Control of replication initiation and heterochromatin formation in *Saccharomyces cerevisiae* by a regulator of meiotic gene expression

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Heterochromatinization at the silent mating-type loci HMR and HML in *Saccharomyces cerevisiae* 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, the protein Sum1, a repressor of meiotic genes during vegetative growth. Sum1 bound both in vitro and in vivo to HML via a functional element within the HML-E silencer, and sum1Δ caused HML derepression. Significantly, Sum1 was also required for origin activity of HML-E, demonstrating a role of Sum1 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. Thus, Sum1 constitutes a novel global regulator of replication initiation in yeast.

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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. While replication initiation factors have been invoked in this process, little is known about its regulation during development.

In the yeast *Saccharomyces cerevisiae*, heterochromatin-like structures are targeted via a set of DNA-binding factors to the silent mating-type loci HMLα and HMRα, the telomeres, and the rDNA locus [Rusche et al. 2003]. For HML and telomeric silencing, these factors include the proteins Rap1 and Abf1, which function as transcriptional activators elsewhere in the genome [Shore and Nasmyth 1987; Kimmerly et al. 1988; Halfter et al. 1989], and the origin recognition complex ORC [Bell et al. 1993; Foss et al. 1993], the replication initiator that is conserved throughout eukaryotes. At the HM loci, the binding sites for these recruitment factors are confined to the so-called silencers E and I that flank the repressed regions. All four HM silencers contain a binding site for ORC; HMR-E and HML-E contain a Rap1-binding site; and HMR-E, HMR-I, and HML-I encompass an Abf1-binding site [Rusche et al. 2003].

While much effort has concentrated on analysis of the HMR silencers, one functional domain within the HML-E silencer has escaped molecular characterization. The HML-E silencer requires a region termed the D element for full silencing activity [Mahoney et al. 1991]. This 93-bp sequence was identified more than a decade ago by deletion analysis, but contains no binding motifs for any of the known targeting factors. Thus, the function of HML-D so far has remained elusive.

Interestingly, the silencers all have the ability to confer replication initiation to plasmids that lack an origin, a feature that is termed ARS (autonomous replicative sequence) activity. However, only HMR-E and HMR-I...
are origins of replication at their native chromosomal locations [Rivier and Rine 1992, Rivier et al. 1999], whereas the HML silencers are inactivated by replication forks initiating at early-firing origins in the vicinity [Sharma et al. 2001], and hence are not active in the chromosome. In line with this, replication initiation by ORC at the silencers is dispensable for silencing [Ehrenhofer-Murray et al. 1995]. Rather, ORC function in silencing requires that the Orc1 subunit physically contact Sir1 [Triolo and Stenglanz 1996], which together with Rap1 interacts with the structural components of yeast heterochromatin, Sir2, Sir3, and Sir4. Sir2 is a NAD+-dependent histone deacetylase (HDAC) that deacetylates the N-terminal tails of histones H3 and H4 [Imai et al. 2000], thereby establishing the chromatin modification state necessary for Sir3 and Sir4 binding [Hecht et al. 1995]. Thus, the Sir2/3/4 complex polymerizes across the chromosome to create silent chromatin [Hoppe et al. 2002, Rusche et al. 2002]. Significantly, the heterochromatin-targeting function of ORC is conserved in metazoans. Drosophila ORC1 interacts with heterochromatin protein 1 [HP-1], and mutations in DmORC2 cause a loss of position effect variegation, a form of heterochromatin-mediated silencing in flies [Pak et al. 1997].

