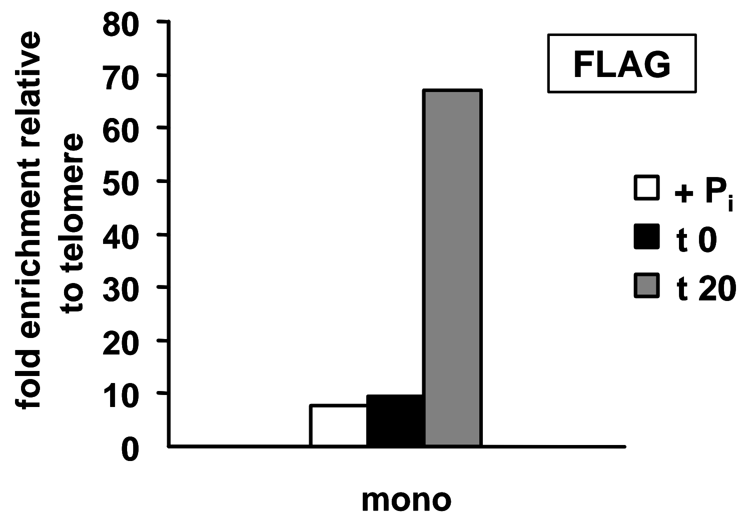


Figure 17. During *PHO5* promoter reassembly, FLAG-tagged histone H3 is incorporated to give rise to a canonical -2 nucleosome.
USY 6 was induced by phosphate starvation for 5 hours and expression of FLAG-H3 was turned on in the last hour of induction. Promoter closure kinetics and ChIP were done as before, with the exception that the chromatin of the cell lysates was not sonified, but fragmented by MNase digestion.
A. Varying amounts of MNase were used (lanes 1-5: 25, 50, 100, 200, 400 U/ml MNase). Cell lysates corresponding to repressive conditions (+Pi), after 5 hours of phosphate starvation (t0) and after 20 min of rerepression (t20) were analysed in a 2% TBE agarose gel after MNase digestion. Depending on the MNase concentration, digests yielded a mixture of mono-, di- and trinucleosomes (T), mono- and dinucleosomes (D) and mainly mononucleosomes (M). For ChIP analysis the MNase concentration yielding mainly mononucleomes (*) was chosen. The outermost lanes show a 123 bp ladder (Gibco) as a size standard.

This lysate was then subjected to anti-MYC / anti-FLAG ChIP, the same way I did with the sonified cell lysate. The primers for real-time PCR analysis (*PHO5* UASp2) hybridize within the -2 nucleosome of the *PHO5* promoter. For that reason, amplification of the corresponding PCR fragment after ChIP can only occur when the underlying DNA has been previously protected by the presence of a canonical nucleosome at that site. Contrary to the UASp2 primers, the telomere primers probably hybridize within a region of little nucleosome positioning. This drastically increases the probability of the DNA fragment corresponding to the UASp2 amplicon to be protected from exhaustive MNase digestion when compared to a DNA fragment corresponding to the telomere region and is reflected in increased levels of ChIP signals. The enrichment in anti-FLAG ChIP signal we observe after the shift to repressive conditions (t 20) is consistent with the one previously found using sonified cell lysates (Fig. 17B). The data therefore argue for the integration of FLAG-H3 into a canonical nucleosome upon promoter reassembly. When monitoring MYC-H3 abundance in the -2 nucleosome, previous results could be reproduced, proving that histones are cleared from the

PHO5 promoter in the course of activation (compare Fig. 17C, $+P_i$ and t0). Upon rerepression, MYC-histones become incorporated into the -2 nucleosome (t20). However, in the presence of FLAG-H3 expression, only 50% of the incorporated histones correspond to the MYC-tagged variant, which is in agreement with previous findings (compare to Fig. 15).

B.



C.

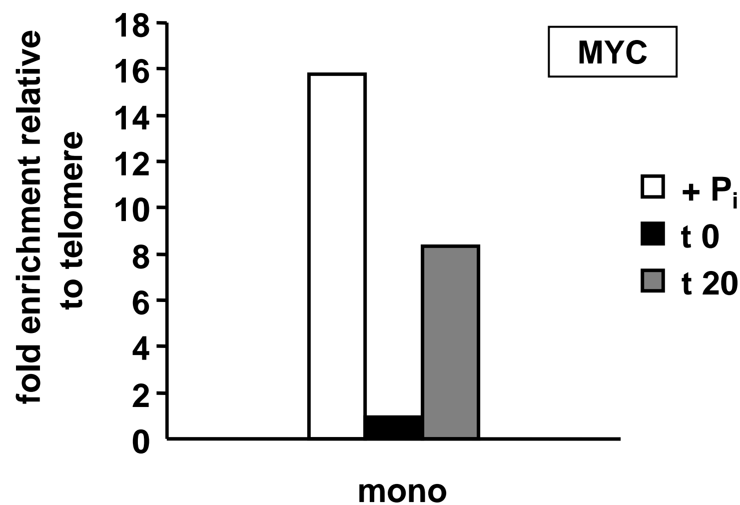


Figure 17 B and C. MYC-H3 and FLAG-H3 levels at the nucleosome -2 were monitored by anti-MYC / anti-FLAG ChIP and real-time PCR using the *PHO5* UASp2 primers. Data were normalized to input DNA and an amplicon in the telomere region.

1.8. *PHO5* promoter reassembly is delayed in the *asf1* and *hir1* histone chaperone deletion strains

Histone chaperones are acidic proteins that associate with the rather basic histones, thereby preventing their aggregation with nucleic acids. Chaperones act as vehicles to target histones to sites of nucleosome assembly, e.g. to the replication fork in S-phase or to DNA repair sites throughout the entire cell cycle. This makes them likely candidates for escorting histones from a source in *trans* to the closing *PHO5* promoter. I tested the involvement of histone chaperones in *PHO5* promoter reassembly on the level of transcriptional repression as well as on the level of histone deposition. Firstly, the potential role of various chaperones in histone deposition at the *PHO5* promoter was investigated during the reassembly process. ChIP kinetics using an antibody directed against the C-terminal tail of histone H3 were carried out.

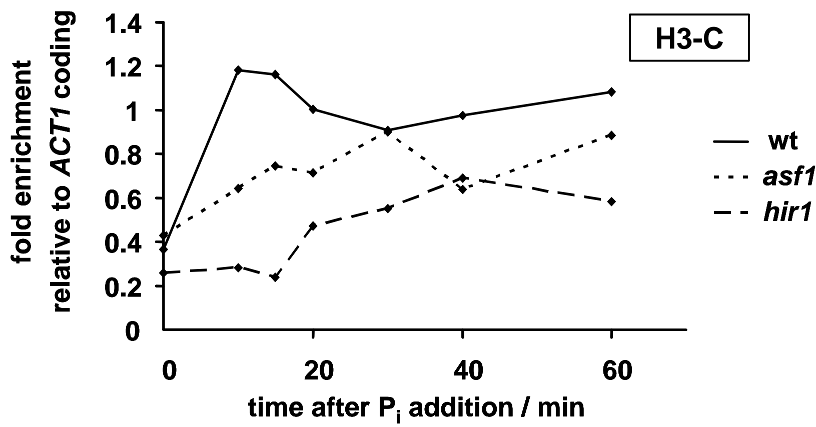
The first class of histone chaperones that were tested for defects in *PHO5* promoter reassembly are involved in replication-independent chromatin assembly. This class of chaperones includes Asf1p and Hir1p.

The yeast histone chaperone mutants *asf1* as well as the *hir* mutants are known to be defective in heterochromatic silencing (Singer et al., 1998; Kaufman et al., 1998), so that normalizing the ChIP signals to a telomere region would not be appropiate in this case. Instead, the coding region of *ACT1* was chosen for normalization of ChIP data.

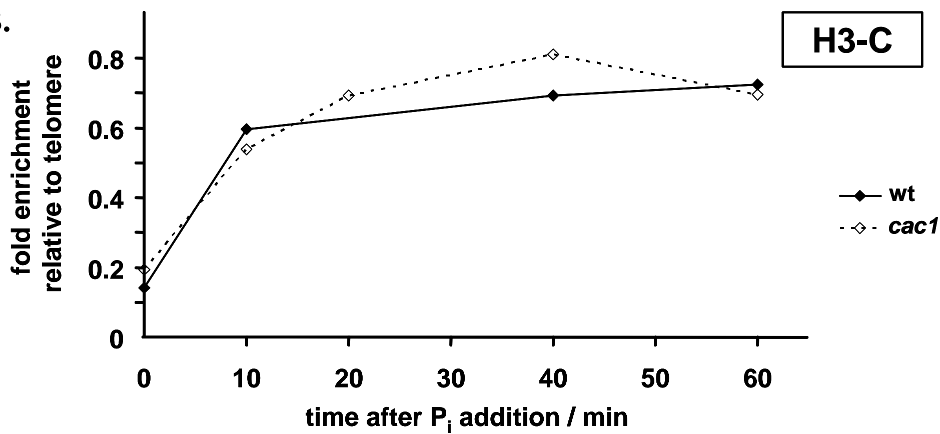
As shown in Fig. 18A, promoter closure in these mutants was severly impaired. In the *asf1* and *hir1* chaperone deletion strains the deposition of histone H3 at the UASp2 site of the *PHO5* promoter occurs with delayed kinetics. I wanted to understand whether the observed kinetic delay is specific for this class of chaperones or occurs generally in all histone chaperone mutants. For comparison, promoter closure kinetics were performed in deletion strains that belong to different classes of histone chaperones, namely *nap1*, an H2A/H2B chaperone, and *cac1*, an H3/H4 chaperone implicated in replication-dependent nucleosome assembly.

No significant differences between the wildtype and the *nap1* or *cac1* mutants could be detected (Fig. 18B/C respectively). This argues against an involvement of these chaperones in promoter closure. At the same time this finding indicates that specific chaperones, which are involved in the replication-independent assembly pathway, are required for proper nucleosome reassembly at the *PHO5* promoter during closure.

A.



B.



C.

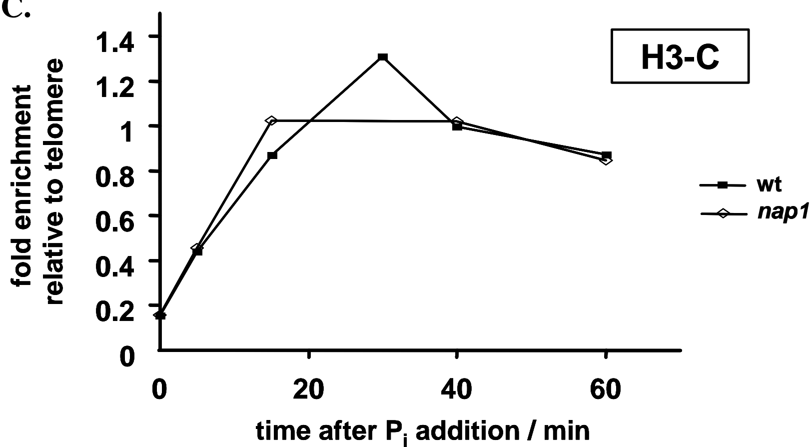


Figure 18. ***PHO5* promoter reassembly is delayed in histone chaperone deletion strains *asf1* and *hir1*.**

A. The histone chaperone deletion strains *asf1*, *hir1* and the isogenic wildtype were induced overnight in phosphate starvation medium. Phosphate was added and aliquots were taken at the indicated time points and subjected to ChIP analysis by using anti-H3 antibodies. Data for the UASp2 amplicon was normalized to input DNA and to an amplicon corresponding to the coding region of *ACT1*.

B. Wildtype and *cac1* were tested as in A., with the difference that an amplicon in the telomere region was chosen for normalization.

C. Wildtype and *nap1* were tested as in B.

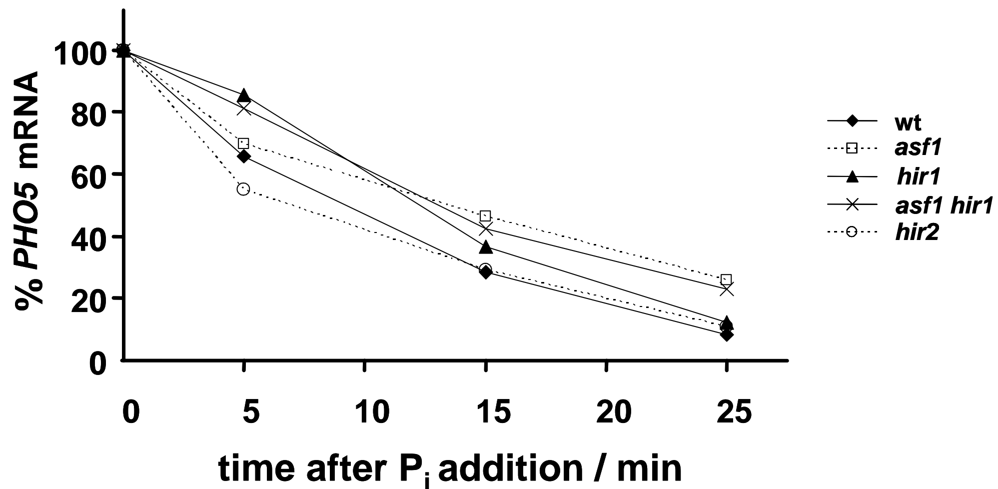


Figure 19. Transcriptional repression of *PHO5* is delayed in *asf1*, *hir1* and *asf1 hir1* strains.
Histone chaperone deletion strains and their isogenic wildtype were induced for 3 hours in phosphate-free medium and then repressed by shifting the cells to phosphate-containing medium. Total RNA was prepared at the indicated time points and analyzed for *PHO5* mRNA by reverse transcriptase PCR. *PHO5* mRNA levels were normalized to levels of *ACT1* mRNA. The normalized *PHO5* mRNA level at 0 min after phosphate addition was set as 100%.

Having discovered the described kinetic delay phenotype in the *asf1* and *hir1* strains, I wanted to confirm this result using a different technique and did so by monitoring *PHO5* mRNA levels during repression by reverse transcriptase PCR.

A time course of repression illustrates how *PHO5* mRNA levels are affected in *asf1, hir1* and *asf1/ hir1* deletion strains (Fig. 19). All three strains exhibit a slight kinetic delay in transcriptional repression and / or degradation of their *PHO5* mRNA compared to the wildtype strain. No synthetic effect was obtained when combining the *asf1* and *hir1* mutations.

The Hir1p and Hir2p proteins are known to cooperate in repressing transcription of histone genes and may act as a complex (Spector et al., 1997). Therefore it is conceivable that the *hir2* deletion may have an effect on *PHO5* repression. However, the *hir2* strain behaved essentially like wildtype, indicating that Hir1p in some cases may exert a repressive effect even in the absence of Hir2p.

1.9. The *SWI/SNF* nucleosome remodeling complex is implicated in rapid *PHO5* promoter reassembly

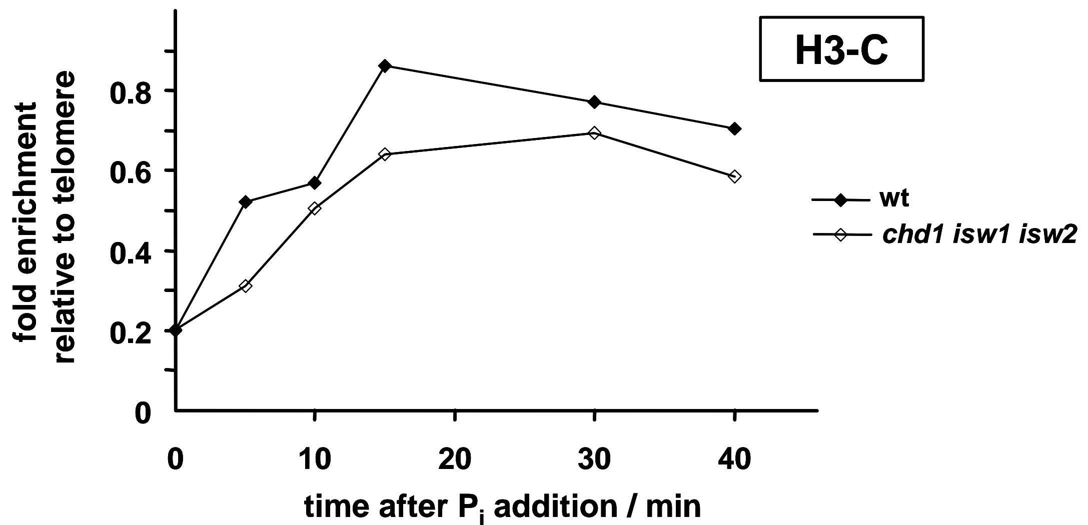
From our previous experiments it became clear that for proper reassembly of nucleosomes at the closing *PHO5* promoter specific histone-binding factors are required. By virtue of their synergistic action with histone chaperones, nucleosome remodelers are obvious candidates for a potential involvement in *PHO5* reassembly. In particular, the *SWI/SNF* complex has been shown to catalyze the transfer of histone octamers onto DNA *in vitro* (Phelan et al., 2000; Owen-Hughes et al., 1996). *In vivo*, results from our own laboratory as well as the Kornberg laboratory have demonstrated histone clearance from the *PHO5* promoter to occur by a *trans*-acting mechanism (Korber et al., 2004; Boeger et al., 2004). In an *snf2* strain, promoter opening is compromised and Snf2p is physically bound to the promoter under phosphate starvation conditions *in vivo* (Dhasarathy and Kladde, 2005). Since a role for Snf2p in *PHO5* activation had been demonstrated, I wanted to find out whether the *SWI/SNF* complex plays a similar role in *PHO5* repression. Moreover, additional nucleosome remodeling machines, whose characteristic mode of action is the relocation of nucleosomes by sliding, were tested (*isw1 isw2 chd1*).

