A role for Sum1 in *HML* silencing and replication initiation in *Saccharomyces cerevisiae*

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Zusammenfassung

In der eukaryotischen Ontogenese ist die Etablierung differenzierungsspezifischer Genexpression eng an die Unterteilung des Genoms in funktionell getrennte Domänen gekoppelt. Solche Domänen lassen entweder erhöhte transkriptionelle Aktivität zu oder unterdrücken sie und werden Eu- bzw. Heterochromatin genannt. Heterochromatin enthält spezielle Proteine, die zur Ausbildung dieser repressiven Chromatinstruktur beitragen. Eine der Hauptfragen in der Heterochromatinbiologie ist, wie solche Proteine rekrutiert werden. Dieser Prozess ist entscheidend damit einzelnde Regionen im Genom koordiniert zeit- und ortsabhängig reprimiert werden können. In *Saccharomyces cerevisiae* entsteht Heterochromatin an den *silent-mating-type* Loci *HMR***a** und *HML*α durch die zielgerichtete Rekrutierung des Sir-Komplexes über eine Gruppe von Proteinen, die an sogenannte *silencer*-DNA Sequenzen binden. In diese Arbeit wird gezeigt, daß das Protein Sum1, bisher bekannt als Repressor meiotischer Gene im vegetativen Zellzyklus, als Heterochromatin-Rekrutierungsfaktor für *HML*α fungiert. Sum1 konnte *in vitro* und *in vivo* an *HML*α über ein funktionelles Element innerhalb des *HML*-E silencers binden und die Deletion von *SUM1* verursachte einen Verlust von Repression an *HML*α.

SUM1 beeinflußte außerdem die Fähigkeit von *HML*-E als Replikationsstartpunkt (*origin*) zu agieren, was eine Rolle von Sum1 in der Replikation nahelegt. Die Beobachtung, daß *orc2-1* und *orc5-1* mit *sum1*Δ synthetisch lethal waren und daß *cdc6-1*, *cdc7-1* oder *cdc45-1* mit *sum1*Δ einen synthetischen Wachstumsdefekt aufwiesen unterstützt die Vermutung, daß *SUM1* eine globale Rolle in der Replikationsinitiation besitzt. In einer genomweiten Suche wurden ARS Elemente gefunden, die sowohl Sum1 als auch ORC rekrutieren. Dabei konnte gezeigt werden, daß die Replikationsaktivität dieser ARS Elemente von Sum1 bzw. Sum1 Bindungsstellen abhängig war. Als Repressor von meiosespezifischen Genen interagiert Sum1 oft mit der Histondeacetylase Hst1. In diesem Zusammenhang konnte gezeigt werden, daß *SUM1*-regulierte *origins* ebenfalls *HST1* zur vollen Aktivität benötigten. Zusammenfassend schlagen wir Sum1 als neuartigen Modulator für die Replikationsinitiation an einer Untergruppe chromosomaler Replikationsstartpunkte vor.

Schlagworte:

Chromatin, Silencing, Replikationsstartpunkt, Replikation, ARS, Sum1, Hst1

Abstract

The division of eukaryotic chromatin into functionally distinct domains is critical to implement gene expression programs that drive the development of multicellular organisms. Regions termed euchromatin exist in the genome that are generally conducive to transcription, whereas heterochromatin contains specialized chromatin binding proteins that repress transcription in these regions. A central question in heterochromatin biology is how the heterochromatin factors are targeted to specific genomic regions, a process that is crucial to ensure that the designated domains, and only they, are repressed in the appropriate spatial and temporal fashion. In *Saccharomyces cerevisiae* heterochromatinization at the silent mating-type loci *HMR***a** and *HML*α is achieved by targeting the Sir complex to these regions via a set of anchor proteins that bind to the silencers. Here, we have identified a novel heterochromatin targeting factor for *HML*α, the protein Sum1, a repressor of meiotic genes during vegetative growth. Sum1 bound both *in vitro* and *in vivo* to *HML*α via a functional element within the *HML*-E silencer, and deletion of *SUM1* caused *HML*α derepression. Significantly *SUM1* was also required for origin activity of *HML*-E, suggesting a role of Sum1 in replication initiation. Our observations of a synthetic lethality between *orc2-1* or *orc5-1* and *sum1*Δ as well as a synthetic growth defect of *cdc6-1*, *cdc7-1* and *cdc45-1* with *sum1Δ* support the notion that *SUM1* has a global role in replication initiation. In a genome-wide search for Sum1-regulated origins, we identified a set of autonomous replicative sequences (ARS elements) that bound both the origin recognition complex and Sum1. Full initiation activity of these origins required Sum1, and their origin activity was decreased upon removal of the Sum1 binding site. In its role as a repressor of meiosis specific genes, Sum1 often works in concert with the histone deacetylase Hst1. We found that *SUM1*-regulated origins also required *HST1* for full activity. Taken together we propose that Sum1 is a novel replication initiation modulator for a subset of chromosomal origins.

Keywords:
Chromatin, silencing, origin, replication, ARS, Sum1, Hst1

Contents

1 Introduction

The following work addresses a molecular link between two major cellular processes: heterochromatin formation and replication initiation. Since these processes are connected to chromatin organisation, an introductory chapter will focus on chromatin biology. Moreover, the establishment of heterochromatin will be reviewed, with a particular focus on current knowledge of silencing in *Saccharomyces cerevisiae*. The connection of heterochromatin formation and replication initiation is reflected by the fact that numerous proteins are involved in both processes. Therefore, a second part of this introduction discusses principles of replication and factors involved in this process.

1.1 Epigenetics and regulation of gene expression

Tight control of gene expression is of pivotal importance during the life of a cell. Besides housekeeping genes that are constantly expressed, various genes are only used at specific stages of the cell cycle or under particular environmental conditions. Also, during the process of differentiation in higher organisms, expression and repression of only the appropriate subset of genes at a given time or cell type is fundamental. To accomplish this, mechanisms have evolved to switch genes on and off. Organisms have developed a large set of proteins that bind to DNA in a sequence-specific manner and positively or negatively influence transcription. These proteins are called transcription factors or transcriptional repressors. However, besides the dynamic properties of transcriptional regulation, daughter cells of a specific cell type can also “remember” the expression pattern of their mother cells. This is of particular importance in highly specialized cell types. The process of inheriting a specific gene expression pattern without changing the genomic sequence is called epigenetics (reviewed in (Hendrich and Willard, 1995)). Epigenetic mechanisms influence a broad spectrum of cellular processes ranging from gene expression control to replication origin choice in metazoa or gene silencing in eukaryotes. Gene silencing can be distinguished from promoter-specific gene repression in that it acts in a regional and gene independent manner. It leads to transcriptional inactivation of whole chromosomal areas by densely packing chromatin to inhibit access for DNA binding proteins or factors of the transcriptional

machinery. Therefore chromatin plays an important role in determining the transcriptional status of a gene.

1.2 Chromatin and gene expression

Chromatin has traditionally been divided into two main classes based on structural and functional criteria. Euchromatin contains the majority of the genes, both actively transcribed and quiescent. Heterochomatin is transcriptionally silent and contains large regions of repetitive DNA sequence (reviewed in (Grewal and Moazed, 2003)). It was first described in light microscopic studies of moss nuclei as the part of chromatin that remains condensed throughout the cell cycle (Heitz, 1928). Coexistence of heterochromatin and transcriptional inactivation is observed in polytene chromosomes in salivary glands of *Drosophila melanogaster*. These structures consist of more than 1000 identical chromosomes that align and form a giant chromosome. Examination by light microscopy shows a pattern of bands and also lateral "puffs" on the chromosomes that are associated with high transcriptional activity. After transcription of the genes within the puffed domains these areas are again compacted into bands (reviewed in (Zhimulev, et al., 2004)).

Transcriptional repression by heterochromatin is sequence-independent, which means that localization of a gene within the genome is as important for its expression as the composition of its promoter elements. One prominent example for a locus specific effect for gene expression is a phenomenon called position effect variegation (PEV) in *D.melanogaster* (Muller, 1930). The *white*$^+$ (w^+) gene encodes a factor responsible for red color development in the facets of the eye. Red-white mosaic phenotypes are caused by a chromosomal position effect in which an X-ray induced rearrangement breakpoint placed the w^+ gene from its normal euchromatic location to the vicinity of heterochromatin. Some rearrangements lead to heterochromatin-mediated silencing of this gene resulting in the occurrence of large patches of red and white facets in the adult eyes. This pattern of variegation suggests that a decision to express or repress the w^+ gene is made early during tissue developement and is maintained in a metastable state through multiple cell divisions (reviewed in (Wakimoto, 1998)).

The phenomenon of transcriptional silencing of genes during development is not restricted to dipteria but found in an increasing number of other organisms including humans. For example in mammalian females one X-chromosome is transcriptionally silenced. Somatic cells of females contain two X-chromosomes, while male cells contain one X and one Y

chromosome. Female cells compensate the extra X-chromosome dose by inactivating one of them which happens in early embryonic development. The inactivated X-chromosome can be seen during interphase as a distinct structure called Barr body and most of its DNA is not transcribed (reviewed in (Chow and Brown, 2003)).

Generally, centromeres and telomeres as well as many intergenic regions in mammalian genomes consist of constitutive heterochromatin (Perrod and Gasser, 2003). All these areas contain a large number of highly repetitive DNA and heterochromatinization is thought to inhibit recombination events. Also repeated gene arrays such as the genes coding for ribosomal RNAs (rDNA) and transposons are subject to transcriptional silencing (Walsh, et al., 1998). Presumably 90% of the mammalian genome is transcriptionally silent in differentiated cells (Perrod and Gasser, 2003). Part of it is constitutive heterochromatin whereas the rest is assigned to differentiation specific events.

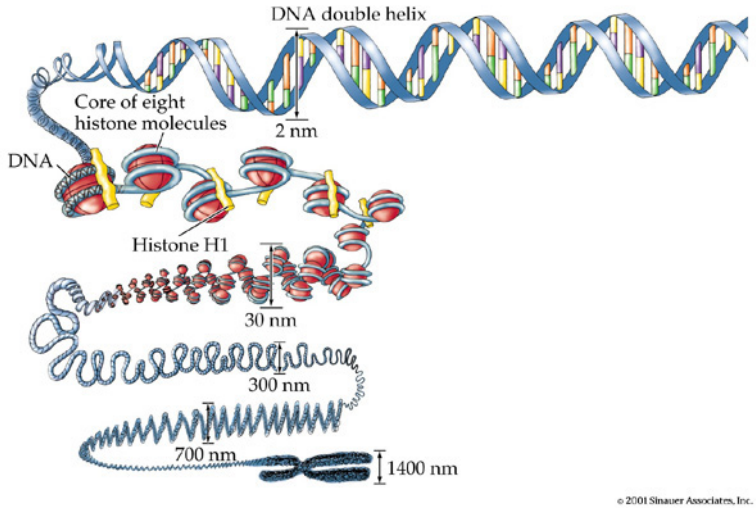

Figure 1.1: Schematic representation of various DNA compaction levels. Details are described in the text. Image adapted from Sinauer Associates, 2001.

1.3 Chromatin composition

Genome sizes vary greatly among eukaryotic organisms. For example the genome of the bakers yeast *Saccharomyces cerevisiae* consists of ~12.5 million basepairs (bp), whereas the whisk fern (*Psilotum nudum*) has its genomic information stored in 2.5×10^{11} bp which is 20.000 times the genome size of *S.cerevisiae*. Since DNA is a linear molecule, a prime

question is how it can be packed in a space-saving way but be concurrently kept flexible enough to allow recombination, replication, transcription or repair. Given the human genome with more than 6.4 billion basepairs (bp) per diploid cell, some 2 meters of DNA molecule have to fit into each cell nucleus. To accomplish this, different levels of DNA compaction have evolved. DNA in eukaryotes does not exist as a naked molecule but is organized within an array of proteins, predominantly histones, into a structure called chromatin. The basic structural unit of chromatin is the nucleosome. 146 bp of DNA are wrapped in 1.75 turns around an octamer of histone proteins which consists of two copies each of histones H2A, H2B, H3 and H4 (Luger, et al., 1997).

Histones are small basic proteins of 103 to 230 amino acids and are highly conserved throughout the eukaryotic world. A nucleosome is present every 200±40 bp and because of its electron microscopic appearance this structure is termed “beads on a string”. However there are several higher-order compaction levels. Arrays of nucleosomes are proposed to fold into a fiber of 30 nm diameter upon incorporation of the linker histone H1. Chromatin which is not actively transcribed exists predominantly in the condensed 30 nm fiber form, whereas actively transcribed chromatin is thought to assume the “beads on a string” form. Higher order compaction levels include association of loops of the 30 nm fiber to a scaffold of non-histone proteins (Paulson and Laemmli, 1977). Folding of this scaffold into a helix and further packing of this helical structure produces the highly condensed structure characteristic of metaphase chromosomes (Fig. 1.1). Therefore accessibility of the genetic information is necessarily connected to mechanisms that either work on chromatin or are influenced by chromatin. Two main enzymatic activities can be distinguished that regulate chromatin access: chromatin remodeling complexes that help to move or remove nucleosomes, and chromatin modifying complexes that modify histones.

1.4 Mechanisms that alter chromatin properties

1.4.1 Chromatin remodeling

Biochemical and genetic experiments showed that nucleosomes are repressive for transcription (Laybourn and Kadonaga, 1991; Workman, et al., 1991). Since promoter elements and other transcriptional enhancers on the DNA level can be occupied by

nucleosomes, several mechanisms have evolved that provide access to DNA without biochemically modifying structural components of chromatin. Factors involved are called chromatin remodeling factors and are defined by their ability to either move or remove nucleosomes along a particular DNA sequence or to create a state of altered histone-DNA interaction (reviewed in (Becker and Horz, 2002) and (Sif, 2004)). Principally, all chromatin remodeling complexes use the energy of ATP hydrolysis to loosen the contact between DNA and histones. The first identified chromatin remodeler was the SWI/SNF complex of *S.cerevisiae* (Hirschhorn, et al., 1992). Nucleosome displacement by Swi2/Snf2 occurs by sliding or tracking nucleosomes along the DNA (Whitehouse, et al., 1999). How this is carried out from a mechanistic point of view is not entirely clear but the RSC complex (remodels the structure of chromatin), a close Swi2/Snf2 homologue, is thought to break DNA–histone contacts, generating a 'wave of DNA' that propagates around the nucleosome (Saha, et al., 2002). Besides their roles in transcriptional control there is strong evidence that they are also involved in replication, repair, and recombination and can interact with histone acetyltransferases, histone deacetylases or histone methyltransferases that biochemically modify chromatin.

1.4.2 Chromatin modifications

Chromatin modifications can occur either on DNA or on histones in their chromosomal context. Some of these modifications are associated with transcriptional activation whereas others lead to transcriptional repression. The fact that some modifications are reversible creates an additional layer of flexibility beyond the DNA sequence level. DNA methylation directly acts on DNA and is widely conserved among eukaryotes. Methylation of cytosine residues within CpG islands on gene promoters is a primary epigenetic event that acts to suppress gene expression (reviewed in (Bird, 2002)). DNA methylation accounts for the specific repression of genes in differentiated cells but also for the stable silencing of transposable elements (Lippman, et al., 2004).

Other chromatin modifying events target histones and include acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation. Histones consist of a globular core domain and N- or C-terminal tails. When assembled into nucleosomes, the histone tails protrude unordered from the nucleosome, exposing 20-35 residues (Luger, et al., 1997). Histone tails as well as the histone core are subject to a large number of

posttranslational modifications. It seems likely that nearly every histone residue that is accessible to solvent may be a target for posttranslational modification.

A universal epigenetic mark in eukaryotic genomes is the acetylation of lysine residues at the ε-NH_3^+ position of the side chain. It is carried out by protein complexes called histone acetyltransferases (HATs). HATs can either act genomewide or on a local basis which is also dependent on factors that recruit these enzymes to the histones.

Histone acetylation is a reversible process and accordingly histone deacetylases (HDACs) have been isolated. Like histone acetyltransferases, HDACs have different specificities and can act either globally or locally. For example in *S.cerevisiae* Rpd3 deacetylates histones on a genomewide basis (Vogelauer, et al., 2000) thereby deacetylating lysine residues of H3 and H4 N-termini, whereas Sir2 is only found at transcriptionally silent regions and specifically removes acetylgroups of H4 K16, thus antagonizing the HAT Sas2 which acetylates the same residue (Suka, et al., 2002). Homologues of Sir2, the Hst proteins Hst1-4 (homologue of Sir two) have been identified and described (Brachmann, et al., 1995; Derbyshire, et al., 1996). Hst1 and Hst2 are active histone deacetylases and share substrate specificity with Sir2 (Sutton, et al., 2001). Interestingly, overexpression of Hst1 can partially restore *HMR***a** silencing in a *sir2Δ* strain (Brachmann, et al., 1995). Hst1 is even able to totally restore *HMR***a** silencing in this strain background when targeted to *HMR*-E (Rusche and Rine, 2001; Sutton, et al., 2001). So far, wild-type Hst1 has been found to act in transcriptional repression of meiotic genes during mitosis (this will be discussed in more detail in chapter 1.8.2).

Generally, hyperacetylated histones are associated with transcriptionally active chromatin whereas hypoacetylated histones correlate with heterochromatic regions. The process of acetylation and deacetylation is highly dynamic and some of these modifying complexes antagonize each other in a steady state equilibrium. For instance Sir2 acts at telomeric histones, whereas Sas2 targets them outside the telomeric region. Disruption of the Sir2/Sas2 equilibrium leads to either spreading of K16 deacetylation telomere-distal or spreading of K16 acetylation telomere-proximal (Kimura, et al., 2002; Suka, et al., 2002).

Histone acetylation may also affect DNA replication since the human MYST family HAT HBO1 interacts with the replication licencing factor Mcm2 (Burke, et al., 2001) and with human Orc1 (Iizuka and Stillman, 1999). Likewise, histone deacetylation has been implicated in negative regulation of replication. For example, in yeast the deletion of the HDAC Rpd3 leads to early activation of late origins (Aparicio, et al., 2004; Vogelauer, et al., 2002). Also,

deletion of the heterochromatin associated HDAC Sir2 can positively influence the activity of a subset of replication origins (Pappas, et al., 2004).

Another important histone modification is histone methylation which can occur both on the tails and the core of the protein. Lysine and arginine residues are targets of histone methyl transferases (HMT) and a variety of these modification have been implicated in transcription control. For example methylation of K4 and K36 at histone H3 is associated with transcriptional activity, whereas methylation of K9 at histone H3 is involved in the formation of stable repressive heterochromatin (Peters, et al., 2001). Notably the ε-NH_3^+ group of lysine can be either mono-, di- or trimethylated, adding an additional layer of complexity to control processes. Histone methylation is also reversible (Cuthbert, et al., 2004; Shi, et al., 2004; Wang, et al., 2004) and therefore similar dynamics in this type of modification are expected as with histone acetylation.

Interestingly histone modifications do not occur independently but can influence each other. Modification of one specific amino acid residue can induce or inhibit other histone modifications either in the immediate vicinity or on neighboring histones (Lo, et al., 2001; Ng, et al., 2002). In addition, other events like chromatin remodeling are controlled by modifications on histones. For example the activity of Chd1, part of the SAGA and SLIK chromatin remodeling complexes, is dependent on methylation of K4 at histone H3 (Pray-Grant, et al., 2005). The growing number of histone modifications that influence important cellular processes has lead to the proposal of a "histone code", which may have universal regulative function (Jenuwein and Allis, 2001).

1.5 Silencing in *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* has been one of the prime model organisms to study heterochromatin for many years. Heterochromatic regions are found in three domains of the yeast genome consisting of the two silent mating type loci *HML*α and *HMR***a**, the telomeres and the rRNA encoding DNA (rDNA). These domains have many features in common with heterochromatin of higher organisms as seen in position effect variegation in *Drosophila* or X chromosome inactivation in humans. In this respect silenced areas of the yeast genome are replicated late in S-phase, contain hypoacetylated nucleosomes and are generally restrictive to DNA modifying enzymes. Also numerous proteins involved in heterochromatin formation of

yeast have homologues in higher eukaryotes that are involved in the same processes. Silencing in yeast has a function in regulating the expression of genes and moreover, it protects repetitive sequences as found at the telomeres and the rDNA locus from homologous recombination. While the involvement of the Sir2 protein in the process is common to all three silenced regions, the mechanisms of recruitment are different.

1.5.1 Silencing at the *HM* loci

The *HM* loci are part of the mating type determination system of *S.cerevisiae*. Yeast cells can either assume the **a** or the α mating type and only haploids of different mating types are able to mate and form diploids. The mating type is determined by alternative alleles of a single locus located close to the center of chromosome III called the mating type locus (*MAT*) (Fig. 1.2A). The *MAT*α allele determines cells of the α mating type and the *MAT***a** allele gives rise to cells of the **a** mating type. Both of these alleles contain genes for regulatory proteins that control the expression of factors which specify the functional differences between the two cell types (reviewed in (Herskowitz, et al., 1992)). In naturally occuring *S.cerevisiae* populations individual cells can interconvert their mating type from **a** to α or vice versa as frequently as once per generation in a gene conversion event catalyzed by the protein HO endonuclease. In laboratory strains, this conversion is abolished due to deletion of this endonuclease. Two additional copies of mating type information, the cryptic mating type loci, serve as donors during the HO endonuclease mating type conversion and are located on the same chromosome close to the telomeres (Fig. 1.2A). Situated left of the centromere is *HML* (homothallic mating left) that carries α mating type information, while *HMR* (homothallic mating right) is located to the right of the centromere and contains **a** mating type information. Since simultaneous expression of opposite mating type factors is a cellular signal for diploidy and thus sterility, both *HM* loci are transcriptionally silenced and embedded in a heterochromatic region.

A number of *trans*-acting proteins as well as *cis*-acting regulatory DNA sequences called silencers that flank the *HM* loci are responsible for heterochromatin formation. In deletion experiments on *HMR***a**, loss of the upstream silencer lead to complete derepression of *HMR***a**, whereas loss of the downstream silencer had no apparent effect on silencing but sensitized it for additional mutations. Therefore, the former was termed E for essential and the latter I for important (Brand, et al., 1985) (Fig. 1.2A). In contrast, either the E- or the I-silencer at *HML*α

are able to achieve silencing in the absence of the other (Mahoney and Broach, 1989). Silencers are about 150bp in length and contain protein binding sites for the proteins Rap1 (repressor-activator protein 1), Abf1 (autonomous replicative sequence (ARS) binding factor 1) and ORC (origin recognition complex). While Rap1 and Abf1 binding site occurrence and composition is variable, ORC binding sites are present at each silencer (Fig. 1.2A). Interestingly, each of these proteins has distinct roles in the cell elsewhere, Rap1 and Abf1 being general transcription factors (Planta, et al., 1995) and ORC being a protein complex important for replication initiation (reviewed in (Bell and Dutta, 2002)). At the silencers their function is to serve as anchor sites for the superordinate Sir-family proteins which ultimately create heterochromatin across the region.

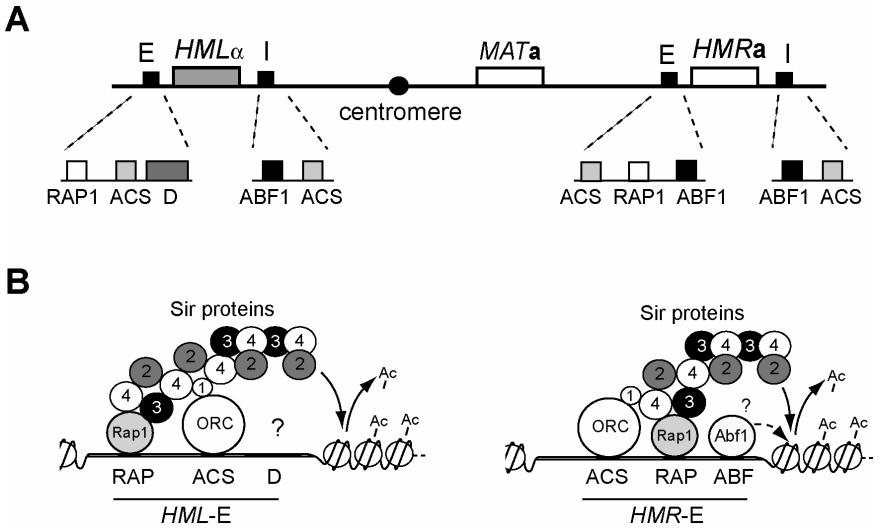

Fig. 1.2: Schematic representation of mating-type loci and silencers
(A) Localization of the *MAT* locus and the silenced *HM* loci on chromosome III. Each of the *HM* loci is flanked by one E and one I silencer which is composed of binding sites for ORC, Rap1 and Abf1. (B) Nucleation of heterochromatin at *HML*-E and *HMR*-E. Sir-proteins bind the anchor proteins at the silencer and spread across the region thereby creating heterochromatin.