Heterochromatin shares several features with gene-specific repression, a locally confined process in which transcriptional repressors bind to sites upstream of the gene to be repressed and recruit HDACs to alter histone acetylation patterns. One such example is the Sum1 protein, which acts as a repressor of a subset of meiotic genes during the mitotic cell cycle [Xie et al. 1999]. In this function, Sum1 recruits the Sir2 homolog Hst1 to deacetylate histones in the promoter region of some, but not all of the Sum1-repressed genes [McCord et al. 2003]. Moreover, Sum1 and Hst1 both interact with the tethering factor Rfm1 [repression factor of MSE [middle sporulation element]], thus targeting Hst1 to a subset of Sum1-regulated genes [McCord et al. 2003]. Intriguingly, Sum1 has been implicated in heterochromatin formation in an unusual context in that a dominant allele of SUM1, SUM1-1 [Livi et al. 1990], renders HM silencing dependent on the HDAC Hst1, but not Sir2 or the other Sir proteins [Rusche and Rine 2001; Sutton et al. 2001]. The mutant phenotype is caused by a single amino acid change [Chi and Shore 1996; Rusche and Rine 2001] that improves the ability of the Sum1 protein to interact with ORC at the HM silencers, such that Sum1-1 recruits Hst1 instead of Sir2 to establish repression [Rusche and Rine 2001; Sutton et al. 2001]. However, wild-type Sum1 so far has not been implicated in silencing. Importantly, while ORC is the replication initiator, an ORC-binding site alone is not sufficient for replicator function. Rather, ARSs and origins contain multiple determinants that enhance initiation activity. All origins contain a perfect or close match to the ARS consensus sequence (ACS), the ORC-binding site. In addition, the ARS1 origin, perhaps the best-studied ARS sequence, contains three so-called B elements that stimulate autonomous replication [Marahrens and Stillman 1992].

The sequence closest to the ACS, B1, cooperates in ORC binding [Lee and Bell 1997]; B2 is required for loading of the MCM complex [Zou and Stillman 2000; Wilmes and Bell 2002], and B3 is bound by Abf1 [Diffley and Stillman 1988]. 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]. Also, Mcm1-binding sites at a distance from the ACS influence initiation at subtelomeric sequences [Chang et al. 2004]. Notably, there is little sequence conservation between individual ARSs outside of the ACS, supporting the notion that there are many ways that cells can assemble functional origins, and that many of these modular origin determinants remain to be characterized.

In this study, we undertook a molecular characterization of the D element within HML-E. This led us to the discovery that the Sum1 protein bound HML-D in vitro and in vivo and was required for full HML repression. Thus, Sum1 acts not only in local, gene-specific repression at early-meiotic genes, but also was important for the creation of more extensive silenced domains. In addition, we uncovered a role for Sum1 as a modulator of replication initiation at HML-E as well as a subset of other chromosomal origins. In line with this, we found that sum1Δ was synthetically lethal with orc2-1 and orc5-1 and caused synthetic growth defects in cdc6-1, cdc7-1, and cdc45-1 strains. These findings underscore the variability of auxiliary determinants at replication origins and in heterochromatin recruitment and provide insight into the regulation of both origin usage and the creation of silent domains under varying conditions.

Results

Defining a core region within the D element of HML-E

Previous deletion analysis of the HML-E silencer defined three functional elements within a 150-bp region that are essential for silencer function: a Rap1-binding site (RAP), an ORC-binding site [ARS consensus sequence, ACS], and a third region termed the D element [Fig. 1A; Mahoney et al. 1991]. These elements were defined by deletions of the HML-E silencer in a strain lacking HML-I. When removed individually, they resulted in little depression, whereas the deletion of any two elements caused a severe loss of silencing [Fig. 1B; Mahoney et al. 1991]. The D element, as it is currently outlined, spans 93 bp and starts 6 bp centromere-proximal to the ACS. It thus represents a relatively large, unmapped DNA segment with no assigned function. Since all other HM silencer elements are binding sites for proteins or protein complexes, we hypothesized that HML-D also harbors a binding site for an as-yet-unidentified silencing factor or complex, which we sought to identify in this study.

Factor-binding sites usually comprise 15–20 bp of DNA sequence. In a first set of experiments, we therefore asked whether D function could be narrowed down...
silencing (Fig. 1C). These results suggested that deletion of the D2 element (termed d2a) caused HML derepression just like the D2 deletion did (Fig. 1D). 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. 1D), indicating that sequences necessary for D function were present in both elements.

We furthermore determined whether the D2 element was not only necessary, but also sufficient for silencing. To this end, we measured the HMLα silencing properties of a strain that had all of the D element removed except for the D2 sequence. We found that the D2 element alone was as efficient in HMLα silencing as the entire D element (Fig. 1D). Taken together, these results showed that the D2 region was the core sequence of the D element and that it was both necessary and sufficient for full D function. 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.