Yeast strains deleted for the various ATPase subunits were induced in no-phosphate medium overnight. ChIP kinetics of promoter closure were performed using an antibody that recognizes the C-terminal part of histone H3. Fig.20A and B show the results of these experiments.

Compared to the baseline level of histone H3 under phosphate starvation conditions (=t0), we observe a maximally 3.5-fold enrichment of H3-ChIP signal in the triple mutant *isw1 isw2 chd1* and a maximally 4-fold enrichment H3-ChIP signal in the wildtype strain when shifting the cultures to repressive conditions (Fig. 20A). Based on this we conclude that there is no major defect in *PHO5* promoter reassembly in the triple mutant *isw1 isw2 chd1*. In contrast to this finding, reassembly was compromised in an *snf2* strain (Fig. 20B). This is manifested in a pronounced kinetic delay in promoter closure with an enrichment of H3-ChIP signal of maximally 1.5 fold over baseline H3 levels in the first hour after repression. The wildtype instead exhibits a maximally 4-5-fold increase in ChIP signal over baseline H3 levels in the first hour after repression. Since a similar delay had been observed in an *asf1* mutant (see Fig.

18A), I asked whether Snf2 acts independently of or in concert with Asf1. Combining the *asf1* with the *snf2* deletion did not exacerbate the kinetic delay phenotype (Fig. 20A).

A.



B.

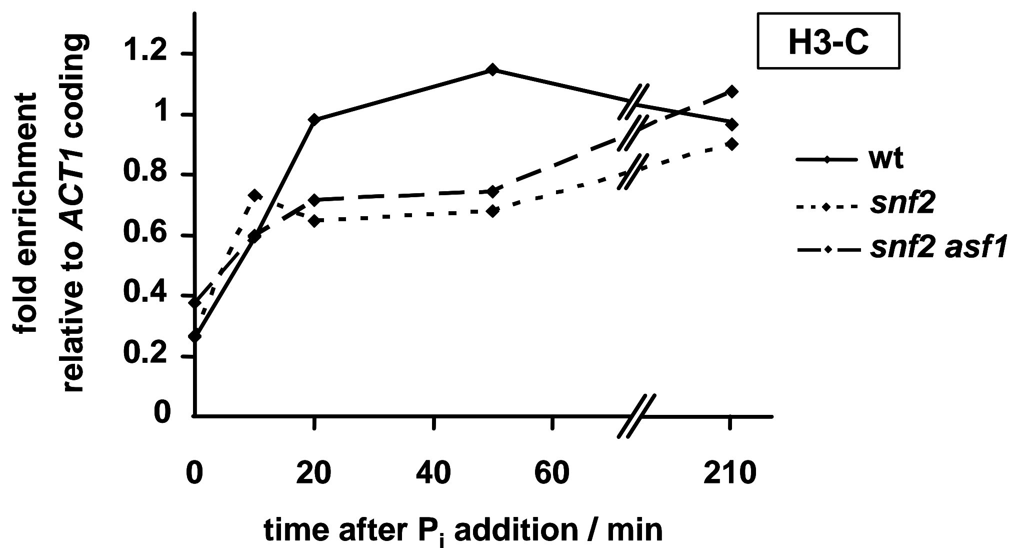


Figure 20. *PHO5* promoter reassembly is delayed in the nucleosome remodeling mutant strain *snf2,* but not in an *isw1 isw2 chd1* strain.

A. A yeast strain deleted for the ATPase subunits of various nucleosome remodeling complexes (*isw1 isw2 chd1*) and its isogenic wildtype were induced in phosphate-free medium overnight. Phosphate was added and aliquots were taken at the indicated time points and subjected to ChIP analysis by using anti-H3 antibodies. Data for the UASp2 amplicon was normalized to input DNA and to an amplicon corresponding to a telomere region. This experiment was carried out once.

B. Wildtype, *snf2* and *snf2 asf1* strains were tested as in A, with the difference that an amplicon in the coding region of *ACT1* was chosen for normalization. This experiment has been performed twice.

For the ChIP data analysis of the double mutant *asf1/ snf2*, again the *ACT1* amplicon was chosen for normalization, as the *asf1* strain exhibits defects in heterochromatin silencing. Nonetheless, when normalizing ChIP data for the *snf2* mutant to a telomere region, essentially the same result (data not shown) was obtained as when normalizing to *ACT1*.

1.10. *PHO5* promoter reassembly is not affected in strains lacking one allele of histone H3 and H4

The yeast genome harbours two alleles of the histone genes encoding H2A, H2B, H3 and H4, summing up to the following 4 histone gene pairs: *HHT1-HHF1*, *HHT2-HHF2*, *HTA1-HTB1*, *HTA2-HTB2*. Expression of such a histone gene pair is controlled from a divergent promoter driving the combined transcription of either H3 and H4 or H2A and H2B (Osley, 1991). Biologically this coupled transcriptional regulation makes sense, as building up a histone octamer consisting of an H3/H4 tetramer and two H2A/H2B dimers requires equal amounts of H3/H4 and H2A/H2B, respectively. Transcription and synthesis of the canonical histones are tightly regulated and, with a few exceptions, restricted to the S-phase of the cell cycle (Gunjan et al., 2005). Outside S-phase, the Hir repressor proteins regulate histone gene transcription by binding to *cis*-acting elements in 3 of the 4 histone gene promoters (Osley and Lycan, 1987; Osley et al., 1986). In addition to the negative control exerted by the Hir proteins during the G1 and G2 phase of the cell cycle, histone genes are activated just before entry into S-phase (Osley, 1991). These two control mechanisms ensure that a sensitive balance is kept between the *de novo* synthesis of DNA and histones, and at the same time the accumulation of excess histones is prevented. Even though regulation of the two gene pairs of H3/H4 involves the very same repressors and activators, they behave differently concerning their expression, i.e. the *HHT2-HHF2* allele expresses 7-fold more than the *HHT1-HHF1* allele (Cross and Smith, 1988). Deletion of the respective pairs *HHT1-HHF1* and *HHT2-HHF2* also leads to the appearance of different phenotypes. Deleting pair one renders the yeast strain sensitive to nitrogen starvation, while deleting pair two does not result in this phenotype (A. Verreault, personal communication). The different behaviour of the two histone gene pairs raises the interesting question of why do two gene pairs exist? One intriguing possibility explaining this may be that one pair is used for replication-independent incorporation, while the other one is used during S-phase and coupled to replication.

Chromatin reassembly at the closing *PHO5* promoter occurs truly independent of replication. Hence, our system allows us to test whether a specific copy of H3-H4 is preferentially incorporated in a replication-independent manner. To this end, we monitored *PHO5* promoter reassembly in strains lacking either copy one (*HHT1-HHF1*) or copy two (*HHT2-HHF2*) of H3-H4. ChIP kinetics were performed as before by using an antibody directed against the C-terminus of H3. It should be noted that for the normalization in this experiment again the telomere region was chosen. However, it is not known how the deletion of one histone gene allel affects histone density at the telomere region. Therefore the telomere may not be an optimal control region.

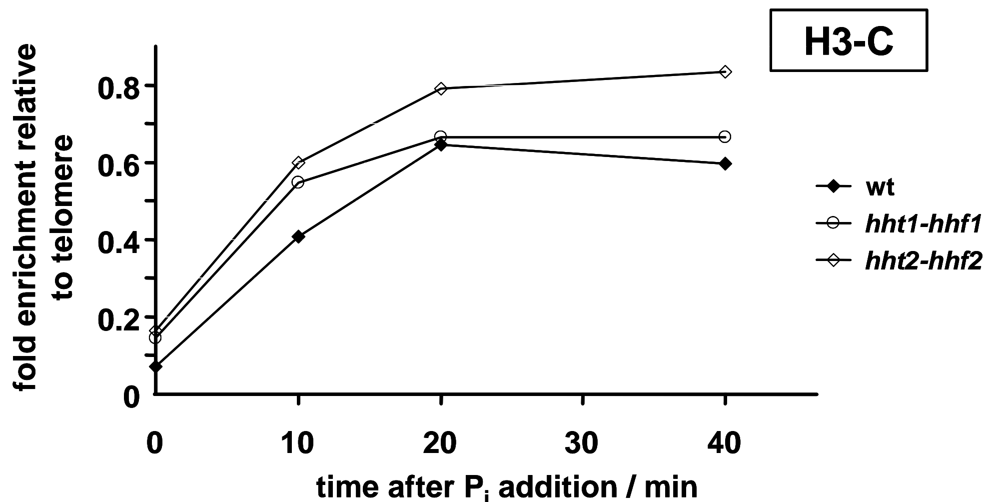


Figure 21. *PHO5* promoter reassembly is not affected in strains lacking either copy one or copy two of the histone H3-H4 allele.
Yeast strains lacking either gene copy one of H3-H4 (*hht1-hhf1*) or gene copy two of H3-H4 (*hht2-hhf2*), along with their isogenic wildtype were induced for 5 hours in phosphate starvation medium. Phosphate was added and aliquots were taken at the indicated time points and subjected to ChIP analysis by using anti-H3 antibodies. Data for the UASp2 amplicon was normalized to input DNA and to an amplicon corresponding to a telomere region.

The ChIP analyses of both mutants, lacking either copy 1 or copy 2 of H3-H4, look almost identical to the wildtype kinetics (Fig. 21). From this data we conclude that the absence of one H3-H4 pair does not hinder promoter reassembly in any way. Unfortunately, no preferential incorporation of one of the two H3-H4 copies during *PHO5* promoter reassembly could be detected.

2. Chromatin reassembly at the *PHO8* and the *PHO84* promoters during repression

2.1. Histones are incorporated in *trans* during reassembly of the *PHO8* and *PHO84* promoters

Having discovered the *trans*-mode of histone incorporation at the *PHO5* promoter, we were eager to investigate whether this chromatin assembly mechanism in *trans* is unique to the *PHO5* promoter or does also hold true for other promoters in the course of their repression. The main challenge in this regard is that there are only few examples of genes with such clearly defined requirements for their activation and repression as it is the case for *PHO5*. For most yeast genes the signals triggering the transition between their transcriptional on and off states are still poorly understood. For that reason genes were analyzed that are also regulated by phosphate levels and that had been previously characterized in our laboratory.

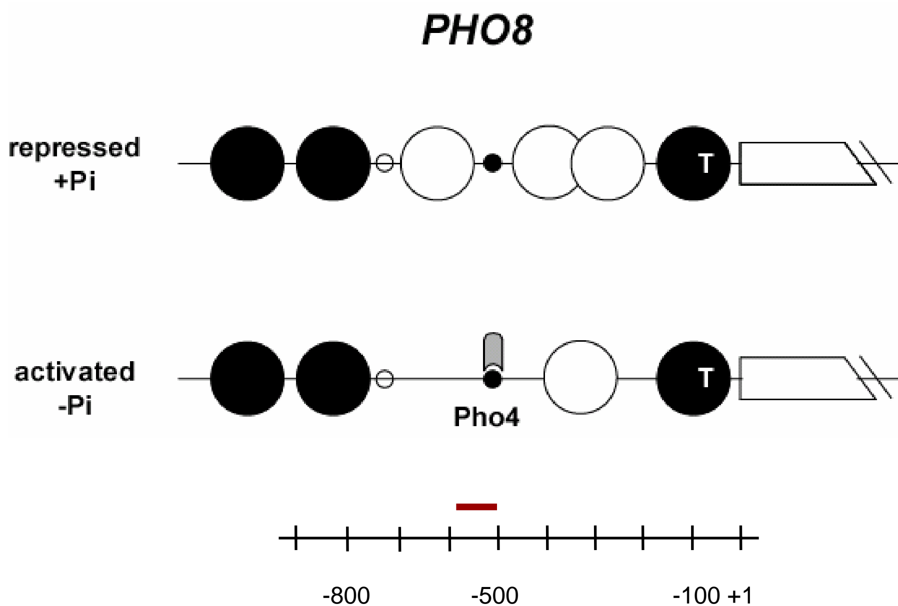


Figure 22. Schematic of the *PHO8* promoter in the repressed and activated state.
The amplicon used for monitoring nucleosome occupancy by ChIP is drawn in red and hybridizes around 500 bp upstream of the transcriptional start of the *PHO8* gene. The scale indicates the distance from the *PHO8* transcriptional start site in bp.

The *PHO8*, *PHO84* and *PHO5* genes share a common link, i.e. all three genes are activated through phosphate starvation, evoking the binding of the transcriptional activator Pho4p to intrinsic UASp elements in the promoter region of these genes. During activation, histones are

evicted from the *PHO8* (Fig. 22) and the *PHO84* promoters, giving rise to a nuclease hypersensitive site, as was shown by ChIP and DNase I digestion (Reinke and Hörz, 2003) and unpublished data). In order to monitor reassembly of the *PHO8* and *PHO84* promoters upon repression, we used the same experimental setup as for *PHO5* (see outline Fig. 11). Thereby, the very same chromatin extracts were used as for the ChIP experiments monitoring *PHO5* promoter closure.

FLAG-H3 levels in the course of *PHO* repression were followed at the promoter regions of the *PHO8* and the *PHO84* gene by ChIP. I found that the FLAG-H3 signal at both promoters progressively increased upon addition of phosphate to the phosphate starved cell culture (Fig. 23 A and B). At the *PHO84* promoter the anti-FLAG ChIP signal increased maximally 4-fold over the baseline levels of FLAG-H3 (Fig. 23A), whereas at the *PHO8* promoter the observed increase was maximally 2.5-fold over baseline (Fig. 23B). It is known that at the *PHO8* promoter on average 1-2 nucleosomes get displaced upon activation (Reinke and Hörz, 2003). The more pronounced increase of FLAG-H3 at the *PHO84* compared to the *PHO8* promoter may reflect that the hypersensitive site at the *PHO84* promoter is more extended than at *PHO8*. Accordingly, more histones appear to be needed to fill up the corresponding promoter region. This result has to be confirmed by DNAse I digestion though. Abolishing FLAG-H3 expression (0 min gal) resulted in a failure to detect FLAG-H3 at the two promoters upon repression. The kinetics of FLAG-H3 incorporation are similarly rapid as the ones observed at *PHO5*. Therefore, chromatin reassembly at the *PHO8* and the *PHO84* promoters probably occurs also in a replication-independent fashion.

Analogous to the experiment described in Fig.15, anti-MYC ChIP again served as a tool to measure to which extent FLAG-histones are deposited at the closing *PHO8* and *PHO84* promoters. MYC-H3 levels were determined in the presence and in the absence of FLAG-H3 synthesis. When FLAG-H3 was not expressed, I found a dramatic signal increase of anti-MYC ChIP signal at the *PHO84* promoter (Fig. 24A), reaching a maximum after 60 minutes in high phosphate medium. Synthesis of FLAG-H3 led to a competition and accordingly to a reduction of this signal of about 75%. The data indicate that a major portion of the histones, probably more than 50%, for *PHO84* reassembly originate from a source in *trans*.

In the case of *PHO8*, the scenario is essentially the same, with MYC-H3 competing with FLAG-H3 for incorporation. Whereas in the absence of FLAG-histones there is a 5-fold enrichment of MYC-H3 over baseline value (=t0) to a final value of about 1, only a final

value of 0.4 is reached in the presence of FLAG-H3 (Fig. 24B), speaking for a *trans*-deposition of histones also at the *PHO8* promoter.