The composition of binding sites within the silencers has been well studied in the past years. However, a sequence of approximately 100bp termed the D element in close vicinity to the Rap1 and ORC binding site was identified at the *HML*-E silencer, but has not been molecularly characterized (Mahoney, et al., 1991) (Fig. 1.2A). Natural silencers are redundant in function and therefore, deletion of any one of the binding sites still allows repression, whereas deletion of any two of these sites abolishes silencing (Mahoney and Broach, 1989).

At *HML*-E the D element showed the same redundancy upon deletion with simultaneous deletion of the Rap1 or the ORC binding site in the absence of *HML*-I (Mahoney, et al., 1991). Therefore a binding site for another, yet unidentified silencing protein might be contained within the D element of *HML*-E. The identification of this factor was the goal of this thesis.

One interesting issue is how the binding sites for three factors with independent roles in the cell can create a silencer element. The observation of a redundant function within silencers lead to the current view that while each of these proteins has some affinity for one or more Sir proteins only the close juxtapostion of two or three of these factors can create a sufficiently high local concentration of Sir proteins to sustain silencing. Consistent with this model, arrays of multiple Rap1 binding sites recruit Sir proteins to telomeres (Cockell, et al., 1995; Hecht, et al., 1996) and can create artificial silencers (Stavenhagen and Zakian, 1994).

The Sir proteins are the major effectors of heterochromatin formation. Sir3 and Sir4 (and to a limited extent Sir1) consitute structural components of heterochromatin while Sir2 contributes to the process via its activity as an NAD+ (nicotin adenine dinucleotide) dependent histone deacetylase. Sir2 targets and deacetylates K9 and 14 of H3 and K16 of H4 *in vitro* (Imai, et al., 2000) and Sir2, Sir3 and Sir4 are essential for the silencing process (Rine and Herskowitz, 1987). Sir1 is not essential but aids in the establishment of silenced regions in that it acts as a bridging factor between ORC and Sir4 (Triolo and Sternglanz, 1996). The establishment of a silenced region is thought to occur in a stepwise process of Sir protein polymerization across the region (Hoppe, et al., 2002) (Fig. 1.2B). The anchor proteins Rap1, Abf1 and ORC are bound to the silencers and assemble the nucleation sites. Sir2 and Sir4 form heterodimers and bind to them via interaction of Sir4 with Sir1 and Rap1. Sir3 can bind the silencers independently via interactions with Rap1 and Sir4. Sir2 which is now localized to the immediate vicinity of nucleosomes starts to deacetylate lysine residues at the tails of histones H3 and H4. Exposure of unacetylated histone tails leads to binding of Sir3 and Sir4 thus recruiting more Sir2/Sir4 complex to this locus (Rusche, et al., 2002). These processes of ongoing deacetylation and polymerization are completed when the Sir proteins emanating from both silencers meet. Although important for the establishment of silenced *HM* loci the role of the Abf1 binding sites at any of the *HM* silencers except *HML*-E is not clear since so far Abf1 was not found to interact with any of the other proteins implicated in silencing. However, Abf1 has the ability to alter chromatin organization (Venditti, et al., 1994) and

could therefore aid in silencing by allowing easier loading of other silencing proteins (Miyake, et al., 2002).

Heterochromatin nucleating from the silencers generally spreads to both sides across the region. To limit it to the designated areas, boundary elements have evolved that block this spreading. For example actively transcribed genes can constitute such a barrier (Bi, 2002; Donze, et al., 1999) since mutations in the respective promoter region or in polymerase factors abolish this barrier function. This could be due to parts of a stably bound polymerase complex forming a physical obstacle to the spreading of heterochromatin. Also the enzymatic activity of HATs or chromatin remodeling factors at the promoter can counteract the propagation of histone deacetylation (Oki, et al., 2004; Suka, et al., 2002). However, spreading of heterochromatin can also be unidirectional as it is the case at the *HML*-I silencer which causes heterochromatin to spread only in direction of the α-genes (Bi, et al., 1999).

Interestingly all silencers can also confer autonomous replication to plasmids but only *HMR*-E and -I are true chromosomal origins (Dubey, et al., 1991). This discrepancy is explained by the fact that the *HML* silencers, although capable of initiating replication, are passively replicated by an early initiating replication origin in the vicinity (Sharma, et al., 2001). ORC and to a lesser extent Abf1 and Rap1 binding are responsible for the bimodular ability of the silencers to nucleate silencing and to initiate replication (Fox, et al., 1995).

1.5.2 Silencing at the telomeres and the rDNA locus

Due to the fact that telomeres constitute the extreme end of the chromosome and contain a single stranded DNA overhang, they are subject to degradation or fusion with telomeres of other chromosomes. However, this is prevented by the creation of heterochromatin in these domains. The mechanistics of heterochromatin formation are similar to those of the *HM* loci. The telomere ends consist of tandem $C_{1-3}A/TG_{1-3}$ repeats that are free of nucleosomes and are bound by Rap1 at 10-20 copies per telomere (Gilson, et al., 1993; Wright, et al., 1992). Here, it is the multitude of Rap1 copies that constitutes anchor sites for the recruitment of the Sir2/Sir4 heterodimer and Sir3, which ultimately leads to the same concerted event of histone deacetylation and Sir complex spreading as described for the *HM* loci (Luo, et al., 2002). Thereby heterochromatin can spread up to 3kb inwards and silence reporter genes inserted at telomere proximal locations. Telomeric $C_{1-3}A/TG_{1-3}$ repeats are also able to silence reporter genes when placed elsewhere in the genome although a higher repeat copy number is

necessary (Stavenhagen and Zakian, 1994). This is due to the fact that natural telomeres contain an additional silencing anchor, an ORC and an Abf1 binding site within a sequence of ~500bp called the CoreX. This region is located subtelomerically as part of a larger X repeat element which is present at most telomeres. Silencing at natural telomeres is discontinuous and is enhanced around subtelomeric CoreX elements. Since Rap1 and ORC are in close proximity at the *HM* silencers to establish stable silencing, a current hypothesis for telomeric silencing is that Rap1-Sir clusters contact the ORC-Sir complex at CoreX by forming a foldback loop structure (Strahl-Bolsinger, et al., 1997).

Silencing at rDNA is different from that of telomeres and the *HM* loci. The *S.cerevisiae* locus coding for ribosomal RNA (rDNA locus) consists of a 9.1kb sequence that is repeated 100 to 200 times (Petes and Botstein, 1977). Each rDNA repeat encodes 35S rDNA, which is the precursor to the 25S, 18S, and 5.8S rRNA and is transcribed by polymerase I, and the 5S rRNA which is transcribed by polymerase III. These two genes are separated by the nontranscribed spacers NTS1 and NTS2. Only a fraction of the rDNA genes are transcribed at a given time and the majority remains silenced by the action of Sir2 (Smith and Boeke, 1997). Sir2 in this context does not act via Sir4 in but is part of the nucleolar RENT (regulator of nucleolar silencing and telophase) complex (Straight, et al., 1999). RENT also contains Net1, which is required for association of Sir2 with the rDNA, and Cdc14, a phosphatase required for mitotic exit. However the mechanistic aspects of rDNA silencing are still unclear.

1.6 Replication initiation

In *S.cerevisiae* and *Drosophila* a shared feature between replication initiation and some types of heterochromatin formation is the fact that both require ORC binding at their nucleation sites (Foss, et al., 1993; Huang, et al., 1998; Pak, et al., 1997). Moreover, ORC is essential for replication initiation in all eukaryotes studied to date (Bell and Dutta, 2002). This chapter gives a general overview on this topic with a particular interest in origins of replication as nucleation sites for replication. The first part addresses the composition of replication origins in yeast and metazoa (chapter 1.6.1) while the second part aims to outline the events that occur at the origin during the time of replication initiation (chapter 1.6.2).

1.6.1 Origins of replication

Origins of replication as starting points for DNA replication are scattered across the genome in eukaryotes. The origin number varies from about 300-400 in *Saccharomyces cerevisiae* (Raghuraman, et al., 2001; Wyrick, et al., 2001) to an estimated ten thousand in humans (Gilbert, 2001). While in *S. cerevisiae* a DNA consensus sequence (the ORC binding site) specifies origins of replication, to date no specific DNA sequence could be assigned to general origin function in any of the other eukaryotes tested.

An origin of budding yeast spans a sequence of 150 to 200 base pairs and is able to support autonomous replication of plasmids. These autonomously replicating sequences (ARS) do not share obvious homology to each other except for an essential consensus sequence of 11 basepairs (WTTTAYRTTTW). This sequence called the A element or the ARS consensus sequence (ACS) is essential but not sufficient for full origin function (Celniker, et al., 1984). Notably, an expanded 17 basepair ACS (EACS: WWWWTTTAYRTTTWGTT) has been described that more effectively predicts *in vivo* ARS elements (Theis and Newlon, 1997). The A element is bound by the origin recognition complex (ORC) in a sequence specific manner (Diffley and Cocker, 1992). Flanking the A element there are one or more B elements which are important but not essential for ARS activity (Marahrens and Stillman, 1992). ARS1, possibly the best studied yeast origin contains three B elements (Marahrens and Stillman, 1992) (Fig. 1.3). The B1 element is closest to the ACS and cooperates in ORC binding (Lee and Bell, 1997). B2 is required for loading of the MCM (minichromosome maintenance) complex (Wilmes and Bell, 2002; Zou and Stillman, 2000), and B3 is bound by Abf1 (Diffley and Stillman, 1988). The B2 element often overlaps with DNA unwinding elements (DUEs) that are presumably melted during replication initiation (Matsumoto and Ishimi, 1994). Abf1 is an accessory factor for origin function at a subset of chromosomal replication origins (Eisenberg, et al., 1988; Rhode, et al., 1992). Abf1 sites are found in several origins, and in three of the four *HM* silencers (Kimmerly, et al., 1988).

Analysis of other yeast ARS elements suggest that many origins may share at least the conserved A and B1 elements that form the ORC binding site (Fig. 1.3). Apart from that, additional B elements are very variable in size and location. Also highly individual sequence elements have been found like the REN1501 enhancer, an ARS element present at ARS1501 which is important for full origin function (Raychaudhuri, et al., 1997).

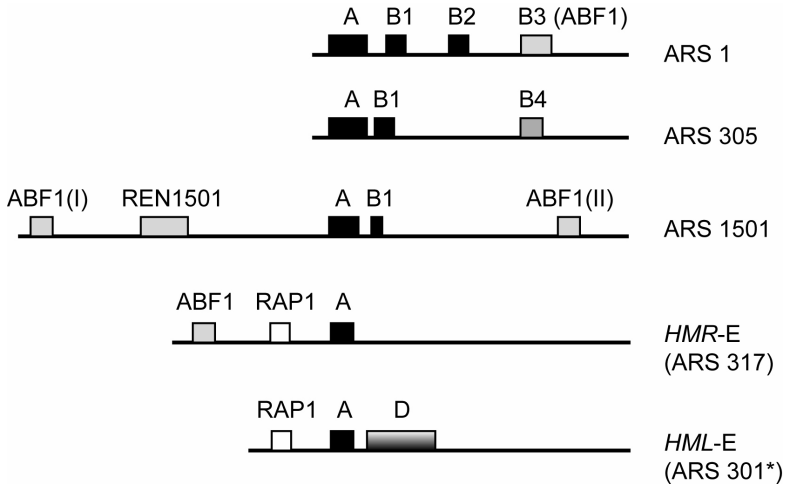

Fig. 1.3: Comparison of ARS elements in *S.cerevisiae*
ARS 1 as described by (Marahrens and Stillman, 1992); ARS305 (Huang and Kowalski, 1996), the B4 element is of unknown function; ARS1501 (Raychaudhuri, et al., 1997); *HMR*-E (ARS317) an inefficient ARS (Rivier and Rine, 1992); *HML*-E (ARS301) (Mahoney, et al., 1991). Asterisk: ARS301 does not initiate replication in its chromosomal context.

In budding yeast prediction of ARS sites on a DNA sequence level is difficult since the occurrence of an ACS alone is not sufficient for ARS activity and B elements do not share sequence homology. There are more than 10 000 matches of the ACS in the yeast genome, but only 300-400 among them serve as an ARS site (Breier, et al., 2004). One posibility to identify new origins is to test the origin activity of an ARS containing DNA sequence in individual experiments (Fangman and Brewer, 1991; Stinchcomb, et al., 1979). However, the majority of origins has been identified based on prediction methods that addressed additional hallmarks for origin activity. One large scale experiment searched for simultaneous *in vivo* presence of ORC and the MCM complex because these two complexes are present on active origins (see chapter 1.6.2) (Wyrick, et al., 2001). In another study the genomewide replication timing profile of *S.cerevisiae* was created using density transfer experiments. Regions that doubled their DNA sequences earlier than the surrounding area were presumed to be *in vivo* origins of replication (Raghuraman, et al., 2001). Although these two approaches greatly advanced the knowledge of origin location, more work is necessary to clarify why particular ACS containing sequences are origins and others are not. A number of recent studies suggest that in addition to an ARS sequence many more factors affect the activity of an origin (see also chapter 1.7).

In other eukaryotes ARS sequences are less well defined. While origins in fission yeast are loosely determined by their high A-T content (Clyne and Kelly, 1995; Okuno, et al., 1999), metazoan origins cannot be identified on DNA sequence level. Instead of that in metazoans sites of replication initiation can appear both in small defined sequence areas (Toledo, et al., 1998) or large initiation zones of 10 to 50kb (Dijkwel, et al., 2002). Still the question remains why the origins in more complex eukaryotes are not specified by a certain sequence element. An explanation may come from the fact that origins are generally distributed in non-transcribed intergenic regions, most probably to avoid undesired interference of the replication and the transcription machinery. In budding yeast with its heavily transcribed genome there would be a selective pressure for origins to locate to nontranscribed regions. An advantage to target replication initiation to those regions would therefore be the evolution of specific DNA sequences as markers for nucleation sites of replication (Brewer, 1994). Metazoans with significantly larger genomes but unproportionally more untranscribed regions would not need such a specific sequence since a stochastic distribution of nucleation sites would still lead to sufficient origin spacing. This hyphothesis can also explain the findings of distinct initiation loci and initiation zones in metazoans. Most solitary origin sites have been identified within loci containing multiple genes (Abdurashidova, et al., 2000; Aladjem, et al., 1998; Toledo, et al., 1998). By contrast, broad initiation zones consisting of multiple inefficient origins are observed at loci where there are large intergenic regions (Dijkwel, et al., 2002; Ina, et al., 2001; Little, et al., 1993).

1.6.2 Events during replication initiation

Principally every origin has the potential to initiate replication once per cell cycle. *In vivo* only a subset of origins initiates per S-phase. This is because some origins can assemble proteins important for initiation faster than others. They are called early origins and replication forks emanating from them migrate across sites containing later origins before these have a chance to fire. The creation of an active origin can be divided into four different phases (Fig. 1.4):

The first is the binding of ORC to the ACS within an origin (Fig. 1.4 (1)) (Bell and Stillman, 1992). In *S.cerevisiae* ORC remains bound to the origins throughout the cell cycle probably to mark them as sites of replication initiation (Aparicio, et al., 1997; Diffley, et al., 1994).

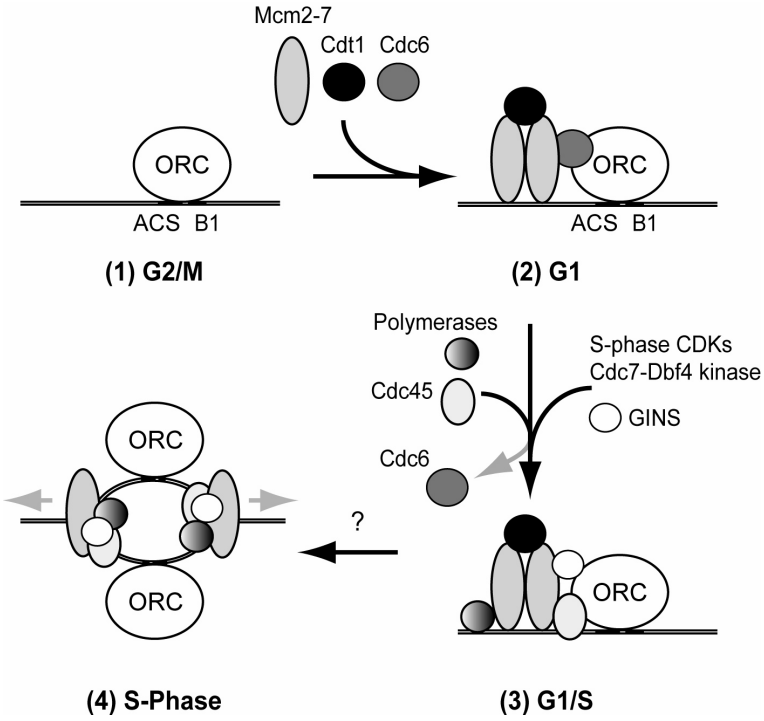

Fig. 1.4: Different phases of replication initiation. Details are described in the text.

In the second phase, ORC promotes assembly of a multiprotein complex called the pre-replicative complex (pre-RC) at origins (Fig. 1.2, (2)) (Diffley, et al., 1994). Pre-RC formation starts during G1 phase and requires minimally ORC, Cdc6, Cdt1 and the MCM (Mcm2-7) complex (Bell and Dutta, 2002). The pre-RCs license origins to initiate replication once per cell cycle and disassemble after the origin has started replicating in S phase. During pre-RC formation Cdc6 directly interacts with ORC and is required together with Cdt1 to recruit the MCM complex, a putative DNA helicase (Bell and Dutta, 2002; Lei and Tye, 2001).

In the third phase, additional proteins join the pre-RC immediately prior to replication initiation. These include Cdc45, the recently described four subunit GINS complex and the replicative DNA polymerases (Fig. 1.2, (3)) (Takayama, et al., 2003). Cyclin-dependent kinase (CDK) activity is required for Cdc45 to associate with the pre-RC (Zou, et al., 1997), and the association of Cdc45 with origins coincides with their time of activation - late origins recruit Cdc45 later in S-phae than earlier origins (Aparicio, et al., 1999). The Cdc7-Dbf4 kinase also associates with chromatin just prior to S-phase and interacts with ORC (Duncker, et al., 2002; Weinreich and Stillman, 1999). The following activation of the putative MCM

helicase and the Polα-primase involves at least 20 polypeptides that must assembly coordinately within the chromatin context of hundreds of origins throughout the *S.cerevisiae* genome.

The fourth phase, origin initiation, signals the beginning of S-phase, and involves localized DNA unwinding at the origin and the start of DNA polymerization. The mechanism of origin unwinding and the initiation of DNA synthesis are poorly understood in eukaryotes and might occur simultaneously with the association of some of the factors outlined in phase three. As the replication starts, Cdc45 and MCM become associated with replication forks. To avoid that origins initiate replication repeatedly within one S-phase, ORC, the MCM complex and Cdc6 are prevented from re-establihing pre-RCs. This is achieved by the activity of CDK, which is upregulated during S and G2/M phase (Drury, et al., 2000; Labib, et al., 1999; Nguyen, et al., 2001). Therefore CDK has the dual role to activate the pre-RC by promoting the binding of Cdc45 and simultaneously prevent new pre-RC assembly until the next G1 phase.

In summary, replication initiation at a given origin is a complex process requiring multiple proteins binding in several steps. It is conceivable that each individual step could be enhanced or inhibited by particular chromatin environments.

1.7 Regulation of replication initiation

There are many more factors besides origin structure that determine origin activity. Therefore the efficiency and timing of replication initiation for a given origin is always a sum of these factors that influence each other. The following chapter gives an overview on events that have been implicated in the regulation of replication initiation:

Chromosome localization

In yeast a number of observations suggest that the localization of an origin within the chromosome is important for its activity. For example placing a large 15kb fragment containing the late initiating ARS501 to a plasmid renders it to an early active origin (Ferguson, et al., 1991). Conversely, placing the effective and early initiating origin ARS1 near ARS501 on the chromosome causes ARS1 to fire as late as ARS501. Interestingly, ARS501 is located 27kb away from the right telomere of chromosome V, suggesting that

telomeres can exert a negative position effect on origin activity. In fact, whole genome replication timing experiments revealed that origins located proximal to telomeres (~40kb or less) are often activated later in S-phase (Raghuraman, et al., 2001).
However, not only the position relative to a telomere but also to other chromosomal regions determine the time of origin activity. For example a cluster of late initiating origins at chromosome XIV (ARS1411-ARS1414) is located more than 150kb away from telomeres. ARS1412 and ARS1413 sites function as early origins on plasmids but a considerable amount of surrounding DNA is necessary to recapitulate the late origin activation time (Friedman, et al., 1996). This indicates that large chromosomal areas distinct from the ARS sites are required to determine different activation times.

Chromatin structure

The telomere binding Ku protein complex (Ku) was shown to control the late activation of ARS501 and other telomere proximal origins (Cosgrove, et al., 2002). Ku is required for the telomere position effect (TPE) in *S.cerevisiae* (Mishra and Shore, 1999) but the effect of Ku on ARS501 firing is independent of Sir proteins (Cosgrove, et al., 2002). Thus, a specialized chromatin domain established by Ku might be responsible for modulating the timing of origins over a long distance. One role of Ku is to tether telomeres to the nuclear envelope (Laroche, et al., 1998) and perinuclear position appears to correlate with late replication in mammalian cells (Heun, et al., 2001). This lead to the theory that Ku influences the activity of origins by locating them in a nuclear compartment containing chromatin modifying factors that can establish a late-activation domain (Gilbert, 2001).
The Sir protein complex also affects origin activity by establishing a specialized chromatin structure. The telomeric X elements contain an inactive ARS and serve along with telomeric repeats as nucleation sites for Sir dependent heterochromatin formation at the telomeres (see chapter 1.5.2). Deletion of Sir3 revealed that telomeric sequence of chromosome V replicates earlier because of earlier initiation of a nearby ARS and activation of the inactive X element ARS (Stevenson and Gottschling, 1999).
However the regulation of origin activity by the Sir proteins appears not only to be restricted to regions that are repressed by Sir-mediated heterochromatin. A recent study found that deletion of the HDAC Sir2 rescued the general replication defect of a *cdc6-4* mutation (Pappas, et al., 2004). This could be caused by a loss of deacetylase activity around certain

origins. Interestingly, direct targeting of the HAT Gcn5 to the late initiating origin ARS1412 could significantly advance its timing of initiation (Vogelauer, et al., 2002).
Also deletion of the Rpd3, a global histone deacetylase, advanced replication of the entire genome (Vogelauer, et al., 2002). This was caused by a preferential advancement of late firing origins that also showed an increased acetylation status. It will be interesting to learn whether acetylation of origin regions coincides generally with a facilitated assembly of active origins.

Nucleosome positioning

While the influence of nucleosome movement and positioning in transcriptional regulation is well established, a picture is emerging that these events are also involved in the regulation of origin activity (Lipford and Bell, 2001; Simpson, 1990; Thoma, et al., 1984). At ARS1 the binding of Abf1 and ORC results in a nucleosome free zone across the origin and mutations in the Abf1 binding site can lead to nucleosome repositioning and reduced origin function (Venditti, et al., 1994). Similarly, forced movement of ARS1 into the central core of a nucleosome significantly reduced its origin activity (Simpson, 1990). Interestingly, if a nucleosome that normally constitutes one boundary of ARS1 is missing, origin function of ARS1 is also impaired. However, not the ability of ORC but of the MCM complex (Mcm2-7) to bind the origin is compromised. This points towards the influence of nucleosome positioning in events like late pre-RC formation or replication elongation (Lipford and Bell, 2001).
Origin activity is not only dependent on the close binding of nucleosome positioning factors but can also be regulated by factors binding to DNA within a distance of several hundred basepaires. For example the presence of multiple binding sites for the protein Mcm1 in a domain up to 600bp upstream of the telomeric ARS120 was shown to be important for full origin activity (Chang, et al., 2004). Since the binding of Mcm1 induces a 66° bend in the DNA (Acton, et al., 1997; West and Sharrocks, 1999) a theory is that multiple Mcm1 molecules in this domain induce a loop-like tertiary structure that could help to exclude nucleosomes from the area thus providing an environment for pre-RC assembly. Alternatively this tertiary complex could limit the region where ORC can bind to enhance its specificity for the ACS. Other DNA binding factors also regulate origin activity. For example stability of

plasmids carrying the ARS *HMR*-E as sole origin is compromised if a binding site for Rap1 within *HMR*-E is mutated (Kimmerly, et al., 1988).