Figure 1. Identification of a D-element core region within the HML-E silencer. (A) Schematic representation of the HMLα 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 HMLα 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. (C) 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 MATa HML-E ACS- ΔI strain background (Aey3395). The mean values of at least three independent experiments are shown. (D) D2 was both necessary and sufficient for HML-E silencing. Loss of silencing in HML-E ACS- ΔI strains was measured for the 14-bp 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).

to a shorter DNA segment. This was achieved by systematically introducing 6–12-bp deletions across D and determining whether they caused HMLα derepression in a MATa strain lacking HML-I and carrying a mutation in the ACS of HML-E. Silencing in these strains was evaluated by determining their mating ability, because derepression of HMLα in MATa strains results in coexpression of a and α information, thus causing a nonmating phenotype. As in the original study (Mahoney et al. 1991), deletion of a sequence essential for D function in this experimental setup should lead to the same full derepression as deletion of the complete D element. Significantly, we found that a 10-bp deletion termed D2 that lies 16 bp centromere-proximal to the ACS (position 133–143, numbering system based on Feldman et al. 1984), was sufficient to mimic the effect of a full D-element deletion. Removing other areas of D had no effect on HML silencing (Fig. 1C). These results suggested that sequences essential for D function were located within the D2 fragment.

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 base pair by transition in a 14-bp region that contained D2, thus maintaining the purine/pyrimidine composition of the original area. We found that the fully mutated 14-bp area (termed d2) caused HML derepression just like the D2 deletion did (Fig. 1D). 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. 1D), indicating that sequences necessary for D function were present in both elements.

sum1Δ caused HML derepression and was epistatic to the D element

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 HML, but not HMR, because only HML, but not HMR, contains a D element. More specifically, this mutation is expected to cause strong derepression only when HML 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 HML silencing, we observed that a deletion of SUM1 exactly matched the genetic predictions for the D-binding factor. Since Sum1 is a DNA-binding protein that, albeit only in its mutant form, has been implicated in silencing, we hypothesized that Sum1 might be the D-binding factor. To address this, we tested whether sum1Δ might cause HM derepression when ORC function was compromised by a NAT1 deletion. Nat1 is a component of NatA, an N-terminal acetyltransferase that acetylates Orc1, which is required for ORC silencing function (Geissenhoner et al. 2004). We found that sum1Δ caused strong derepression at HML, but not HMR [Fig. 2A]. This suggested that Sum1 acted in a parallel pathway to ORC in HML silencing. We therefore investigated the effect of sum1Δ in the presence of mutations at HML. Significantly, sum1Δ caused a strong loss of HML silencing when RAP or ACS of HML-E was deleted [Fig. 2A]. However, sum1Δ did not generally weaken HML silencing, because it did not
cause derepression when the D element was deleted. Thus, sum1Δ affected HML silencing as predicted for the D-binding factor in that it caused derepression when HML silencing was compromised and was epistatic to a deletion of the D element.

The Sum1 protein has previously been characterized as a DNA-binding protein that functions as a repressor of a subset of meiotic genes during vegetative growth [Xie et al. 1999]. A consensus Sum1-binding sequence has been defined [DSYGWCAYWDW] [Pierce et al. 2003], although this sequence is not found at all native Sum1-binding sites and is also not present at HML-E. Normal Sum1 has so far not been implicated in silencing, but the mutant Sum1-1 version [Laurenson and Rine 1991] is able to interact with ORC at the HML-E silencer and recruits Rfm1 and Hst1 to establish a Sir-independent form of silencing [Chi and Shore 1996; Rusche and Rine 2001; Sutton et al. 2001; McCord et al. 2003]. Intriguingly, Sum1-1 does not suppress sirΔ silencing defects at HML in some strain backgrounds [Laurenson and Rine 1991], whereas it does in others [Livi et al. 1990]. Our results suggested that the normal Sum1 protein acted in silencing by binding to a nonconsensus binding site within the D element of HML-E. It is not surprising that a role for Sum1 at HML has so far not been observed [Chi and Shore 1996], because its effect is only apparent when HML silencing is weakened.