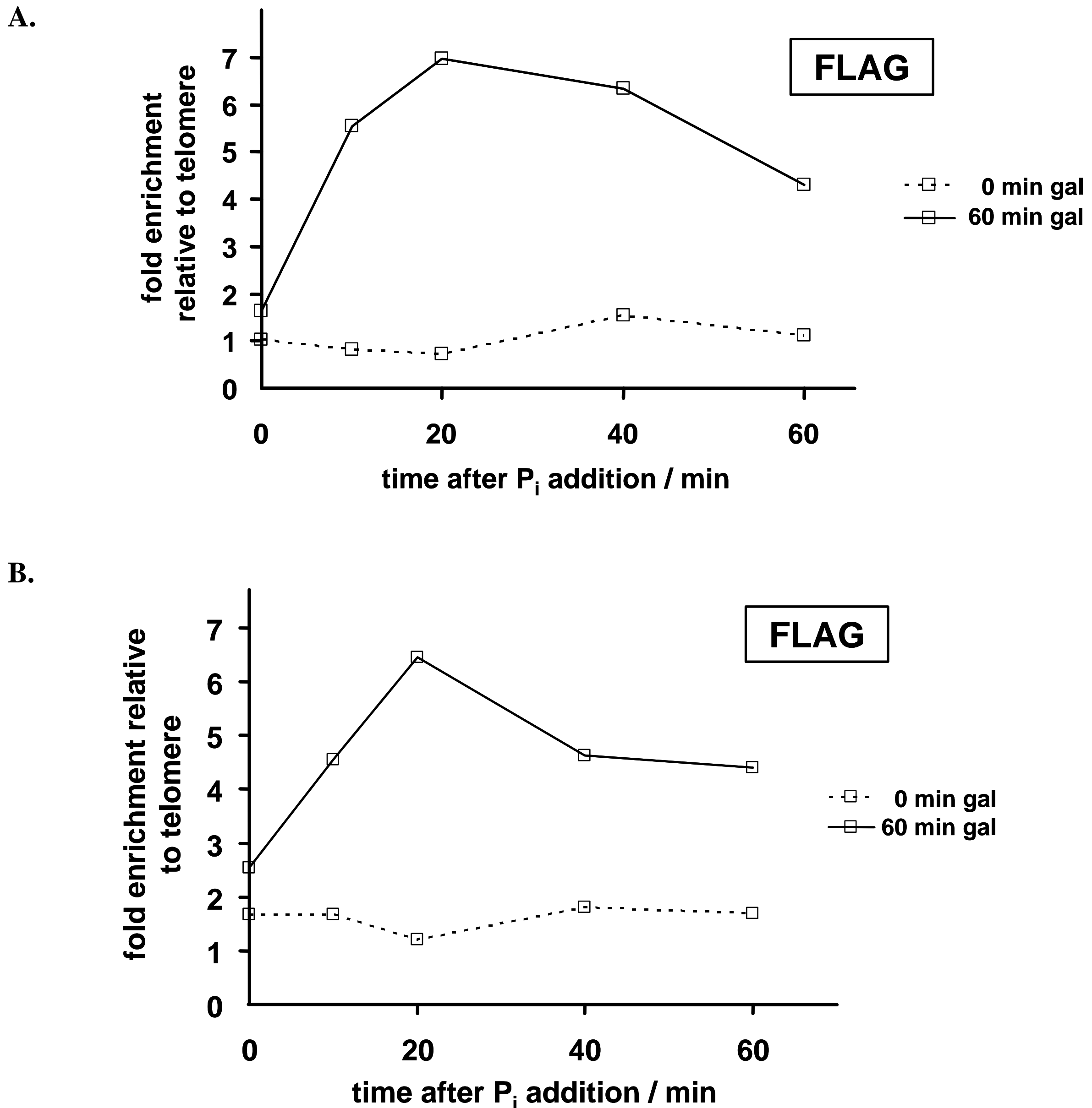


Figure 23. FLAG-H3 is specifically incorporated at the *PHO84* and the *PHO8* promoter upon repression.
A. Strain USY6 was induced for 5 hours in phosphate-free medium leading to the opening of the *PHO5* promoter. FLAG-H3 expression was turned on in the last hour of phosphate starvation. At time point t0, promoter closure was initiated by adding phosphate to the culture. FLAG-H3 levels were followed at the indicated time points by anti-FLAG ChIP using amplicons corresponding to either the *PHO84* promoter. ChIP data were normalized to input DNA and to an amplicon in the telomere region.
B. As A., but an amplicon corresponding to the *PHO8* promoter region was used.

A.

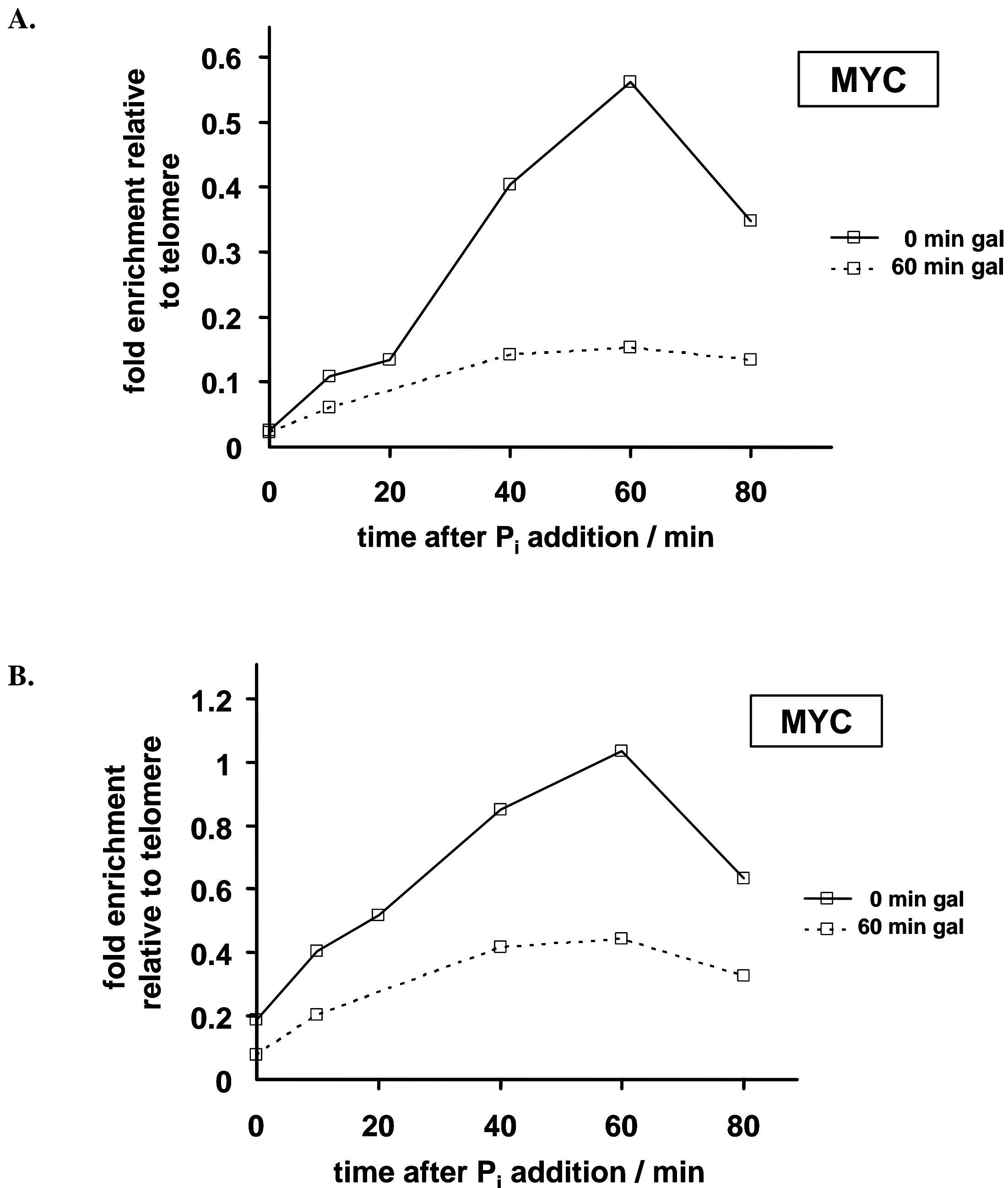


Figure 24. FLAG-H3 competes significantly with MYC-H3 for incorporation during *PHO84* and the *PHO8* promoter reassembly.

A. Strain USY6 was induced by phosphate starvation for 5 hours and FLAG-H3 expression was turned on in the last hour of induction. At time point t0, phosphate was added to initiate promoter closure. MYC-H3 levels were determined by anti-MYC ChIP analysis at the indicated time points using an amplicon corresponding to the *PHO84* promoter. ChIP data were normalized to input DNA and to an amplicon in the telomere region.

B. As A., but an amplicon corresponding to the *PHO8* promoter region was used.

Notably, MYC-H3 levels decrease again at time point t 80 min. This effect may be connected to replication. After the shift to high phosphate conditions yeast cells re-enter S-phase. 80 min after the medium shift the cells probably have completed the replication of their genome. During this process, a re-distribution of MYC- and FLAG-histones may take place, which could account for the observed change in MYC-H3 levels.

In summary, promoter closure of the three phosphate regulated promoters we investigated, namely *PHO5*, *PHO8* and *PHO84*, occurs very rapidly. Promoter reassembly requires the incorporation of histones at the hypersensitive sites of the respective promoters. Regarding the mechanism of promoter reassembly, evidence is provided for incorporation of histones from a source in *trans*. Moreover, replication appears not to be necessary for the reassembly reaction.

3. Transcriptional activation of *PHO5* in an *asf1* strain is dependent on the phosphate concentration of the medium

3.1. Transcriptional activation of the *PHO5* gene is delayed in an *asf1* strain

During the past years one main focus of research in our laboratory was to identify factors that are required for activation of the *PHO5* gene. Besides the transactivator Pho4 itself, to date none of the candidates tested was essential for *PHO5* activation. However, in the course of these studies, multiple factors were found to assist in the activation process. For example, lack of the the nucleosome remodeler Snf2p or the histone acetyltransferase Gcn5p, leads to a delay in promoter chromatin remodelling and concomitantly to a delayed eviction of histones from the promoter (Neef and Kladde, 2003; Barbaric et al., 2001). It is intuitive that the displacement of histones in *trans* might be mediated by histone acceptor proteins. Histone chaperones are therefore likely to play also a role in chromatin disassembly at the *PHO5* promoter upon activation. To evaluate such a function, *asf1* and *hir1* strains were assayed for defects in *PHO5* induction. An easy way to determine whether *PHO5* activation is impaired is to measure the activity of the *PHO5* gene product, an acid phosphatase, in the course of induction. This method of determining protein levels is of course not ideal, but rather practical since acid phosphatase is a very stable molecule (Haguenauer-Tsapis and Hinnen, 1984).

The outcome of this analysis is depicted in Fig. 25. Compared to the wildtype strain, the *asf1* deletion exhibited slower kinetics of *PHO5* activation after the shift to phosphate-free medium (Fig. 25A). At no time point, the *asf1* mutant could be induced to wildtype enzyme activity levels. The observed effect was specific for Asf1p, as the *hir1* disrupted strain did not give any kinetic delay phenotype (data not shown).

A study from the Tyler group also investigated the involvement of Asf1p in *PHO5* induction. In contrast to our data, they find Asf1p to be essential for *PHO5* promoter opening (Adkins et al., 2004). One reason explaining this discrepancy could be that Tyler and coworkers use <u>low</u> phosphate medium for *PHO5* induction whereas we use <u>no</u> phosphate medium in our experiments. Accordingly, one possible hypothesis would be that *PHO5* can not be activated under growth conditions, where the medium is not completely free of phosphate. I tested whether the conflicting data sets can be explained by comparing *PHO5* induction in an *asf1* strain in medium containing different phosphate concentrations.

Coming from high phosphate medium (=off state), wildtype and *asf1* strains were shifted to medium containing different phosphate concentrations and the cultures were grown overnight, followed by acid phosphatase measurements (Fig. 25B). The time period of 17 hours is sufficient to fully activate *PHO5* in phosphate-free medium. The result of this analysis is really clear-cut and strongly favours the hypothesis that an *asf1* mutant can only be fully induced when the growth medium is absolutely free of phosphate. Even the abundance of trace amounts of phosphate (0.05 mM) dramatically decreases the acid phosphatase activity of an *asf1* strain to only 60U, compared to about 500U under no phosphate conditions. In contrast to the *asf1* mutant, the wildtype can be induced to comparably high levels, even at a phosphate concentration of 0.4 mM. However, at a concentration of 1mM phosphate, the wildtype can no longer be induced to a significant degree.

Another possibility explaining this difference between wildtype and *asf1* under the above described growth conditions would be that the stability of the gene product, the acid phosphatase, is markedly reduced in an *asf1* strain compared to the wildtype. In order to completely confirm the hypothesis that *PHO5* cannot be fully activated in an *asf1* strain when there are residual amounts of phosphate in the medium, DNAseI digestions would have to be performed. As an alternative, mRNA level analysis could be used to confirm that *PHO5* is hardly transcribed.

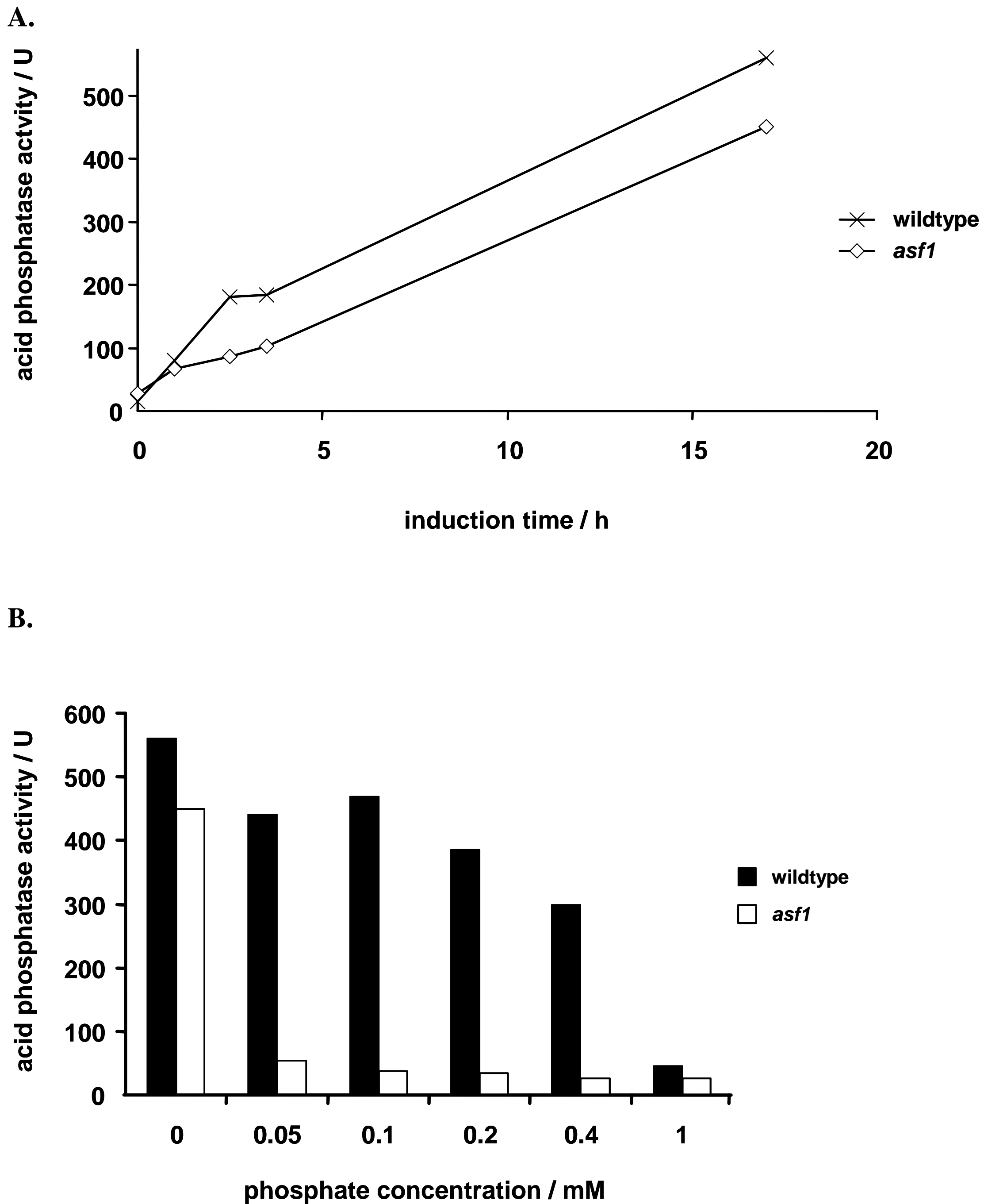


Figure 25. Transcriptional activation of *PHO5* in an *asf1* strain is dependent on the phosphate concentration of the medium.

A. An *asf1* strain and its isogenic wildtype were grown in high phosphate medium (8 mM). They were then harvested, washed and shifted to medium containing no phosphate. Aliquots were taken at the indicated time points and *PHO5* induction was determined by measuring acid phosphatase activity. This experiment was repeated once.

B. An *asf1* strain and its isogenic wildtype were grown in high phosphate medium, washed and shifted to media containing various different phosphate concentrations, ranging from 0 mM to 1 mM phosphate. Acid phosphatase activity was measured after 17 hours growth in the indicated media.