Given the importance of the correct position of a nucleosome, protein complexes that can move or remove nucleosomes might also play a role in origin activity. Several chromatin remodelling factors like the chromatin accessibility complex (CHRAC) in *Drosophila* or SWI/SNF of *S.cerevisiae* and humans have been implicated in origin activity (Alexiadis, et al., 1998; Flanagan and Peterson, 1999). For example the human SWI/SNF complex interacts with the human papilloma virus E1 replication protein and is involved in efficient replication of papilloma virus DNA (Lee, et al., 1999). Also mutations in the budding yeast SWI/SNF remodeling complex compromises plasmid stability of plasmids carrying some origins (Flanagan and Peterson, 1999). Interestingly, while ARS1 activity is unaffected in this SWI/SNF mutant, a deletion of its Abf1 binding site renders this ARS1 dependent on SWI/SNF suggesting that Abf1 and SWI/SNF have redundant roles in the modulation of origin activity.

1.8 Proteins investigated in this work

The following two chapters provide information on the proteins Sum1, Hst1 and Rfm1 that had been the focus of this study. Since Sum1 is implicated in multiple cellular roles, more detailed sub-chapters will individually refer to these roles.

1.8.1 Sum1

Sum1 in silencing

More than a decade before the wild-type function of the protein Sum1 was described, a mutant allele of *SUM1*, *SUM1-1* puzzled researchers in the field of transcriptional silencing. In a screen for mutant suppressors of a loss of function allele of *SIR2*, a strong suppressing candidate allele was isolated. Since the screen was initially carried out with a *SIR2* mutant called *MAR1-1*, the identified allele received the name *SUM1* (suppressor of mar) (Klar, et al., 1985). Silencing at the *HM* loci can be disrupted or compromised by mutating or deleting any member of the SIR family genes (Fig. 1.5A). Surprisingly the identified mutant allele *SUM1-1* was able to reestablish silencing of both *HM* loci in any *sir* mutant (Klar, et al., 1985; Laurenson and Rine, 1991). It also suppressed several other mutations that impair *HM* locus

repression like mutations in *HHF2*, a gene for histone H4 or deletional weakening of the *HMR*-E silencer. Interestingly the ability of *SUM1-1* to repress *HMLα* in *sir2Δ* was decreased by 100fold in comparison to *HMR***a** and *SUM1-1* required either *HMR*-E or -I for full suppression of *HMR***a** (Laurenson and Rine, 1991; Sutton, et al., 2001). *SUM1-1* was also not able to overcome the telomeric derepression effect that occurs in a *sir2Δ* strain or upon introduction of the *rap1-17* mutant allele, while wild-type repression of telomeric silencing could be slightly improved (Chi and Shore, 1996).

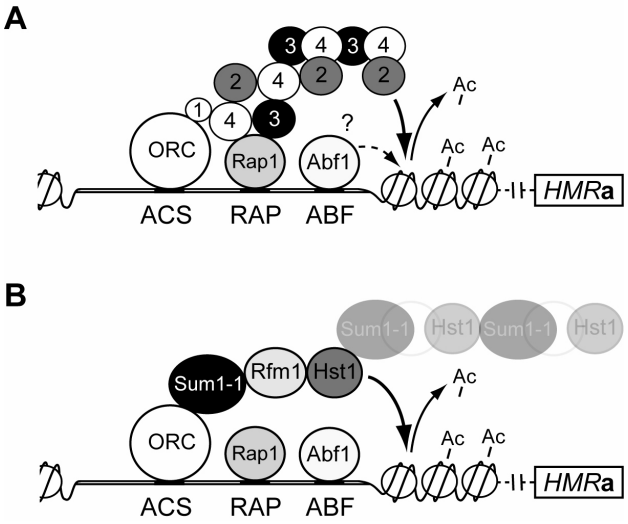

Fig. 1.5: Sum1-1 can establish silencing at *HMR*a independently of the Sir-proteins
(A) Schematic representation of Sir mediated silencing of *HMR*a. (B) Schematic representation of Sir-independent silencing of Sum1-1 via Rfm1 and the HDAC Hst1.

Also notable was the finding that *SUM1-1* mutants exhibit reduced viability and a significantly increased chromosome loss rate compared to wild-type cells. Cloning of the *SUM1-1* gene revealed a single missense mutation at codon 974 in the predicted 1062 aa of the gene *SUM1*, which resulted in a threonine-to-isoleucine change and was responsible for the dominant *SUM1-1* suppressor phenotype (Chi and Shore, 1996). It was later found that Sum1-1 as well as Sum1 recruit the Sir2 homologue Hst1 (homologue of Sir two) via a bridging factor Rfm1 (repression factor of MSEs) (McCord, et al., 2003; Rusche and Rine, 2001; Sutton, et al., 2001; Xie, et al., 1999). In addition Sum1-1 considerably enhanced an interaction with ORC at the silencer and therefore recruited the HDAC Hst1 to the silencer. It was hypothezised that the strong ORC-Sum1-1 interaction enabled Hst1 to deacetylate

histone tails at *HMR***a** analogous to Sir2. Since Hst1 as well as Sum1-1 were not only found at the silencer but also across the entire *HMR***a** locus it was assumed that these proteins were able to establish an alternative repressive structure that could spread along a chromosome like the Sir-protein complex (Rusche and Rine, 2001; Sutton, et al., 2001) (Fig. 1.5B). Interaction of Sum1-1 and ORC was probably via the N-terminus of Orc1 since a mutant allele of Orc1 that misses the 235 N-terminal amino acids eliminated the ability of Sum1-1 to silence the *HM* loci (Rusche and Rine, 2001).

So far, wild-type Sum1 has not been implicated in silencing since overexpression of *SUM1* did not lead to the *SUM1-1* phenotype and this phenotype was decreased if *SUM1* was coexpressed from plasmids. Therefore it was hypothesized that the *SUM1-1* mutation was not of increased function (hypermorphic) but rather of new function (neomorphic) (Chi and Shore, 1996). Additional investigations revealed that Sum1 was localized to the nucleus and was neither essential for normal growth nor transcriptional repression at the telomeres or the *HM* loci. In *sum1Δ* strains only a very mild derepression effect was observed at *HMR***a** in a sensitized assay where the *ADE2* gene replaced *HMR***a** and the E silencer was compromised by deletion of the ACS or the Abf1 binding site (Chi and Shore, 1996).

Sum1 in gene specific repression and meiosis

Although the mutant allele *SUM1-1* was a repressor of *HM* silencing defects, the wild-type gene product appeared not to be required for silencing. Instead, Sum1 was found to be a repressor for a number of middle sporulation specific genes during vegetative growth and in the early and late phase of sporulation (Lindgren, et al., 2000; Pak and Segall, 2002; Pak and Segall, 2002; Pierce, et al., 2003; Xie, et al., 1999). Sporulation specific genes can broadly be divided into early, middle or late categories based on the timing of their expression (Mitchell, 1994). In this respect middle sporulation specific genes are expressed as cells exit prophase, enter the nuclear division and assemble spores. More than 150 genes are induced around that time (Chu, et al., 1998). Activation of these genes is carried out by the Ndt80 transcription factor that binds to a conserved sequence termed the middle sporulation element (MSE) found in many middle sporulation gene promoters (Chu, et al., 1998; Ozsarac, et al., 1997). The MSEs of a subset of these genes is also bound by Sum1 during vegetative growth and in the early and late stages of meiosis which leads to their repression. This repression is brought about through Sum1´s recruitment of the histone deacetylase Hst1 (Xie, et al., 1999).

Comparison of sequence requirements for MSE binding revealed very similar but distinct consensus sequences for Sum1 (DSYGWCAYWDW) and Ndt80 (VNDNCRCAAW), so Ndt80 binding sites can be contained within Sum1 binding sites. Also subtle basepair changes within the MSE can alter the individual affinities of Sum1 or Ndt80 to the respective MSE site (Pierce, et al., 2003). Notably only a subset of middle sporulation genes was derepressed in *sum1Δ* strains, and some sporulation unrelated genes were affected (Pierce, et al., 2003). However, Sum1-repressed middle sporulation genes are further divided into two subclasses: one class whose repression is also dependent on Hst1 and Rfm1 and the other which is only Sum1 dependent (McCord, et al., 2003). The reason why not all MSE containing genes were derepressed in a *sum1Δ* strain is probably because some Sum1 repressed genes require additional activation by the meiosis specific Ndt80 transcription factor while others are not a target of Sum1. Also at some genes additional regulatory sequences such as URS1 (upstream regulatory sequence 1) are present. URS1 is mitotically bound by the Ume6-Rpd3-Sin3 repressor complex that prevents expression in a *sum1Δ* strain (Kadosh and Struhl, 1997; Pak and Segall, 2002). This, and the fact that Ndt80 itself contains MSE sites which are partly targets of Sum1, points towards a carefully controlled execution programm for meiotic genes (Pak and Segall, 2002).

1.8.2 Hst1 and Rfm1

All eukaryotic species examined to date have multiple homologues of Sir two (HSTs), which share a highly conserved globular core domain. In *S.cerevisiae* the family of HST proteins consists of four members *HST1* to *HST4* (Brachmann, et al., 1995; Derbyshire, et al., 1996). Yeast Hst1 is the closest relative to Sir2, showing 63% overall identity and 82% identity in the conserved core. Like Sir2 it exhibits NAD+ dependent deacetylase activity on K16 of histone H4 (Sutton, et al., 2001). Disruption of *HST1* has no phenotype regarding mechanisms in which *SIR2* has a role, namely, regional silencing of *HML*α (Brachmann, et al., 1995) or in rDNA recombination (Derbyshire, et al., 1996). However *HST1* overexpression can partially restore *HMR***a** silencing in a *sir2*Δ strain (Brachmann, et al., 1995). The other HST members have varying silencing phenotypes. For example the cytosolic *HST2* which also shows NAD+ dependent deacetylase activity improves rDNA silencing but reduces telomeric silencing when overexpressed, probably by competing with Sir2 for a limiting ligand (Perrod, et al.,

2001). *HST3* and *HST4* double mutants are defective in telomeric silencing and chromosome maintenance (Brachmann, et al., 1995).

Cellular Hst1 occurs in two different complexes: in the SET3 complex (Set3C) and in complex with Rfm1 and Sum1 (Pijnappel, et al., 2001; Xie, et al., 1999). Set3C consists of Set3, Snt1, YIL112w, Sif2, Cpr1 and two histone deacetylases, Hos2 and Hst1. It acts as a meiosis specific repressor for a set of genes that are expressed at the early middle- and middle stage of meiosis (Chu, et al., 1998; Pijnappel, et al., 2001). The repressive properties of Set3C are dependent on Hos1 and Hst1 but Hst1 is only weakly associated with Set3C (Pijnappel, et al., 2001). The second complex is the Hst1-Rfm1-Sum1 complex. As pointed out above, Hst1 almost exclusively interacts with Sum1 through Rfm1 (McCord, et al., 2003). It represses many but not all of the middle sporulation genes that are bound by Sum1 during vegetative growth (Xie, et al., 1999). Hst1 is also the enzymatically active component responsible for histone deacetylation and silencing at the *HM* loci in the *SUM1-1* mutant (Rusche and Rine, 2001; Sutton, et al., 2001).

Interestingly, Hst1 as an NAD+ dependent deacetylase is also involved in biosynthesis and maintenance of cellular NAD+ levels. Hst1 has a relatively low affinity to NAD+ and represses via Sum1 key factors for the *de novo* synthesis of NAD+. When NAD+ levels decrease, the repressive properties of Hst1 diminish and repression at these genes is abrogated so that NAD+ biosynthesis can take place (Bedalov, et al., 2003). By this simple feedback loop maintenance of particular cellular NAD+ levels are ensured.

1.9 Outline of this thesis

Silencers flanking the *HM* loci are among the determinants for the establishment of a transcriptionally silent domain across these regions. It has been shown that they consist of a set of binding sites for proteins that in turn can recruit the Sir-family proteins to this loci. Sir proteins are subsequently able to polymerize across the region, thereby deacetylating histone tails and creating heterochromatin. Thus, silencers provide the targeting sites for heterochromatin nucleation.

While the *HMR*-E silencer had been thoroughly mapped in the past, one sequence element within the *HML*-E silencer escaped molecular characterization. Deletional studies at *HML*-E had discovered a Rap1, an ORC binding site and a sequence of 93bp termed the D element. Deletion of each of these sites individually lead to minor derepression of $HML\alpha$. Deletion of any combination of two of these sites however resulted in total loss of $HML\alpha$ silencing (Mahoney, et al., 1991). This lead to the assumption, that the D element contained a binding site for a yet unidentified silencing protein.

The aim of this study was to identify a protein that binds the D element. Specifically, we found a minimal sequence element within the D element that was essential for D function. This element spanned 14 basepairs and was termed D2 element. We further identified a factor, the transcriptional repressor Sum1, that acted genetically via the D element. Using *in vitro* binding assays we showed that Sum1 bound the D element and that this binding was mediated via D2. *In vivo* assays further confirmed this finding, suggesting that Sum1 is the assumed factor that aids in the establishment of silencing at *HML*-E.

All silencers can act as origins of replication if replaced on plasmids. We found that Sum1 was necessary for full origin function of *HML*-E but not *HMR*-E and other origins lacking a Sum1 binding site. We (and others) found that $sum1\Delta$ was synthetically lethal with a mutation in the second largest ORC subunit, *orc2-1* (Suter, et al., 2004). Since this allele has severely reduced genomewide replication initiation efficiency (Foss, et al., 1993), this and the effect of Sum1 on origin activity of *HML*-E pointed towards a general relevance for Sum1 in replication initiation. This predicts a larger number of origins that are bound and affected by Sum1. Using the datasets of two *in vivo* binding studies (Lee, et al., 2002; Wyrick, et al., 2001), we identified a number of origins, that were bound by ORC and Sum1. Examination of several of these origins revealed that their activity on plasmids was indeed dependent on

SUM1. Furthermore we found for a subset of these origins that full activity in their chromosomal context was also dependent on Sum1.

As Sum1 in its role as transcriptional repressor often acts in concert with the HDAC Hst1, we tested the influence of *hst1Δ* on the activity of the origins of our dataset. Like Sum1, Hst1 also was able to positively regulate the activity of these origins. Since *hst1Δ*, like Sum1, also was synthetically lethal with *orc2-1* (Suter, et al., 2004), this pointed towards an important function of Sum1 and Hst1 in replication initiation for a subset of origins in the genome.

In summary, we provide evidence for two novel findings: (1) Sum1 is binding the D2 sequence within the D element and aids in the establishment of heterochromatin at *HML*. (2) Sum1 and Hst1 are general replication factors that are important for the activity for a subset of origins.

2 Materials and Methods

2.1 *E.coli* strains

TOP 10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 *lac*X74 *rec*A1 *ara*D139 (*ara- leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen, chemically competent)

DH5alpha: F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ$^-$ (Invitrogen, chemically competent)

BL 21 CodonPlus (DE3) RIL: F$^-$ *omp*T *hsd*S($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* λ(DE3) *end*A Hte [*argU ileY leuW* Cam^r] (Stratagene, chemically competent)

2.2 Growth conditions and media

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

2.3 Yeast strain construction

Yeast strains

Yeast strains used in this study are given in Table 2.1. Strains were generated either by direct deletion or by chromosomal integration of the sequence area of interest. Alternatively, strains were derived from crosses between strains from the laboratory stock. Strain AEY3474 was generated by transformation of AEY1558 with *Afl*II digested pAE223. Expression of the tagged Orc2 protein was confirmed by Western blotting using anti-polyhistidine antibody (Sigma). To generate strain AEY3552, pAE1163 was linearized in *HML* with *Bsu*36I and AEY2 was transformed to uracil prototrophy. Yeast transformations were according to (Klebe, et al., 1983) or by the lithium acetate procedure according to (Ito, et al., 1983).

Gene disruption

Gene knockouts were performed with the *kanMX* or *HIS3MX* module according to the guidelines for EUROFAN (Wach, et al., 1997; Wach, et al., 1994). Knockout strains were verified by PCR analysis. For genomic introduction of *HML*-E mutated alleles, the two step gene replacement technique was used. In brief the entire *HML*α locus was replaced by the

URA3 gene by transformation of AEY2 with an *URA3* PCR-product that carried primer-mediated homologous sequences of the respective genomic region resulting in the *hmlΔ::URA3* strain AEY3387. To construct *HML*-E mutant strains, *HML*-E mutant plasmid derivates (see chapter 2.5) were digested with *Apa*LI/*Hin*dIII, thus releasing a 4.2kb fragment that contained the *HMLα* locus and adjacent regions including *HML*-E. AEY3387 was transformed with these fragments and plated on 5-FOA plates to select for integrants that had lost the *URA3* gene at *HML*. Successful integration was verified via PCR analysis.

Sporulation and dissection of asci

For crosses, cell material of the two parental strains grown overnight was mixed together in a drop of water on YPD plates. After 8 h of incubation at 30°C (23°C for temperature sensitive strains) the cells were streaked out on selective medium for diploids. To induce the formation of spores, diploids were plated on sporulation medium (Sherman, 1991) and incubated at 30°C for 2-3 days or at 23°C for 3-4 days. Next, a loopful of asci was incubated in 10 µl zymolyase buffer (1M sorbitol, 0.1 M Na Citrate, 60 mM EDTA, pH 8.0, 5 mg/ml zymolyase (Seikagaku Corp.)) for 10 min. at RT. The zymolyase reaction was stopped by adding 250 µl H_2O. The ascospores were then dissected on YPD plates using a micromanipulator (Narishige) connected to a Zeiss Axioscope FS microscope. Plates were incubated at 30°C or 23°C for 3-5 days.

2.4 Yeast strains

Table 2.1: *S. cerevisiae* strains used in this study

Strain	Genotype	Source[a]
AEY2	*MAT***a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* (W303-1A)	
AEY80	*MAT***a** *nat1Δ::LEU2*	R.Sternglanz
AEY264	*MAT***a** *his4*	
AEY265	*MATα his4*	
AEY279	*MATα cdc7-1 ADE2 lys2Δ*	J.Rine
AEY373	*MATα cdc45-1*	
AEY1223	*MAT***a** *sum1Δ::URA3*	D.Shore
AEY1227	*MATα nat1Δ::LEU2*	
AEY3006	*MATα sum1Δ::URA3*	
AEY3008	*MATα nat1Δ::LEU2 sum1Δ::URA3*	
AEY3010	*MAT***a** *nat1Δ::LEU2 sum1Δ::URA3*	
AEY3358	*MAT***a** *sum1Δ::HisMX*	
AEY3362	*MAT***a** *sum1Δ::HisMX orc2-1* + p*URA3-ORC2*	
AEY3368	*MAT***a** *sum1Δ::HisMX orc2-1* + p*LEU2-ORC2*	
AEY3385	*MAT***a** *sum1Δ::HisMX orc2-1* + p*TRP1-SUM1*	
AEY3387	*hmlΔ::URA3*	
AEY3388	*MAT***a** *HML*-ΔI	
AEY3391	*MAT***a** *HML*-E Δ123-216 (ΔD) ΔI	
AEY3395	*MAT***a** *HML*-E ACS$^-$ ΔI	
AEY3398	*MAT***a** *HML*-E ACS$^-$ Δ123-216 (ΔD) ΔI	
AEY3401	*MAT***a** *HML*-E ACS$^-$ Δ123-132 (ΔD1) ΔI	
AEY3404	*MAT***a** *HML*-E ACS$^-$ Δ133-142 (ΔD2) ΔI	
AEY3407	*MAT***a** *HML*-E ACS$^-$ Δ143-154 (ΔD3) ΔI	
AEY3410	*MAT***a** *HML*-E ACS$^-$ Δ155-160 (ΔD4) ΔI	
AEY3412	*MAT***a** *HML*-E ACS$^-$ Δ161-166 (ΔD5) ΔI	

AEY3416	*MAT***a** *HML*-E ACS$^-$ Δ167-176 (ΔD6) ΔI	
AEY3419	*MAT***a** *HML*-E ACS$^-$ Δ177-186 (ΔD7) ΔI	
AEY3422	*MAT***a** *HML*-E ACS$^-$ Δ187-198 (ΔD8) ΔI	
AEY3424	*MAT***a** *HML*-E ACS$^-$ Δ199-210 (ΔD9) ΔI	
AEY3426	*MAT***a** *HML*-E ACS$^-$ mutated 133-146 (*d2*) ΔI	
AEY3430	*MAT***a** *HML*-E ACS$^-$ mutated 133-139 (*d2a*) ΔI	
AEY3434	*MAT***a** *HML*-E ACS$^-$ mutated 140-146 (*d2b*) ΔI	
AEY3476	*MAT***a** *HML*-E ΔD *HML*-ΔI *sum1Δ::HisMX*	
AEY3535	*MAT***a** *sum1Δ::HisMX sir4Δ::kanMX*	
AEY3536	*MAT***a** *sir4Δ::kanMX*	
AEY3542	*MAT***a** *cdc7-1*	
AEY3543	*MAT***a** *cdc7-1*	
AEY3544	*MAT*α *cdc7-1 sum1Δ::HisMX*	
AEY3545	*MAT***a** *cdc7-1 sum1Δ::HisMX*	
AEY3546	*MAT***a** *sum1Δ::HisMX*	
AEY3548	*MAT*α *cdc45-1 sum1Δ::HisMX*	
AEY3549	*MAT*α *cdc45-1 sum1Δ::HisMX*	
AEY3550	*MAT*α *cdc45-1 sum1Δ::HisMX*	
AEY3551	*MAT*α *sum1Δ::HisMX*	
AEY3552	*MAT***a** *HML*-E ACS$^-$ Δ147-216 (ΔD3-D9) ΔI *:: URA3*	
AEY565	*MAT***a** *ade2-101 his3Δ200 leu2-Δ1 trp1Δ1 ura3-52 lys2-801 ppr1Δ::HIS3*	O.Aparicio
AEY1558	*MAT***a** *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112*	B. Jones
AEY3474	AEY1558 *ORC2-6xHis*	
AEY1281	*MAT***a** *can1-100 ade2-1 his5-2 leu2-3,112 lys1-1 trp5-48 ura3-52 HML-*EΔ90-95 (ΔRAP) *HML*-IΔ242	J. Broach
AEY1282	*MAT***a** *HML*-EΔ123-216 (ΔD)	J. Broach
AEY1283	*MAT***a** *HML*-EΔ107-216 (ΔACSΔD)	J. Broach
AEY1313	*MAT***a** *HML*-EΔ107-119 (ΔACS)	J. Broach
AEY3193	AEY1313 *sum1Δ::kanMX*	
AEY3202	AEY1283 *sum1Δ::kanMX*	
AEY3204	AEY1281 *sum1Δ::kanMX*	
AEY3480	AEY1281 *rfm1Δ::kanMX*	
AEY3482	AEY1313 *rfm1Δ::kanMX*	
AEY3484	AEY1283 *rfm1Δ::kanMX*	
AEY3486	AEY1281 *hst1Δ::kanMX*	
AEY3488	AEY1313 *hst1Δ::kanMX*	
AEY3490	AEY1283 *hst1Δ::kanMX*	
AEY600	*cdc6-1 MAT***a** *leu2-3,112 lys2Δ ura3-1*	D.Koshland
AEY3537	*cdc6-1* [b]	
AEY3538	*cdc6-1* [b]	
AEY3539	*cdc6-1 sum1Δ::HIS*MX [b]	
AEY3540	*cdc6-1 sum1Δ:: HIS*MX [b]	
AEY3541	*cdc6-1 sum1Δ:: HIS*MX [b]	
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Resgen Inc.

