Tight protein–DNA interactions favor gene silencing

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The heterochromatin-like structure formed by the yeast silent information regulator complex (SIR) represses transcription at the silent mating type loci and telomeres. Here, we report that tight protein–DNA complexes induce ectopic recruitment of the SIR complex, promoting gene silencing and changes in subnuclear localization when *cis*-acting elements are nearby. Importantly, lack of the replication fork-associated helicase Rrm3 enhances this induced gene repression. Additionally, Sir3 and Sir4 are enriched genome-wide at natural replication pause sites, including tRNA genes. Consistently, inserting a tRNA gene promotes SIR-mediated silencing of a nearby gene. These results reveal that replication stress arising from tight DNA–protein interactions favors heterochromatin formation.

Supplemental material is available for this article.

In budding yeast, repressed chromatin is generated at telomeres and cryptic mating type loci (*HM*) by the recruitment of a complex of silent information regulators; namely, Sir2, Sir3, and Sir4 (Rusche et al. 2003; Moazed et al. 2004). These silent loci are preferentially located at the nuclear envelope, forming foci that sequester SIR factors (Gotta et al. 1996) in a manner comparable with heterochromatic chromocenters, which sequester HP1 in higher eukaryotes (Maison and Almouzni 2004). At *HM* loci, repression is nucleated by short silencer elements that flank the target genes. These elements contain combinations of binding sites for Rap1, Abf1, Sum1, and the origin recognition complex (ORC) (Rusche et al. 2003; Irlbacher et al. 2005). Notably, all of these factors also have functions outside silencing: Rap1 and Abf1 act as activators at many gene promoters, Sum1 is a specific repressor of meiotic genes also involved in the control of replication initiation, and ORC is the replication initiator that binds both active and inactive origins of replication.

One issue is how the combination of binding sites for factors with independent roles in the cell can create a silencer able to nucleate gene silencing. The current view is that the property of silencers emerges from the close juxtaposition of these factors, three of which have been shown to interact with one or more Sir proteins (Rusche et al. 2003). No single binding site for any one of these factors by itself can create a sufficiently high local concentration of Sir proteins to sustain silencing, but in combination, these factors can.

Here we show that tight DNA–protein interactions can contribute to the formation of silent chromatin. Importantly, this effect is increased in the absence of the Rrm3 helicase, known to facilitate replication crossing non-histone protein–DNA complexes (Ivessa et al. 2003), thus revealing a novel mechanism linking replication stress with gene repression.

Results and Discussion

The efficiency of *HM* silencers is sensitive to the local SIR concentration and thus to the distance to telomeres, which are located in compartments of the nucleoplasm enriched in silencing factors (Taddei et al. 2010). Accordingly, the *ADE2* reporter gene flanked by two functional silencers of the left *HM* (*HML*; referred to as *lys2::E-ADE-I*) is weakly repressed by the SIR complex when integrated at the *LYS2* locus, located 339 kb away from a telomere (Fig. 1A,B; Maillet et al. 2001). Unexpectedly, we found that integration of an array of 256 lac operators (*lacO*) (Robinett et al. 1996) 1.5 kb upstream of this construct was sufficient to reduce *ADE2* expression, as seen by both a colony color assay and quantitative RT–PCR (Fig. 1A,B). This transcriptional repression was observed only in strains expressing the LacI (*lacO*-binding protein) fused or not to GFP (Fig. 1C; Supplemental Fig. S1A). A similar *ADE2* repression was observed when a *tet* operator (*tetO*) array was integrated at this locus in a strain expressing the *tetO*-binding protein TetR-GFP (Fig. 1A). Thus, arrays of sequences bound by either the LacI or the TetR proteins induce the repression of a reporter gene flanked by silencer elements away from a "heterochromatic environment."

Importantly, this repression depended on an intact SIR complex and required the presence of the E and I silencers flanking the reporter gene (Fig. 1B,D; Supplemental Fig. S1B). Thus, protein-bound arrays act as protosilencers, defined in *Saccharomyces cerevisiae* as DNA elements incapable of establishing silencing on their own but able to cooperate with a silencer to locally promote the formation and maintenance of a heterochromatin-like structure (Fourel et al. 2002). Consistent with this notion, *lacO* or *tetO* arrays bound by their cognate proteins induced silencing when integrated as far as 8 kb away from the silencer, although the silencing decreased with increasing distance between the *lacO* and silencer (Supplemental Fig. S1C). Furthermore, this protosilencer effect correlated directly with the number of *lacO* units in the array (Supplemental Fig. S1D).