IV. Discussion

1. Histones are incorporated to rebuild the inactive chromatin structure of the *PHO5* promoter upon repression

Over the years the *PHO5* promoter has become one of the best-studied systems illustrating the existence of a link between nuclease hypersensitive sites and transcriptional activity. The current model for *PHO5* activation involves the binding of the *trans*-activator Pho4p to the UASp1 site that is located in a nucleosome-free hypersensitive stretch of promoter DNA. Pho4p binding evokes concomitant remodeling and leads to the removal of nucleosome -2 and subsequent binding of Pho4p also to the second UASp element, UASp2 (Reinke and Hörz, 2004). This example demonstrates how hypersensitive sites can be used as entry points for elements of the transcriptional machinery.

Conversely the *PHO5* gene is also one of the first examples demonstrating the powerful influence of positioned nucleosomes on repressing basal gene expression. Repression of *PHO5* is alleviated when yeast cells are depleted for histone H4, indicating that nucleosomes are required for transcriptionally silencing *PHO5* under high phosphate conditions (Han et al., 1988). Consistent results were obtained in a study by Straka and Hörz, where a fragment from the *PHO5* promoter corresponding to one of the positioned nucleosomes (nucleosome -2) was replaced by a DNA fragment from African green monkey alpha satellite DNA. This well-defined DNA stretch stably associates with histones to yield a uniquely positioned nucleosome. Even though the UASp1 site is still accessible for the activator Pho4p, the -2 nucleosome can not be remodeled any more under activating conditions (Straka and Hörz, 1991). Failure to remove this persistent nucleosome from the *PHO5* promoter region severely reduces the levels to which the *PHO5* gene can be induced.

When looking at the activation of yeast genes, *PHO5* served once more as an excellent model. In a pioneering study Reinke and Hörz demonstrated nucleosomal clearance at the *PHO5* promoter region in the course of phosphate induction (Boeger et al., 2003; Reinke and Hörz, 2003). Histone eviction is not restricted to *PHO5*, but can rather be observed on a genome-wide scale. Two independent groups found the depletion of nucleosomes at promoters to be a

general phenomenon that is often associated with active transcription (Bernstein et al., 2004; Lee et al., 2004).

In summary, the data from the literature suggest a close relationship on the one hand between nucleosome depletion from promoters in the course of transcriptional activation, and on the other hand between the deposition of nucleosomes at promoters and the transcriptional shutdown of genes.

Along this line, in this study I provide evidence that rerepression of the yeast *PHO5* gene is indeed accompanied by the incorporation of histones at the closing promoter.

Throughout all the ChIP experiments that were carried out, a region of the *PHO5* promoter was monitored that corresponded to the -2 nucleosome of the *PHO5* promoter. This particular nucleosome has been previously shown to become preferentially remodeled upon activation, compared to the -1 and -4 promoter nucleosomes (Boeger et al., 2003). Therefore, the UASp2 element localized within this nucleosome represents a site that is maximally depleted for nucleosomes in the active promoter state. The readdition of phosphate to a phosphate starved cell culture results in a rapid increase in H4 ChIP signal at the *PHO5* UASp2 site (Fig. 10, page 33). This indicates that *PHO5* promoter reassembly is accompanied by the deposition of at least the H3-H4 tetramer, since H3 is always found to be tightly complexed with H4 (Kornberg and Thomas, 1974). In agreement with this, results from the Tyler group confirmed the deposition of histones during reassembly of the *PHO5* promoter (Adkins et al., 2004). Tyler and coworkers also followed histone deposition at the *PHO5* promoter in the course of *PHO* repression by ChIP using an anti-H3 C-terminal antibody. In their hands promoter reassembly occured with slower kinetics than the ones I observed. Their data argue that the *PHO5* promoter gets equipped with a full complement of histones after around 2 hours. In my observations, however, promoter closure is already completed half an hour after the shift to high phosphate conditions. This discrepancy may be due to the fact that the Tyler group used different growth media in their experiments.

My result is strengthened by the analysis of the *PHO5* mRNA levels (Fig. 7, page 29). This analysis demonstrates that transcriptional repression of *PHO5* occurs with kinetics matching the kinetics of histone incorporation in the course of reassembly quite well. Concerning *PHO5* mRNA stability, almost all of the mRNA has disappeared 30 minutes after readdition of phosphate. Despite this efficient degradation of *PHO5* mRNA, the corresponding gene product, Pho5p, is more robust and persists for comparably longer times. In a wildtype

situation, relatively high levels of acid phosphatase activity (300 U) can be detected even 3 hours after rerepression (Adkins et al., 2004). Therefore, the determination of Pho5p protein levels would not be an appropriate tool to monitor the transcriptional repression of *PHO5*.
In summary, the deposition of histones at the active *PHO5* promoter appears to be part of an efficient mechanism to shut off *PHO5* transcription upon the shift to repressive conditions. The speed with which the promoter reassembly reaction is accomplished is striking. Whereas the activation process of the *PHO5* gene takes around 6 hours (Barbaric et al., 2001), its transcriptional shutdown is completed after about 30 minutes.
Such a fast and efficient mechanism of gene repression is probably very favourable for the organism, since it allows yeast cells to rapidly adapt to changing growth conditions and prevent the wasteful synthesis of gene products that are no longer required.

2. Histones for reassembly of the *PHO5*, *PHO8* and *PHO84* promoters originate, at least in part, from a histone source in *trans*

Investigating the fate of promoter nucleosomes upon activation at the three phosphate regulated promoters, *PHO5*, *PHO8* and *PHO84,* has been one of the central questions in our laboratory. In the course of *PHO* induction nucleosomes get displaced from the respective promoters giving rise to a nuclease hypersensitive site (Boeger et al., 2003; Reinke and Hörz, 2003) and unpublished results). The mechanism accounting for the observed nucleosome loss operates, at least in case of the *PHO5* promoter, in *trans* meaning that nucleosomes are completely disassembled and histones lose contact to the underlying promoter DNA (Boeger et al., 2004; Korber et al., 2004). Based on these findings we reasoned that a similar *trans* mechanism could also be implicated in promoter reassembly of *PHO5*, *PHO8* and *PHO84*. Alternatively, one could also think of repositioning of the *PHO* promoter nucleosomes by a sliding mechanism.
Making use of the histone double tag strategy, I could unambiguously show that at least part of the histones for promoter reassembly at *PHO5*, *PHO8* and *PHO84* originates from a soluble histone pool, i.e. from a histone source in *trans*. This is manifested by the incorporation of FLAG-histones at the reassembling promoters as shown in Figs. 13 and 23A/B (pages 37 and 53 respectively). In a further step, MYC-ChIP experiments investigating

the incorporation of MYC-histones (representing the chromatin-bound histone fraction) in the presence and in the absence of FLAG-histone synthesis assisted in determining the extent of *trans* incorporation of histones (representing the soluble histone fraction) at the respective promoters. I found that the amount of MYC-histones that are deposited at *PHO5*, *PHO8* and *PHO84* is shifted to a significant degree (around 50% in the case of *PHO5* and *PHO8,* and around 75% in the case of *PHO84*) downwards when FLAG-histones are synthesized in parallel (Figs. 15 and 24A/B, pages 40 and 54 respectively). This dataset conclusively demonstrates the competition between MYC-H3 and FLAG-H3 for incorporation at the closing *PHO* promoters. At the same time this finding rules out our concerns that FLAG-H3 constitutes only a minor portion of the total amount of histones used for reassembly. It rather proves the opposite, namely that FLAG-histones represent 50-75% of the total deposited histones at the *PHO* promoters. The experiments where I explicitely probe for the abundance of canonical nucleosomes show that these immunoprecipitated histones are not just binding non-specifically to the *PHO5* promoter DNA, but are rather part of canonical nucleosomes (Fig. 17, page 43). However, it is impossible to draw definite conclusions about the remainder of the deposited histones corresponding to the MYC-H3 variant. MYC-histones may originate from a source in *cis* or in *trans*. They are on the one hand present in the chromatin fraction, on the other hand they may also be part of the soluble histone pool. Therefore the MYC-histone deposition we observe at the closing promoters could be due to incorporation of MYC-H3 arising from the histone pool. Alternatively, the increase in MYC-ChIP signal could be the outcome of nucleosomes sliding in *cis* onto the promoters from regions neighbouring the promoters.

Obviously, one of the major concerns regarding our experimental setup where FLAG-histone synthesis is driven by the strong galactose-inducible promoter is that FLAG-H3 may be strongly overexpressed compared to the physiological situation where H3 expression is regulated by the wildtype H3-H4 promoter. In this case our system may be flooded with FLAG-histones and distort the results regarding *cis/ trans* incorporation of histones at the *PHO5* promoter. However, data from the literature makes this scenario is unlikely. Histone levels in yeast are highly regulated. Surplus histones have been shown to be toxic for yeast cells. Therefore, a Rad53p-dependent mechanism drives the degradation of overexpressed histones to ensure the proper balance between DNA and histone synthesis (Gunjan and Verreault, 2003). Another argument speaking against the massive overexpression FLAG-

histones is that one would expect much higher levels of FLAG-H3 deposition at the closing *PHO5* promoter. In particular, the competition experiment where MYC-H3 competes to a significant level with FLAG-H3 indicates that there are similar amounts of FLAG- and MYC-histones present in the soluble pool.

Taken together, my data could be reconciled with a mechanism that catalyzes the incorporation of histones at the reassembling *PHO* promoters to 100% from a source in *trans*. Such a situation would imply the existence of a mixture of MYC- and FLAG-tagged histones in the soluble histone pool. The existence of such a mixture is likely, as cells require some of the histones that are synthesized during S-phase to remain in the soluble pool, in order to e.g. repair chromatin at DNA lesions throughout the entire cell cycle. Upon phosphate addition, both histone variants would be shuttled from the soluble histone pool to the promoter regions of the *PHO* promoters (as depicted in Fig. 26). Subsequently both histone variants would be detected by anti-MYC / anti-FLAG-ChIP at the closing promoters, which is what I actually observed.

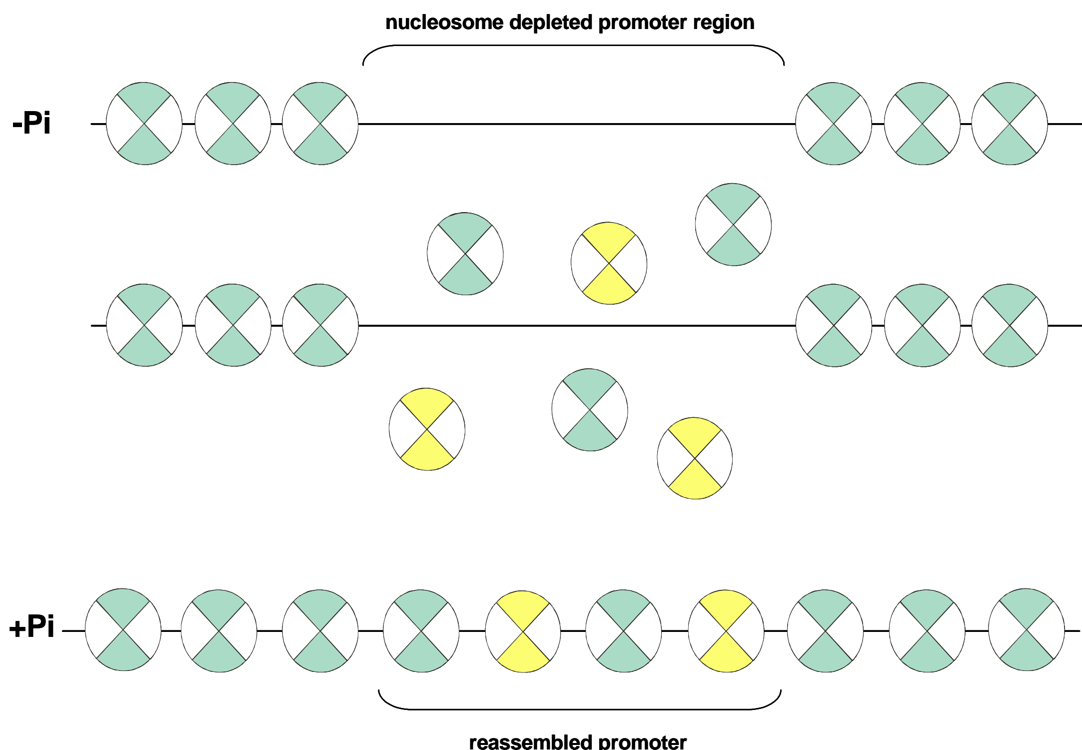


Figure 26. Schematic illustrating a potential mechanism replacing *PHO* promoter nucleosomes upon repression to 100% from a histone source in *trans*.

For simplification only the H3-H4 tetramer is shown. Green-white circles denote octamers made up of MYC-H3 and untagged H4, which on the one hand neighbour the *PHO* promoter region, but are also present in the soluble histone pool. Yellow-white triangles denote octamers made up of FLAG-H3 and untagged H4.

In an alternative line of argumentation, my results could be interpreted in a different way, namely that promoter closure does not make use of a single mechanism, but rather of a combination of two mechanisms. In this model, part of the nucleosomes used to fill up the hypersensitive sites would be deposited from a source in *trans*, whereas the remainder of the histones required for reassembly would be translocated from a source in *cis* (Fig. 27). Applying solely the ChIP technique it is difficult discriminate between the two possible scenarios I described above.

Nonetheless, it is clear that *PHO* promoter reassembly occurs, at least in part, by a mechanism acting in *trans*.

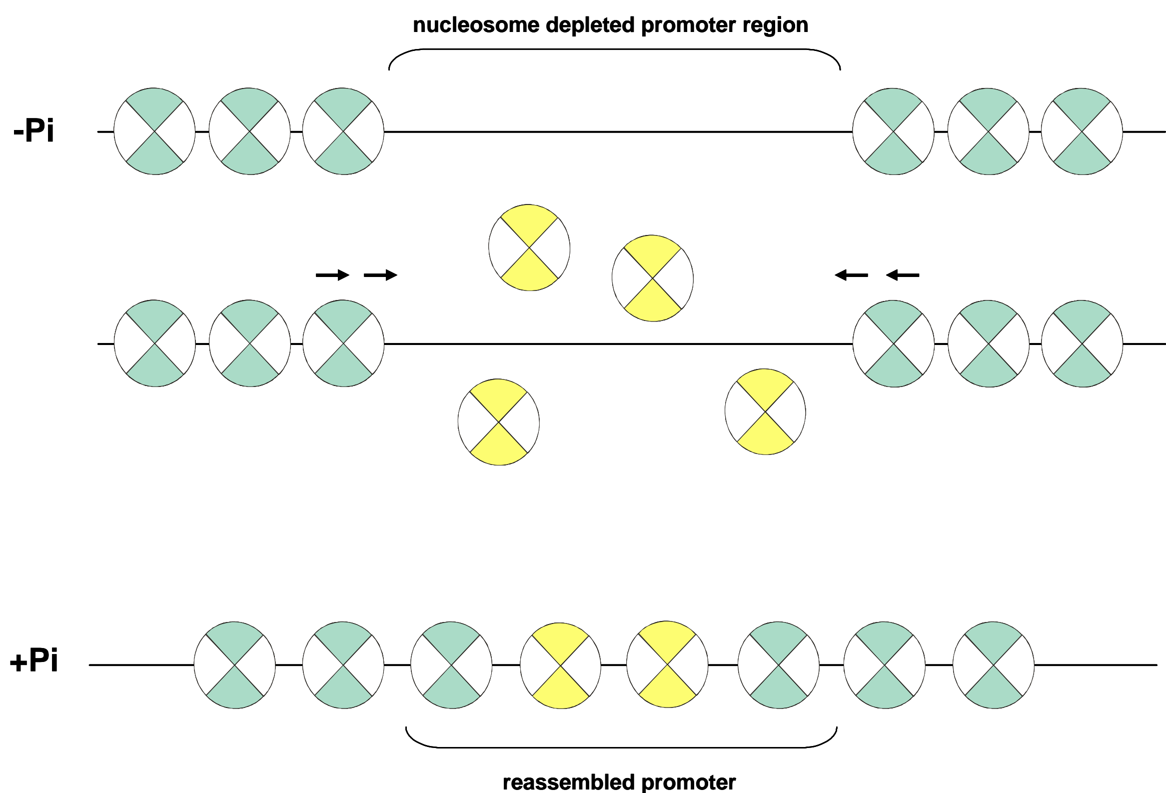


Figure 27. Schematic illustrating a potential scenario, in which *PHO* promoter nucleosomes are replaced by a combination of two mechanisms, acting partially in *trans* and partially in *cis*.

For simplification only the H3-H4 tetramer is shown and MYC-tagged H3 histones that may be present in the soluble histone pool are omitted. Green-white circles denote nucleosomes that consist of MYC-H3 and untagged H4, yellow-white circles denote nucleosomes that consist of FLAG-H3 and untagged H4.