As Sum1 in some instances recruits Rfm1 and Hst1 to repress transcription [McCord et al. 2003], we asked whether they were required like Sum1 to silence HML fully. However, while deletion of RFM1 showed a general weakening of HML silencing at each of the single silencer deletion strains, the deletion of HST1 did not cause HML derepression [Fig. 2B]. This indicated that Rfm1 had a role in HML silencing beyond Sum1, and that Sum1 did not cooperate with Hst1 in this context.

**Sum1 bound specifically to the D element within 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 purified full-length Sum1 [6xHis-tagged at the N terminus] from bacteria and performed electrophoretic mobility shift assays (EMSA) with HML-E DNA. As a control, the purified 6xHis-Sum1 shifted DNA of a known Sum1-binding sequence, the SMK1 promoter [Xie et al. 1999], toward a slower mobility [Fig. 3D, lanes 1,2]. Importantly, 6xHis-Sum1 also caused a 220-bp HML-E fragment to migrate more slowly [Fig. 3A, lanes 1,2], indicating that Sum1 bound to HML-E. This binding was competed away by adding unlabeled HML-E DNA, but not by adding an unspecific 210-bp INO1 promoter region fragment, indicating specificity for HML-E [Fig. 3A, lanes 3,4]. Sum1 also did not bind the INO1 fragment in an individual binding assay [Fig. 3A, lanes 5,6]. 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. 3A, lanes 7,8].

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 were mutated [Fig. 3B, lanes 1–8]. 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 134-bp wild-type HML-E fragment bound Sum1 [Fig. 3C, lanes 1,2], binding was strongly decreased with a fragment of HML-E lacking 10 bp of D2 [Fig. 3C, lanes 3,4]. However, the binding was not as strongly reduced as with a complete deletion of the D element [Fig. 3C, lanes 5,6], indicating that sequences surrounding D2 influenced the binding affinity of Sum1.

Since Sum1 bound two unrelated sequences, SMK1 and HML-D, 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. 3D, lanes 2–4], thus showing a competition between the two fragments for Sum1 binding. In summary, these experiments showed that Sum1 bound HML-E in vitro in a D2-dependent fashion.

**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
Figure 3. D-specific binding of Sum1 to HML-E in vitro and in vivo. (A) Sum1 bound in vitro to HML-E, but not the INO1 promoter region. [Left] A radioactively labeled 220-bp HML-E fragment was incubated without protein [lane 1] or with 0.1 µM bacterially expressed 6xHis-Sum1 [lanes 2–4]. For competition experiments, unlabeled DNA of HML-E [specific competitor, lane 3] or a 210-bp INO1 fragment [unspecific competitor, lane 4] was added. DNA-protein complexes were resolved on a polyacrylamide gel and labeled DNA was visualized by autoradiography. [Right] Sum1 did not bind INO1 DNA, and bacterially expressed 6xHis β-galactosidase [β-Gal] did not bind HML-E DNA. [Upper arrow] Protein–DNA complex. [Lower arrow] Free DNA. [B] Binding of Sum1 to HML-E required the D element. Mutant versions of HML-E were incubated with Sum1 (+) or without protein [−] and gel-electrophoresed as in A. HML-E DNA containing a mutation in the ACS site is termed ACS– [lanes 3, 4, 7, 8], and HML-E DNA with deletion of the 93-bp D element is termed DΔ [lanes 5–8]. To maintain DNA size in the Δ derivates, the deleted D element was substituted for the genomic 3′-region of equivalent length. All DNA fragments were –220 bp. [C] 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 134-bp wild-type HML-E fragment containing the ACS and the D2 element [lanes 1, 2], [D2Δ] HML-E without the D2 element [lanes 3, 4], [DΔ1] a 140-bp HML-E fragment lacking the entire D element [lanes 5, 6]. [D] Competition between SMK1 and HML-E for Sum1 binding. A radioactively labeled double-stranded 19-bp fragment containing the MSE site of the SMK1 promoter was incubated without protein [lane 1] or with 0.1 µM 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. [E] 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 [pAEl032]. [Left] (WT) wild-type HMLα [AEl2], [ΔD] HMLΔDΔ1 [AEl3391]. 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), 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. [Right] 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.