In order to test whether LacI-bound *lacO* arrays can act as protosilencers in a different context, we introduced a *lacO* array 1 kb upstream of a mutated version of *HMR* missing the Rap1- and Abf1-binding sites of the E silencer element. This crippled version of *HMR* is unable to nucleate repression of the *URA3* reporter gene introduced at this locus (Chien et al. 1993), as shown by the absence of growth on 5-Fluoroorotic acid (5-FOA) plates. Again,
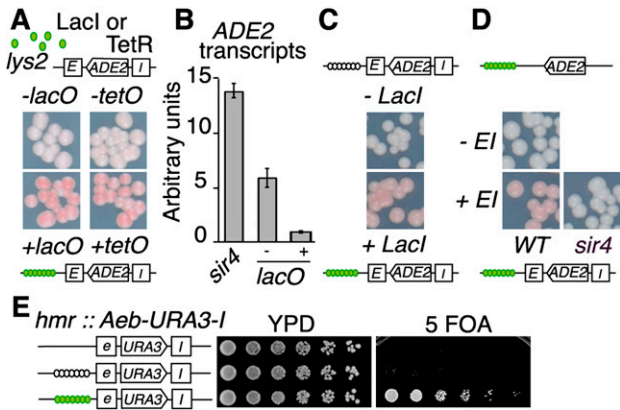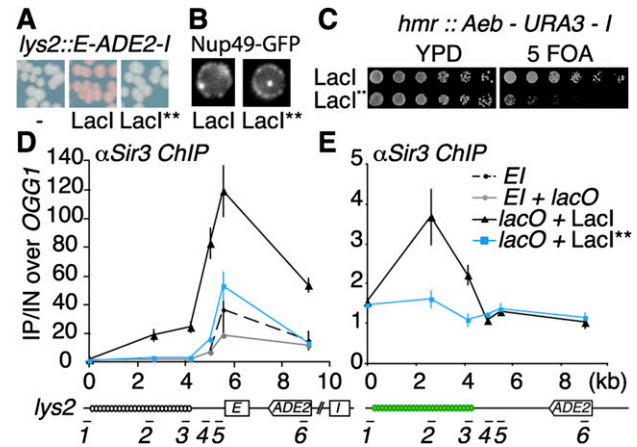
Figure 1. Arrays of DNA–protein complexes cooperate with *cis*-acting elements to induce *SIR*-dependent silencing in budding yeast. (*A*) Yeast cells expressing GFP-LacI (yAT1023) or GFP-TetR (yAT525) fusions bear an *ADE2* gene flanked by the *E* and *I HML* silencers integrated at *LYS2* (*lys2::E-ADE2-I*) to monitor ectopic silencing. These strains form white colonies when *ADE2* is expressed. Integration of 256 *lacO* (yAT58) or 2x112 *tetO* (yAT557) repeats 1.5 kb upstream of the reporter construct in strains expressing the cognate fusion proteins gives rise to pink colonies, indicative of *ADE2* repression. (*B*) *ADE2* mRNA levels were determined by quantitative real-time PCR on mRNA isolated from strains bearing the *E-ADE2-I* reporter system and expressing GFP-LacI in *sir4* mutant (yAT1394) and wild-type context without (yAT1023) or with (yAT58) a *lacO* array integrated at the *LYS2* locus. Shown are *ADE2* transcript levels normalized to *ACT1* mRNA. Error bars denote the SEM of three independent experiments. (*C*) Gene repression induced by *lacO* array insertion requires the expression of the GFP-LacI fusion. *ADE2* repression is monitored as in *A* in strains expressing (yAT752) or not (yAT751) the GFP-LacI fusion. (*D*) *ADE2* expression is monitored as in *A* in strains expressing GFP-LacI fusions with (yAT415) or without (yAT414) silencers flanking the *ADE2* reporter gene in the wild type or *sir4* mutant (yAT502). (*E*) *lacO* array integration at *HMR* induces silencing of *URA3* adjacent to a modified *E* silencer (*hmr::Aeb-URA3*), in which the *Rap1* and *Abf1* elements are replaced by four *lexA*-binding sites (yAT1504). Serial fivefold dilutions of strains, without (yAT1504) or with (yAT1505) a *lacO* array inserted 970 pb upstream of the *hmr::Aeb* silencer and expressing the GFP-LacI fusion (yAT1506) as indicated, are grown on YPD or 5-FOA plates. Growth on 5-FOA reflects *URA3* silencing.