3. Nucleosome remodeling machines that catalyze nucleosome movements in *trans* assist in the rapid reassembly of *PHO5* promoter chromatin

There is an increasing body of evidence that nucleosomes are not static entities but rather exist in a dynamic equilibrium of fully assembled and partially unraveled states (Li et al., 2005; Tomschik et al., 2005). This equilibrium is strongly influenced by the action of histone modifying enzymes and nucleosome remodeling machines. According to the so-called histone code model, posttranslational modifications of the histone tails generate a platform, which is "read" by chromatin-binding proteins (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The association of such proteins, e.g. ATP-dependent nucleosome remodelers or histone acetyltransferases with chromatin subsequently leads to either condensation or decondensation of the nucleoprotein complex (de la Cruz et al., 2005; Jenuwein and Allis, 2001). The molecular basis underlying the recognition of such histone marks is found in specific motifs of the chromatin binding factors. Examples for such recognition modules are the bromodomain and the chromodomain, which mediate the association with specifically acetylated or methylated histone tails, respectively (Bottomley, 2004; Brehm et al., 2004).

The acetylation of specific lysines in the histone H3 and H4 tails often appears to be a prerequisite for remodeling of promoters as it is exemplified at the *PHO5* promoter (Nourani et al., 2004; Reinke and Hörz, 2003). The acetylation can stabilize SWI/SNF binding to the promoter thereby facilitating remodeling and concomitant removal of nucleosomes in the promoter region (Hassan et al., 2001).

By virtue of their capacity to determine nucleosome dynamics, nucleosome remodeling factors appeared to be obvious candidates for reassembling *PHO5* promoter chromatin. Therefore, representative remodeling mutants were tested for their impact on *PHO5* promoter closure.

Interestingly, only those nucleosome remodelers (Snf2p), which are capable of catalyzing the transfer of histones onto DNA templates in trans (Phelan et al., 2000), played a role in rapid reassembly of the *PHO5* promoter (Fig. 20B, page 48). On the other hand, factors whose characteristic mode of action is the translocation of octamers by sliding in cis (Isw1p, Isw2p, Chd1p) did not exhibit such a defect (Fig. 20A, page 48).These results further corroborate the hypothesis that histones are shuttled from a soluble histone pool to the *PHO5* promoter

region, making the nucleosome sliding mechanism an unlikely explanation for the reassembly.

What remains unresolved is the question whether the SWI/SNF complex is also involved in targeting the histones to the reassembling *PHO5* promoter. Under phosphate starvation conditions (= activated state) Snf2p is physically associated with the *PHO5* promoter, as was judged by ChIP experiments (Dhasarathy and Kladde, 2005). Upon repression, Snf2p may stay transiently bound at the promoter and recruit histones or additional factors that are required for nucleosome reassembly.

Carrying the hypothesis that in particular *trans* acting remodelers contribute to rapid promoter reassembly one step further, we speculate that also other factors being able to catalyze nucleosome movements in trans, e.g. the RSC complex (Lorch et al., 1999a; Lorch et al., 2006) may be implicated in *PHO5* promoter reassembly. However, the role of RSC in *PHO5* repression is somewhat complicated to investigate, as yeast strains lacking this remodeling complex are not viable (Cairns et al., 1996).

Clearly there is a strong redundancy among the factors contributing to *PHO5* repression. The lack of one factor does not abolish, but rather slows down the speed with which promoter closure occurs. This redundancy can be explained by the fact that there are at least 5 different nucleosome remodeling complexes in yeast, many of which have been shown to play direct roles in repressing genes. The *RSC* complex has been shown to be essential for proper nucleosome positioning of the yeast *CHA1* promoter and for transcriptional repression of the corresponding gene (Moreira and Holmberg, 1999). Factors belonging to the *ISWI* class of remodelers can repress gene expression by positioning nucleosomes at critical promoter sites of the *POT1* and *REC104* genes (Fazzio and Tsukiyama, 2003).

These examples illustrate how nucleosome remodelers repress gene transcription by either translocating nucleosomes in *cis*, or by rebuilding repressive nucleosome structures using a histone source in *trans*.

From the data we collected on the mechanism of *PHO5* repression using the histone double-tag strain, it remains formally possible that MYC-tagged nucleosomes that are associated with the DNA template are exchanged by FLAG-histones from the soluble histone pool in the process of sliding along the DNA. However, I think this is unlikely for the following reason. The original concept of nucleosome sliding, as it is catalyzed for example by *ISWI* remodeling machines, implies that the histone octamer remains intact (Eberharter and Becker, 2004).

Nucleosomes are repositioned with respect to the underlying DNA, but no disassembly with concomitant histone transfer is observed (Längst and Becker, 2001). A recent study reports the involvement of the histone H2A/H2B chaperone Nap1p in assisting nucleosome sliding (Park et al., 2005). However, even though sliding in some cases may be accompanied by the loss of H2A/H2B dimers, the integrity of the H3/H4 tetramer is probably maintained. As in this study I look at H3-, and not H2A/H2B- incorporation from a source in *trans*, the data can hardly be reconciled with a sliding mechanism.

Finally, there is an alternative machinery that has the capacity of remodeling nucleosomes and that could theoretically account for the replacement of *PHO5* promoter nucleosomes from a source in *trans*. Proteins like e.g. components of the FACT complex characteristically restore chromatin in the wake of the transcribing RNA polymerase (Belotserkovskaya and Reinberg, 2004). So far, none of these mutants involved in transcriptional elongation have been analyzed for defects in *PHO* promoter reassembly. To date there is no evidence for transcription through the *PHO5* promoter. However, this has been reported for many other genes (Johnson et al., 2005). The possibility that the transcriptional machinery is needed for the opposite effect, namely the repression of a gene, seems somewhat counter-intuitive to me. Moreover, the transition of inactive *PHO5* promoter chromatin to the active state is independent of transcription, as it can also occur in a TATA-less promoter (Fascher et al., 1993). The same principle holds probably true also for the opposite process, as in the TATA-mutant strain repressive chromatin can be rebuilt at the *PHO5* promoter. Whether the kinetics of promoter closure are affected in transcriptional elongation mutants, e.g. *pob3* or *spt16*, remains subject of further investigations.

4. *PHO* promoter reassembly is replication-independent

First evidence that repression of the *PHO5* gene could take place independently of replication stems from an early study in our laboratory (Schmid et al., 1992). By nuclease accessibility analysis Schmid et al. demonstrated that the chromatin transition to the inactive state of the *PHO5* promoter occurs in the absence of cell division. It remained unclear whether replication had an influence on the speed with which the promoter was reassembled, as promoter chromatin was analyzed several hours after the shift to repressive conditions. The initial

experiment of this study, where *PHO5* mRNA levels were determined (Fig. 7, page 29) illustrated that *PHO* repression occured with fast kinetics and is completed within 30 minutes. In light of this result, the role of replication in *PHO5* promoter reassembly had to be reinvestigated by performing kinetics of repression also at earlier timepoints after readdition of phosphate.

As was previously discussed, phosphate starved yeast cultures arrest in the G1-phase due to the nutrient limitating conditions. When following promoter closure in such growth-arrested cells by ChIP, I find that *PHO* repression is accomplished within a strikingly short period of time (30-40 minutes). Regardless of which antibody was used, either against the C-terminus of histones H3/H4, or against the MYC-/FLAG-epitope, the incorporation of histones at the closing *PHO* promoters was essentially the same. (Figs. 10, 12, 13, pages 33, 36, 37 respectively). This observation was true for all *PHO* promoters we analyzed, *PHO5*, *PHO8* and *PHO84* (Figs. 23, 24, pages 53 and 54 respectively).

The time span of 30-40 minutes is relatively short when compared to the time a yeast cell requires to duplicate its genome. A typical cell cycle of the histone double tag strain USY6 is completed after about 120 minutes (data not shown). This is slightly longer than the doubling time of a wildtype yeast strain, which is about 90 minutes. I ascribe the difference to the fact that growth in the double tag strain is mildly impaired due to the existence of solely tagged versions of histone H3. Similar observations concerning the prolonged doubling time of yeast strains carrying tagged histone versions have also been made in other studies (Alain Verreault, personal communication).

The rapid kinetics of *PHO* promoter closure indicate that cell division is not a prerequisite for *PHO* repression. The short time period during which the *PHO* genes are shut off does not allow for one round of replication. In the case of *PHO5*, this finding is expected and in agreement with the results from the article by Schmid et al. Here, I also show that *PHO8* and *PHO84* accomplish the transition to the inactive promoter state in the absence of replication.

The mechanism of packaging promoter regions into chromatin upon changes in growth conditions obviously facilitates rapid repression of the corresponding gene. In case of the *PHO* genes, their repression is accomplished so fast that it constitutes a short-term repressional mechanism relative to the well-studied long-term repressive mechanisms as e.g. silencing of heterochromatin. This means of switching off genes must be particulary important for a unicellular organism like yeast, as it ensures the competence to promptly

respond to varying environmental conditions, e.g. the limitation of nutrients in the medium. There are other examples for such fast responding repressive mechanisms in yeast. Transcriptional repression of the *GAL* genes upon the shift to glucose-containing medium is also completed within a very short time window, illustrating its replication-independence (Bash and Lohr, 2001). Due to the compact genome organization of the unicellular eukaryote *S. cerevisiae*, the majority of its genome is actively transcribed with only a minor portion being maintained in a silent state, e.g. the telomeres, the centromers, the rDNA repeats and the mating type loci (Moazed et al., 2004; Perrod and Gasser, 2003). Mechanisms other than short-term repression contribute to the maintenance of the silenced heterochromatic regions in yeast. The starting point for the establishment of heterochromatin in *S. cerevisiae* are the Sir (Silencing information regulator) proteins binding to silencer elements on the DNA (Rusche et al., 2003). Subsequently, histone modifying activities (e.g. histone acetylases and/or histone methylases) play an important role in this process, as they mark nucleosomes for silencing and evoke preferential binding of the Sir proteins to such modified nucleosomes, which then leads to the spreading of heterochromatin (Suka et al., 2002). Replication appears to be required for the inheritance of the heterochromatic domain (Miller and Nasmyth, 1984), and multiple rounds of cell divisions are required to establish a fully silenced domain (Katan-Khaykovich and Struhl, 2005). This picture of maintaining and propagating silenced chromatin states by long-term repression is analogous to what is observed in higher eukaryotes (Lande-Diner and Cedar, 2005).

5. Histone chaperones are involved in the rapid nucleosome reassembly at the *PHO5* promoter

Histone chaperones are key players in the process of targeting histones to sites of chromatin assembly. Examples for physiologically relevant histone chaperones in *S. cerevisiae* are Asf1p, Cac1p, Cac2p, Cac3p, Nap1p and the Hir proteins (Gunjan et al., 2005). The classification of these chaperones occurs based on their preferred substrate specificity and their dependency on replication. Nap1p exhibits a distinct specificity for histones H2A and H2B and also promotes the import of its substrate histones into the nucleus (Mosammaparast et al., 2002). In contrast to the H2A-H2B chaperone Nap1p, the CAF1 complex and Asf1p

preferentially catalyze the assembly of histones H3 and H4 into nucleosomes (Tyler, 2002). Concerning their roles in the two major assembly pathways, CAF1 has been shown to be implicated in the replication-coupled branch of chromatin assembly (Krude and Keller, 2001). A potential role for the Hir proteins in the second branch, namely the replication-independent chromatin assembly, is suggested by two lines of evidence. Firstly, the human homologue of the yeast Hir proteins, HIRA, is implicated in the replication-independent chromatin assembly (Tagami et al., 2004). Secondly, disrupting the *HIR* genes in yeast leads to defects in the chromatin assembly at certain regions of the yeast genome (Kaufman et al., 1998). Asf1p is presumed to be the factor delivering histones H3 and H4 for both pathways and therefore takes part in both pathways (Mello and Almouzni, 2001; Tagami et al., 2004).

An interesting question in the context of *PHO5* promoter reassembly was if any the known histone chaperones can assist the loading of nucleosomes. Therefore, all the above described chaperones have been analyzed for their impact on *PHO5* promoter closure.

The result shown in Fig. 18, page 45, illustrates that only a subset of chaperones is important for rapid *PHO5* promoter reassembly upon repression. As was judged by ChIP, the deletion of the *NAP1* or *CAC1* gene did not affect *PHO5* repression (Fig. 18B and C, page 45). The disruption of *HIR2* or *HIR3* did also not result in delayed kinetics of *PHO5* repression. This was determined by the analysis of *PHO5* mRNA levels (Fig. 19, page 46).

On the other hand, yeast strains that lacked either Asf1p or Hir1p displayed a pronounced kinetic delay in histone H3 incorporation during *PHO5* promoter reassembly (Fig. 18A, page 45). Both chaperones serve important functions in the process of replication-independent chromatin assembly. In this regard, the outcome of our ChIP analysis reconfirms previous results that *PHO5* reassembly occurs indeed independently of replication (Schmid et al., 1992). This notion is further supported by the result that histone chaperones that are typically active during the S-phase of the cell cycle (e.g. Cac1p) behave like wildtype strains concerning *PHO5* repression (Fig. 18B, page 45).

I failed to identify a single chaperone or remodeler that is essential for *PHO5* promoter reassembly. Even combining various deletions in chaperone genes with deletions in remodeler genes (e.g. *snf2 asf1*) did not lead to a synthetic phenotype (see Fig. 20B, page 48). This suggests that Snf2p and Asf1p act in the same pathway. Since *PHO5* repression is not completely abolished in the *snf2* and *asf1* deletion strains, redundancy exists both on the level of the remodeler as well as on the level of the histone chaperone. Apparently an alternative

trans-acting pathway can compensate for the loss of Snf2p and Asf1p. For example the abundant remodeling complex RSC might cooperate with an alternative histone chaperone. Conceivably, reassembly of *PHO5* promoter chromatin may not only require H3/H4 histone chaperones, but also H2A/H2B chaperones. The only chaperone with H2A/H2B specificity that was tested was Nap1p. Deletion of *NAP1* did not have any effect on *PHO5* repression indicating that there are other redundant factors dealing with H2A/H2B deposition.

6. Are histone chaperones limiting in our system?

As newly synthesized histones are aggregation prone and probably not stable, they need acceptor molecules. Presumably a crucial function of histone chaperones is to stabilize histones in the soluble histone pool. Histone protein levels in *S. cerevisiae* are tightly regulated. There is evidence for a Rad53-dependent mechanism that can, if necessary, degrade excess histones to prevent an imbalance between DNA and histone synthesis (Gunjan and Verreault, 2003). The authors of this study speculate that possibly only free histones are degraded, whereas histones associated with histone chaperones may be protected from degradation.

Additional mechanisms exist dealing with the regulation of levels of active histone chaperone complex in response to increased histone levels. A recent study provides evidence that human cells are able to buffer their pool of excess S phase histones with the help of the chaperone Asf1 (Groth et al., 2005). Apparently Asf1 exists in two forms, an inactive, histone-free form and an active multi-chaperone complex together with histones. Under conditions of replicational stress the majority of Asf1 is mobilized into the histone-containing active complex. In this way the cell makes sure of an "active" pool of histones being immediately available for incorporation upon e.g. DNA repair (Groth et al., 2005).

It is unclear whether the availability of histone chaperones in our system is limiting. In contrast to MYC-H3 expression, the expression of the FLAG-histone is driven by the *GAL*-promoter instead of the wildtype H3-H4 promoter. The induced changes of the histone H3 protein levels in turn might influence the expression of the MYC-H3 gene. I can not rule out that histone levels in our system may be elevated compared to physiological conditions. In any case, most probably only those FLAG-histones are physiologically relevant which are

complexed with a histone chaperone, whereas non-complexed histones are probably prone to degradation.

Concerning the size of the soluble histone pool little is known so far. In the course of this work it became clear that the histone pool in our system does not exclusively contain FLAG-histones, but also MYC-histones. I carried out the competition experiment shown in Fig.15 (page 40) and observed 50% less MYC-H3 incorporation in the presence of FLAG-H3. The extent of FLAG-H3 and MYC-H3 deposition at the -2 nucleosome upon repression may reflect their respective relative concentrations in the soluble histone pool. Accordingly, equal amounts of FLAG-H3 : MYC-H3 incorporation at the -2 nucleosome argue for a 1 : 1 ratio of FLAG-H3 : MYC-H3 in the soluble histone pool. It could be that newly synthesized FLAG-H3 is preferentially degraded, as the cellular chaperone concentration and / or the competition of FLAG-H3 with MYC-H3 for binding to a histone chaperone is not sufficient to provide all FLAG-histones with acceptor molecules. In contrast to FLAG-H3, soluble MYC-H3 originating from the last round of replication could be preferentially protected from degradation as it still exists in an active chaperone complex. This could explain the observed 1 : 1 ratio of “active” FLAG-H3 to MYC-H3, even though the FLAG-histone expression is under the control of the *GAL1/10* promoter. This promoter is probably stronger than the endogenous H3-H4 promoter.