[a]Unless indicated otherwise, strains were constructed during the course of this study or were from the laboratory strain collection. Groups of strains between horizontal lines are isogenic.
[b]segregant from AEY600 x AEY3358

2.5 Plasmid construction

E.coli transformation by plasmids was carried out with TOP10 or DH5α strains according to the manufacturers´ protocols. Plasmid DNA was extracted from *E.coli* by the alkaline lysis procedure (Sambrook, et al., 1989), and further purification using the Qiagen plasmid kits. Plasmids from yeast were isolated using the protocol of Jacques Paysan: 1.5ml yeast culture was grown to saturation, pelleted and incubated in 200µl zymolyase solution (1.2M sorbitol, 0.1M KPO_4 pH 7.5, 400µg/ml zymolyase) for 2h at 37°C. Then plasmids were isolated by alkaline lysis with the Qiagen plasmid kit continuing with 400µl Buffer 2.
Plasmids used in this study are listed in Table 2.2. To generate pAE419 and pAE421 a *Spe*I/*Pst*I fragment of *HML*-E was blunt ended using Klenow enzyme and ligated into the SmaI site of pAE370. To generate pAE440 and pAE442 a 140bp *Eco*RI fragment of *HML*-E was blunt ended and ligated into the SmaI site of pAE370. The directionality of the ligations was checked by sequencing. Deletions in the ACS site or the D element of *HML*-E were introduced using the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA) generating the plasmids pAE735-736 and pAE739-740. To generate the mutant D-element versions of *HML*-E, a 5.17kb *Hind*III/*BamH*I fragment of *HML*-E was ligated into the *Hind*III/*Bam*HI site of pUC18 (pAE1033). The *HML*-I deletion, the *HML*-E ACS-site mutation and the small deletions across the D-element were introduced using the Quick Change site-directed mutagenesis kit from Stratagene. *HML*-I was deleted in pAE1033 according to (Mahoney, et al., 1991) to generate pAE1034. Mutation of the *HML*-E ACS site was achieved by replacing the central part of its 11bp sequence by a *Kpn*I restriction site in pAE1034 to generate pAE1035. For the D-element-core screen, deletions of 10bp were introduced to create the $D1^-$ to $D3^-$ plasmids. To construct plasmids $D4^-$ to $D9^-$, 6 to 12 basepairs were replaced by a *Sac*II restriction site in the specified sequences. The numbering system shown in Table 2.2 is based on (Feldman, et al., 1984). The mutation *d2* affecting the D2 element was introduced by replacing the wild-type 14bp sequence TTTTCGGCACGGAC by AATACCGGAGGCAG, thus creating pAE1050. Derivates of pAE1050, where the first 7 bp (*d2a*) or last 7bp (*d2b*) of the D2 sequence were mutated as indicated above are pAE1051 and 1052. To create the minimal D plasmid, 68bp downstream of D2 were deleted to generate pAE1162. The parent plasmid of the resulting plasmids pAE1036 through 1052 and pAE1162 was pAE1035. As a control, the entire 5.17 KB *Bam*HI/*Hin*dIII fragment containing the *HML* locus on the final plasmids was sequenced. For genomic insertion of the minimal D element, pAE1162 was digested with *Hin*dIII/*Bam*HI and the resulting insert ligated into the *Hin*dIII/*Bam*HI site of pRS306, resulting in pAE1163. The N-terminally tagged 6xHis-*SUM1* expression plasmid for bacterial expression was constructed by ligating a PCR-generated fragment of the complete *SUM1* open reading frame (ORF) into the *Nde*I/*Bam*HI sites of pET15b (Novagen) to generate pAE1054. The N-terminally 6xmyc tagged *SUM1* expression plasmid was constructed as follows: the 500bp upstream promoter region of *SUM1* was PCR amplified with flanking *Kpn*I and *Xho*I sites and ligated into the *Kpn*I/*Xho*I sites of pAE469. pAE469 was derived from pRS306 and carries six c-myc tags in its *Xho*I/*Not*I site. Next, the entire *SUM1* ORF was PCR amplified with flanking *Not*I and *Sac*II sites and ligated into the *Not*I/*Sac*II sites of the previous construct, which placed the *SUM1* ORF downstream of the 6xc-myc sequence. To generate a 2µ-version, the promoter-6xmyc-*SUM1* sequence was released from the previous construct by *Kpn*I/*Sac*II digestion and ligated into the *Kpn*I/*Sac*II digested multiple cloning site of pRS424. The resulting construct pAE1032 was verified by sequence analysis, and the expression of the recombinant protein in yeast was confirmed by Western blot of yeast transformants using an anti-myc antibody. To generate plasmids for the

plasmid loss tests of ARS606, ARS1223 and ARS1511, the ARS1013-1 carrying plasmid pAE1078 was digested with *Eco*RI/*Hind*III to release ARS1013-1 and blunt ended using Klenow enzyme. The above mentioned ARS sites were PCR amplified from genomic DNA of AEY2 using the primer pairs ARS606fw: GTCTTCTTGATAATTCTGTGGGCGC, ARS606rv: GTCTTGCCTTAGGACTCAGCCAGG for ARS606, ARS1223up: CTTGAGT-CAAGTTCAGAGTAATTTTCGG, ARS1223down: CCCATTTGACGCAAGGCAATTTC-CCTG for ARS1223 and ARS1511up: CGACCCTGCAGCAGCTGCTCAG, ARS1511down: CCAGCTCATCTGCAGCTGCC for ARS1511. Amplicons were subcloned into the TOPO-TA xl vector (Invitrogen). For ligation into the plasmid backbone of pAE1076, they were released from TOPO by *Sac*I/*Xho*I digestion and blunt ended using Klenow enzyme. The resulting constructs pAE1126 (ARS606), pAE1130 (ARS1223) and pAE1135 (ARS1511) were verified by sequence analysis. To generate plasmids that carried ectopic Sum1 binding sites upstream of ARS1013-2, pAE1080 was digested with *Hin*dIII and blunt ended using Klenow enzyme. Inserts were either a 56bp oligonucleotide containing 4xD2 sequence resulting in pAE1159 or a PCR product derived from pAE1035 (*HML*-D) using primers HML-Eup: GGTGTATCGCAATGGAATG and HML-Edown: CCCGAAATCGATAATAA-TGGCC resulting in pAE1160. The *SMK1* promoter insert was PCR generated from genomic DNA using the primers for *SMK1* as used in the chromatin immunoprecipitation PCR which resulted in pAE1161.

Table 2.2: Plasmids used in this study

Plasmid	Description	Source[a]
pAE 53	pRS315 *ORC2*	J.Rine
pAE 168	pRS316 *HML*α	J.Rine
pAE 223	YIplac128-GPD-His_6-*ORC2*	
pAE 298	YIp5-CEN6 ss*HMR*-E	J.Rine
pAE 299	YIp5-CEN6 *HMR*-E	J.Rine
pAE 469	pRS306 6xmyc	
pAE 370	pRS315 *URA3* **a**1 *HMR*-I	
pAE 374	pRS315 *HMR*-E *URA3* **a**1 *HMR*-I	
pAE 419	pRS315 *HML*-E *URA3* **a**1 *HMR*-I	
pAE 421	pRS315 *HML*-E revers *URA3* **a**1 *HMR*-I	
pAE 440	pRS315 *HML*-E ΔD *URA3* **a**1 *HMR*-I	
pAE 442	pRS315 *HML*-E ΔD revers *URA3* **a**1 *HMR*-I	
pAE 735	pRS315 *HML*-E ΔACS *URA3* **a**1 *HMR*-I	
pAE 736	pRS315 *HML*-E ΔACS ΔD *URA3* **a**1 *HMR*-I	
pAE 739	pRS315 *HML*-E ΔACS revers *URA3* **a**1 *HMR*-I	
pAE 740	pRS315 *HML*-E ΔACS ΔD revers *URA3* **a**1 *HMR*-I	
pAE 1032	p*TRP1* 6xmyc*SUM1*	
pAE 1033	pUC18 *HML*α	
pAE 1034	pUC18 *HML*α *HML*-ΔI	
pAE 1035	pUC18 *HML*α *HML*-E ACS^- *HML*-ΔI	
pAE 1036	pUC18 *HML*α *HML*-E Δ123-216 (ΔD) *HML*-ΔI	
pAE 1038	pUC18 *HML*α *HML*-E ACS^- Δ123-216 (ΔD) *HML*-ΔI	
pAE 1039	pUC18 *HML*α *HML*-E ACS^- Δ123-132 (ΔD1) *HML*-ΔI	
pAE 1040	pUC18 *HML*α *HML*-E ACS^- Δ133-142 (ΔD2) *HML*-ΔI	
pAE 1041	pUC18 *HML*α *HML*-E ACS^- Δ143-154 (ΔD3) *HML*-ΔI	
pAE 1042	pUC18 *HML*α *HML*-E ACS^- Δ155-160 (ΔD4) *HML*-ΔI	

pAE 1045	pUC18 *HML*α *HML*-E ACS$^-$ Δ161-166 (ΔD5) *HML*-ΔI	
pAE 1046	pUC18 *HML*α *HML*-E ACS$^-$ Δ167-176 (ΔD6) *HML*-ΔI	
pAE 1047	pUC18 *HML*α *HML*-E ACS$^-$ Δ177-186 (ΔD7) *HML*-ΔI	
pAE 1048	pUC18 *HML*α *HML*-E ACS$^-$ Δ187-198 (ΔD8) *HML*-ΔI	
pAE 1049	pUC18 *HML*α *HML*-E ACS$^-$ Δ199-210 (ΔD9) *HML*-ΔI	
pAE 1050	pUC18 *HML*α *HML*-E ACS$^-$ mutated 133-146 (*d2*) *HML*-ΔI	
pAE 1051	pUC18 *HML*α *HML*-E ACS$^-$ mutated 133-139 (*d2a*) *HML*-ΔI	
pAE 1052	pUC18 *HML*α *HML*-E ACS$^-$ mutated 140-146 (*d2b*) *HML*-ΔI	
pAE 1054	pET15B-*SUM1*	
pAE 1076	CEN4-*URA3* + ARS1012	O. Aparicio
pAE 1078	CEN4-*URA3* + ARS1013-1	O. Aparicio
pAE 1080	CEN4-*URA3* + ARS1013-2	O. Aparicio
pAE 1081	CEN4-*URA3* + ARS1013-3	O. Aparicio
pAE 1119	pMW311 *HML*-E	J. Huberman
pAE 1123	pRS426 *HML*α	
pAE 1126	CEN4-*URA3* + ARS606	
pAE 1130	CEN4-*URA3* + ARS1223	
pAE 1135	CEN4-*URA3* + ARS1511	
pAE 1159	CEN4-*URA3* + ARS1013-2 + 4xD2	
pAE 1160	CEN4-*URA3* + ARS1013-2 + HML-E ACS-	
pAE 1161	CEN4-*URA3* + ARS1013-2 + SMK1promoter	
pAE1162	pUC18 *HML*α *HML*-E ACS$^-$ Δ147-216 (ΔD3-D9) *HML*-ΔI	
pAE1163	pRS306 *HML*α *HML*-E ACS$^-$ Δ147-216 (ΔD3-D9) *HML*-ΔI	

[a]Unless indicated otherwise, plasmids were constructed during the course of this study or were from the laboratory plasmid collection.

2.6 Silencing assays

Mating assays were performed using AEY264 (*MAT***a** *his4*) and AEY265 (*MAT*α *his4*) as mating-type tester strains. For qualitative mating assays (patch mating), strains were grown on YPD plates overnight and replica-plated to a lawn of the respective tester strain on YM medium selective for diploids. After 2-4d of incubation, the yield of diploids indicated the mating efficiency of the strain. Quantitative mating assays were performed as described (Ehrenhofer-Murray, et al., 1997). All quantitative mating efficiencies are the average of at least two independent determinations and were normalized to the wild-type srain AEY2.

2.7 Plasmid loss assay

Plasmid loss rates were determined in strains AEY2 and AEY2 *sum1Δ*::*HIS*MX. Transformants were grown into stationary phase in minimal medium lacking uracil before inoculating into rich medium (YPD) that was further supplemented with adenine, histidine, leucine, lysine, tryptophan and uracil at standard concentrations. The initial fraction of cells that contained the plasmid (F_i) was determined by plating dilutions of the new culture onto solid medium either containing or lacking uracil. After at least 12 doublings at 30°C with shaking, the final fraction of cells that contained the plasmid (F_f) was determined in the same way. The loss rate (L) was calculated as $1\text{-}10^m$, where $m = [\log(F_f) - \log(F_i)]$/number of cell divisions (Dillin and Rine, 1997). The loss rate is therefore equivalent to the fraction of daughter cells that have received no plasmid during the previous cell division

2.8 Yeast extracts for Western blotting

Approximately 10 OD of cells were harvested and washed once in ice cold TBS. Then the cells were resuspended in 250µl lysis buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1mM PMSF, 1x "complete" proteinase inhibitor (Roche Diagnostics)) and glass beads (0.5mm diameter) were added slightly below liquid level. Cells were disrupted by vortexing at full speed for 3 min followed by 2 min incubation on ice, this step was repeated once. The resulting lysate was pipetted off the glass beads and centrifuged for 10 minutes at 14,000 rpm in a 5415C Eppendorf table centrifuge. The supernatant was collected and 15-50µl were mixed with the appropriate amount of 4 x Laemmli buffer (see (Sambrook, et al., 1989)), incubated for 5 min at 70°C and applied on SDS gels. Alternatively the samples were stored at -80°C.

2.9 SDS PAGE and Immunoblotting

Proteins were separated by SDS-PAGE in Tris-glycine buffer according to standard methods (Laemmli, 1970). Transfer to nitrocellulose membranes (Pharmacia) was accomplished by blotting with the BIO-RAD Tank Transfer System for 1 hour at 0.8 mA/cm^2 in 39 mM glycine; 48 mM Tris; 0.037% SDS; 20% methanol. The nitrocellulose membrane was subsequently blocked for 1 hour at RT in 5% milk/TBS-T (50 mM Tris-HCl pH7.5; 150 mM NaCl; 0,05% Tween-20). After incubation over-night at 4°C with the primary antibody (concentrations see below) in 5% milk/ TBS-T, the blot was washed 4 times for 5 minutes with TBS-T. It was then incubated with the appropriate secondary antibody (concentrations see below) in 5% milk/ TBS-T for 30-60 minutes at RT. After washing 6 times for 5 minutes with TBS-T the SuperSignal West Pico Chemiluminescent Substrate from Pierce was used for immunochemical detection.
Anti-epitope antibodies were purchased from Invitrogen (α-myc; 1:5,000) and Sigma (α-polyhistidine 1:3000). Secondary antibodies conjugated to horseradish peroxidase were purchased from Sigma (sheep α-mouse; 1:1000 and goat α-guinea pig; 1:10,000). Antibodies were used for Western blotting in the concentrations as indicated.

2.10 Co-Immunoprecipitation

Yeast extracts from the protease deficient strains AEY1558 and AEY3474 were prepared as follows: Per co-immunoprecipitation experiment, 50 OD of cells were suspended in 500µl lysis buffer (50mM Tris-HCl pH7.5, 140mM NaCl, 1% Triton X-100, 0,1% ND-40, 1mM PMSF, 1x "complete" proteinase inhibitor (Roche Diagnostics), 1mM DTT) and disrupted with glass beads for 5min at 4°C using a vortex mixer. The lysate was cleared by centrifugation for 10min at 4°C. Antibody (α-myc, Invitrogen or α-poly-His, Sigma) was added to the cleared lysate and incubated overnight at 4°C with shaking. For experiments excluding DNA mediated co-immunoprecipitation, ethidiumbromide was added to a concentraion of 100µg/ml to the lysate prior to the addition of antibody. Subsequently, G-Sepharose 4-FF beads (Pharmacia) were added to the lysate-antibody mix and incubated for 1h. Immunoprecipitates were collected by brief centrifugation and washed three times with lysis buffer. The resulting precipitate was resuspended in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting according to standard protocols.

2.11 Electrophoretic mobility shift assays (EMSA)

Protein expression and purification

For bacterial expression of Sum1, N-terminal 6xHis-tagged Sum1 was generated by inserting *SUM1* into the *Nde*I/*Bam*HI-site of plasmid pET15B (Novagen) resulting in pAE1054. BL21 Codon Plus (DE3)-RIL cells (Stratagene) were transformed with pAE1054. Liquid cell culture (1300 ml LB-Amp, 2% glucose) was grown to mid-log phase (OD 0.5) at 37°C and subsequently mixed with 700 ml LB-Amp, 2% glucose of 15°C. Sum1-expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 0.5mM. To reduce protein degradation, ethanol was added to a final concentration of 2%. To minimize the risk for inclusion body formation, the cells were allowed to grow at 15°C for 16h. *LacZ* expression was induced by addition of 1mM IPTG to a mid-log culture of 300ml and subsequently incubated at 37°C for 3h. Protein extraction under native conditions and purification was essentially done as described in protocols 9+12 of the QIAexpressionist™ handbook (Qiagen, 2003). Cells were harvested and resuspended in 15 ml lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 10mM imidazole, 1% Triton X-100, PMSF 1mM, 1x proteinase inhibitor mix). Cell lysis was carried out by sonication (3x 20sec at 40% sonicator capacity) and the lysate was cleared by centrifugation. 2 ml 50% Ni-NTA matrix was then added to the supernatant and incubated with rotation for 1h at 4°C. The suspension was loaded onto a column and washed twice with 10ml washing buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 20mM imidazole). The matrix bound fraction of the lysate was eluted by adding 10 x 500µl buffer (see washing buffer) containing 250mM imidazole and collected separately. Protein containing fractions were pooled and dialyzed overnight at 4°C against protein dilution buffer (20mM Tris-HCl pH 8, 50mM NaCl and 1mM EDTA). To increase the protein concentration centricon tubes (Amicon) with an exclusion threshold of 10 kDalton were used according to the manufacturers guidelines.

Probe preparation

Probe preparation and EMSAs were essentially carried out as previously described (Xie, et al., 1999). For EMSAs, PCR fragments of the respective regions were amplified from AEY2 (for the *HML*-E wt and the *INO1* sequence), AEY3395 (for the *HML*-E ACS$^-$ sequence), AEY3391 (for the *HML*-E DΔ sequence) or AEY3398 (for the *HML*-E ACS$^-$DΔ sequence) and were purified and and diluted to equal concentrations. The primer sequence for the *HML*-E wt and *HML*-E ACS$^-$ PCR reaction was: GGTGTATCGCAATGGAATG (HML-E up) and CCCGAAATCGATAATAA (HML-E down). The reverse primer for the *HML*-E ACS$^-$DΔ PCR reaction was GTTTACATTTCATTCTATGTGCGCTAG (HML-E downII). For the INO1 PCR product, primers TGTTCTGTTGTCGGGTTCC (INOup) and GTAGTCTT-GAACAGTGGGCG (INOdown) were used.. For experiments described in Fig. 3.5B, PCR primers for *HML*-E wild-type and *HML*-E D2Δ were GGGTTTTTGATTTTTTTATGTTTTT-TTTAAAACATTAAAG (HML-EACSfw) and HML-Edown. For *HML*-E DΔ, primers were GGGTTTTTGATTTTTTTATGTTTTTTTTAAATCGATTTCG (HML-E D$^-$fw), and HML-E downII. For *SMK1* sequence binding, the oligonucleotide sequence was CCACTAATTTGT-GACACTT (with corresponding antiparallel oligonucleotide). Radioactive labeling was done by polynucleotide kinase catalyzed addition of [γ-^{32}P]-ATP to 5´-ends of the double stranded DNA. Separation of unincorporated nucleotides was accomplished by gel filtration using Nick™ Sephadex G-50 columns (Amersham Biosciences) or Micro Bio-Spin P-30 columns (Biorad) according to the manufacturers guidelines. The specific activity of the DNA-probes was determined by a scintillation counter.

EMSA

Binding reactions for the protein preparations were carried out in 10 mM Tris-HCl pH 7.5, 40 mM NaCl, 4 mM $MgCl_2$, 6% (w/v) glycerol, 10µg/ml of sonicated salmon sperm DNA and ^{32}P-labeled DNA sequence (10 000 counts per minute) in a total volume of 20µl at room temperature. Competition experiments were performed by premixing a 400fold molar excess of unlabeled *HML*-E DNA or the unspecific *INO1*-DNA with protein extract before the addition of labelled *HML*-E DNA probe. Incubation time was 20min prior to loading on gels. Samples were analyzed on 20cm 6% polyacrylamide gels (run in 0.5xTBE buffer for 120 min at 200V). Gels were dried after electrophoresis, exposed to a storage phosphor screen (Molecular Dynamics) and scanned on a phosphorimager (Molecular Dynamics).

2.12 Chromatin immunoprecipitations

Chromatin immunoprecipitations (ChIP) were performed essentially as described (Rusche and Rine, 2001), except that mouse anti-myc antibody (Invitrogen) at 4µg per sample and protein G sepharose beads were used. Crosslinking was carried out in 1xTBS with 10mM dimethyl-adipimidate (DMA, Pierce) for 45min at room temperature and subsequently in 1xTBS with 1% formaldehyde for 30min (Kurdistani and Grunstein, 2003). PCR reactions were performed using 1.25 units of Taq DNA polymerase (Promega), with 3mM $MgCl_2$, 0.25mM dNTPs and 0.5 µM per primer. Samples were cycled 28 times for 15sec at 94°C, 20 sec at 54°C and 2.5 min at 72°C. The oligonucleotides used are described in (Rusche and Rine, 2001), except *HML*-EdownII: GTTTACATTTCATTCTATGTGCGCTAG which was used as reverse primer for *HML*-E sequence amplification.

2.13 *In vivo* replication-origin assay

The replication-origin assay was carried out according to (Fangman and Brewer, 1991) and is described below in brief:.

Preparation of genomic 2-D DNA

a.) Culture growth and harvesting

Per replication origin test 1 liter each of AEY2 and AEY3358 was grown in liquid YPD to an OD_{600} of 1.0 to 1.5 either at 18°C or at 30°C. For harvesting, 1L centrifuge bottles were prepared with 100ml dry-ice frozen EDTA-glycerol solution (0.2M EDTA pH 8.0, 20% glycerol in water) each. Prior to harvesting, the cell culture was treated with azide at a concentration of 1g/liter and 900ml were subsequently poured into the precooled centrifuge bottles and incubated until the EDTA-glycerol solution was completely thawed. Cells were pelleted by centrifugation in a GS3 rotor (Sorvall) for 5 min at 5000 rpm at 4°C. The pellet was washed in 10m ice cold water. Cells were resuspended in 6ml Nuclear Isolation Buffer, NIB (17% glycerol, 50mM MOPS, 150mM K-COOH, 2mM $MgCl_2$, 500µM spermidine and 150µM spermine, adjusted to pH 7.2) and frozen at -80°C for DNA isolation.

b.) Nucleus extract preparation

Cells in NIB were mixed with an equal amount of glass beads in a 50ml conical bottomed plastic tube. Cell disruption was carried out by multiple cycles (20 to 25) of 30 seconds vortexing at full speed and 30 seconds incubation on ice. The cell nuclei-containing supernatant was recovered and the glass beads were washed twice with 8ml NIB per washing step. Supernatants from each washing step were combined and centrifuged in an SS-34 rotor (Sorvall) at 8000 rpm, 4°C for 10 min. The resulting pellet was resuspended at $2x10^9$ cell

equivalents/ml in 50mM Tris, 50mM EDTA, 100mM NaCl, pH 8.0. Cell nucleus disruption was achieved by adding the detergent N-Laurylsarcosine to a final concentration of 1.5%. Subsequently proteinase K was added to a final concentration of 300µg/ml and incubated at 37°C for 1h. Cell nucleus ghosts were removed upon centrifugation for 5 min at 4°C and 5000 rpm using a SS-34 rotor.
c.) High molecular weight DNA preparation
The supernatant from the previous step was recovered and mixed with 1.05g CsCl/ml. Hoechst 33258 dye was added to a final concentration of 125µg/ml, the samples were transfered to ultracentrifuge tubes and centrifuged at 50.000 g for 17-24h. High molecular weight DNA was visualized by long wave ultraviolett light and recovered with a syringe. Subsequently the DNA was precipitated by addition of 3 volumes of 70% ethanol and gentle swiveling of the reaction tube. The precipitate was washed with 70% ethanol, air dried and dissolved in 200µl TE per liter yeast culture for 3-4 days at 4°C.

2-D gel electrophoresis
Restriction enzymes were chosen, that resulted in a fragment of 4kb-5kb carrying the suspected replication origin in its center. For analysis of ARS 1013, 250µg DNA of strains grown at 30°C was digested with *Bfu*AI, which generates a 4.25kB fragment. For analysis of ARS606, 100µg DNA of strains grown at 18°C or 30°C was digested with *Nar*I and *Sfo*I, generating a fragment of 4.3kb. For analysis of ARS 607, 100µg DNA of strains grown at 18°C was digested with *Apa*LI and *Sac*I, which generates a 4.2kB fragment. For analysis of ARS1511, 100µg DNA of strains grown at 18°C was digested with *Pst*I, generating a fragment of 5.7kb. Per origin, DNA was digested in a volume of 500µl. 100-200 units of restriction enzyme were used and after an incubation time of 1h at 37°C again 100-200 units of restriction enzyme was added and incubated for another hour at 37°C. To enrich single-stranded replication intermediates BND-Cellulose (Benzoylated Naphtoylated DEAE Cellulose, Sigma St. Louis, USA) was used according to J.A. Huberman. After precipitation the DNA was resuspended in 30µl TE for 2h on ice. DNA was separated on a 15cm 0,4% TBE agarose gel in 1xTBE buffer for 18h at 2,5-3V/cm and 4°C. DNA between 1n and 2n of the tested fragment was cut out, cast into a 1% TBE agarose gel at a 90° turned angle and run for 18h at 50V and 4°C to resolve the DNA in its second dimension.