integration of the *lacO* array in a strain expressing the LacI protein induced the silencing of this locus (Fig. 1E). Therefore, LacI-bound *lacO* arrays are not only able to favor silencing mediated by the ectopic *HML* silencers, but are also able to compensate for the absence of *cis* elements essential for silencing at *HMR*. Moreover, a LacI/*lacO* array integrated 8.3 kb from the telomere VI-R was also able to cooperate with the telomeric TG repeats to induce gene silencing of the *URA3* reporter gene located 6 kb downstream (Supplemental Fig. S1E). Thus, the protosilencer activity of the LacI-bound *lacO* array appears to be a general phenomenon that can affect different reporter genes at different loci.

Since the combinations of either *lacO*/LacI or *tetO*/TetR showed similar protosilencer activities despite the absence of any homology between these operators or their binding proteins, we reasoned that the common feature responsible for this effect could be the strong affinity of these proteins for their binding sites (Lewis 2005; Ramos et al. 2005). We tested this hypothesis by reducing this binding affinity. First, we used tetracycline to compete with the TetR/*tetO* interaction and found that 1µg/mL was sufficient to suppress the protosilencer effect (Supplemental Fig. S2A).

We next tested a set of LacI variants expected to have weak affinity for their binding sequences based on previous studies (Falcon and Matthews 1999). Notably, one of these variants, LacI** (see the Materials and Methods), enabled the detection of a *lacO* array in vivo without interfering with the expression of a neighboring silencer-flanked reporter gene (Fig. 2A,B; Supplemental Fig. S2B,C), both at the ectopic *HML* silencers and at the crippled *HMR* locus (Fig. 2C). These findings strongly argue that the effect of the *lacO*/LacI or *tetO*/TetR arrays on silencing is due to the high affinity of these proteins for their DNA-binding sequence.

To check whether the repression induced by the LacI-bound *lacO* array reflects an increase in Sir protein recruitment, we performed chromatin immunoprecipitation (ChIP) experiments to monitor the association of Sir3 and Sir4 at this locus. We observed a basal recruitment of Sir3 and Sir4 at the *E* silencer and at the *ADE2* reporter gene flanked by the *HML* silencers with or without an unbound *lacO* array located 1.5 kb upstream of the silencers (Fig. 2D; Supplemental Fig. S2D). This basal recruitment is consistent with the 2.3-fold repression of the reporter gene by the SIR complex (Fig. 1B). Strikingly, both Sir3 and Sir4 recruitment increased dramatically when the *lacO* array was bound by the commonly used LacI but not when bound by the LacI** variant (Fig. 2D; Supplemental Fig. S2D). Thus, the close proximity of a tightly bound *lacO*

Figure 2. Arrays of tight DNA–protein complexes recruit the SIR complex. (*A*) *lacO* array bound by a LacI variant with low affinity does not induce silencing of a silencer-flanked reporter gene. Expression of the *ADE2* reporter gene at *LYS2* is monitored as in Figure 1A, in strains expressing (yAT752) or not (yAT751) the commonly used GFP-LacI or its variant, GFP-LacI** (yAT755). (*B*) *lacO* arrays integrated at the *LYS2* locus are detected with GFP-LacI (yAT752) or its LacI** variant (yAT755) in strains expressing the GFP-Nup49 fusion. (*C*) Silencing is monitored at the *hmr::Aeb-URA3* reporter as in Figure 1E in strains expressing either version of the GFP-LacI as indicated (yAT1506 and yAT1507). (*D,E*) Sir3 association with chromatin assessed by ChIP using antibodies against Sir3. Primers are described in Supplemental Table S2. Primer pair (1) was used as a reference (0 kb) to plot ChIP results according to the distance to this primer pair. (*D*) LacI-bound array increases Sir3 recruitment at the *LYS2* locus bearing the silencer-flanked *ADE2* reporter gene (yAT826). In contrast, unbound (yAT825) or LacI**-bound (yAT827) *lacO* arrays show Sir3 recruitment similar to that in wild-type strain *lys2::E-ADE2-I* (yAT1023). (*E*) Sir3 recruitment is detected by ChIP at a *lacO* array in a strain expressing the LacI protein (yAT995) but not the LacI** variant (yAT996) in the absence of silencer at the *LYS2* locus. Error bars denote the SEM of three independent experiments.