7. What could be the machinery that reassembles yeast promoter regions?

A yet unresolved issue is the question whether the reassembly of the *PHO5* promoter upon repression is achieved via a targeted mechanism. As one possibility I speculate that chromatin assembly occurs constitutively and genome-wide by default, i.e. without locus-specific targeting. There may be some kind of enzymatic machinery scanning the chromosomes for loss of nucleosomes. At certain underassembled regions as e.g. at active promoters, the action of transcriptional activators and the transcriptional apparatus may prevent chromatin assembly by such a "default” machinery to keep these regions actively nucleosome-free. Once the gene is repressed again and the counteracting factors have left the promoter, the "default" chromatin assembly machinery may reload nucleosomes onto the DNA and restore chromatin structure in this region.

In the case of *PHO5*, the addition of phosphate to the medium leads to the dissociation of the transcriptional activator Pho4p from its two binding sites at the *PHO5* promoter and to the shutdown of transcription (Lenburg and O´Shea, 1996; Svaren and Hörz, 1997). This could enable the "default" chromatin assembly machinery to restore *PHO5* promoter chromatin.
Such a scenario is somewhat reminiscent to the situation in which nucleosomes are reloaded in the aftermath of RNA polymerases. But in contrast to such a transcription-mediated mechanism, the reassembly of underassembled promoter regions in the yeast genome probably does not require the passage of polymerases. Nonetheless it is conceivable that other protein complexes may travel on the chromatin template and detect nucleosome loss during this process.
In an alternative model chromatin reassembly at the *PHO5* promoter could be mediated by factors that have been involved in the opening of the chromatin structure and that are still bound in the vicinity of the promoter via a Pho4 independent mechanism, e.g. via binding of the bromodomain of SWI/SNF to acetylated histone tails (Hassan et al., 2002) (depicted in Fig. 28).
Such a model is supported by the following observations: Previously we and others have found that SWI/SNF is involved in opening of the *PHO5* promoter and recently, Snf2p has been shown to be physically associated with the promoter region under activating conditions (Dhasarathy and Kladde, 2005; Neef and Kladde, 2003). Biochemical and genetic analyses from *Drosophila* provide evidence for an interaction between the SWI/SNF homologue Brahma and the histone chaperone Asf1 (Moshkin et al., 2002). It is therefore possible that Snf2p is indeed recruiting Asf1p (and maybe other factors) to the closing *PHO5* promoter in order to reload nucleosomes onto the hypersensitive site upon repression. Whether Asf1p is physically associated with the *PHO5* promoter region remains to be investigated. Adkins and coworkers tried to chromatin immunoprecipitate six differently tagged versions of Asf1p at the *PHO5* promoter (Adkins et al., 2004). However, they failed to detect binding of this chaperone to the promoter region and to any other region. This could be due to a transient or unstable interaction of Asf1p with the promoter DNA. Alternatively, Asf1p may not bind chromatin directly, but rather indirectly via other factors like chromatin remodeling factors. Such an interaction could impossibly be unraveled by the DNA-protein crosslinker formaldehyde, but rather would require the use of protein-protein crosslinking agents, like DMA (dimethyladipinate).

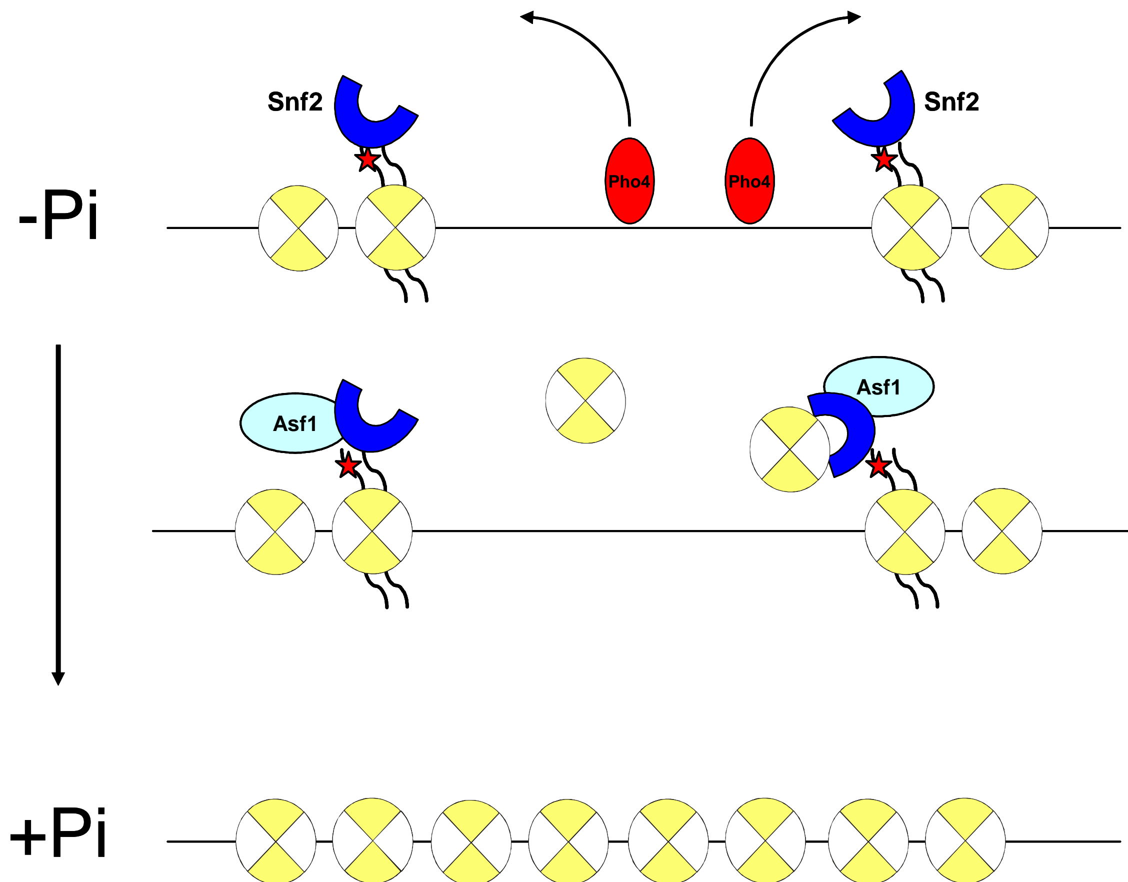


Figure 28. Potential model illustrating how histones may be targeted to the *PHO5* promoter upon repression.
Upon the shift to repressive conditions, the transactivator Pho4 dissociates from the *PHO5* promoter region. The remodelling factor Snf2 stays transiently tethered to chromatin in the vicinity of the promoter via its interaction with acetylated histone tails. There Snf2 recruits Asf1 (and possibly other factors) in order to rebuild *PHO5* promoter nucleosomes.

8. Induction of *PHO5* in an *asf1* strain is strictly dependent on the phosphate concentration of the medium

Many of the cofactors implicated in the activation of the *PHO5* and *PHO8* genes belong to classes of enzymes which specifically act on a chromatin substrate (Gcn5p, Snf2p) (Barbaric et al., 2001; Neef and Kladde, 2003). By the same token, early experiments of this study concentrated on the influence of histone chaperones on *PHO5* induction. I found that among the candidates tested, a yeast strain disrupted for the histone chaperone gene *ASF1* displayed a marked delay in the synthesis of the *PHO5* gene product, especially at the early timepoints

after the shift to phosphate-free growth conditions (Fig. 25A, page 57). Interestingly, by slightly increasing the phosphate concentration in the *PHO* activating medium, this kinetic delay can be shifted to a complete inability to activate the *PHO5* gene in an *asf1* strain (Fig. 25B, page 57). Similar observations have been made in the Tyler laboratory. There, the authors find an *asf1* strain to be totally deficient in disassembling chromatin at the *PHO5* and *PHO8* promoters upon induction (Adkins et al., 2004). Their experimental setup involved the induction of the *PHO* genes with medium that is chemically depleted for phosphate. After the depletion some residual phosphate is left over in the growth medium. This consequently prevents the activation of *PHO5* and *PHO8* altogether. Conversely, the use of no phosphate medium brings about *PHO5* activation in the *asf1* strain, albeit with slower induction kinetics. When our laboratory monitored histone loss in an *asf1* strain by ChIP, we found the rate of histone eviction to be decreased at the *PHO5* and the *PHO8* promoters upon activation (Korber et al., 2006). After prolonged induction however, chromatin remodeling proceeds in an *asf1* strain as in a wildtype and results in the complete opening of *PHO5* and *PHO8* promoter chromatin.

Taken together, the data indicate that Asf1p plays a substantial role in the activation of the *PHO5* and the *PHO8* genes. However, it is not essential for chromatin disassembly under maximally inducing conditions, i.e. in phosphate-free medium. This idea is favoured by data from a high-throughput screen for novel factors which are important for *PHO5* expression under no phosphate conditions. Huang and O´Shea thereby identified nine novel genes that are implicated in *PHO5* activation, with *ASF1* not being among them (Huang and O'Shea, 2005).

The dependency of the *PHO* genes on cofactors (e.g. histone chaperones) correlates with the nature of the activating conditions. Dhasarathy and Kladde reported that submaximally inducing conditions (low phosphate instead of no phosphate) lead to a stronger dependency on the coactivators Snf2p and Gcn5p (Dhasarathy and Kladde, 2005).

These results clearly illustrate the contribution of Asf1p to the disassembly of promoter chromatin as it is exemplified by the *PHO5* and *PHO8* genes. Most likely this function of the histone chaperone is not restricted to the reported promoters but rather a phenomenon that can be observed genome-wide (Adkins and Tyler, 2004; Ramey et al., 2004).

9. Outlook

The histone double-tag strategy provides us with a tool to distinguish between histones originating from the chromatin fraction and histones arising from the soluble histone pool. Of course this novel method might be used in various ways.

One potential application could be to determine in which regions of the yeast genome replication-independent nucleosome assembly occurs. By using synchronization procedures that abolish replication (e.g. arrest in G1 with α-factor), chromosomal loci can be identified that are subject to histone deposition in the course of transcription or during DNA repair.

Moreover, analogous to the generation of the histone double tag strain USY6, yeast strains could be created that harbour galactose-inducible, FLAG-tagged versions of various histone variants, e.g. H2AZ. Inducing the expression of these tagged variants outside of S-phase in combination with ChIP experiments could allow us to investigate in which regions of the genome these variants will become incorporated. Alternatively, the *PHO* promoters could be loaded with specific histone variants, like H2AZ, in the course of their repression. In a next step it could be asked whether such packaged promoters can be opened up again. In this way we could gain novel insights into the functional roles of specific histone variants in the configuration of either repressive or activate chromatin domains.

V. Summary

In previous studies it has been demonstrated that histones are cleared from the *PHO5* and *PHO8* promoters upon activation. The mechanism accounting for this loss of histones has been shown to operate in a *trans* manner. In the course of rerepression, the inactive chromatin structure of the *PHO5* and *PHO8* promoters is restored. An open question remaining to be addressed was whether the histones required for rebuilding the inactive promoters are translocated to the promoter region by a sliding mechanism or by the de-novo synthesis of nucleosomes using histones from the soluble histone pool.
The goal of this study has therefore been to elucidate the mechanisms responsible for rebuilding nucleosomes at the *PHO5* promoter upon rerepression.
In this work, I could unambiguously show that histones are incorporated at the *PHO5* promoter upon repression. Regarding the source of these histones, I provide evidence that a significant fraction of the deposited histones originate from a soluble histone pool, i.e. a histone source in *trans*. Promoter closure occurs with strikingly rapid kinetics and is independent of replication. In agreement with the finding that *PHO5* repression does not require cell division, I found that histone chaperones which are associated with replication-independent nucleosome assembly are important for rapid *PHO5* promoter closure. Strains deleted for histone chaperones involved in replication-dependent nucleosome assembly did not exhibit any defect in promoter closure. Other factors contributing to rapid *PHO5* repression turned out to be nucleosome remodelers, whose characteristic mode of action is chromatin assembly in *trans.* Nucleosome remodeling mutants typically catalyzing nucleosome movements in *cis* are not implicated in *PHO5* promoter reassembly. The phenomenon of *trans*-deposition of histones upon repression is not restricted to the *PHO5* promoter but is also found at two other phosphate regulated promoters, *PHO8* and *PHO84*. By its rapid mode of action, this mechanism contributes to efficiently shutting off transcription. This might also hold true for other yeast genes.
In the second part of this work I present results that indicate a role for the histone chaperone Asf1p in the activation of the *PHO5* gene. Interestingly, the induction of *PHO5* in an *asf1* mutant is dependent on the phosphate concentration of the growth medium. Full induction

occurs only when the medium is completely free of phosphate. The abundance of even trace amounts of phosphate precludes *PHO5* activation altogether.

VI. References

Adkins,M.W., Howar,S.R., and Tyler,J.K. (2004). Chromatin Disassembly Mediated by the Histone Chaperone Asf1 Is Essential for Transcriptional Activation of the Yeast PHO5 and PHO8 Genes. Mol. Cell *14*, 657-666.

Adkins,M.W. and Tyler,J.K. (2004). The histone chaperone Asf1p mediates global chromatin disassembly in vivo. J. Biol. Chem. *279*, 52069-52074.

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

Akey,C.W. and Luger,K. (2003). Histone chaperones and nucleosome assembly. Curr. Opin. Struct. Biol. *13*, 6-14.

Almer,A., Rudolph,H., Hinnen,A., and Hörz,W. (1986). Removal of positioned nucleosomes from the yeast *PHO5* promoter upon *PHO5* induction releases additional upstream activating DNA elements. EMBO J. *5*, 2689-2696.

Almouzni,G. and Mechali,M. (1988). Assembly of spaced chromatin involvement of ATP and DNA topoisomerase activity. EMBO J. *7*, 4355-4365.

Barbaric,S., Münsterkötter,M., Goding,C., and Hörz,W. (1998). Cooperative Pho2-Pho4 interactions at the *PHO5* promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol. Cell. Biol. *18*, 2629-2639.

Barbaric,S., Walker,J., Schmid,A., Svejstrup,J.Q., and Hörz,W. (2001). Increasing the rate of chromatin remodeling and gene activation - a novel role for the histone acetyltransferase Gcn5. EMBO J. *20*, 4944-4951.

Bash,R. and Lohr,D. (2001). Yeast chromatin structure and regulation of GAL gene expression. Prog. Nucleic Acid Res. Mol. Biol. *65*, 197-259.

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

Belotserkovskaya,R., Oh,S., Bondarenko,V.A., Orphanides,G., Studitsky,V.M., and Reinberg,D. (2003). FACT facilitates transcription-dependent nucleosome alteration. Science *301*, 1090-1093.

Belotserkovskaya,R. and Reinberg,D. (2004). Facts about FACT and transcript elongation through chromatin. Curr. Opin. Genet. Dev. *14*, 139-146.

Bernstein,B.E., Liu,C.L., Humphrey,E.L., Perlstein,E.O., and Schreiber,S.L. (2004). Global nucleosome occupancy in yeast. Genome Biol. *5*, R62.

Boeger,H., Griesenbeck,J., Strattan,J.S., and Kornberg,R.D. (2003). Nucleosomes unfold completely at a transcriptionally active promoter. Mol. Cell *11*, 1587-1598.

Boeger,H., Griesenbeck,J., Strattan,J.S., and Kornberg,R.D. (2004). Removal of promoter nucleosomes by disassembly rather than sliding in vivo. Mol. Cell *14*, 667-673.

Bottomley,M.J. (2004). Structures of protein domains that create or recognize histone modifications. EMBO Rep. *5*, 464-469.

Brehm,A., Tufteland,K.R., Aasland,R., and Becker,P.B. (2004). The many colours of chromodomains. Bioessays *26*, 133-140.

Bruno,M., Flaus,A., Stockdale,C., Rencurel,C., Ferreira,H., and Owen-Hughes,T. (2003). Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. Mol. Cell *12*, 1599-1606.

Cairns,B.R. (2005). Chromatin remodeling complexes: strength in diversity, precision through specialization. Curr. Opin. Genet. Dev. *15*, 185-190.

Cairns,B.R., Lorch,Y., Li,Y., Zhang,M.C., Lacomis,L., Erdjument-Bromage,H., Tempst,P., Du,J., Laurent,B., and Kornberg,R.D. (1996). RSC, an essential, abundant chromatin-remodeling complex. Cell *87*, 1249-1260.

Cairns,B.R., Schlichter,A., Erdjument-Bromage,H., Tempst,P., Kornberg,R.D., and Winston,F. (1999). Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. Mol. Cell *4*, 715-723.

Chadwick,B.P. and Willard,H.F. (2002). Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome. J. Cell Biol. *157*, 1113-1123.

Cross,S.L. and Smith,M.M. (1988). Comparison of the structure and cell cycle expression of mRNAs encoded by two histone H3-H4 loci in Saccharomyces cerevisiae. Mol. Cell. Biol. *8*, 945-954.

de,l.C., X, Lois,S., Sanchez-Molina,S., and Martinez-Balbas,M.A. (2005). Do protein motifs read the histone code? Bioessays *27*, 164-175.

Dhasarathy,A. and Kladde,M.P. (2005). Promoter occupancy is a major determinant of chromatin remodeling enzyme requirements. Mol. Cell. Biol. *25*, 2698-2707.