Southern blotting and hybridization
A rectangle containing the twodimensionally separated DNA structures was cut out from the gel and blotted onto a Zeta-Probe GT membrane (BioRad, Hercules CA) via Southern blot. The membran was hybridized with one or two [α-^{32}P]-dCTP labeled probes. ARS1013 was detected using two probes: a 1.2-kB *Eco*RI-*Hin*dIII fragment of pAE1078 and a 380-bp *Eco*RI-*Sac*I fragment of pAE1081. ARS606 was detected by a 490bp probe, amplified via PCR from genomic DNA. The primer sequence was ARS606up: GGTCTTCTTGATA-ATTCTGTGGGCGC and ARS606dn: TGTCTTGCCTTAGGACTCA-GCCACC generated from genomic DNA. ARS607 was detected using a 1145bp probe amplified via PCR from genomic DNA. The primer sequence was ARS607up: GCTCTAGAAGTAGTTCTAGTGGG and ARS607dn: GGCCTAATAGGAGTAACTACGGG. ARS1511 was detected by a 653bp probe, amplified via PCR from genomic DNA. The primer sequence was ARS1511up: CTCTACTACTACAACTATTCCCACTGG and ARS1511dn: GACATATTGTGCCTCAA-CTCTTGCAG. After washing the membrane was exposed to a storage phosphor screen (Molecular Dynamics) and scanned on a phosphorimager (Molecular Dynamics).

3 Results

3.1 Definition of a core region within the D element

The *HM* loci are flanked by the E- and I-silencers that contain a number of protein binding sites (Fig. 3.1A). At the *HML*-E-silencer, three functional elements essential for silencing have been defined within a region of 150bp. Deletion experiments uncovered the presence of a Rap1- and an ORC-binding site and the so called D element (Mahoney, et al., 1991) (Fig. 3.1A). In a strain lacking the *HML* I-silencer, deletion of any one of these regions leads to minor loss but deletion of any two of these regions leads to a complete loss of silencing at *HML*α (Fig. 3.1B) (Mahoney, et al., 1991). Unlike the Rap1- and the ORC binding site, the D element was so far uncharacterized and consisted of a large 93bp sequence stretch. Since all other essential silencer elements contain protein binding sites we hypothesized that the D element might also harbor an unidentified binding site for a silencing protein which we sought to identify in this study.

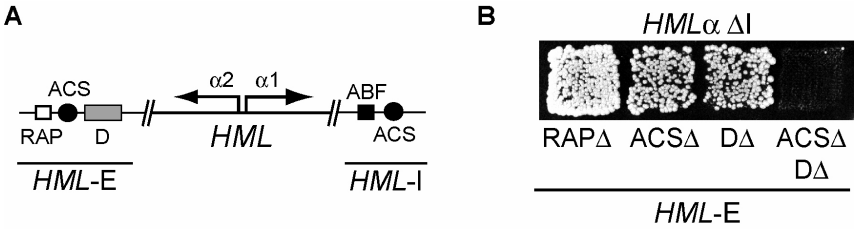

Figure 3.1: Silencing properties of the D-element at the *HML*-E silencer.
(A) Schematic representation of the *HML*α locus on the left arm of chromosome III. Location and elements of the silencers *HML*-E and *HML*-I are indicated (RAP = Rap1 binding site, ACS = ORC binding site, D = D element, ABF = ABF binding site). (B) Redundancy of *HML*-E silencer elements. Loss of *HML*α silencing in *HML*-E silencer deletion mutants was measured as loss of a-mating ability in a patch mating assay. All strains were *HML*-ΔI.

In a first set of experiments, we asked whether the D element could be narrowed down to a smaller DNA segment, because protein binding motifs usually occupy a sequence stretch of 10 to 20 basepairs. A deletion of this core element should lead to the same silencing defect as deletion of the complete D element. Similar to the original study, we used a strain that carried a deletion of *HML*-I and a mutation at the ORC binding site (ACS^-) and inserted 6-12bp deletions or mutations in D. In this situation, silencing is compromised such that any further

weakening by disruption of D element function should lead to total loss of silencing at *HML*α. Expression of *HML*α in a *MAT***a** strain leads to simultaneous presence of α- and **a** information, which results in a non-mating phenotype. Therefore, the *HML*α silencing properties of individual D mutants were evaluated as their ability to mate and form diploids with a *MAT*α tester strain. Indeed deletion of a 10bp segment within D termed D2, located 16bp centromere proximal to the ACS (position 133-143, numbering system based on (Feldman, et al., 1984)), mimicked the deletion of the full D element (Fig. 3.2A). Deletion of other segments within D did not alter the silencing properties of the ACS$^-$ strain (Fig. 3.2A). These results suggested that sequences essential for D function were located within the D2 element.

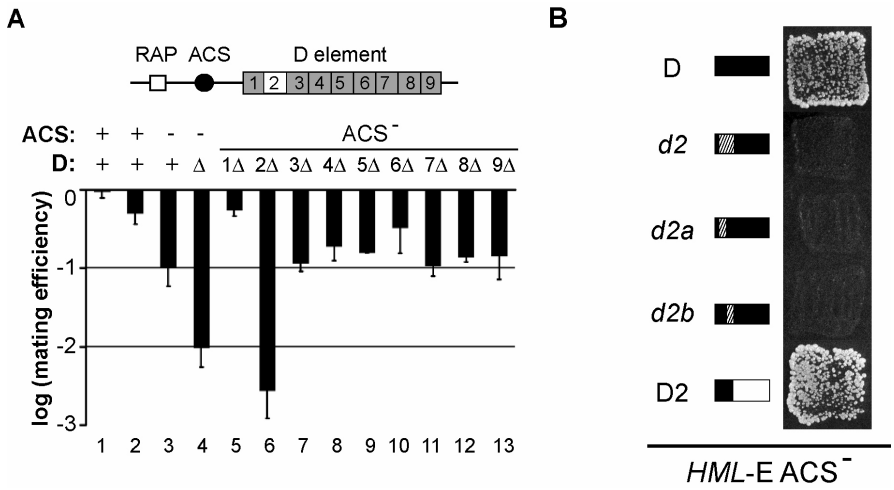

Figure 3.2: Identification of a D-element core region within the *HML*-E silencer.
(A) D2 was the core element of D. Quantitative mating assays were performed to compare the effect on silencing of different D element deletions in a *MAT*a *HML*-E ACS$^-$ ΔI strain background (AEY3395, lane 3). Lane 1: *MAT*a *HML*-E wild-type (AEY2), lane 2: *MAT*a *HML*-E ΔI (AEY3388). The mean values of at least three independent experiments are shown. (B) D2 was both necessary and sufficient for *HML*-E function. Loss of silencing in *HML*-E ACS$^-$ ΔI strains was measured for the 14bp sequence element containing the D2 element (D, AEY3395), mutations in the entire (*d2*, AEY3426), the first (*d2a*, AEY3430) or the second (*d2b*, AEY3434) half of the D2 element, or with the D2 element remaining as the sole D sequence at *HML*-E (D2, AEY3552).

We next asked whether D2 function could be abrogated by mutating rather than deleting the sequence, because a sequence deletion might not just remove a protein binding site, but could alter silencing by other means, for instance by changing nucleosome position and chromatin

architecture. Therefore, we mutated every other basepair by transition in a 14-bp region that contained D2, thus maintaining the purin/pyrimidine composition of the original area. We found that the fully mutated 14-bp area (termed *d2*) caused *HMLα* derepression just like the D2 deletion (Fig. 3.2B). We used the same strategy to individually mutate the first or the second seven bp of this region (termed *d2a* and *d2b*). Both *d2a* and *d2b* lead to a complete derepression of *HMLα* (Fig. 3.2B), indicating that sequences necessary for D function were present in both elements.

As the D2 integrity was necessary for D function we next asked whether the presence of D2 alone without accompanying D sequence might suffice for D function. To this end we constructed a *HML*-E ACS$^-$ ΔI strain that had all D element sequence downstream of D2 removed and assessed its *HMLα* silencing properties. When compared to a strain carrying the complete D element, no difference in mating capability was dectectable (Fig. 3.2B). This indicated that D2 was also sufficient for the execution of D function.

Taken together, these results showed that the D2 region was the core sequence of the D element. Furthermore, because this element is comparable in length to the Rap1 and ORC binding sites, this suggested that D2 contained a binding site for a protein (complex) essential for silencing.

3.2 Genetic interaction of *SUM1* and the D element

3.2.1 *sum1Δ* caused *HMLα* derepression

We next sought to genetically identify the hypothesized D binding factor. One prediction for a mutation or deletion in the gene encoding this factor is that it causes derepression when silencing is compromised at *HMLα*, but not *HMR***a**, because only *HMLα*, but not *HMR***a**, contains a D element. More specifically, this mutation is expected to cause strong derepression only when *HMLα* silencing is weakened for instance by mutations in RAP or ACS of *HML*-E in an *HML*-ΔI background. In short, removal of the D binding factor is expected to have the same silencing phenotypes as mutation of its binding site in the *HML*-E silencer and should be epistatic to the binding site deletion.

In genetic crosses to characterize *HMLα* silencing, we observed that a deletion of *SUM1* exactly matched the genetic predictions for the D binding factor. This observation was made

in experiments to elucidate the role of the N-terminal acetyltransferase NatA at Orc1 (Geissenhoner, et al., 2004). In the course of this study, double mutants of *nat1Δ*, a member of the NatA complex, and *sum1Δ* were generated and routinely assayed for *HM* silencing defects. Surprisingly the *nat1Δ sum1Δ* mutant exhibited strong derepression at *HMLα*, but not *HMR***a** (*nat1Δ,* Fig 3.3A). Deletion of *NAT1* alone leads to weakened *HM* silencing (Geissenhoner, et al., 2004) which is a pre-requisite to uncover redundant silencing mechanisms. Since Sum1 is a known DNA binding protein it was possible that the silencing phenotype of *sum1Δ* was mediated via the D element.

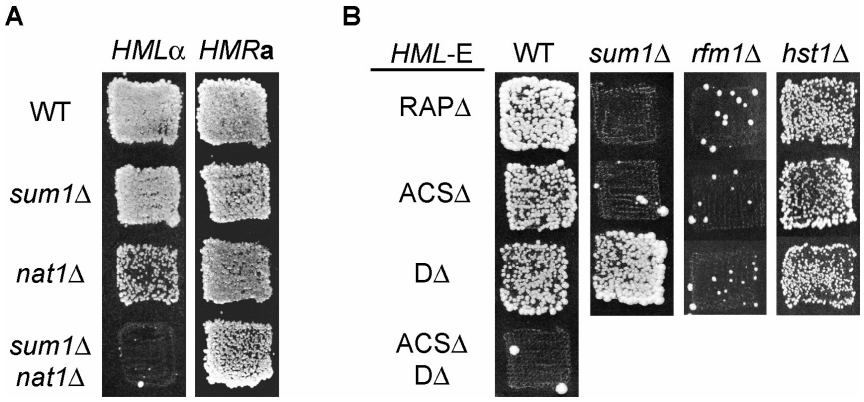

Figure 3.3: *SUM1* was required for *HMLα* silencing and was epistatic to the D element.
(A) Repression of *HMLα* or *HMR*a in strains deleted for *SUM1*, *NAT1* or both was measured by patch mating assays. (B) *SUM1*, but not *RFM1* or *HST1* was genetically linked to *HML*-D. *HMLα* silencing of *sum1Δ*, *rfmΔ* or *hst1Δ* strains in combination with silencer element deletions at *HML*-E is shown by patch mating assays.

3.2.2 *sum1Δ* was epistatic to the D element

Since *NAT1* deletion weakened *HM* locus repression by compromising ORC function (Geissenhoner, et al., 2004), the additional silencing defect of a *SUM1* deletion suggested that Sum1 acted in a parallel pathway to ORC. We therefore investigated the effect of *sum1Δ* in the presence of mutations at *HML*-E. Based on the finding of (Mahoney, et al., 1991), full derepression of *HMLα* is only expected if any two of the three silencer elements are compromised. This can be achieved either by deleting the binding site in *cis*, or by mutating the respective gene coding for the binding protein in *trans*. We thus combined a deletion of *SUM1* with individual deletions of any of the E silencer elements and assessed *HMLα*

silencing. If Sum1 acted via D, the only *cis-trans* combination without silencing defect should be the one with a D element deletion since then only one silencer element would be impaired. Significantly, *sum1Δ* caused a strong loss of *HMLα* silencing when RAP or ACS of *HML*-E were deleted (Fig. 3.3B). However, *sum1Δ* did not generally weaken *HMLα* silencing, because it did not cause derepression when the D element was deleted. Thus, *sum1Δ* affected silencing of *HMLα* as predicted for the D binding factor in that it caused derepression when *HMLα* silencing was compromised and was epistatic to a deletion of the D element.

3.2.3 *SUM1* dependent *HMLα* silencing was independent of *HST1* and *RFM1*

Previously, the Sum1 protein has been characterized as mitotic repressor for a set of genes that are upregulated in the middle stages of meiosis (Xie, et al., 1999). In this function, Sum1 binds a regulatory DNA sequence, the middle sporulation element (MSE), which is present at the promoters of these genes and recruits the histone deacetylase Hst1 via a bridging protein Rfm1 (McCord, et al., 2003; Xie, et al., 1999). Although wild-type Sum1 has so far not been implicated in silencing a mutant allele of Sum1, Sum1-1 is able to confer silencing to the *HM* loci in a *sirΔ* background (Chi and Shore, 1996; Laurenson and Rine, 1991; Livi, et al., 1990). In this role, Sum1-1 exerts its function by binding ORC and by recruiting Rfm1 and Hst1 (McCord, et al., 2003; Rusche and Rine, 2001; Sutton, et al., 2001).

Our results suggested that normal Sum1 indeed had a role in *HMLα* silencing and bound a sequence element within *HML*-D, although the MSE consensus sequence was not present at the D element. However, it was previously shown that not all Sum1 repressed middle sporulation genes contain an MSE at the promoter (Pierce, et al., 2003), and likewise, not all Sum1 repressed genes require Hst1 and Rfm1 for repression (McCord, et al., 2003). Nonetheless it was conceivable that Sum1 exerted its silencing function at *HMLα* via these two proteins. To address this question we carried out epistasis experiments of *RFM1* and *HST1* with *HMLα* alleles as done with *SUM1*. However, while deletion of *RFM1* showed a general weakening of *HMLα* silencing at each of the single silencer deletion strains, the deletion of *HST1* did not cause *HMLα* derepression (Fig. 3.3B). This indicated that Rfm1 had a role in *HMLα* silencing beyond Sum1, and that Sum1 did not cooperate with Hst1 in this context.

3.3 Sum1 bound specifically to the D element within *HML*-E

3.3.1 *In vitro* binding of Sum1 to *HML*-E

To test the notion that the Sum1 protein was the D binding factor, we asked whether Sum1 was able to bind *HML*-E *in vitro*. To this end, we performed electrophoretic mobility shift assays (EMSA) with purified full length Sum1 (6xHis-tagged at the N-terminus) from *E.coli* and *HML*-E DNA. As a control, the purified 6xHis-Sum1 shifted DNA of a known Sum1 binding sequence, the MSE containing *SMK1* promoter (Xie, et al., 1999), towards a slower mobility (Fig. 3.4B, lane 1 and 2).

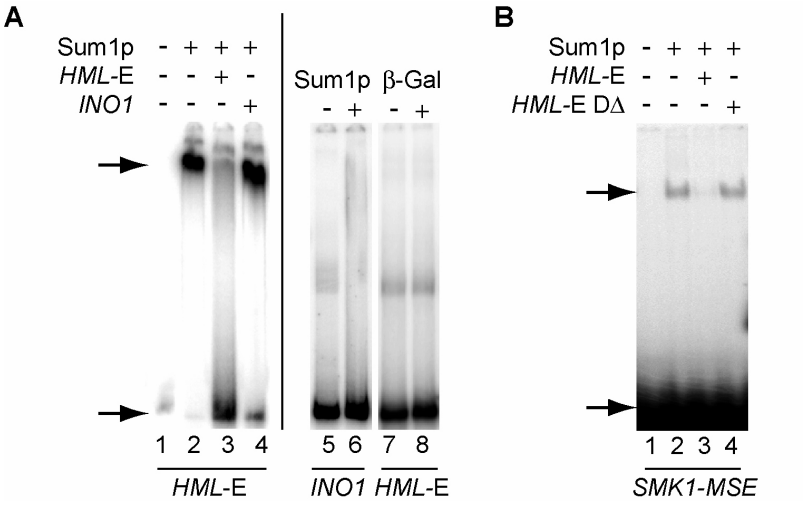

Figure 3.4: Binding of Sum1 to *HML*-E *in vitro*
(A) Sum1 bound *in vitro* to *HML*-E, but not the *INO1* promoter region. (Left) A radioactively labeled 220bp *HML*-E fragment was incubated without protein (lane 1) or with 0,1µM of bacterially expressed 6xHis-Sum1 (lanes 2-4). For competition experiments, unlabeled DNA of *HML*-E (specific competitor, lane 3) or a 210bp *INO1* fragment (unspecific competitor, lane 4) was added. DNA-protein complexes were resolved on a polyacrylamide gel and labeled DNA visualized by autoradiography. (Right) Sum1 did not bind *INO1* DNA, and bacterially expressed 6xHis-β-galactosidase (β-Gal) did not bind *HML*-E DNA. (B) Competition between *SMK1* and *HML*-E for Sum1 binding. A radioactively labeled double-stranded 19bp fragment containing the MSE site of the *SMK1* promoter was incubated without protein (lane 1) or with 0,1µM of bacterially expressed 6xHis-Sum1 (lanes 2-4). For competition experiments, unlabeled DNA of *HML*-E (specific competitor, lane 3) or *HML*-E DΔ was added. Upper arrow: protein-DNA complex, lower arrow: free DNA.

Importantly, 6xHis-Sum1 also caused a 220bp *HML*-E fragment to migrate more slowly (Fig. 3.4A, lane 1 and 2), indicating that Sum1 bound to *HML*-E. This binding was competed away by adding a molar excess of unlabeled *HML*-E DNA, but not by adding an unspecific 210bp

INO1 promoter region fragment, indicating specificity for *HML*-E (Fig. 3.4A, lane 3 and 4). Sum1 also did not bind the *INO1* fragment in an individual binding assay (Fig. 3.4A, lane 5 and 6). Also, the binding ability was unrelated to the 6xHis affinity tag, because 6xHis-tagged β-galactosidase was unable to bind to *HML*-E DNA (Fig. 3.4A, lane 7 and 8).

Since Sum1 bound two unrelated sequences, *SMK1* (containing MSE) and *HML*-D (without MSE), we were interested to determine whether the two sequences could compete with each other for Sum1 binding. Significantly, the mobility shift of Sum1 with *SMK1* DNA was competed away by addition of a molar excess of *HML*-E, but not by the same amount of *HML*-E lacking the D element (Fig. 3.4B, lanes 2 to 4), thus showing a competition between the two fragments for Sum1 binding.

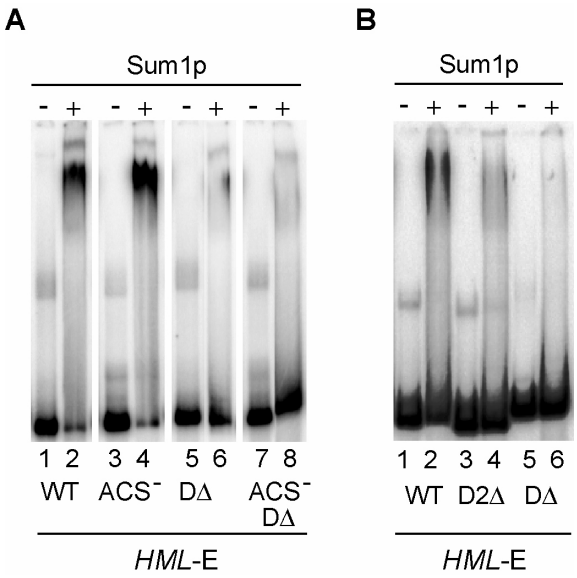

Figure 3.5: D-specific binding of Sum1 *in vitro*
(A) Binding of Sum1 to *HML*-E required the D element. Mutant versions of *HML*-E were incubated with Sum1(+) or without protein (-) and gel-electrophorezed as in Fig. 3.4. *HML*-E DNA containing a mutation in the ACS site is termed ACS$^-$ (lanes 3,4,7,8), *HML*-E DNA with deletion of the 93bp D element is termed DΔ (lanes 5, 6, 7, 8). To maintain DNA size in the DΔ derivates, the deleted D element was substituted for the genomic 3´-region of equivalent length. All DNA fragments were ~220bp. (B) Binding of Sum1 to *HML*-E required the D2 element. Mutant versions of *HML*-E were incubated with Sum1(+) or without protein (-) as in (A). WT, a 134bp wild-type *HML*-E fragment containing the ACS and the D element (lanes 1, 2). D2Δ, *HML*-E without the D2 element (lanes 3, 4). DΔ, a 140bp *HML*-E fragment lacking the entire D element (lanes 5,6).

To test whether the Sum1-mediated mobility shift of *HML*-E DNA depended on the D element, we performed a series of EMSAs with mutated *HML*-E DNA. Whereas a shift was visible both with wild-type *HML*-E and *HML*-E with the ACS mutated, it was abolished when either the D element alone or the ACS and the D element together where mutated (Fig. 3.5A, lanes 1 to 8). This showed that Sum1 required the D element in order to bind to *HML*-E. We also attempted EMSAs of Sum1 with a 14-bp fragment containing the D2 element. However, Sum1 was unable to bind to this short sequence (data not shown), indicating that neighboring sequences within the D element were necessary for full binding of Sum1 *in vitro*.

To further test the involvement of D2 in Sum1 binding, we determined how the deletion of D2 affected the ability of *HML*-E to bind Sum1. Whereas a 134bp wild-type *HML*-E fragment bound Sum1 (Fig. 3.5B, lanes 1 and 2), binding was strongly decreased with a fragment of *HML*-E lacking 10bp of D2 (Fig. 3.5B, lanes 3 and 4). However, the binding was not as strongly reduced as with a complete deletion of the D element (Fig. 3.5, lanes 5 and 6), indicating that sequences surrounding D2 influenced the binding affinity of Sum1. In summary, these experiments showed that Sum1 bound *HML*-E *in vitro* in a D2-dependent fashion.

3.3.2 *In vivo* localization of Sum1 at *HML*-E

We next asked whether Sum1 bound to *HML*-E *in vivo*. To this end, we performed chromatin immunoprecipitation (ChIP) experiments with 6xmyc tagged Sum1. In the precipitates, we observed a weak, but consistent 2.5-fold enrichment of *HML*-E DNA in the presence of the α-myc antibody as compared to ChIPs without antibody (Fig. 3.6A) or in strains lacking myc-tagged Sum1 (data not shown). In the same precipitates, the *SMK1* promoter, a known binding region for Sum1, was enriched 8-fold (Fig. 3.6A, WT), whereas the unrelated *SSC1* gene promoter was not enriched (data not shown). We next tested whether the *HML*-E enrichment was dependent on the D element. We reasoned that if Sum1 bound the D element *in vivo*, there should be no antibody specific enrichment of *HML*-E in a strain deleted for the D element. To this end, we performed ChIP analysis in a strain with an additional deletion of *HML*-D. In these experiments the difference in *HML*-E enrichment in the fractions with or without antibody was indistinguishable (Fig. 3.6A, ΔD).

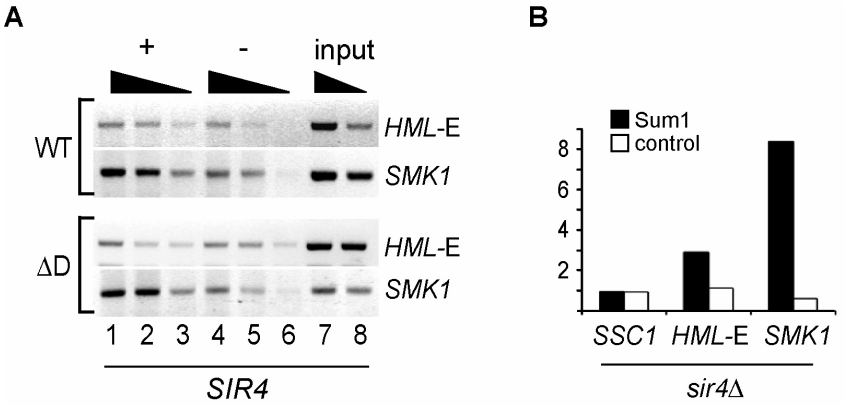

Figure 3.6: *In vivo* localization of Sum1 at *HML*-E
(A) Sum1 was associated *in vivo* with *HML*-E in a D-element dependent manner. ChIPs were performed on *sum1Δ* strains containing a 2μ plasmid carrying N-terminally 6xmyc tagged *SUM1* under control of its own promoter (pAE1032). WT: wild-type *HML*α (AEY2); ΔD: *HML*ΔDΔI (AEY3391). DNA was immunoprecipitated with (+) or without (-) anti-myc antibody and PCR amplified. A total of 1/50 or 1/100 of the input DNA (lanes 7, 8) or 1/2 (lanes 1, 4), 1/4 (lanes 2, 5) or 1/8 (lanes 3,6) of the immunoprecipitated DNA was analyzed. As a control, the promoter region of the *SMK1* gene was PCR amplified. (B) ChIP was performed in *sir4Δ* strains. Columns indicate the ratio of DNA enrichment with versus without anti-myc antibody (black columns: 6xmyc Sum1; white columns: untagged). The y-axis indicates fold enrichment.