antibody as compared to ChIPs without antibody [Fig. 3E, left] 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 eightfold [Fig. 3E], whereas the unrelated SSCI 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. 3E, ΔD]. The enrichment of Sum1 at HML-E was consistently weaker than that of Sum1 at the SMK1 promoter. One explanation is that there are often multiple Sum1-binding sites at Sum1-repressed meiotic genes [Pierce et al. 2003], whereas HML-E has only one Sum1-binding site. It is also conceivable that Sum1 ChIP at HML-E for some reason is sterically hindered as compared to SMK1, although Sum1 enrichment was no stronger at HML-E in a sir4Δ strain as compared to wild type. Quantitation showed that HML-E and SMK1 enrichment were threefold and eightfold, respectively [Fig. 3E, right]. Also, adding the 6xmyc tag to the C terminus rather than the N terminus did not alter the ability of ChIP 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 HMLα silencing strongly suggests that Sum1 bound in vivo to HML-E via the D element.

sum1Δ 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 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, 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.

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. Noticeably, the sum1Δ strain exhibited a more than twofold higher loss rate of the HML-E plasmid than the wild-type strain (Fig. 4A). This suggested that Sum1 was required for full 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. 4A). 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.

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-labeled 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. 4B), 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 [for review, see Bell and Dutta 2002]. Cdc6 is required in early G1 for chromatin binding of MCM proteins and formation of the prereplicative complex [pre-RC] at origins of replication. Cdc7 is part of the DDK [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. 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 respec-

**Figure 4.** Genetic interactions between SUM1 and replication initiation components. [A] sum1Δ reduced the ARS activity of HML-E. Plasmid loss rates were determined in a wild-type [AEY2] and a sum1Δ [AEY3588] 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. [B] Synthetic lethality of orc2-1 and sum1Δ. An orc2-1 sum1Δ strain carrying an URA3-labeled 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 pURA3–ORC2 plasmid was tested on 5-FOA medium. [C] 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 semipermissive temperature of the respective cdc single mutant. For cdc6-1, strains AEY600, 3358, and AEY3357 to 3541, for cdc7-1 strains AEY3542 to 3546, and for cdc45-1 strains AEY373 and AEY3548 to 3551 were used. Incubation was 3 d for cdc6-1 and cdc7-1 and 6 d for cdc45-1. A asterisk indicates the parental strain, which was not isogenic to the sum1Δ strain. [D] Coimmunoprecipitation of Sum1 and Orc2. Strains AEY1558 [+] and AEY3474 [6×His-Orc2, +] carried a 6×myc–Sum1 2µ plasmid [pAE1032] and a HMLa [pAE1123] 2µ plasmid. Precipitates were analyzed by SDS-PAGE and immunoblotting using anti-myc-antibody. [Lanes 1,2] Input. [Lanes 3,4] Immunoprecipitation [IP]. [Lanes 5,6] Supernatant [sup].

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tive semipermissive growth temperature (Fig. 4C). Since these mutations impair replication initiation, our findings further supported the notion that Sum1 played a global role in initiation.

Our observation of a role for Sum1 in replication initiation and the genetic interaction between sum1Δ and orc mutations is further bolstered by previous observations of a weak interaction between Orc3 and Sum1 in vivo (Sutton et al. 2001). We further extended this finding in that we observed coimmunoprecipitation between Orc2 and Sum1 (Fig. 4D). This supports the notion that ORC and Sum1 interact physically at origins of replication.

Sum1 was a replication initiation factor for several origins of replication

A global role for Sum1 in replication initiation predicts that replication origins exist that are also binding sites for Sum1. To search for such sequences, we used the data from two previous studies that identified genome-wide Sum1- and ORC-binding sites using ChIP-mediated microarray analysis (Wyrick et al. 2001; Lee et al. 2002). 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 nonbinding 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. 5A]. Of these, five were located next to a gene that is derepressed in sum1Δ as determined by Pierce et al. (2003), suggesting that they constitute true Sum1-binding regions. One [YJL038C] identified a known ARS, ARS1013 (Wyrick et al. 2001). We asked whether the ARS activity of ARS1013 was affected by Sum1 by testing ARS function of three overlapping ARS1013 fragments [Wyrick et al. 2001] in wild-type and sum1Δ strains [Fig. 5B,C]. Two fragments [ARS1013-1, ARS1013-2] formed pin-prick transformants that failed to grow upon restreaking (data not shown). In contrast, ARS1013-3, which contains several Sum1-binding sites, formed small transformants in wild-type strains and pin-prick transformants in sum1Δ strains. Furthermore, the wild-type transformants formed colonies upon restreaking, whereas the sum1Δ transformants did not [Fig. 5C]. 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 closely [Fig. 5B]. When tested for plasmid maintenance, ARS1012 transformants grew equally well in wild-type and sum1Δ strains [Fig. 5C]. Taken together, these experiments showed that Sum1-binding sites within a replicator improved origin function.