Eberharter,A. and Becker,P.B. (2004). ATP-dependent nucleosome remodelling: factors and functions. J. Cell Sci. *117*, 3707-3711.

Eberharter,A., Ferreira,R., and Becker,P. (2005). Dynamic chromatin: concerted nucleosome remodelling and acetylation. Biol. Chem. *386*, 745-751.

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

Everid,A.C., Small,J.V., and Davies,H.G. (1970). Electron-microscope observations on the structure of condensed chromatin: evidence for orderly arrays of unit threads on the surface of chicken erythrocyte nuclei. J. Cell Sci. *7*, 35-48.

Fascher,K.D., Schmitz,J., and Hörz,W. (1993). Structural and functional requirements for the chromatin transition at the *PHO5* promoter in *Saccharomyces cerevisiae* upon *PHO5* activation. J. Mol. Biol. *231*, 658-667.

Fazzio,T.G., Kooperberg,C., Goldmark,J.P., Neal,C., Basom,R., Delrow,J., and Tsukiyama,T. (2001). Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. Mol. Cell. Biol. *21*, 6450-6460.

Fazzio,T.G. and Tsukiyama,T. (2003). Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. Mol. Cell *12*, 1333-1340.

Formosa,T., Ruone,S., Adams,M.D., Olsen,A.E., Eriksson,P., Yu,Y., Rhoades,A.R., Kaufman,P.D., and Stillman,D.J. (2002). Defects in SPT16 or POB3 (yFACT) in Saccharomyces cerevisiae cause dependence on the Hir/Hpc pathway: polymerase passage may degrade chromatin structure. Genetics *162*, 1557-1571.

Forrester,W.C., Thompson,C., Elder,J.T., and Groudine,M. (1986). A developmentally stable chromatin structure in the human beta-globin gene cluster. Proc. Natl. Acad. Sci. U. S. A *83*, 1359-1363.

Franco,A.A., Lam,W.M., Burgers,P.M., and Kaufman,P.D. (2005). Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C. Genes Dev. *19*, 1365-1375.

Fyodorov,D.V., Blower,M.D., Karpen,G.H., and Kadonaga,J.T. (2004). Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. Genes Dev. *18*, 170-183.

Gietz,D., St.Jean,A., Woods,R.A., and Schiestl,R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. *20*, 1425.

Goldmark,J.P., Fazzio,T.G., Estep,P.W., Church,G.M., and Tsukiyama,T. (2000). The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. Cell *103*, 423-433.

Green,C.M. and Almouzni,G. (2003). Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. EMBO J. *22*, 5163-5174.

Green,E.M., Antczak,A.J., Bailey,A.O., Franco,A.A., Wu,K.J., Yates,J.R., III, and Kaufman,P.D. (2005). Replication-independent histone deposition by the HIR complex and Asf1. Curr. Biol. *15*, 2044-2049.

Groth,A., Ray-Gallet,D., Quivy,J.P., Lukas,J., Bartek,J., and Almouzni,G. (2005). Human Asf1 Regulates the Flow of S Phase Histones during Replicational Stress. Mol. Cell *17*, 301-311.

Gunjan,A., Paik,J., and Verreault,A. (2005). Regulation of histone synthesis and nucleosome assembly. Biochimie *87*, 625-635.

Gunjan,A. and Verreault,A. (2003). A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. Cell *115*, 537-549.

Haguenauer-Tsapis,R. and Hinnen,A. (1984). A deletion that includes the signal peptidase cleavage site impairs processing, glycosylation, and secretion of cell surface yeast acid phosphatase. Mol. Cell. Biol. *4*, 2668-2675.

Han,M., Kim,U.J., Kayne,P., and Grunstein,M. (1988). Depletion of histone H4 and nucleosomes activates the *PHO5* gene in *Saccharomyces cerevisiae*. EMBO J. *7*, 2221-2228.

Hansen,J.C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. Annu. Rev. Biophys. Biomol. Struct. *31*, 361-392.

Hassan,A.H., Neely,K.E., and Workman,J.L. (2001). Histone acetyltransferase complexes stabilize Swi/Snf binding to promoter nucleosomes. Cell *104*, 817-827.

Hassan,A.H., Prochasson,P., Neely,K.E., Galasinski,S.C., Chandy,M., Carrozza,M.J., and Workman,J.L. (2002). Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell *111*, 369-379.

Haushalter,K.A. and Kadonaga,J.T. (2003). Chromatin assembly by DNA-translocating motors. Nat. Rev. Mol. Cell Biol. *4*, 613-620.

Hayes,J.J. and Hansen,J.C. (2001). Nucleosomes and the chromatin fiber. Curr. Opin. Genet. Dev. *11*, 124-129.

Henikoff,S. and Ahmad,K. (2005). Assembly of variant histones into chromatin. Annu. Rev. Cell Dev. Biol. *21*, 133-153.

Henikoff,S., Furuyama,T., and Ahmad,K. (2004). Histone variants, nucleosome assembly and epigenetic inheritance. Trends Genet. *20*, 320-326.

Hoek,M. and Stillman,B. (2003). Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. Proc. Natl. Acad. Sci. U. S. A *100*, 12183-12188.

Horn,P.J. and Peterson,C.L. (2002). Molecular biology. Chromatin higher order folding--wrapping up transcription. Science *297*, 1824-1827.

Huang,S. and O'Shea,E.K. (2005). A systematic high-throughput screen of a yeast deletion collection for mutants defective in PHO5 regulation. Genetics *169*, 1859-1871.

Ishimi,Y., Hirosumi,J., Sato,W., Sugasawa,K., Yokota,S., Hanaoka,F., and Yamada,M. (1984). Purification and initial characterization of a protein which facilitates assembly of nucleosome-like structure from mammalian cells. Eur. J. Biochem. *142*, 431-439.

Ishimi,Y. and Kikuchi,A. (1991). Identification and molecular cloning of yeast homolog of nucleosome assembly protein I which facilitates nucleosome assembly in vitro. J. Biol. Chem. *266*, 7025-7029.

Ito,T., Bulger,M., Pazin,M.J., Kobayashi,R., and Kadonaga,J.T. (1997). ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell *90*, 145-155.

Ito,T., Levenstein,M.E., Fyodorov,D.V., Kutach,A.K., Kobayashi,R., and Kadonaga,J.T. (1999). ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. Genes Dev. *13*, 1529-1539.

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

Johnson,J.M., Edwards,S., Shoemaker,D., and Schadt,E.E. (2005). Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments. Trends Genet. *21*, 93-102.

Kaffman,A., Herskowitz,I., Tjian,R., and O'Shea,E.K. (1994). Phosphorylation of the transcription factor Pho4 by a cyclin-CDK complex, Pho80-Pho85. Science *263*, 1153-1156.

Kaffman,A., Rank,N.M., O'Neill,E.M., Huang,L.S., and O'Shea,E.K. (1998a). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-486.

Kaffman,A., Rank,N.M., and Oshea,E.K. (1998b). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Psel/Kapl21. Genes Dev. *12*, 2673-2683.

Kamakaka,R.T. and Biggins,S. (2005). Histone variants: deviants? Genes Dev. *19*, 295-310.

Kaplan,C.D., Laprade,L., and Winston,F. (2003). Transcription elongation factors repress transcription initiation from cryptic sites. Science *301*, 1096-1099.

Karpov,V.L., Preobrazhenskaya,O.V., and Mirzabekov,A.D. (1984). Chromatin structure of hsp 70 genes, activated by heat shock: selective removal of histones from the coding region and their absence from the 5' region. Cell *36*, 423-431.

Katan-Khaykovich,Y. and Struhl,K. (2005). Heterochromatin formation involves changes in histone modifications over multiple cell generations. EMBO J. *24*, 2138-2149.

Kaufman,P.D., Cohen,J.L., and Osley,M.A. (1998). Hir proteins are required for position-dependent gene silencing in Saccharomyces cerevisiae in the absence of chromatin assembly factor I. Mol. Cell. Biol. *18*, 4793-4806.

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

Keene,M.A., Corces,V., Lowenhaupt,K., and Elgin,S.C. (1981). DNase I hypersensitive sites in Drosophila chromatin occur at the 5' ends of regions of transcription. Proc. Natl. Acad. Sci. U. S. A *78*, 143-146.

Khorasanizadeh,S. (2004). The nucleosome: from genomic organization to genomic regulation. Cell *116*, 259-272.

Kireeva,M.L., Walter,W., Tchernajenko,V., Bondarenko,V., Kashlev,M., and Studitsky,V.M. (2002). Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. Mol. Cell *9*, 541-552.

Kobor,M.S., Venkatasubrahmanyam,S., Meneghini,M.D., Gin,J.W., Jennings,J.L., Link,A.J., Madhani,H.D., and Rine,J. (2004). A Protein Complex Containing the Conserved Swi2/Snf2-Related ATPase Swr1p Deposits Histone Variant H2A.Z into Euchromatin. PLoS. Biol. *2*, E131.

Komeili,A. and O'Shea,E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science *284*, 977-980.

Korber,P., Barbaric,S., Luckenbach,T., Schmid,A., Schermer,U.J., Blaschke,D., and Horz,W. (2006). The histone chaperone Asf1 increases the rate of histone eviction at the yeast PHO5 and PHO8 promoters. J. Biol. Chem.

Korber,P., Luckenbach,T., Blaschke,D., and Horz,W. (2004). Evidence for Histone Eviction in trans upon Induction of the Yeast PHO5 Promoter. Mol. Cell. Biol. *24*, 10965-10974.

Kornberg,R.D. and Thomas,J.O. (1974). Chromatin structure; oligomers of the histones. Science *184*, 865-868.

Krogan,N.J., Keogh,M.C., Datta,N., Sawa,C., Ryan,O.W., Ding,H., Haw,R.A., Pootoolal,J., Tong,A., Canadien,V., Richards,D.P., Wu,X., Emili,A., Hughes,T.R., Buratowski,S., and Greenblatt,J.F. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol. Cell *12*, 1565-1576.

Krude,T. (1995). Chromatin - nucleosome assembly during DNA replication. Curr. Biol. *5*, 1232-1234.

Krude,T. and Keller,C. (2001). Chromatin assembly during S phase: contributions from histone deposition, DNA replication and the cell division cycle. Cell Mol. Life Sci. *58*, 665-672.

Kurdistani,S.K. and Grunstein,M. (2003). In vivo protein-protein and protein-DNA crosslinking for genomewide binding microarray. Methods *31*, 90-95.

Kusch,T., Florens,L., Macdonald,W.H., Swanson,S.K., Glaser,R.L., Yates,J.R., III, Abmayr,S.M., Washburn,M.P., and Workman,J.L. (2004). Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. Science *306*, 2084-2087.

Lande-Diner,L. and Cedar,H. (2005). Silence of the genes--mechanisms of long-term repression. Nat. Rev. Genet. *6*, 648-654.

Längst,G. and Becker,P.B. (2001). Nucleosome mobilization and positioning by ISWI-containing chromatin- remodeling factors. J. Cell Sci. *114*, 2561-2568.

Laskey,R.A., Mills,A.D., Philpott,A., Leno,G.H., Dilworth,S.M., and Dingwall,C. (1993). The role of nucleoplasmin in chromatin assembly and disassembly. Philos. Trans. R. Soc. Lond. [Biol]. *339*, 263-269.

Le,S., Davis,C., Konopka,J.B., and Sternglanz,R. (1997). Two new S-phase-specific genes from Saccharomyces cerevisiae. Yeast *13*, 1029-1042.

Lee,C.K., Shibata,Y., Rao,B., Strahl,B.D., and Lieb,J.D. (2004). Evidence for nucleosome depletion at active regulatory regions genome-wide. Nat. Genet. *36*, 900-905.

Lenburg,M.E. and O´Shea,E.K. (1996). Signaling phosphate starvation. Trends Biochem. Sci. *21*, 383-387.

Li,G., Levitus,M., Bustamante,C., and Widom,J. (2005). Rapid spontaneous accessibility of nucleosomal DNA. Nat. Struct. Mol. Biol. *12*, 46-53.

Linger,J.G. and Tyler,J.K. (2005). The yeast histone chaperone chromatin assembly factor 1 protects against double-strand DNA damaging agents. Genetics.

Logie,C. and Peterson,C.L. (1999). Purification and biochemical properties of yeast SWI/SNF complex. Methods Enzymol. *304*, 726-741.

Lorain,S., Quivy,J.P., MonierGavelle,F., Scamps,C., Lecluse,Y., Almouzni,G., and Lipinski,M. (1998). Core histones and HIRIP3, a novel histone-binding protein, directly interact with WD repeat protein HIRA. Mol. Cell. Biol. *18*, 5546-5556.

Lorch,Y., Maier-Davis,B., and Kornberg,R.D. (2006). Chromatin remodeling by nucleosome disassembly in vitro. Proc. Natl. Acad. Sci. U. S. A *103*, 3090-3093.

Lorch,Y., Zhang,M., and Kornberg,R.D. (1999a). Histone octamer transfer by a chromatin-remodeling complex. Cell *96*, 389-392.

Loyola,A. and Almouzni,G. (2004). Histone chaperones, a supporting role in the limelight. Biochim. Biophys. Acta *1677*, 3-11.

Loyola,A., LeRoy,G., Wang,Y.H., and Reinberg,D. (2001). Reconstitution of recombinant chromatin establishes a requirement for histone-tail modifications during chromatin assembly and transcription. Genes Dev. *15*, 2837-2851.

Luger,K. and Hansen,J.C. (2005). Nucleosome and chromatin fiber dynamics. Curr. Opin. Struct. Biol. *15*, 188-196.

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

Lusser,A. and Kadonaga,J.T. (2003). Chromatin remodeling by ATP-dependent molecular machines. Bioessays *25*, 1192-1200.

Lusser,A., Urwin,D.L., and Kadonaga,J.T. (2005). Distinct activities of CHD1 and ACF in ATP-dependent chromatin assembly. Nat. Struct. Mol. Biol. *12*, 160-166.

Magnaghi,P., Roberts,C., Lorain,S., Lipinski,M., and Scambler,P.J. (1998). HIRA, a mammalian homologue of Saccharomyces cerevisiae transcriptional co-repressors, interacts with Pax3. Nat. Genet. *20*, 74-77.

Martini,E., Roche,D.M., Marheineke,K., Verreault,A., and Almouzni,G. (1998). Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. J. Cell Biol. *143*, 563-575.

McGhee,J.D., Wood,W.I., Dolan,M., Engel,J.D., and Felsenfeld,G. (1981). A 200 base pair region at the 5' end of the chicken adult beta-globin gene is accessible to nuclease digestion. Cell *27*, 45-55.

Mello,J.A. and Almouzni,G. (2001). The ins and outs of nucleosome assembly. Curr. Opin. Genet. Dev. *11*, 136-141.

Mello,J.A., Sillje,H.H., Roche,D.M., Kirschner,D.B., Nigg,E.A., and Almouzni,G. (2002). Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. EMBO Rep. *3*, 329-334.

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

Miller,A.M. and Nasmyth,K.A. (1984). Role of DNA replication in the repression of silent mating type loci in yeast. Nature *312*, 247-251.

Mizuguchi,G., Shen,X., Landry,J., Wu,W.H., Sen,S., and Wu,C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science *303*, 343-348.

Moazed,D., Rudner,A.D., Huang,J., Hoppe,G.J., and Tanny,J.C. (2004). A model for step-wise assembly of heterochromatin in yeast. Novartis. Found. Symp. *259*, 48-56.

Moggs,J.G., Grandi,P., Quivy,J.P., Jonsson,Z.O., Hubscher,U., Becker,P.B., and Almouzni,G. (2000). A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. Mol. Cell. Biol. *20*, 1206-1218.

Mohrmann,L. and Verrijzer,C.P. (2005). Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. Biochim. Biophys. Acta *1681*, 59-73.

Moreira,J.M. and Holmberg,S. (1999). Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling complex RSC. EMBO J. *18*, 2836-2844.

Morrison,A.J., Highland,J., Krogan,N.J., Arbel-Eden,A., Greenblatt,J.F., Haber,J.E., and Shen,X. (2004). INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. Cell *119*, 767-775.

Mosammaparast,N., Ewart,C.S., and Pemberton,L.F. (2002). A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B. EMBO J. *21*, 6527-6538.

Moshkin,Y.M., Armstrong,J.A., Maeda,R.K., Tamkun,J.W., Verrijzer,P., Kennison,J.A., and Karch,F. (2002). Histone chaperone ASF1 cooperates with the Brahma chromatin-remodelling machinery. Genes Dev. *16*, 2621-2626.

Myung,K., Pennaneach,V., Kats,E.S., and Kolodner,R.D. (2003). Saccharomyces cerevisiae chromatin-assembly factors that act during DNA replication function in the maintenance of genome stability. Proc. Natl. Acad. Sci. U. S. A *100*, 6640-6645.

Nabatiyan,A. and Krude,T. (2004). Silencing of chromatin assembly factor 1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. Mol. Cell. Biol. *24*, 2853-2862.