The enrichment of Sum1 at *HML*-E was consistently weaker than that of Sum1 at the *SMK1* promoter. One explanation is that the *SMK1* promoter likely contains more than one Sum1 binding site (Pierce, et al., 2003), whereas *HML*-E has only one Sum1 binding site. To test the possibility that Sum1 ChIP at *HML*-E is sterically hindered due to heterochromatin, we performed co-immunoprecipitations in a *sir4Δ* strain. However Sum1 enrichment was not stronger at *HML*-E in a *sir4Δ* strain than in a wild-type. Quantitation showed that *HML*-E and *SMK1* enrichment were 3- and 8-fold, respectively (Fig. 3.6B). Also, adding the 6xmyc tag to the C- rather than the N-terminus did not alter the ability to chromatin-immunoprecipitate Sum1 at *HML* (data not shown). However, the fact that we observed consistent enrichment, combined with the *in vitro* binding of Sum1 to *HML*-E DNA and the effect of *sum1Δ* on *HML*α silencing strongly suggests that Sum1 bound *in vivo* to *HML*-E via the D element.

3.4 *sum1*Δ decreased origin function of *HML*-E

The presumed Sum1 binding site at the D2 element lies close to the ORC binding site of *HML*-E. Interestingly, other protein binding sites close to ACS sites of replication origins like one for Abf1 at ARS1 strongly influence the ability of such sequences to initiate replication (Marahrens and Stillman, 1992), raising the question whether Sum1 affected *HML*-E origin function. In its chromosomal location, *HML*-E does not initiate replication (Dubey, et al., 1991), because it is inactivated by replication forks emanating from centromere-proximal origins (Sharma, et al., 2001). However, when removed from this context and placed on a plasmid, *HML*-E has ARS activity, meaning that it confers autonomous replication to plasmids lacking an origin. Sharma et al. (2001) showed that deletion of a sequence stretch including the D element abrogated the ARS activity of *HML*-E, indicating that D was required for ARS function.

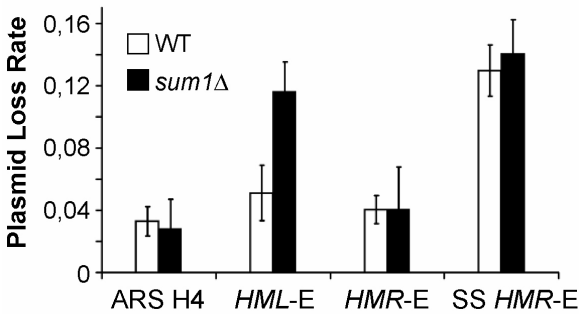

Figure 3.7: *sum1Δ* reduced the ARS activity of *HML*-E.
Plasmid loss rates were determined in a wild-type (AEY2) and a *sum1Δ* (AEY3358) strain. Strains with plasmids carrying ARS H4 (pRS316), *HML*-E (pAE1119), *HMR*-E (pAE229) or the *HMR*-E synthetic silencer *SS HMR*-E (pAE298) as their sole origins were analyzed. The average loss rates obtained from three independent experiments are shown with corresponding error bars.

We now asked how Sum1 affected *HML*-E origin activity by measuring the stability of a plasmid carrying *HML*-E as the sole origin of replication in wild-type and *sum1Δ* strains. Notably, the *sum1Δ* strain exhibited a more than two-fold higher loss rate of the *HML*-E plasmid than the wild-type strain (Fig 3.7). This suggested that Sum1 was required for full replication initiation efficiency of *HML*-E on a plasmid. Furthermore, *sum1Δ* strains grew more slowly than wild-type strains when selecting for the *HML*-E plasmid (data not shown), also indicating that plasmid transmission, probably through reduced origin initiation, was

impaired. In contrast, *sum1Δ* did not affect the stability of plasmids carrying the wild-type or synthetic *HMR*-E silencers as origins. Also, *sum1Δ* did not affect plasmid stability of an ARS H4 plasmid (Fig. 3.7). These results showed that *sum1Δ* did not affect other plasmid functions, for instance CEN function. Also, the effect of *sum1Δ* was restricted to *HML*-E, which was predicted because the D element is not found in the other origins tested. Furthermore, it showed that *sum1Δ* did not simply impair weak origins of replication (like the synthetic *HMR*-E silencer). In summary, these results demonstrated that Sum1 showed a specific effect on origin function of *HML*-E.

3.5 *sum1Δ* interacted genetically with *orc* mutations, *cdc6-1, cdc7-1 and cdc45-1*

The plasmid maintenance defect of *sum1Δ* strains with an *HML*-E-origin plasmid likely reflects a role of Sum1 in replication initiation at this origin. This observation prompted us to ask whether Sum1 might be required more globally for replication initiation and thus might constitute a novel replication initiation factor that aids ORC in initiation at selected chromosomal origins. Significantly, we observed that *sum1Δ* caused lethality in strains with mutations in the ORC subunits Orc2 and Orc5, since we were unable to recover double mutants in genetic crosses between *sum1Δ* and *orc2-1* or *orc5-1* strains (data not shown), which was in agreement with (Suter et al., 2004). The *orc* mutants on their own are temperature sensitive and show reduced firing of chromosomal origins and high plasmid loss (Fox, et al., 1995; Loo, et al., 1995). *sum1Δ orc2-1* double mutants were able to grow when provided with a *URA3*-marked plasmid carrying *ORC2*. However, they were only able to survive on *URA3*-counterselective medium (5-fluoro-orotic acid, 5-FOA) when supplemented with plasmids carrying either *SUM1* or *ORC2* (Fig. 3.8A), showing that the lethality depended on these two genes and that *sum1Δ orc2-1* strains were not inviable due to a germination defect. One interpretation of the synthetic interaction between ORC and *SUM1* is that chromosomal replication initiation in the *orc* mutants is further impaired by the absence of Sum1 such that the cells are unable to survive.

We further assessed genetic interactions between *sum1Δ* and mutations in genes encoding other factors required for replication initiation (reviewed in (Bell and Dutta, 2002)).

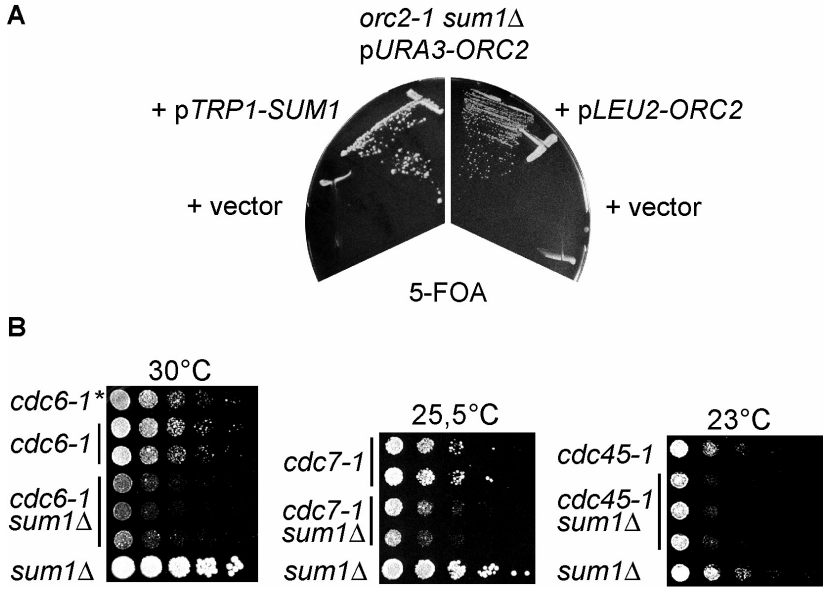

Figure 3.8: Genetic interactions between *SUM1* and replication initiation components.
(A) Synthetic lethality of *orc2-1* and *sum1Δ*. An *orc2-1 sum1Δ* strain carrying an *URA3* marked *ORC2* plasmid (pRS316-*ORC2*) was transformed with a *SUM1* (pAE1032) or an *ORC2* (pAE53) plasmid or the corresponding empty vectors. Its ability to lose the p*URA3-ORC2* plasmid was tested on 5-FOA medium. (B) Synthetic growth defects of *cdc6-1*, *cdc7-1* or *cdc45-1* with *sum1Δ*. Serial dilutions of several segregants from each cross were plated and incubated at the semi-permissive temperature of the respective *cdc* single mutant. For *cdc6-1*, strains AEY600, 3358 and AEY3537 to 3541, for *cdc7-1* strains AEY3542 to 3546 and for *cdc45-1* stains AEY373 and AEY3548 to 3551 were used. Incubation was 3 days for *cdc6-1* and *cdc7-1* and 6 days for *cdc45-1*. *Cdc6-1* marked with an asterisk indicates the parental strain, which was not isogenic to the *sum1Δ* strain.

Cdc6 is required in early G1 for chromatin binding of MCM proteins and formation of the pre-replicative complex (pre-RC) at origins of replication. Cdc7 is part of the DKK (Dbf4 dependent kinase) that is required for the G1/ S-phase transition, perhaps by phosphorylating MCM proteins. Cdc45 plays an important role in the transition from initiation to replication. It is required for association of the DNA polymerases with chromatin and colocalizes with the polymerases at the replication fork (see also chapter 1.6.2). We found that double mutant strains of *sum1Δ* with temperature-sensitive alleles of *CDC6, CDC7* and *CDC45* were viable, but showed a growth defect as compared to the single mutants at their respective semi-permissive growth temperature (Fig. 3.8B). Since these mutations impair replication initiation, our findings further supported the notion that Sum1 played a global role in initiation.

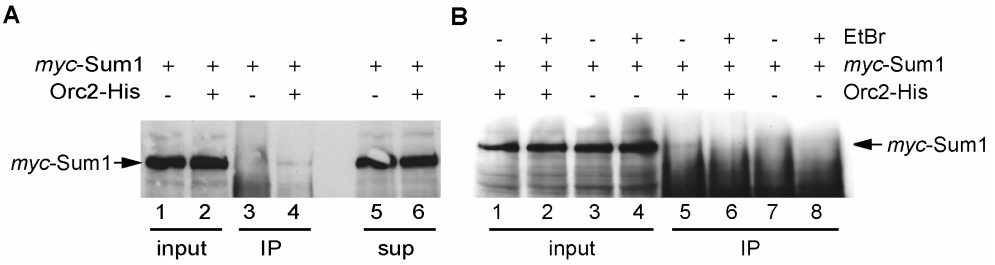

Figure 3.9: Test for physical interaction of Sum1 and Orc2.
(A) Co-immunoprecipitation of Sum1 and Orc2. Strains AEY1558 (-) and AEY3474 (6xHis-Orc2, +) carried a 6xmyc-Sum1 2μ-plasmid (pAE1032) and a *HMLα* (pAE1123) 2μ-plasmid. Precipitates were analyzed by SDS-PAGE and immunoblotting using anti-myc-antibody. Input (lanes 1,2), Immunoprecipitation (IP, lanes 3, 4), Supernatant (sup, lanes 5,6). (B) Co-immunoprecipitation of Sum1 and Orc2 in the presence of ethidiumbromide. Strains and experimental procedures were as described in (A). Where indicated, ethidiumbromid to a concentration of 100μg/ml was added to the lysate (Input, lanes 2, 4) prior to the addition of antibody (IP, lane 6, 8).

Our observation of a role for Sum1 in replication initiation and the genetic interaction between *sum1Δ* and *orc* mutations might be due to a direct interaction of Sum1 and ORC. A previous study reported a weak interaction between Orc3 and Sum1 *in vivo* (Sutton, et al., 2001). We also found a weak interaction between Orc2 and Sum1 by co-IP (Fig. 3.9A, lane 4). However this interaction is possibly mediated via DNA since it was abrogated upon addition of high concentrations of ethidiumbromide, which is thought to disrupt protein-DNA interactions (Fig. 3.9B, lane 5,6). This indicated that Sum1 may not interact directly with ORC. However it could also mean that subpopulations of Sum1 and ORC are located close to each other on DNA sequences.

3.6 Sum1 was a replication initiation factor for several origins of replication

3.6.1 Identification of genomic sites of combined Sum1 and ORC binding

A global role for Sum1 in replication initiation predicts a significant number of replication origins that are also bound by Sum1. To search for such sequences, we used the data from two previous studies that identified genomewide Sum1 and ORC binding sites using ChIP mediated microarray analysis (Lee, et al., 2002; Wyrick, et al., 2001).

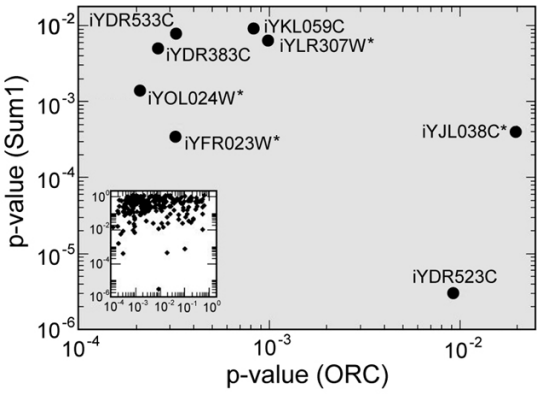

Figure 3.10: Search for intergenic regions that bind both Sum1 and ORC.
Plot of p-values for Sum1 binding (Lee et al. 2002), p<0.01 versus ORC binding (Wyrick et al. 2001), p<0,05. Inserted diagram: all data points. The origin function of intergenic regions marked with an asterisk was tested below.

In both experiments, the authors used the same error model to convert the observed Cy5/Cy3 intensity ratios into p-values (the probability that such a ratio or larger could be observed from a non-binding event). In their large-scale analysis, they imposed a strict prescription (p<0.001) to reduce the number of wrong binding predictions (false positives) at the expense of a higher false negative rate (discarding true binding events). For our purposes, we considered those eight intergenic regions where p(Sum1)<0.01 and p(ORC)<0.05 (Fig. 3.10; Table 3.1).

1	2	3	4	5	6	7
p(Sum1)	p(ORC)	intergenic	Gene name	ARS	*sum1Δ* / WT	*hst1Δ* / WT
5.60E-03	2.60E-04	*iYDR383C*	*NKP1*	ARS433	1.2	1.0
2.90E-06	9.30E-02	*iYDR523C*	*SPS1*	ARS446	21.6	12.6
7.90E-03	3.20E-02	*iYDR533C*	*HSP31*	ARS447	2.3	1.6
3.50E-04	3.20E-04	*iYFR023W*	*PES4*	ARS607	3.4	2.0
4.10E-04	2.00E-02	*iYJL038C*	*YJL038C*	ARS1013	2.8	2.0
9.10E-03	8.40E-04	*iYKL059C*	*MPE1*	ARS1109	0,8	1.0
1.50E-03	1.00E-03	*iYLR307W*	*CDA1*	ARS1223	25.5	6.2
1.40E-03	1.80E-03	*iYOL024W*	*YOL024W*	ARS1511	5.1	1.5

Table 3.1: Genomic loci that are bound by Sum1 at p<0.01 and ORC at p<0.05
For the indicated gene loci, p-values as an estimation of *in vivo* Sum1 binding have been determined by (Lee, et al., 2002) (1) and for ORC binding by (Wyrick, et al., 2001) (2). The indicated ARS sites are located upstream of the respective gene (5). Expression change of the indicated ORF in a *sum1Δ* (6) or *hst1Δ* (7) strain compared to the wild-type (WT) as determined by (Bedalov, et al., 2003).

Of these, five were located upstream of a gene that is derepressed in *sum1*Δ more than 2,5fold as determined by (Bedalov, et al., 2003), suggesting that they constitute true Sum1 binding regions (Table 3.1, column 6).

In a complementary approach we used a binding-motif based sequence search to find origins that require Sum1 for full activity. Initially, we used the consensus sequence for ORC binding (WTTTAYRTTTW) (Broach, et al., 1983) and the sequence of the identified D2 element (TTTTCGGCACGGAC) and searched the genome for co-occurrance of the two sequences within an distance of 200bp or less. One problem in this approach was that there was no consensus sequence available for D2, so sequence variations in other possible D2 elements could only be considered upon allowing a certain number of random mismatches. Except one candidate (Table 3.2, line 1), we found a high number of hits that did not pass subsequent refining steps (see below).

Motif 1-Motif 2	Ch	ARS[(1)]	origin[(2)]	ORF	p-value Sum1[(3)]	*sum1Δ*[(4)]
ORC-D2	7	726	No	*YGR087C*	7.5E-01	1.21
ORC-Sum1	2	229	Probable (5)	*YBR297W*	3.8E-02	1.17
ORC-Sum1	6	606*	Yes (9)	*YFR012W*	3.3E-02	2.35
ORC-Sum1	7	715	Yes (9)	*YGL118C*	2.1E-01	1.10
ORC-Sum1	7	724	Probable (3)	*YGR043C*	2.4E-01	1.37
ORC-Sum1	12	1217	Yes (9)	*YLR178C*	9.3E-01	0.71
ORC-Sum1	12	1227	Probable	*YLR345W*	2.8E-01	0.93
ORC-Sum1	13	1335	No	*YMR325W*	4.4E-05	1.09
ORC-Sum1	16	1609	Yes (8)	*YPLWdelta7*	1.2E-01	n.d.
ORC-Sum1	16	1618	Yes (9)	*YPL087W*	2.5E-02	1.33

Table 3.2: Genomic loci of ORC and Sum1 consensus motif co-occurrence.
(1) The indicated ARS or proposed ARS (pro-ARS) sites are located upstream of the respective ORF. (2) *In vivo* origin activity (Raghuraman, et al., 2001). The authors estimated the probability to be an *in vivo* origin for the respective ARS ranging from 1 to 9. (3) p-value as an indication of *in vivo* Sum1 binding at the indicated ORFs. (4) Expression change of the indicated ORF in a *sum1Δ* strain compared to the wild-type. Ch: chromosome; ORF: ORF downstream of the indicated ARS. The indicated loci are found at a distance of less than 200bp. ARS606 marked with an asterisk has been independently shown to be an active origin of replication.

In a subsequent approach we searched genomewide for areas <200bp that contained the consensus motif of the MSE (DSYGWCAYWDW), a well characterized Sum1 binding site (Pierce, et al., 2003), and the ORC binding site. To exclude random sequences, we checked the resulting ~100 candidates for whether they were *in vivo* targets of ORC and Sum1. As above we used the data from two previous studies that identified genomewide Sum1 and ORC binding sites using ChIP mediated microarray analysis (see Table 3.2, (1) and (3)) (Lee, et al.,

2002; Wyrick, et al., 2001). Additionally, we utilized data on replication timing by (Raghuraman, et al., 2001) to find possible *in vivo* origins (2). Finally we checked whether expression of ORFs adjacent to the loci was upregulated in a *sum1Δ* strain (3) (Bedalov, et al., 2003). Loci that fullfilled at least two of the above mentioned requirements were scored as possible candidates.

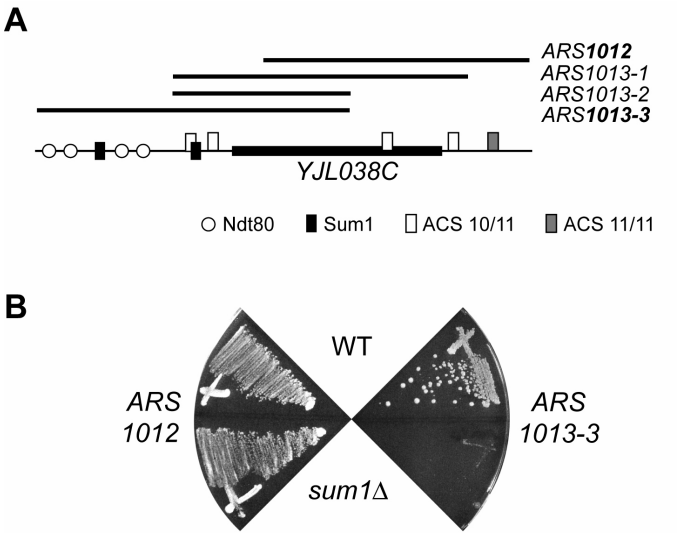

Figure 3.11: Sum1 influenced origin function of ARS1013
(A) Schematic representation of ARS1012 and ARS1013 located at the ORF *YJL038C* on chromosome X. The location of Ndt80 and Sum1 consensus sites (Pierce et al. 2003) and ACS matches is indicated. Bold lines represent fragments whose ARS function was tested. (B) *SUM1* was required for ARS activity of ARS 1013 on plasmids. Strains AEY2 (WT) and AEY3358 (*sum1Δ*) were transformed with *URA-CEN4* plasmids carrying either ARS1012 (pAE1076) or ARS1013-3 (pAE1081) as their sole origin. Transformants obtained upon transformation of ARS1013-1 or ARS1013-2 -*URA-CEN4* plasmids (pAE1078, pAE1080) were not restreakable.

3.6.2 Sum1 influenced origin function of ARS1013

Among the known ARS sites that had been identified in the Sum1-ORC binding screen was ARS1013 (Fig. 3.10, Table 3.1) which mapped to the intergenic region upstream of ORF *YJL038C* (Wyrick, et al., 2001). We asked whether ARS activity of ARS1013 was affected by Sum1 by testing ARS function of three overlapping ARS1013 fragments (Wyrick, et al., 2001) in wt and *sum1*Δ strains (Fig. 3.11A). Two fragments (ARS1013-1, -2) formed pin-prick transformants that failed to grow upon restreaking (data not shown). In contrast, ARS1013-3, which contains several Sum1 bindings sites, formed small transformants in wild-

type strains and pin-prick transformants in *sum1*Δ strains. Furthermore, the wild-type transformants formed colonies upon restreaking, whereas the *sum1Δ* transformants did not (Fig. 3.11B). This demonstrated that ARS function of ARS1013 was improved by the presence of Sum1 binding sites and depended on *SUM1*. Another ARS adjacent to ARS1013, ARS1012, is an active origin of replication (Raghuraman, et al., 2001), but does not contain Sum1 binding sites closeby (Fig. 3.11B). When tested for plasmid maintenance, ARS1012 transformants grew equally well in wild-type and *sum1Δ* strains (Fig. 3.11B). Taken together, these experiments showed that Sum1 binding sites within a replicator improved origin function.

3.6.3 Sum1 binding sites controlled origin function of ARS1013

To further test this notion, we next determined whether Sum1 sites other than those naturally present at ARS1013 could improve ARS function of a weak origin. This was achieved by adding ectopic Sum1 binding sites from *HML*-D (4xD2 or *HML*-D) or the *SMK1* promoter to ARS1013-2 and testing ARS function in wt and *sum1*Δ strains.

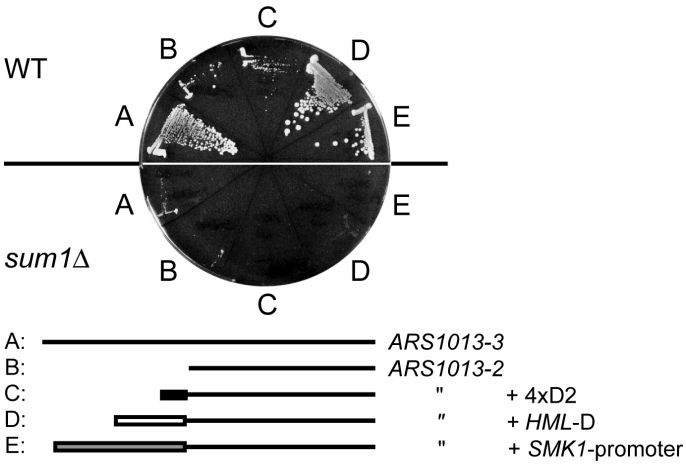

Figure 3.12: Addition of Sum1 binding sites improved the ARS function of ARS1013-2.
Strains AEY2 (WT) and AEY3358 (*sum1Δ*) were transformed with *URA-CEN4* plasmids either carrying ARS1013-3 (pAE1081) or variants of ARS1013-2 containing additional fragments of *HML*-E (4xD2, pAE1159 and *HML*-E ACS$^-$, pAE1160) or the *SMK1* promoter (pAE1161) upstream of the ARS1013-2 fragment.