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 wild-type and sum1Δ strains. Addition of HML-D or the SMK1 pro-
sum1 silencing as well as replication initiation. To investigate this, we measured origin firing of ARS1013 in its native chromosomal location in wild-type and sum1Δ strains by performing two-dimensional origin mapping gels [Fangman and Brewer 1991]. We observed a weak signal indicative of bubble-shaped replication intermediates in the wild-type strain [Fig. 5E, arrow], indicating that ARS1013 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 sum1Δ strain [Fig. 5E]. This showed that Sum1 was required for replication initiation of ARS1013 in its chromosomal environment.

We also determined the plasmid maintenance properties of two other intergenic regions from our data set, iYLR306W [ARS1223] and iYOL024W [ARS1511]. Both were designated “pro-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. 6A]. This showed that the replication capacity of these origins depended on Sum1.

In a complementary approach, we used a binding-motif-based sequence search to find origins that require Sum1 for full activity. Using the consensus sequence for Sum1 binding [DSYGWCAYWDW], we searched the genome for regions where Sum1-binding sites and ACS sites lie within a distance of <200 bp. Among these was the known ARS606. Significantly, we observed that ARS activity of ARS606 strongly depended on Sum1, since sum1Δ transformants containing this ARS did not grow upon restreaking, whereas wild-type transformants did [Fig. 6B]. In summary, these results showed that multiple origins within the yeast genome required Sum1 for full initiation potential, indicating that Sum1 was a general initiation factor.

Discussion

Heterochromatin targeting and replication initiation are mechanistically linked by the observation that ORC, the replication initiator, is required for both processes. Here, we found that the Sum1 protein bound to a functional element of the HML-E silencer and was required for HML silencing as well as for replication initiation at HML-E and several origins of replication in the yeast genome. Thus, Sum1 represents a novel regulator of replication in yeast. In this function, Sum1 may be comparable to Abf1 [Diffley and Stillman 1988; Eisenberg et al. 1988], Rap1 [Kimmerly et al. 1988], or Mcm1 [Chang et al. 2004], which bind to a subset of yeast origins and are required for efficient initiation. A picture emerges where 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 show that Sum1 binds to origins in the vicinity of ORC and interacts physically with ORC. Sum1 binding close to origins may affect nucleosome positioning at the ACS, thus providing a more favorable environment for ORC binding and increasing the likelihood of initiation [Lipford and Bell 2001]. Sum1 may act before pre-RC formation and thus may be necessary for early initiation events. Alternatively, it might function downstream of the pre-RC, for instance, in the transition from initiation to replication. Such a role of Sum1 might be similar to Drosophila Myb [see below], which affects replication initiation, but not the formation of pre-RCs [Beall et al. 2002]. Furthermore, Sum1 may recruit the HDAC Hst1 to origins, such that its influence on initiation may be exerted through chromatin deacetylation, a notion that is supported by the synthetic sick phenotype of an orc2-1
**hst1Δ** strain (Suter et al. 2004). Thus, Hst1 in cooperation with Sum1 may influence both gene repression and origin firing by locally deacetylating histones. Interestingly, other HDACs have been implicated in initiation: The absence of Rpd3 deacetylases causes late origins to fire early [Vogelauer et al. 2002; Aparicio et al. 2004], and Sir2 has a negative role in initiation at selected origins [Pappas et al. 2004]. Thus, the different HDACs seem to have highly individual effects on their target origins.