Nagaich,A.K. and Hager,G.L. (2004). UV laser cross-linking: a real-time assay to study dynamic protein/DNA interactions during chromatin remodeling. Sci. STKE. *2004*, l13.

Nakagawa,T., Bulger,M., Muramatsu,M., and Ito,T. (2001). Multistep chromatin assembly on supercoiled plasmid DNA by nucleosome assembly protein-1 and ATP-utilizing chromatin assembly and remodeling factor. J. Biol. Chem. *276*, 27384-27391.

Neef,D.W. and Kladde,M.P. (2003). Polyphosphate loss promotes SNF/SWI- and Gcn5-dependent mitotic induction of PHO5. Mol. Cell. Biol. *23*, 3788-3797.

Nourani,A., Utley,R.T., Allard,S., and Cote,J. (2004). Recruitment of the NuA4 complex poises the PHO5 promoter for chromatin remodeling and activation. EMBO J. *23*, 2597-2607.

Ogawa,N. and Oshima,Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in Saccharomyces cerevisiae. Mol. Cell. Biol. *10*, 2224-2236.

Olins,D.E. and Olins,A.L. (2003). Chromatin history: our view from the bridge. Nat. Rev. Mol. Cell Biol. *4*, 809-814.

Orphanides,G., LeRoy,G., Chang,C.H., Luse,D.S., and Reinberg,D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell *92*, 105-116.

Osley,M.A. (1991). The regulation of histone synthesis in the cell cycle. Annu. Rev. Biochem. *60*, 827-861.

Osley,M.A., Gould,J., Kim,S., Kane,M.Y., and Hereford,L. (1986). Identification of sequences in a yeast histone promoter involved in periodic transcription. Cell *45*, 537-544.

Osley,M.A. and Lycan,D. (1987). Trans-acting regulatory mutations that alter transcription of Saccharomyces cerevisiae histone genes. Mol. Cell. Biol. *7*, 4204-4210.

Owen-Hughes,T. (2003). Colworth memorial lecture. Pathways for remodelling chromatin. Biochem. Soc. Trans. *31*, 893-905.

Owen-Hughes,T., Utley,R.T., Côté,J., Peterson,C.L., and Workman,J.L. (1996). Persistent site-specific remodeling of a nucleosome array by transient action of the SWI/SNF complex. Science *273*, 513-516.

Palmer,D.K., O'Day,K., Trong,H.L., Charbonneau,H., and Margolis,R.L. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. Proc. Natl. Acad. Sci. U. S. A *88*, 3734-3738.

Park,Y.J., Chodaparambil,J.V., Bao,Y., McBryant,S.J., and Luger,K. (2005). Nucleosome Assembly Protein 1 Exchanges Histone H2A-H2B Dimers and Assists Nucleosome Sliding. J. Biol. Chem. *280*, 1817-1825.

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

Phelan,M.L., Schnitzler,G.R., and Kingston,R.E. (2000). Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases. Mol. Cell. Biol. *20*, 6380-6389.

Pidoux,A.L. and Allshire,R.C. (2004). Kinetochore and heterochromatin domains of the fission yeast centromere. Chromosome. Res. *12*, 521-534.

Prochasson,P., Florens,L., Swanson,S.K., Washburn,M.P., and Workman,J.L. (2005). The HIR corepressor complex binds to nucleosomes generating a distinct protein/DNA complex resistant to remodeling by SWI/SNF. Genes Dev. *19*, 2534-2539.

Quivy,J.P., Roche,D., Kirschner,D., Tagami,H., Nakatani,Y., and Almouzni,G. (2004). A CAF-1 dependent pool of HP1 during heterochromatin duplication. EMBO J. *23*, 3516-3526.

Raisner,R.M., Hartley,P.D., Meneghini,M.D., Bao,M.Z., Liu,C.L., Schreiber,S.L., Rando,O.J., and Madhani,H.D. (2005). Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. Cell *123*, 233-248.

Ramey,C.J., Howar,S., Adkins,M., Linger,J., Spicer,J., and Tyler,J.K. (2004). Activation of the DNA damage checkpoint in yeast lacking the histone chaperone anti-silencing function 1. Mol. Cell. Biol. *24*, 10313-10327.

Reinke,H. and Hörz,W. (2003). Histones are first hyperacetylated and then lose contact with the activated *PHO5* promoter. Mol. Cell *11*, 1599-1607.

Reinke,H. and Hörz,W. (2004). Anatomy of a hypersensitive site. Biochim. Biophys. Acta *1677*, 24-29.

Richard Foy,H. and Hager,G.L. (1987). Sequence-specific positioning of nucleosomes over the steroid- inducible MMTV promoter. EMBO J. *6*, 2321-2328.

Richmond,T.J. and Davey,C.A. (2003). The structure of DNA in the nucleosome core. Nature *423*, 145-150.

Ridgway,P. and Almouzni,G. (2000). CAF-1 and the inheritance of chromatin states: at the crossroads of DNA replication and repair. J. Cell Sci. *113 (Pt 15)*, 2647-2658.

Rusche,L.N., Kirchmaier,A.L., and Rine,J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. *72*, 481-516.

Schmid,A., Fascher,K.D., and Hörz,W. (1992). Nucleosome disruption at the yeast *PHO5* promoter upon *PHO5* induction occurs in the absence of DNA replication. Cell *71*, 853-864.

Schwabish,M.A. and Struhl,K. (2004). Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol. Cell. Biol. *24*, 10111-10117.

Sharp,J.A., Franco,A.A., Osley,M.A., and Kaufman,P.D. (2002). Chromatin assembly factor I and Hir proteins contribute to building functional kinetochores in S. cerevisiae. Genes Dev. *16*, 85-100.

Sherwood,P.W., Tsang,S.V., and Osley,M.A. (1993). Characterization of HIR1 and HIR2, two genes required for regulation of histone gene transcription in Saccharomyces cerevisiae. Mol. Cell. Biol. *13*, 28-38.

Shibahara,K. and Stillman,B. (1999). Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell *96*, 575-585.

Shintomi,K., Iwabuchi,M., Saeki,H., Ura,K., Kishimoto,T., and Ohsumi,K. (2005). Nucleosome assembly protein-1 is a linker histone chaperone in Xenopus eggs. Proc. Natl. Acad. Sci. U. S. A *102*, 8210-8215.

Singer,M.S., Kahana,A., Wolf,A.J., Meisinger,L.L., Peterson,S.E., Goggin,C., Mahowald,M., and Gottschling,D.E. (1998). Identification of high-copy disrupters of telomeric silencing in Saccharomyces cerevisiae. Genetics *150*, 613-632.

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

Smith,S. and Stillman,B. (1989). Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell *58*, 15-25.

Smith,S. and Stillman,B. (1991). Stepwise assembly of chromatin during DNA replication in vitro. EMBO J. *10*, 971-980.

Spector,M.S., Raff,A., DeSilva,H., Lee,K., and Osley,M.A. (1997). Hir1p and Hir2p function as transcriptional corepressors to regulate histone gene transcription in the Saccharomyces cerevisiae cell cycle. Mol. Cell. Biol. *17*, 545-552.

Stillman,B. (1986). Chromatin assembly during SV40 DNA replication in vitro. Cell *45*, 555-565.

Stockdale,C., Flaus,A., Ferreira,H., and Owen-Hughes,T. (2006). Analysis of nucleosome repositioning by yeast ISWI and CHD1 chromatin remodeling complexes. J. Biol. Chem.

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

Straka,C. and Hörz,W. (1991). A functional role for nucleosomes in the repression of a yeast promoter. EMBO J. *10*, 361-368.

Studitsky,V.M., Walter,W., Kireeva,M., Kashlev,M., and Felsenfeld,G. (2004). Chromatin remodeling by RNA polymerases. Trends Biochem. Sci. *29*, 127-135.

Sudarsanam,P., Iyer,V.R., Brown,P.O., and Winston,F. (2000). Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A *97*, 3364-3369.

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

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

Svaren,J. and Hörz,W. (1997). Transcription factors vs nucleosomes: Regulation of the *PHO5* promoter in yeast. Trends Biochem. Sci. *22*, 93-97.

Tagami,H., Ray-Gallet,D., Almouzni,G., and Nakatani,Y. (2004). Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell *116*, 51-61.

Thomas,G.H. and Elgin,S.C.R. (1988). Protein/DNA architecture of the DNase I hypersensitive region of the Drosophila hsp26 promoter. EMBO J. *7*, 2191-2201.

Tomschik,M., Zheng,H., van Holde,K., Zlatanova,J., and Leuba,S.H. (2005). Fast, long-range, reversible conformational fluctuations in nucleosomes revealed by single-pair fluorescence resonance energy transfer. Proc. Natl. Acad. Sci. U. S. A *102*, 3278-3283.

Tran,H.G., Steger,D.J., Iyer,V.R., and Johnson,A.D. (2000). The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. EMBO J. *19*, 2323-2331.

Truss,M., Bartsch,J., Schelbert,A., Hache,R.J.G., and Beato,M. (1995). Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter in vivo. EMBO J. *14*, 1737-1751.

Tsukuda,T., Fleming,A.B., Nickoloff,J.A., and Osley,M.A. (2005). Chromatin remodelling at a DNA double-strand break site in Saccharomyces cerevisiae. Nature *438*, 379-383.

Tuan,D., Solomon,W., Li,Q., and London,I.M. (1985). The "beta-like-globin" gene domain in human erythroid cells. Proc. Natl. Acad. Sci. U. S. A *82*, 6384-6388.

Tyler,J.K. (2002). Chromatin assembly. Cooperation between histone chaperones and ATP-dependent nucleosome remodeling machines. Eur. J. Biochem. *269*, 2268-2274.

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

Tyler,J.K., Collins,K.A., Prasad-Sinha,J., Amiott,E., Bulger,M., Harte,P.J., Kobayashi,R., and Kadonaga,J.T. (2001). Interaction between the Drosophila CAF-1 and ASF1 chromatin assembly factors. Mol. Cell. Biol. *21*, 6574-6584.

Umehara,T., Chimura,T., Ichikawa,N., and Horikoshi,M. (2002). Polyanionic stretch-deleted histone chaperone cia1/Asf1p is functional both in vivo and in vitro. Genes Cells *7*, 59-73.

van Attikum,H., Fritsch,O., Hohn,B., and Gasser,S.M. (2004). Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair. Cell *119*, 777-788.

Verreault,A., Kaufman,P.D., Kobayashi,R., and Stillman,B. (1996). Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. Cell *87*, 95-104.

Woodcock,C.L. and Dimitrov,S. (2001). Higher-order structure of chromatin and chromosomes. Curr. Opin. Genet. Dev. *11*, 130-135.

Wu,C. (1980). The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I. Nature *286*, 854-860.

Xu,H., Kim,U.J., Schuster,T., and Grunstein,M. (1992). Identification of a new set of cell cycle-regulatory genes that regulate S-phase transcription of histone genes in Saccharomyces cerevisiae. Mol. Cell. Biol. *12*, 5249-5259.

Ye,X., Franco,A.A., Santos,H., Nelson,D.M., Kaufman,P.D., and Adams,P.D. (2003). Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. Mol. Cell *11*, 341-351.

Yoon,H.W., Kim,M.C., Lee,S.Y., Hwang,I., Bahk,J.D., Hong,J.C., Ishimi,Y., and Cho,M.J. (1995). Molecular cloning and functional characterization of a cDNA encoding nucleosome assembly protein 1 (NAP-1) from soybean. Mol. Gen. Genet. *249*, 465-473.

Zhang,H., Roberts,D.N., and Cairns,B.R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell *123*, 219-231.

Zhang,W., Bone,J.R., Edmondson,D.G., Turner,B.M., and Roth,S.Y. (1998). Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase. EMBO J. *17*, 3155-3167.

VII. Appendix

1. List of abbreviations

ATP	adenosintriphosphate
bp	base pair(s)
BSA	bovine serum albumin
C-	carboxy-
Cdk	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
DNA	2'-desoxyribonucleic acid
DNase	desoxyribonuclease
EDTA	ethylendiaminetetraacetic acid
e.g.	exempli gratia (for example)
et al.	et alii (and others)
h	hour(s)
i.e.	id est (that is)
IP	immunoprecipitation
kb	kilo base pair(s)
kd	kilo Dalton
min	minute(s)
mRNA	messenger RNA
N-	amino-
PCR	polymerase chain reaction
PolII	RNA polymerase II
PolIII	RNA polymerase III
RNA	ribonucleic acid
Rnase	ribonuclease
RT	room temperature
s	second(s)
S-phase	phase of DNA synthesis

SDS	sodium dodecylsulfate
Tris	α,α,α-Tris-(hydroxymethyl)methylamine
U	units
% v/v	percent per volume
% w/v	percent per weight
wt	wildtype

2. Curriculum Vitae

08. October 1973	• born in Blaj/ Romania
July 1993	• A-Levels, Michaeli-Gymnasium München
January 1994- September 1996	• apprenticeship in a printing office in Munich
November 1996- July 2002	• diploma studies in biology, University of Regensburg, diploma thesis in the laboratory of Dr. Sabine Strahl: "Characterization of signal transduction pathways in *S. cerevisiae* that are induced by O-mannosylation defects"
September 2002-	• research in the laboratory of Dr. Michael Grunstein at the University of Los Angeles, California
since May 2003	• PhD thesis in the laboratory of Dr. Wolfram Hörz, LMU München: "Mechanism of chromatin assembly at the yeast *PHO5* promoter upon repression"

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4. Zusammenfassung

Frühere Untersuchungen haben gezeigt, dass Histone von dem *PHO5* und dem *PHO8* Promotor abgelöst werden, wenn man die entsprechenden Gene durch Phosphathunger induziert. Diesem Phänomen liegt ein *trans*-Mechanismus zugrunde. Werden die *PHO* Gene erneut reprimiert, so wird die inaktive Chromatinstruktur des *PHO5* und des *PHO8* Promotors wieder hergestellt. Zu Beginn dieser Arbeit war unklar, ob die Histone, die zur Wiederherstellung des inaktiven Promotors benötigt werden, über einen *cis*-Mechanismus an den Promotor gelangen oder ob Nukleosomen *de novo* am Promotor synthetisiert werden.
Ziel dieser Arbeit war es den Mechanismus aufzuklären, der dafür sorgt, dass im Zuge der Phosphatrepression Nukleosomen am *PHO5* Promotor eingebaut werden.
Im Verlauf dieser Arbeit konnte ich zeigen, dass tatsächlich Nukleosomen am *PHO5* Promotor inkorporiert werden, wenn man das entsprechende Gen durch Zugabe von Phosphat zum Nährmedium reprimiert. Was den Ursprung dieser Nukleosomen angeht, so gibt es experimentelle Hinweise dafür, dass ein signifikanter Teil aus der löslichen Fraktion, d.h. einer Histonquelle aus *trans*, stammt. Die Repression des *PHO5* Promotors geschieht sehr schnell und ist nicht von einer Replikationsrunde abhängig. Im Einklang mit diesem Ergebnis steht, dass nur diejenigen Histonchaperone zur raschen Inaktivierung des Promotors beitragen, die eine Rolle bei der replikationsunabhängigen Assemblierung von Chromatin spielen. Im Gegensatz dazu haben diejenigen Histonchaperone, die an der der replikationsabhängigen Chromatinassemblierung beteiligt sind, keinen Einfluss auf die Geschwindigkeit, mit der der *PHO5* Promotor assembliert wird. Weiterhin sind Nukleosomen-Remodulierungsfaktoren an der raschen Repression des *PHO5* Promotors beteiligt. Interessanterweise gehören diese Faktoren einer Klasse von Nukleosomen-Remodulierungsmaschinen an, die die Assemblierung von Chromatin unter Zuhilfenahme eines *trans*-Mechanismus katalysieren. Nukleosomen-Remodulierungsfaktoren, die typischerweise Nukleosomen in *cis* repositionieren, haben keinen Einfluss auf die Geschwindigkeit, mit der der inaktive *PHO5* Promotor wieder hergestellt wird. Die *trans*-Inkorporation von Histonen konnte nicht nur am *PHO5* Promotor, sondern auch an zwei anderen phosphatregulierten Promotoren beobachtet werden, dem *PHO8* und dem *PHO84* Promotor. Dadurch, dass die Schliessung der Promotoren so rasch vonstatten geht, trägt dieser

Mechanismus zur effizienten Abschaltung der Genexpression bei. Es ist vorstellbar, dass ein ähnlicher Mechanismus auch bei anderen Genen in Hefe eine Rolle spielt.
Im zweiten Teil dieser Arbeit zeige ich Ergebnisse, die auf eine Rolle des Histonchaperons Asf1p bei der Aktivierung des *PHO5* Gens hindeuten. Interessanterweise ist die Aktivierung von *PHO5* in einem *asf1* deletierten Stamm abhängig von der Phosphatkonzentration des Nährmediums. Vollständige Induktion erfolgt nur dann, wenn das Medium absolut phosphatfrei ist. Selbst sehr geringe Mengen von Phosphat verhindern die Aktivierung von *PHO5* vollständig.