Addition of *HML*-D or the *SMK1* promoter significantly improved ARS function, and the improvement was completely dependent on Sum1 (Fig. 3.12), which showed that Sum1 sites from alternative sources had the ability to increase replication initiation of a plasmid origin. Addition of four D2 elements barely increased initiation, suggesting that the D2 element was too minimal for Sum1 binding in this context.

3.6.4 Sum1 affected the chromosomal origin activity of ARS1013

Our observation that Sum1 influenced plasmid stability suggested that it might also affect chromosomal replication initiation of Sum1-binding origins. To investigate this, we measured origin firing of ARS1013 in its native chromosomal location in wt and *sum1*Δ strains by performing two-dimensional origin mapping gels (Fangman and Brewer, 1991).

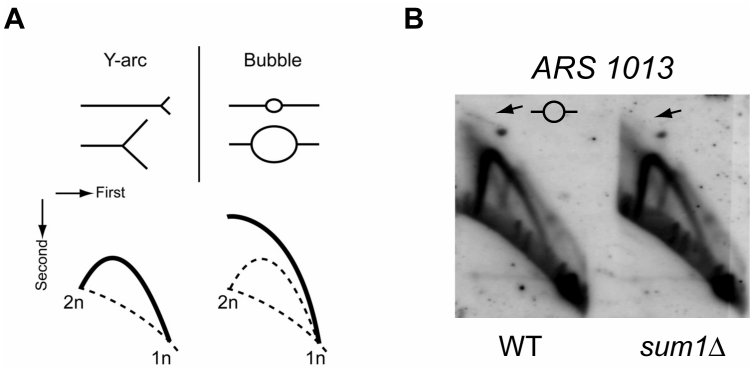

Figure 3.13: Sum1 was required for chromosomal origin activity of ARS1013
(A) Schematic representation of hybridization patterns of DNA harboring a chromosomally active or inactive origin after two dimensional gel electrophoresis (Y-arc: passively replicated fragment, bubble-arc: fragment carrying an active origin of replication).
(B) Sum1 was required for chromosomal origin activity of ARS1013. The appearance of bubble shaped replication intermediates indicative of chromosomal initiation (arrows) was measured by 2-D gel electrophoresis and Southern hybridization in a wild-type (AEY2) and *sum1Δ* (AEY3358) strain.

The principle of origin mapping by using two-dimensional gel-electrophoresis is based on the fact that actively replicating DNA migrates differently from passively replicating DNA in an agarose gel. To visualize this difference, DNA fragments carrying a suspected origin of replication are generated by restriction enzyme digestion of genomic DNA and separated in a two-dimensional gel run. After transfer to a membrane via Southern blot, the fragment is

detected with a radioactively labeled DNA and the migration pattern is visualized by autoradiography. If an active origin is present on the fragment, two replication forks are emanating outwards from the origin thus creating a bubble shaped structure. Passively replicated DNA shows only one replication fork migrating through the fragment creating a characteristic Y-shape (Fig. 3.13A). Since the tested DNA is obtained from a pool of cells, the fragments represent a pool of possible replication stages ranging from not yet replicated DNA to almost replicated DNA with doubled DNA content. A 2D-migration pattern resembling a strongly bent arc therefore represents passively replicated Y-DNA (Fig. 3.13A left) while actively replicated bubble-DNA is indicated by a lightly bent arc pattern (Fig. 3.13A right). Simultaneous presence of the two migration patterns indicates that the examined ARS does not initiate replication in all cells.

Upon testing ARS1013 we observed a weak signal indicative of bubble-shaped replication intermediates along with strong signal for Y-arc shaped replication intermediates in the wild-type strain (Fig. 3.13B, arrow), indicating that ARS1013 was only active in a fraction of cells and thus, that it was an inefficient chromosomal origin. This was expected, because ARS1013 lies close to ARS1012, which has stronger ARS activity than ARS1013 and therefore probably initiates in the majority of cell cycles and inactivates ARS1013. However, this signal was absent in the *sum1*Δ strain (Fig. 3.13B). This showed that Sum1 was required for replication initiation of ARS1013 in its chromosomal environment.

3.6.5 Sum1 influenced origin function of selected origins

We also determined the plasmid maintenance properties of other intergenic regions from our dataset (see Table 3.1). The intergenic regions i*YLR307W* (ARS1223) and i*YOL024W* (ARS1511) were both designated “proposed ARS” (pro-ARS) by Wyrick et al (2001) due to their ability to bind ORC and Mcm proteins. However, their ARS activity so far has not been tested. We selected these regions, because they colocalize with probable *in vivo* origins of replication as determined by genome-wide density transfer experiments (Raghuraman, et al., 2001). In a plasmid maintenance assay, we found that ARS1223 and ARS1511 indeed conferred autonomous replication to an origin-less plasmid, and that they displayed a significantly increased plasmid loss rate in *sum1Δ* cells as compared to wild-type cells (Fig. 3.14A). This showed that the replication capacity of these origins depended on Sum1.

Additionally we tested ARS606 that had been identified by our consensus sequence search (see Table 3.2). When assaying plasmid stability of plasmids carrying ARS606 as the only origin, we observed that ARS activity of ARS606 strongly depended on Sum1. *sum1Δ* transformants containing this ARS did not grow upon restreaking, whereas wild-type transformants did (Fig. 3.14B).

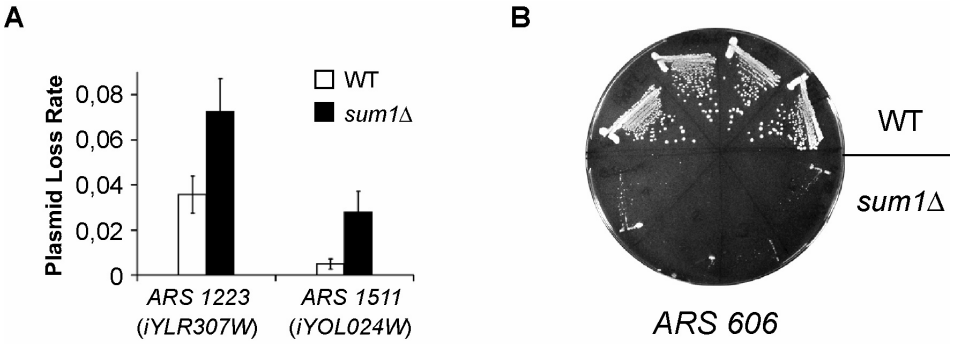

Figure 3.14: Sum1 affected ARS activity of selected origins of replication.
(A) ARS1223 and ARS1511 required *SUM1* for full ARS activity. Plasmid loss rates were determined in a wild-type, WT (AEY2) and a *sum1Δ* (AEY3358) strain. Strains with *URA-CEN4* plasmids carrying ARS1223 (pAE1130) or ARS1511 (pAE1135) as their sole origins where analyzed. The average loss rates obtained from three independent experiments are shown with corresponding error bars. The loss rate in *sum1Δ* strains was approximately 2-fold (ARS1223) and 5.5-fold (ARS1511) higher than in wild-type strains. (B) ARS activity of ARS606 was dependent on *SUM1*. Strains AEY2 (WT) and AEY3358 (*sum1Δ*) were transformed with *URA-CEN4* plasmids carrying ARS606 (pAE1126) as their sole origin and streaked on a -Ura plate.

3.6.6 Sum1 affected origin function at chromosomes

Since ARS1511 and ARS606 exhibited a strong dependence on *SUM1* if tested on plasmids it was of interest to test the *SUM1* dependence of these origins at their native chromosomal location. To this end we measured origin firing of ARS606 and ARS1511 in wt and *sum1Δ* strains by performing two-dimensional origin mapping gels (Fig. 3.15). ARS607 was additionally chosen, because it located to an intergenic region that also co-localized with a known Sum1 binding site (Fig. 3.10, *iYDR523C*). In a first approach we measured origin activity of ARS606 in strains grown at normal temperature (30°C). However the amount of origin firing of ARS606 was indistinguishable in wild-type and *sum1Δ* strains. Signal quantification revealed that both strains exhibited a comparable ratio of bubble shaped to Y-arc shaped replication intermediates (Fig 3.15A, 30°C and Table 3.3). Since *sum1Δ* strains

show a mild growth defect at low temperature (data not shown) we reasoned that an existing difference in origin activity might be enhanced at lower temperature.

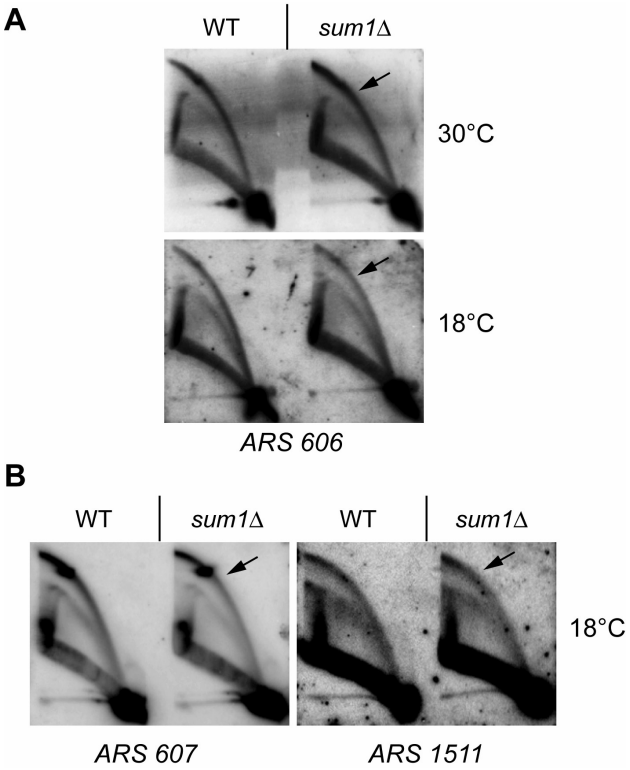

Figure 3.15: Chromosomal origin activity of selected ARS sites in wild-type and *sum1Δ* strains. (A) *SUM1* improved chromosomal origin activity of ARS606. The ratio of bubble- to Y-arc shaped replication intermediates was compared by 2-D gel electrophoresis and Southern hybridization in a wild-type (AEY2) and *sum1Δ* (AEY3358) strain grown at 30°C or at 18°C. Bubble shaped replication intermediates in *sum1Δ* are marked with an arrow. (B) Chromosomal origin activity of ARS607 and ARS1511 in wild-type (AEY2) and *sum1Δ* (AEY3358) strains grown at 18°C. Bubble shaped replication intermediates of the *sum1Δ* autoradiogram are marked with an arrow.

To this end we performed the experiment with DNA from strains grown at 18°C. In fact the *sum1Δ* strain showed a slightly reduced ratio of bubble to Y-arc signal compared to the wild-type (Fig. 3.15A, 18°C and table 3.1), indicating that at 18°C ARS606 was less often active in the *sum1Δ* strain. However signal intensity ratios for ARS607 and ARS1511 were indistinguishable between wild-type and *sum1Δ* strains (Fig. 3.15B, table 3.1).

This either indicated that the difference was only prominent enough at ARS606 to be visualized by the method of 2D gel electrophoresis mapping, or that ARS607 and ARS1511 are not affected by Sum1 in their chromosomal location. However, the observed influence of

Sum1 on chromosomal origin activity of ARS1013 and ARS606 along with the synthetic phenotypes of *sum1Δ* with replication initiation proteins (see chapter 3.5) showed that Sum1 acted as a global initiation factor.

Investigated ARS	WT [B/Y]	*sum1Δ* [B/Y]
ARS 606, 30°C	6.35	7.52
ARS 606, 18°C	4.46	0.91
ARS 607, 18°C	3.39	4.80
ARS 1511, 18°C	1.26	1.39

Table 3.3: Sum1 affected chromosomal origin activity of ARS606 at low temperature.
The signal intensity ratio of bubble to Y-arc [B/Y] intermediates is indicated. The signal intensity per arc of a representative section in each autoradiogram in Fig. 3.14 was quantified and normalized to background noise. Signal quantification was carried out using the ImageQuant™ 1.1 software (Molecular Dynamics, CA).

3.7 Hst1 affected Sum1-modulated replication origins

In our search for replication origins that are bound by Sum1 we mostly selected candidate origins that were located upstream of genes that are derepressed in a *sum1*Δ strain (see chapter 3.6). Since Sum1 acts in concert with Hst1 in a subset of these genes (Table 3.1(7)) (Bedalov, et al., 2003; McCord, et al., 2003), we asked whether Hst1 might also affect the ability of our selected origins to initiate replication. In a plasmid maintenance assay, we found that ARS1223 and ARS1511 displayed a significantly increased plasmid loss rate in *hst1Δ* cells as compared to wild-type cells (Fig. 3.16A). Notably the magnitude of plasmid loss in *hst1Δ* cells (ARS1223: 1.9fold; ARS1511: 5.4fold) was almost identical to that in *sum1Δ* cells (ARS1223: 2fold; ARS1511: 5.5fold, Fig.3.14A). This suggested that these two proteins acted in concert at the tested origins.

We also tested the involvement of Hst1 in origin activity of ARS1013 which was shown to be dependent on Sum1 (see chapter 3.6 and Fig. 3.11B and 3.13B). When assaying the ARS function of overlapping ARS1013 fragments in *hst1Δ* cells we found - as with Sum1 - that fragment 1013-2 formed pin-prick transformants (data not shown). ARS activity of fragment 1013-3, which contains several Sum1 binding sites, however, was completely dependent on Hst1, since *hst1Δ* transformants failed to grow upon restreaking whereas wild-type

transformants did (Fig. 3.16B). This demonstrated that ARS function of ARS1013 was both dependent on the presence of Sum1 binding sites and Hst1.

ARS606 was identified independently of our first search (see Fig.3.10), and ORFs in the vicinity of this origin are only weakly affected in *sum1Δ* cells (Bedalov, et al., 2003) (Table 3.2). Nevertheless, its activity in plasmid loss assays was strongly dependent on Sum1 (Fig. 3.14B). This prompted us to ask whether Hst1 also affected the activity of this ARS. Indeed we observed that origin activity of ARS606 strongly depended on Hst1, since *hst1Δ* transformants containing this ARS did not grow upon restreaking, whereas wild-type transformants did (Fig. 3.16C left).

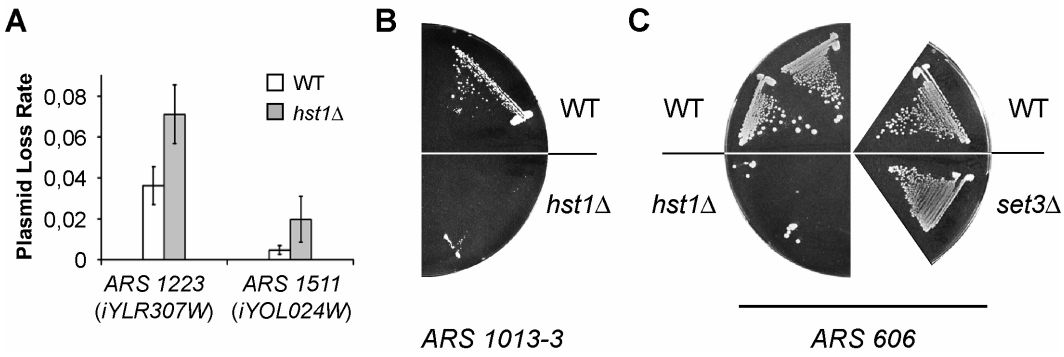

Figure 3.16: Hst1 affected ARS activity of Sum1 modulated origins of replication.
(A) ARS1223 and ARS1511 required *HST1* for full ARS activity. Plasmid loss rates were determined in a wild-type, WT (AEY2) and a *hst1Δ* (AEY1499) strain. The experimental procedure was as described in Fig. 3.14A. The loss rate in *hst1Δ* strains was approximately 1,9 fold (ARS1223) and 5,4 fold (ARS1511) higher than in wild-type strains. (B) *HST1* was required for ARS activity of ARS 1013 on plasmids. Strains AEY2 (WT) and AEY1499 (*hst1Δ*) were transformed with a *URA-CEN4* plasmid carrying ARS1013-3 (pAE1081) as its sole origin. Transformants obtained upon transformation of a ARS1013-2 -*URA-CEN4* plasmid (pAE1080) were not restreakable. (C) ARS activity of ARS606 was dependent on *HST1* but not on *SET3*. (Left) Strains AEY2 (WT) and AEY1499 (*hst1Δ*) or (Right) strains BY4741 (WT) and BY4741 *set3Δ* (*set3Δ*) were transformed with *URA-CEN4* plasmids carrying ARS606 (pAE1126) as their sole origin and streaked on a -Ura plate.

Hst1 was previously shown to occur in two different protein complexes. In one case, it is in complex with Sum1 and Rfm1 (McCord, et al., 2003; Pijnappel, et al., 2001) and the other case, it is in the Set3 complex (Pijnappel, et al., 2001). The multisubunit Set3 complex acts as a histone deacetylase and functions as a repressor of genes during the early/middle stages of the sporulation program (Pijnappel, et al., 2001). Therefore it was conceivable that the Set3 complex might be involved in modulating the activity of Sum1-regulated origins. Since the

protein Set3 is the central component of the multi-subunit Set3 complex, we assayed ARS606 activity in a *set3Δ* strain. However, growth of *set3Δ* transformants was indistinguishable from that of wild-type transformants (Fig. 3.16C right), indicating that the Set3 complex was not involved in origin activity of Sum1/Hst1 affected ARS sites.

Considering that *hst1Δ*, like *sum1Δ*, is synthetically lethal with *orc2-1* and *orc5-1* (Suter, et al., 2004) these results indicate that Hst1 also plays a significant role in replication initiation of a notable set of replication origins.

4 Discussion

An important question in heterochromatin biology is how its formation is targeted to the right location. In *S.cerevisiae*, the presence of a set of DNA binding proteins serves as nucleation point for the spreading of heterochromatin. In this work, we identified a new anchor protein for this purpose, the protein Sum1, which was shown to bind a sequence element within the *HML*-E silencer. Heterochromatin targeting and replication initiation are mechanistically linked by the observation that ORC, the replication initiator, is required for both processes. Here, we show that Sum1 was also required for replication initiation of several origins of replication in the yeast genome. Since the Sum1 protein has previously been identified to be a mitotic repressor for a set of middle meiotic genes, our results notably expand the knowledge about this protein and identify it as a novel regulator of replication and silencing in yeast.

4.1 Sum1 in silencing

Previous studies described a role of the mutant Sum1-1 protein but not the wild-type Sum1 in transcriptional silenicing at both *HM* loci (Chi and Shore, 1996; Klar, et al., 1985; Laurenson and Rine, 1991). Sum1-1, through its interaction with the HDAC Hst1 is thereby able to establish an alternative type of heterochromatin that is independent of the Sir-proteins (Rusche and Rine, 2001; Sutton, et al., 2001) (Fig. 4.1B left). In this study we show that the wild-type Sum1 protein is also implicated in silencing of *HML*α, though not *HMR***a**. In this role Sum1 binds to a sequence element within the D element, termed D2 at the *HML*-E silencer.

This finding extends the current knowledge on biological functions of wild-type Sum1 that to date was only known as mitotic transcriptional repressor for a set of middle meiosis specific genes (Xie, et al., 1999). As a transcriptional repressor, Sum1 often acts in concert with Rfm1 and Hst1 (Fig. 4.1A, left). The histone deacetylase activity of Hst1 is thereby important for the repressive properties of this protein complex. In contrast, we found that the activity of Sum1 as a silencing protein at *HML*-E was independent of Hst1. This is surprising, especially in light of the observed dependence of Sum1-1 on Hst1 in silencing the *HM* loci. However, Sum1 as a transcriptional repressor does not always act via Hst1. Microarray analysis of *sum1Δ*, *hst1Δ* and *rfm1Δ* strains revealed that of the 146 genes that were derepressed in a

sum1Δ strain only 55 were also derepressed in *hst1Δ* and *rfm1Δ* strains (McCord, et al., 2003). This shows that Sum1 has the potential to repress transcription independently of Hst1. Interestingly, genes that are repressed both by Sum1 and Hst1 are significantly stronger derepressed in a *sum1Δ* than in an *hst1Δ* strain (McCord, et al., 2003; Xie, et al., 1999). These data indicate either that Sum1 has intrinsic repressive properties or that Sum1 can interact with an additional factor that has repressive properties (Fig. 4.1A, right). Sum1 interacts with Rfm1, but Rfm1 serves exclusively as bridging protein between Sum1 and Hst1, which was determined genetically and biochemically (McCord, et al., 2003). In its role as a silencer protein Sum1 may interact with Sir2 or may stabilize the establishment of the Sir-protein complex at *HMLα*. In fact one study using a specialized repression system showed an indirect dependence of Sir2 repression to Sum1 presence (Xie, et al., 1999). However, in numerous protein-protein interaction screens the Sir proteins have not been shown to interact with Sum1. Nevertheless it would be interesting to directly address the question of a Sum1-Sir2 interaction or to search for Sum1 interactors in a yeast two-hybrid screen.

However, Sum1 could also act as silencer protein at *HML*-E as Abf1 does at *HMR*-E. Abf1 was so far not found to interact with any of the other proteins implicated in silencing and yet it is important for full repression at *HMR*. Besides the fact that it is a transcriptional activator elsewhere, Abf1 has been shown to posses nucleosome positioning activity (Lipford and Bell, 2001). Sum1 could also possess this activity and act comparably to Abf1 at the silencer, thus supporting heterochromatin formation (Fig. 4.1B right).

In its role as transcriptional repressor Sum1 often binds to a sequence element called the MSE upstream of middle sporulation genes (Xie, et al., 1999). A consensus sequence for the MSE has been determined (Pierce, et al., 2003) but the identified D2 sequence of *HML*-D does not contain this consensus sequence. This could mean that Sum1 binds to a non-consensus sequence at D2. Perhaps, accessory sequence elements outside D2 also aid in binding. In line with this, in electrophoretic mobility shift assays (EMSA) we observed binding of Sum1 to a sequence that contained the 93 basepair D element but we were unable to detect binding of Sum1 to an oligonucleotide that solely contained the 14 basepair D2.

While *in vitro*, bacterially expressed and purified full length Sum1 bound *HML*-E DNA with high affinity, the *in vivo* ChIP assay showed only a weak binding of tagged Sum1 to *HML*-E. We used different experimental approaches to improve the *in vivo* Sum1 binding at *HML*-E. This included the addition of the c-myc tag to either end of Sum1, prevention of

heterochromatin formation across *HML* by the deletion of Sir4, or ChIPs at different timepoints of the cell cycle, but the enrichment of Sum1 to *HML*-E remained weak. Possible interpretations are that Sum1 *in vivo* binds weakly to the non-consensus sequence D2 element, or that Sum1 binds transiently to D2 and is influenced by factors that are not dependent on the cell cycle. Alternatively, using an affinity tag different from the c-myc tag may yield higher enrichment of *HML*-E in ChIP assays.

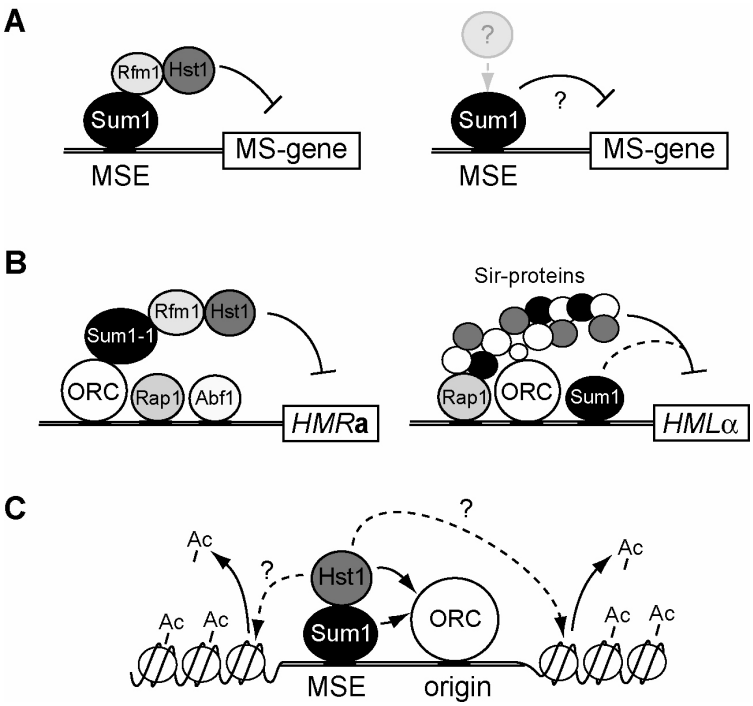

Fig. 4.1: The different facets of Sum1
(A) Sum1 functions as a repressor for middle sporulation (MS) genes either in concert with Rfm1 and Hst1 (Left) or without Rfm1/Hst1 (Right). (B) Sum1 in silencing. (Left) The mutant allele Sum1-1 can repress *HMR*a in the absence of the Sir proteins. (Right) Wild-type Sum1 aids in the establishment of silencing at *HMLα* upon binding the D2 element. (C) Sum1 in replication initiation. Sum1 and Hst1 regulate the activity of a subset of origins. The ability of Hst1 to deacetylate histone tails at the surrounding nucleosomes might be involved in this regulation.