The observation of synthetic phenotypes between *orc* or *cdc* mutations and *sum1Δ* suggests that *sum1Δ*, although on its own not lethal [Chi and Shore 1996], compromises replication initiation such that it is incompatible with reduced initiation. That the phenotypes are more severe with *orc* than with the *cdc* mutations may point toward a direct function of Sum1 with ORC, but could also be due to differences in severity of the mutant alleles. It is also possible that *sum1Δ* additionally affects other processes that become essential in *orc* mutants, for instance, sister chromatid cohesion [Suter et al. 2004]. A global effect for *sum1Δ* on 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: 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 in our first approach [Pierce et al. 2003]. Thus, several parameters restricted 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] Equally, 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 *SMKI* promoter [Xie et al. 1999], is not identified by this data set [Lee et al. 2002]. 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. [5] Origins with co-occurrence of ORC and Sum1 binding may also lie within coding regions. Taken together, it seems likely that several more Sum1-regulated origins exist that await identification.

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, concurrently with premeiotic S phase, and are lowest in the middle stages [Lindgren et al. 2000]. This raises the question of 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 origin usage thus 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]. Whether Sum1 affects premeiotic replication will depend upon how mechanistically it functions in initiation. For instance, if it is required for pre-RC formation, Sum1 levels at the time of pre-RC formation must be low enough to inhibit the process.

In this study, we furthermore identified wild-type Sum1 as an anchoring factor for heterochromatin at *HML*. This finding extends previous work that had solely recognized a function for the mutant Sum1-1, but not for wild-type Sum1, in silencing. Interestingly, whereas Sum1-1 recruits Hst1 instead of Sir2 to establish repression [Rusche and Rine 2001; Sutton et al. 2001], Sum1 at *HML* apparently does not. Perhaps Sum1 binds differently to *HML*-D than it does to MSE elements, such that it now preferentially recruits Sir2 rather than Hst1 to the *HML* locus. That Sum1 can function without Hst1 is reflected in the finding that Sum1 can also exert its repressive function independently of Hst1 at a subset of middle-meiotic genes [McCord et al. 2003]. In its silencing role, Sum1 most likely interacts with other silencing factors, for instance, the Sir silencing complex, to stabilize the nucleation of silent chromatin at *HML*. Sum1 so far has been known as a local, gene-specific repressor of meiotic genes [Xie et al. 1999]. 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, jibes with an earlier, elegant observation that silencing can be made dependent on the environmental conditions [Shei and Broach 1995]. In this study, *HM* silencers transposed to the *MAT* locus could elicit silencing on glucose-containing medium, but this silencing was relieved on nonfermentable 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, such that silencing is abrogated. Interestingly, other silencer-binding proteins like Rap1 and Abf1 function as transcriptional activators rather than as repressors elsewhere in the genome [Shore and Nasmyth 1987; Halfter et al. 1989]. 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 [Kerrigan et al. 1991; Brown et al. 1998].

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 multicycl-
lular organisms exercise control over replication and het-
rochromatin formation during development. Metazo-
ans use differential origin patterns to replicate a given
chromosomal area depending on the cell type. For ex-
ample, 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 dif-
ferentiated 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 substi-

tuent 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 chro-

mion loci of follicle cells, where Myb is required for site-
specific DNA replication leading to gene amplification
[Beall et al. 2002]. Perhaps other eukaryotic replication
modulators exist that are functionally related to Sum1,
are expressed in the early stages of development, and
which, 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 in-
creasing the distance between origins. Conver- sely, 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 hetero-
chromatinization 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 for-
mation 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 dif-
ferentially control replication and the chromatin state of
their genome during different stages of development. A fu-
ture challenge will be to identify such eukaryotic regu-
lators and to investigate how they integrate the processes
of replication initiation and heterochromatin formation.

Materials and methods

Yeast strains and plasmids are described in the Supplemental
Material.

Silencing assays

Quantitative and quantitative mating assays were performed as
described [Ehrenhofer-Murray et al. 1997] using AYE264 [MATa
his4] and AYE265 [MATa his4] as mating tester strains. All
quantitative mating efficiencies are the average of at least two
independent determinations and were normalized to the wild-
type strain AYE 2.

Plasmid loss rates

Plasmid loss rates were determined in strains AYE2 and
AYE3358 (sum1Δ::HisMX) according to Dillin and Rine (1997).

The loss rate is equivalent to the fraction of daughter cells that
have received no plasmids during the previous cell division.