array favors the recruitment of silencing factors at a reporter gene flanked by silencer elements.

Intriguingly, we observed that Sir3 and Sir4 were significantly enriched along the LacI-bound *lacO* array. We thus tested whether tightly bound arrays had the ability to recruit the SIR complex in the absence of a neighboring silencer. Indeed, we found that Sir3 was enriched 3.5-fold above the background level at *lacO* arrays in cells expressing the common LacI but not in cells expressing the LacI** variant (Fig. 2E). Although this recruitment alone was not sufficient to increase the silencing of the reporter gene (Supplemental Fig. S1B), it could cooperate with silencer elements to reach the threshold of SIR recruitment necessary for silencing.

Since silent chromatin is often found at the nuclear periphery, we wondered whether SIR recruitment at LacI-bound *lacO* arrays affected the localization of the tagged locus. We thus compared the localization of the *HML* silencers flanking *ADE2* when introduced at the *lacO*-tagged *LYS2* locus in strains expressing either LacI or LacI** fused to GFP. The locus was found enriched at the nuclear periphery (zone 1) in strains expressing the GFP-LacI fusion, while it was found mostly in the nuclear interior (zone 3) in strains expressing the LacI** variant (Fig. 3A,B). Thus, arrays of protein tightly bound to DNA can affect both the transcription and the localization of the tagged locus.

Previous studies have shown that silencing can be either a cause or a consequence of perinuclear anchoring (Andrulis et al. 1998; Taddei et al. 2004). We first asked whether the silencing induced by *lacO*/LacI arrays could stem from the perinuclear anchoring of the locus by bringing it to an area of high Sir protein concentration. Following this hypothesis, *lacO*/LacI arrays should not

induce silencing in a *yku70* mutant, in which the SIR complex is not concentrated at the nuclear periphery (Laroche et al. 1998). However, the *lacO*/LacI-induced silencing was still efficient in this mutant, arguing that the concentration of SIR at the nuclear periphery was not necessary to induce silencing in this context and that the silencing is not a direct consequence of perinuclear localization in this case (Supplemental Fig. S3A).

Reciprocally, we tested whether the formation of silent chromatin induced by the *lacO*/LacI array could be the cause of its perinuclear anchoring. Indeed, we showed previously that recruiting Sir4 to an internal locus is sufficient to induce its perinuclear localization (Taddei et al. 2004). Accordingly, we found that *SIR4* deletion restored the internal localization of the *lacO*/LacI-tagged locus (Fig. 3C), while deleting *SIR3* or *SIR2* had a weaker effect (Supplemental Fig. S3B). These results suggested that the recruitment of Sir4 is responsible for the perinuclear localization of the locus.

We next sought the mechanism responsible for the recruitment of the SIR complex at arrays of tight DNA–protein complexes. Interestingly, LacI-bound arrays have been shown to induce replication fork stalling in bacteria (Possoz et al. 2006). We thus explored the possibility that replication stress was responsible for this recruitment. Indeed, previous reports showed that sites of DNA stress such as double-strand breaks or chromatin stretching recruit SIR proteins (Martin et al. 1999; Mills et al. 1999; Thrower and Bloom 2001). Strains bearing a LacI-bound *lacO* array exhibited no cell cycle defect, indicating that, if these arrays induce replicative stress, it is likely to be transient and to not be detected by the checkpoint machinery, as shown for a single replication block in *Schizosaccharomyces pombe* (Lambert et al. 2005). Consistent with the notion that LacI-bound arrays create a replicative stress, we found that the stability of these arrays were compromised in strains deleted for components of the checkpoint machinery (Supplemental Fig. S4A), indicating that the cells have difficulty in replicating LacI-bound arrays. Similarly, *lacO* arrays became highly unstable in the absence of the Rrm3 DNA helicase (Fig. 4A), which helps replication forks move across protein–DNA complexes (Ivessa et al. 2003).