4.2 Sum1 in replication initiation

A second, unexpected finding of this study is that Sum1 was not only a novel anchor protein at *HML*-E to silence *HMLα*, but also a regulator of replication initiation. In this function, Sum1 may be comparable to Rap1 (Kimmerly, et al., 1988), Mcm1 (Chang, et al., 2004) or Abf1 (Diffley and Stillman, 1988; Eisenberg, et al., 1988) which bind to a subset of yeast

origins and are required for efficient initiation. A picture emerges in which yeast replication origins, in addition to ORC, bind an accessory factor that enhances initiation, with different subsets of origins being bound by different modulators.

How does Sum1 promote replication initiation? We showed that Sum1-binding sites in the vicinity of origins and simultaneous presence of Sum1 are important for their replication activity. Data of (Lee, et al., 2002) and our data for *HML*-E indicated that Sum1 is physically present at origins whose activity is regulated by Sum1. Finally, the ectopic addition of Sum1 binding sites to an inactive origin could render it active, and this activity was also Sum1 dependent. Thus, in regulating origin activity Sum1 is functionally comparable to Abf1 (Diffley and Stillman, 1988; Eisenberg, et al., 1988), which also shares its ability to be a silencing protein (Kimmerly, et al., 1988). Abf1 binding repositions nuclesosomes both *in vivo* and *in vitro* (Lipford and Bell, 2001). This leads to a region of favourably positioned nucleosomes around the ACS and increases the likelihood of replication initiation. The same could be true for Sum1, though additional experiments will directly have to address this question. Alternatively, Sum1 could be implicated in facilitating the binding of ORC to the ARS site. It is conceivable that Sum1 interacts physically with ORC at the origin such that this interaction is further stabilized by the presence of a Sum1 binding site nearby. In light of this one could re-interpret our co-immunoprecipitation experiment where a Sum1-ORC interaction was abolished upon addition of an agent that can destroy protein-DNA interactions. Abolishing the Sum1-DNA interaction might have weakened the Sum1-ORC interaction to an extent that it was not possible to detect it via co-immunoprecipitation. However, it is also possible, that Sum1 aids in events like the formation of the pre-RC or at the transition of pre-RC formation to replication initiation in that it stabilizes the assembly of involved protein complexes.

We also found that deletion of Hst1 had a negative effect on origin activity at Sum1-regulated origins. Interestingly, in *hst1Δ* strains the origin function was impaired to a comparable extent as in *sum1Δ* strains. This suggests that both proteins affect origin activity by related mechanisms. The fact that *HST1* in its role as transcriptional repressor acts almost exclusively via *SUM1* (McCord, et al., 2003; Robert, et al., 2004) and that the individual deletions of *HST1* or *SUM1* are synthetically lethal with mutant *orc* alleles (Suter, et al., 2004) suggests that Hst1 also interacts with Sum1 at origins. To confirm this hypothesis, the effect of double mutants on origin activity needs to be quantified and compared to that of the individual

mutants. Also, it will be interesting to investigate the involvelment of Rfm1, a bridging factor between Sum1 and Hst1, in origin activity.

Since Hst1 is an HDAC (Sutton, et al., 2001), the influence of Hst1 on initiation may be exerted through chromatin deacetylation (Fig. 4.1C). In fact, HDAC activity at an origin had previously been linked to its activity. Nucleosomes adjacent to OriP, the replication origin of the Epstein Barr virus, were shown to be deacetylated, presumably by human HDAC1/2, at the G1/S transition but not at other times of the cell cycle (Zhou, et al., 2005). How histone deacetylation around origins promotes their replication initiation capacity is not yet clear but (Zhou, et al., 2005) showed that the chromatin remodeling complex SNFh2 was bound to the origin concurrently to HDAC1/2 and was important for origin activity. Thus it is conceivable that deacetylation of origins by Hst1 is also a prerequisite for the association of a chromatin remodeling factor which in turn leads to increased DNA accessibility and facilitates pre-RC assembly. In line with this, it has previously been shown that acetylation of lysine 16 at histone H4 influences the function of ISWI, another chromatin remodeler (Corona, et al., 2002). However, although Hst1 is a histone deacetylase, another possibility is that it might deacetylate a non-histone protein such as ORC, other pre-RC components or a regulator of initiation thereby activating replication initiation. Hst1 is a homologue of Sir2, and mammalian Sir2 homologues have been shown to deacetylate non-histone proteins such as p53 (Luo, et al., 2001).

Interestingly, two other HDACs have been implicated in replication initiation: The absence of Rpd3 deacetylation causes late origins to fire early (Aparicio, et al., 2004; Vogelauer, et al., 2002), and Sir2 has a negative role in initiation at selected origins (Pappas, et al., 2004). This is an apparent paradox, since (histone) deacetylation by Hst1 causes increased firing at the origins we tested. However the authors investigated origins unrelated to the set of origins identified in this work. Perhaps there are different classes of origins which are also subject to differential regulation by histone deacetylases. Regulation of origin initiation is known to be context dependent, and it would be interesting to test our set of origins in *rpd3Δ* strains. To test the effect of Sir2 on origins of our selection one could target Sir2 fused to a Gal4 binding-domain to an origin that carries Gal4 binding sites instead of Sum1 binding sites. When targeted to our set of origins Sir2 may behave much like Hst1 since there are several indications that Sir2 under some circumstances can substitute for Hst1. For example, one study showed that Hst1 *in vitro* is able to deacetylate K16 at histone H4 just as Sir2 (Sutton,

et al., 2001). Another study, that used the MSE of *SMK1* fused upstream of a *LacZ* reporter gene showed that expression of this gene was repressed under wild-type conditions but activated in a *sum1Δ* or *hst1Δ* strain (Xie, et al., 1999). Overexpression of Sir2 in the *hst1Δ* strain partially re-established repression of *LacZ*, indicating that Sir2 could take over the task of Hst1 to a certain extent (Xie, et al., 1999).

The observation of synthetic phenotypes between *orc* and *cdc* mutations and *sum1Δ* suggests that *SUM1* may have a global role in replication initiation. *SUM1*, although it is not lethal if deleted (Chi and Shore, 1996), may have a supportive function at a number of origins. The observed synthetic phenotypes of *sum1Δ* and mutant alleles of replication proteins suggest that deletion of *SUM1* may compromise replication initiation such that it is incompatible with reduced initiation ability. A reason why the synthetic phenotype of *sum1Δ* and *orc2-1* was more severe than the one of *sum1Δ* and the *cdc* mutations could either be a direct function of Sum1 with ORC, but could also be due to differences in severity of the mutant alleles.

Hst1 might have a comparably important role in replication since independent observations found a synthetic lethal phenotype between *orc* mutations and *hst1Δ* (Suter, et al., 2004). However, it is also possible that *sum1Δ* and *hst1Δ* additionally affect other processes that become essential in *orc* mutants, for instance sister chromatid cohesion (Suter, et al., 2004).

An increased plasmid loss as observed in *sum1Δ* and *hst1Δ* can also result if factors involved in sister chromatin cohesion are impaired. This could be caused by inefficient function at CEN sequences on the plasmid. We can rule this out for Sum1 because we used identical plasmid backbone sequences (including CEN) in some of our ARS assays and also scored origins, whose activity was equally high in wild-type and *sum1Δ* strains (i.e. ARS1012). This indicates, that Sum1 did not affect CEN function.

A global effect for *sum1Δ* on replication initiation suggests that the number of Sum1-modulated origins must be sufficiently high to cause cell death in *orc2-1 sum1Δ* mutants, but our predicted set of possible Sum1-affected origins shows only few such origins. However, our mode of prediction was quite stringent: For our first approach, in addition to a requirement to be bound by both ORC and Sum1, we only scored origins upstream of genes that were derepressed in a *sum1Δ* strain (Pierce, et al., 2003). Thus, several parameters restricted here our origin identification: 1) The ChIP-on-chip analysis for ORC binding sites has probably not identified all sites, since (Breier, et al., 2004) found sequences by

computational analysis that were not in the ORC binding data set (Wyrick, et al., 2001) but were active origins in the ARS assay. This is also reflected by the fact, that we found another Sum1-dependent ARS, ARS606, by an independent search for Sum1 and ORC binding site colocalization. 2) Similarly, the p-value prescription of the binding experiment may also exclude intergenic regions with real binding of Sum1. For instance, one known Sum1 binding site, the MSE within the *SMK1* promoter (Xie, et al., 1999), was bound according to (Lee, et al., 2002) at a p-value of 0.22 which was more than one decimal power beyond our cutoff p-value of 0.01 and hence did not score in our search. Also, microarray analysis may only be sensitive enough to find locations with multiple Sum1 binding sites, as is the case for many Sum1-regulated genes (Pierce, et al., 2003), whereas origins may contain only one Sum1 binding site, as is the case for *HML*-E. 3) There may be Sum1 binding sites that do not regulate the neighboring gene, but may be part of an origin. 4) The Sum1 binding site may be at a longer distance from the ACS, and 5) origins with co-occurrence of ORC and Sum1 binding may also lie within coding regions.

In our second approach to find Sum1-ORC binding site co-occurrences *in silico* we initially obtained ~100 possible candidates. In several stringent refinement steps we applied much of the above mentioned large scale data to evaluate whether the selected loci might be *in vivo* loci of co-occurrance. Thus many of these shortcomings also affect this selection and might explain why we only obtained 10 possible candidates of which we tested ARS606. Taken together, it seems likely that several more Sum1-regulated origins exist that await identification.

4.3 Sum1 as a cell programm-dependent replication initiator?

So far, Sum1 was solely considered a repressor of meiotic genes. Our work now demonstrates that Sum1 has a global function in replication initiation. One notable aspect about the involvement of Sum1 in replication is its regulation during meiosis. While constant throughout the mitotic cell cycle, Sum1 protein levels dramatically decrease during the early stages of meiosis, probably concomitantly with premeiotic S phase, and are lowest in the middle stages (Lindgren, et al., 2000). This raises the question how Sum1-affected origins initiate in premeiotic replication. Perhaps the absence of Sum1 leads to a delayed or a reduced firing rate at selected origins, and thus origin usage may be reduced in meiotic cells, which is

in agreement with the observation that *sum1Δ* diploids progress slightly slower than wild-type into meiosis (Lindgren, et al., 2000). The decrease of Sum1 levels is most probably accomplished by targeted protein degradation since mRNA levels remain constant throughout meiosis (Lindgren, et al., 2000). It is necessary to determine the exact kinetics of this degradation since meiotic replication initiates in the early meiotic stages. Sum1 levels must be low enough at the time of pre-RC formation to inhibit this process. An experimental approach to address this question would be to have a synchronyzed cell population proceed into meiosis and to determine *in vivo* origin activity of a Sum1-regulated origin in premeiotic S-phase.

In contrast to Sum1, expression of *HST1* and *RFM1* is significantly increased during that time. Since Sum1 is the targeting factor for Hst1, availability of Hst1 and/or Rfm1 might lead to interaction with another cofactor to reestablish repression of genes that were specifically induced during the earlier stages of meiosis (Chu, et al., 1998).

The fact that Sum1 is repressed in meiosis, which in yeast is induced by depriving cells of glucose, and that Sum1 is required for *HML* silencing, is in agreement with an earlier, elegant observation that silencing can be made dependent on environmental conditions (Shei and Broach, 1995). In this study, *HM* silencers transposed to the *MAT* locus could repress *MAT* if grown on glucose-containing medium, but this silencing was relieved on non-fermentable carbon sources such as are used to induce meiosis. In light of our results, one interpretation of this observation is that Sum1 is no longer present under these conditions, so that silencing is abrogated. However, in the original study, not only *HML*-E, which contains a Sum1 binding site, but also *HMR*-E, which lacks Sum1 binding sites, showed this effect. Perhaps there are as yet unrecognized binding sites for environmentally regulated proteins at *HMR*-E that aid in *HMR* silencing. However reestablishment of silencing after a shift back to glucose-containing medium exhibited a long lag in *HML*-E (Shei and Broach, 1995). Thus one might hypothesize that upon shift to conditions favourable for *SUM1*, a certain lag time would be expected until Sum1 protein is present again and *HML*-E can fully exert its repressive properties. Interestingly, in the early/middle stages of meiosis the protein kinase Ime2, one of the general meiosis regulators, negatively regulates Sum1 repression at a promoter (Pak and Segall, 2002). It was speculated that Ime2 marks Sum1 for targeted degradation by phosphorylation of Sum1 (Pak and Segall, 2002). Therefore it would be worthwile to test this hypothesis by observing mitotic Sum1 levels in a strain that carries an inducible copy of Ime2 on plasmids. If Sum1 levels decreased upon induction of Ime2 it would be interesting to test the influence

of targeted Sum1 degradation on *HML*-E silencer activity in the experimental setup done by Shei and Broach (Shei and Broach, 1995).

Interestingly, other silencer binding proteins like Rap1 and Abf1 function as transcriptional activators rather than repressors elsewhere in the genome (Halfter, et al., 1989; Shore and Nasmyth, 1987). This situation is paralleled in higher eukaryotes in that the recruitment of Polycomb group complexes to Polycomb response elements (PREs) to maintain homeotic gene repression involves proteins like GAGA and Pho that can function as transcriptional activators as well as repressors (Brown, et al., 1998; Kerrigan, et al., 1991).

On a broader perspective, the finding that a factor, whose expression is regulated by the cell program (i.e. meiosis vs mitosis), influences replication initiation and silencing in yeast, can be compared to the way multicellular organisms exercise control over replication and heterochromatin formation during development. Metazoans use differential origin patterns to replicate a given chromosomal area depending on the cell type. For example, *Drosophila* embryonic cells have a much broader use of origins than cells of later stages, probably in order to complete the early cell cycles faster than in more differentiated cells, which must accommodate their cell cycle to the respective tissue environment (Sasaki, et al., 1999). Also, the spacing between meiotic origins in the newt *Triturus cristatus* is much longer than in mitotic cells, and accordingly, premeiotic S phase is substantially longer than the mitotic S phase (Callan, 1974). The function of Sum1 at yeast origins may be analogous to that of *Drosophila* Myb at replication origins in the chorion loci of follicle cells, where Myb is required for site-specific DNA replication leading to gene amplification (Beall, et al., 2002).

On an amino-acid sequence level there are no homologues of Sum1 in budding yeast and in more complex eukaryotes. Syntenic homologues in two other fungi, *AAL045C* of *Ashbya gossypii* (ATCC 10895) (Dietrich, et al., 2004) and an unnamed ORF in *Kluyveromyces lactis* have been found by sequence comparison but their role is unknown to date. The Sum1 protein exhibits only few distinct domains. Two AT-hook domains indicating a DNA binding protein had been identified at pos. aa 204-216 and aa 326-338 (Aravind and Landsman, 1998). Also a coiled coil domain was predicted at pos. aa 155-170. In light of the absence of sequence homologues in larger eukaryotes perhaps other eukaryotic replication modulators exist that are functionally related to Sum1. They might be expressed in the early stages of development and, in cooperation with ORC, activate origins that are silent in their absence. The down-regulation of these hypothesized factors would reduce origin usage, thus contributing to the

lengthening of the cell cycle by increasing the distance between origins. Conversely, origins could be activated differentially in specialized cell types or in meiosis by regulating the expression of origin accessory factors. In summary, the modulation of heterochromatinization and replication initiation by regulating an accessory factor could constitute an economical way for an organism to control origin usage and heterochromatin formation during development and differentiation.

In conclusion, we propose a model for the regulation of origin choice and usage as well as heterochromatin formation during meiosis and differentiation. We present data that a factor that is repressed in meiosis is required for replication initiation at several origins and for gene silencing in yeast. We propose that larger eukaryotes use this mechanism of regulating an accessory factor to differentially control replication and the chromatin state of their genome during different stages of development. A future challenge will be to identify such eukaryotic regulators and to investigate how they integrate the processes of replication initiation and heterochromatin formation.

5 Appendix

The D-element silencer screen in the *"silencing cassette"*- plasmid

To search for silencing active sequences within the D element of *HML*-E, we initially used a plasmid carrying the "silencing cassette" (pAE370) that had been previously developed (Grunweller and Ehrenhofer-Murray, 2002). Using this cassette it is possible to assess the capability of DNA sequences to confer silencing to reporter genes. The silencing cassette consists of two components: (1) the *URA3* gene, whose expression can be monitored by growth of transformants on uracil-lacking medium and whose repression can be monitored by growth of transformants on medium containing the drug 5-FOA, and (2) the mating-type gene **a**1, whose expression leads to a non-mating phenotype in a *MAT*α strain that can be measured in mating assays. The effect of silencing on *URA3* is sensitized by utilizing strain AEY565 that lacks the *trans*-activator of *URA3*, Ppr1 (Roy, et al., 1990). The silencing cassette is flanked on one side by the *HMR*-I silencer (Fig. 5.1), which on its own does not confer silencing, but is capable of supporting silencing by weak silencers. On the other side of the cassette it is possible to insert potential silencers and test their silencing activity. For our purpose of screening *HML*-D we wanted to compare the silencing activity of *HML*-E with that of *HML*-E lacking small fragments of *HML*-D.

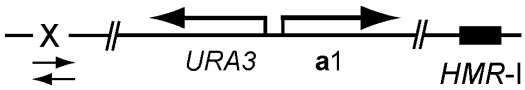

Fig. 5.1: Schematic representation of the silencing cassette.
X indicates the location for the tested sequences. Arrows indicate, that the silencing properties of a DNA sequence can depend on its direction relatively to the reporter genes.

The properties of the silencing cassette are such that in the absence of a silencer, transformants are completely Ura^+ and FOA sensitive, indicating full expression of *URA3*, despite the presence of *HMR*-I (Fig. 5.2A line 2). In the presence of a silencer (Fig. 5.2A, WT line 1), transformants are FOA resistant but at the same time $URA3^+$, indicating that *URA3* is still expressed in a portion of the (Grunweller and Ehrenhofer-Murray, 2002). In our experiment *HML*-E did also confer silencing to *URA3* in the above mentioned fashion regardless of its orientation towards the reporter gene (Fig. 5.2, line 3 and data not shown).

However if the *HML*-E silencer lacking the D element was inserted into the plasmid *URA3* repression in the transformants was not discernable from that of the complete *HML*-E silencer (Fig. 5.2, line 3, 4). This was also observed in a tester strain of the opposite mating type (pAE2225, data not shown). Thus we were not able to visualize subtle effects on silencing capacity at *HML*-E.

In a second approach we wanted to exploit the observation of (Mahoney, et al., 1991) that removal of any one of the silencer elements at *HML*-E results only in little derepression at *HML*α whereas removal of any two of the silencer elements leads to total loss of silencing. We reasoned that if silencing at the silencer cassette is compromised by a deletion of the ACS site of *HML*-E any further removal of silencer elements, for example *HML*-D should lead to total loss of silencing. However deleting the ACS site alone completely abolished the ability of the *HML*-E silencer to confer silencing to the silencing cassette regardless of the orientation (Fig. 5.2A, lines 5-8).

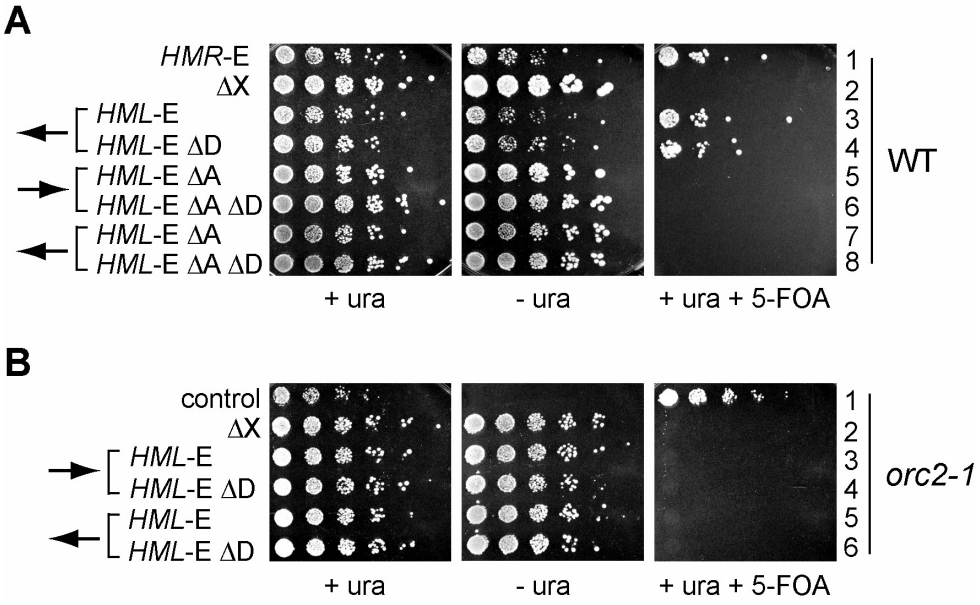

Fig. 5.2: *HML*-E properties in the silencing cassette
(A) *HML*-E silencing was independent of D but dependent on the ACS. Serial dilutions of transformants with plasmids carrying the indicated sequence elements at the silencer cassette were plated and incubated at 30°C for 4 days (+ura) and 7 days (-ura, +ura +5-FOA) respectively. ΔX: no sequence element was ligated into X. ΔA: The ACS site was deleted. Plasmids were 1: pAE374, 2: pAE369, 3: pAE421, 4: pAE442, 5: pAE735, 6: pAE736, 7: pAE739, 8: pAE740; (B) *HML*-E silencing was abolished in an *orc2-1* strain. Control: CEN6-*LEU2* plasmid as a control for plasmid maintenance. Plasmids were 1: pRS315, 2: pAE369, 3: pAE419, 4: pAE440, 5: pAE421, 6: pAE442.

This was in contrast to *HML*-E deletion experiments done previously (Mahoney, et al., 1991), where an ACS deletion alone was not sufficient to cause full derepression at *HMLα*. However the deletions there had been introduced genomically and the necessity for a silencer element could be altered on a plasmid.

Binding of ORC to the ACS is important for the establishment of silencing but mutant alleles of ORC with reduced silencing properties exist. We reasoned that performing the *HML*-D screen in an ORC mutant strain might circumvent the strong effect of an complete ACS deletion. The *orc2-1* allele represents such a mutation. *orc2-1* haploids are severly but not completely mating defective, indicating that this mutation is strongly affecting silencing at the *HM* loci (Foss, et al., 1993). We transformed AEY565 that harboured an *orc2-1* mutation with the wild-type *HML*-E and *HML*-E ΔD silencing-cassette plasmids and measured *URA3* silencing. However, as in an *HML*-E ΔACS plasmid, silencing was completely abolished in this strain background. Therefore we considered the plasmid based silencer cassette as inappropriate for our plan to search for a silencing active core element within D. We proceeded with a screening method, where mutations or deletion were genomically introduced into the D element as outlined in chapter 3.1.

6 References

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Abbreviations

2-D	two dimensional
5-FOA	5-fluoro-orotic acid
aa	amino acids
ACS	ARS consensus sequence
ARS	autonomously replicating sequence
bp	basepair
ChIP	Chromatin Immunoprecipitation
Co-IP	Co-Immunoprecipitation
EMSA	electrophoretic mobility shift assay
HAT	histone acetyltransferase
HDAC	histone deacetylase
HM	homothallic (referring to HML and HMR)
HML	homothallic mating left
HMR	homothallic mating right
HMT	histone methyltransferase
Hst	homologue of Sir two
MAT	mating type locus
MCM	minichromosome maintenance
MSE	middle sporulation element
NAD	nicotine adenine dinucleotide
NAT	N-terminal acetyltransferase
OD	optical density
ORC	origin recognition complex
ORF	open reading frame
PCR	polymerase chain reaction
PEV	position effect variegation
Rfm	repression factor of MSEs
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
Sir	silent information regulator
Sum	suppressor of mar
ts	temperature sensitive
WT	wild-type

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Publications

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Poster abstracts

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

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