EMSA

6xHis-Sum1 used for EMAS was prepared from BL21 Codon
Plus cells transformed with pAE1054. Cells were grown to mid-
log phase, protein expression was induced by addition of isoprop-
yl-β-D-thiogalactopyranoside (IPTG) to 0.5 mM, and cells were
allowed to grow overnight at 15 °C. Cell lysis was carried out by
sonication, and the protein was purified using a Ni-NTA col-
umn (QIAGEN). The protein was eluted with 250 mM imidaz-
ol and the eluate was dialyzed overnight at 4 °C against 20 mM
Tris-HCl (pH 8), 50 mM NaCl, and 1 mM EDTA. To increase the
protein concentration, centri-on tubes (Millipore) were used
according to the manufacturer’s guidelines. Probe preparation
and EMAS were carried out as previously described [Xie et al.
1999]. For EMAS, PCR fragments of the respective regions were
amplified from AYE2 [for the HML-E wild type and the INO1
sequence], AYE3395 [for the HML-E ACS sequence], AYE3391
[for the HML-E Dα sequence], AYE3398 [for the HML-E ACS
Dα sequence], or AYE3404 [for the HML-E D2 sequence] and
were purified and labeled as described [Xie et al. 1999]. The
primer sequence for the HML-E ACS Dα PCR reaction was GGTGTATCGCAATGGAAAT [HML-E up]
and CCCGAATTCGATAATAA [HML-E down]. The reverse
primer for the HML-E ACS Dα PCR reaction was GTTGA
CATTTCTTCTATGCGCGCAAAT [HML-E downII]. For the
INO1 PCR product, primers TTGTTCTTGTGTCGGGTTC
[INOup] and GTAGTCTGATGCGCGCGGCC [INOdown]
were used. For Figure 3C, PCR primers for HML-E wild-type
and HML-E DΔ were GCCGTTATGCTATTATGATGCT
AAAACATTTAAG [HML-E ACSfw] and HML-Edown. For
HML-E DΔ, primers were GCCGTTATGCTATTATGATGCT
AAAACATTTAAG [HML-E ACSfw] and HML-Edown. For
SMK1 sequence binding, the oligonucleotide sequence was
CCCTAACGTGACATCTT with corresponding antiparal-
lel oligonucleotide.

ChIPs

ChIPs were performed essentially as described [Rusche and Rine
2001], except that mouse anti-myc antibody [Invitrogen] at 4 µg
per sample and protein-G Sepharose beads were used. Cross-
linking was carried out in 1× TBS with 10 mM dimethyl-adip-
imidate [DMA, Pierce] for 45 min at room temperature and sub-
sequently in 1× TBS with 1% formaldehyde for 30 min [Kurd-
istani and Grunstein 2003]. PCR reactions were performed
using 1.25 units of Taq DNA polymerase (Promega), with 3 mM
MgCl2, 0.25 mM dNTPs, and 0.5 µM each primer. Samples were
cycled 28 times for 15 sec 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 [GTGTACATTCATTCTAG
TGTGCCGGC] used as a reverse primer for HML-E sequence amplification.

Immunoprecipitations

Yeast extracts from the protease-deficient strains AEY1558 and
AYE3474 were prepared as follows: Per coimmunoprecipitation
experiment, 50 OD of cells were suspended in 500 µL of lysis
buffer (50 mM Tris-HCl at pH 7.5, 140 mM NaCl, 1% Triton
X-100, 0.1% ND-40, 1 mM PMSF, 1x "complete" proteinase
inhibitor [Roche Diagnostics], 1 mM EDTA) and disrupted with
glass beads for 5 min using a vortex mixer. The lysate was
separated by centrifugation. Antibody a-myc [Invitrogen] or
α-poly-His [Sigma] was added to the lysate and incubated overnight at 4°C with shaking. Subsequently, G- Sepharose 4-FF beads [Pharmacia] were added to the lysate-antibody mix and incubated for 1 h. 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.

**Two-dimensional origin mapping gels**

Replication intermediates were isolated and analyzed as described (Fangman and Brewer 1991). DNA was digested with BsuAI, which generates a 4.25-kb fragment of ARS1013. ARS1013 was detected using two probes: a 1.2-kb EcoRI–HindIII fragment of pAE1078 and a 380-bp EcoRI–Sacl fragment of pAE1081.

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