Importantly, we found that, contrary to the situation in wild-type strains, in an *RRM3*-deleted strain, a single LacI-bound *lacO* was sufficient to induce SIR-dependent silencing of the neighboring silencer-flanked reporter gene inserted at the *LYS2* locus (Fig. 4A–B). Moreover, this repression was dependent on *SIR4* and the presence of the flanking silencers (Supplemental Fig. S4B,C). In contrast, no detectable silencing occurred in the absence of the *lacO* sequence in an *rrm3* mutant strain expressing LacI, or in the presence of 120 *lacO* bound by the LacI** variant, ruling out an indirect effect of *RRM3* deletion on silencing (Fig. 4A,B). Thus, a single *lacO*, tightly bound by a LacI dimer, is sufficient to induce silencing in the absence of the Rrm3 DNA helicase, strongly indicating that a replicative stress is at the origin of SIR complex recruitment. To further test this hypothesis, we monitored the effect of replacing the LacI-bound *lacO* array by a tRNA gene [tS(AGA)E, referred to as *tDNA*], known to induce replication pausing that is increased in the absence of Rrm3 (Ivessa et al. 2003). Strikingly, we found that this tRNA gene induced a weak but reproducible decrease in the expression of the silencer-flanked *ADE2* gene whose
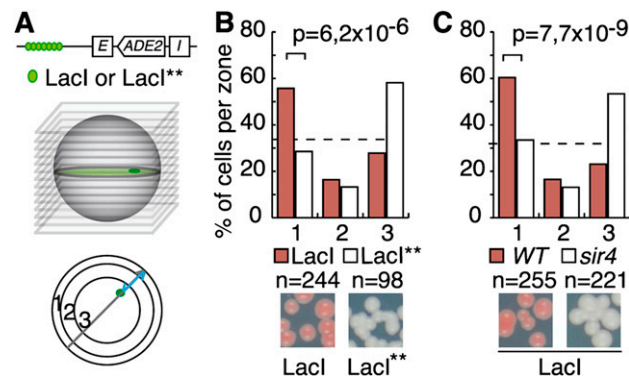
Figure 3. Tightly bound *lacO* arrays cooperate with *cis*-acting elements to induce Sir4-dependent perinuclear localization. (*A*) Position of the *LYS2*-tagged locus relative to the nuclear envelope. Z-stacks were acquired from strains bearing a *lacO* array inserted at the *LYS2* locus and expressing Nup49-GFP and either the GFP-LacI or the GFP-LacI** mutant. The localization of the tagged locus in one of the three equal concentric zones was scored on the corresponding focal plane (Hediger et al. 2002). (*B*) Tightly bound *lacO* arrays induce the perinuclear association of the *lys2::E-ADE2-I* locus. This locus is localized either mostly at the nuclear periphery or mostly in the nuclear interior in wild-type cells expressing the common GFP-LacI (yAT752) or its variant, GFP-LacI** (yAT755), respectively. The colony color of the same strains is shown at the *bottom* of the graph. (*C*) The perinuclear localization of the LacI-tagged *lys2::E-ADE2-I* locus depends on *SIR4*. (*Bottom*) Subnuclear localization of the *lys2::E-ADE2-I* and the colony color as in *B* for the wild type (yAT58) and its *sir4* derivative (yAT502) are shown.
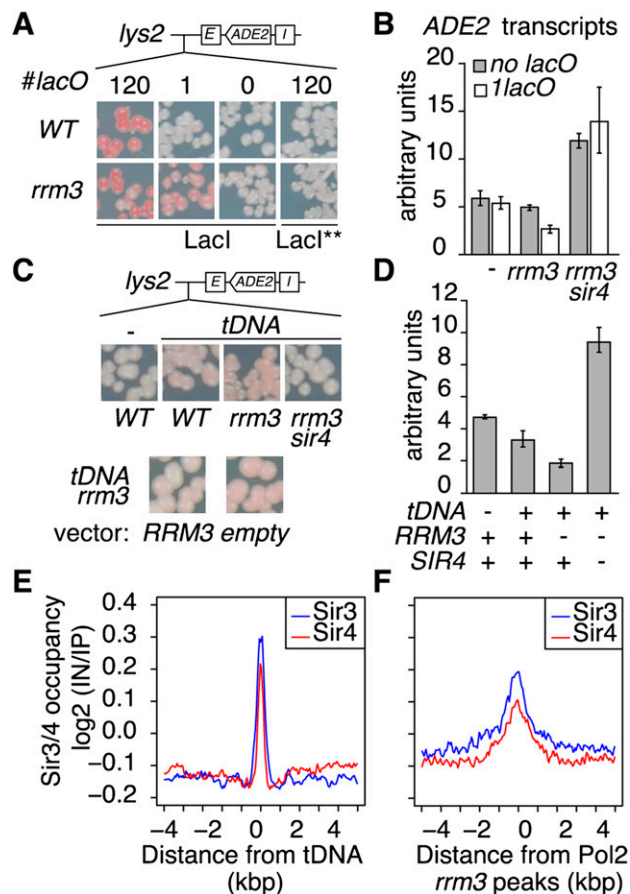
Figure 4. *rrm3*-sensitive replication pause sites induce SIR recruitment. Expression of the *ADE2* reporter gene at *LYS2* was monitored as in Figure 1. (*A,B*) A single LacI-bound *lac* operator is sufficient to promote silencing in the absence of the Rrm3 DNA helicase. (*A*) Colony color assay performed in the wild-type (yAT826, yAT528, yAT1023, and yAT827) or *rrm3* mutant (yAT945, yAT944, yAT1024, and yAT886) strains expressing either LacI or the LacI** variant and bearing 120, one, or no *lacO* sites inserted 1.5 kb upstream of the *E-ADE2-I* reporter gene at the *LYS2* locus. (*B*) *ADE2* expression monitored in the wild-type strain with (yAT528) or without (yAT1023) a single *lacO*/LacI complex integrated upstream of the *lys2::E-ADE2-I* construct in *rrm3* (yAT944 and yAT1024, respectively) or *rrm3 sir4* mutants (yAT1425 and yAT1384, respectively) as indicated. (*C,D*) Integration of a tRNA gene (*tDNA*) favors *SIR4*-dependent silencing in the absence of Rrm3. (*C, top* panel) *ADE2* expression monitored in strains with (yAT1294) or without (yAT1023) a *tDNA* integrated 5.7 kb upstream of the *lys2::E-ADE2-I* construct in wild-type strains or in *rrm3* (yAT1295) or *rrm3 sir4* (yAT1300) mutants as indicated. Expression of a wild-type copy of *RRM3* rescues the increased silencing observed in the *rrm3* mutant strain. (*D*) *ADE2* mRNA levels were determined by quantitative real-time PCR on mRNA isolated from the strains shown in *C*. (*E*) Sir3 and Sir4 are enriched at *tDNAs*. Mean profiles of Sir3/Sir4 occupancy (Sperling and Grunstein 2009) around *tDNAs*. The enrichment profiles were computed as the log2 score of immunoprecipitate versus input, on both sides of each *tDNA* center (annotations from the *Saccharomyces* Genome Database), in 50-base-pair adjacent windows as in Sperling and Grunstein (2009). (*F*) Sir3 and Sir4 are enriched at *rrm3*-sensitive replication pause sites. Mean profiles of Sir3 and Sir4 occupancy (Sperling and Grunstein 2009) were computed as in *E* around DNA Pol2 peak centers detected in *RRM3*-deleted strains (Azvolinsky et al. 2009).

promoter was located 5.7 kb downstream (Fig. 4C,D). Supporting the hypothesis that this transcriptional silencing stems from a replicative stress, *ADE2* repression

further increased in the absence of Rrm3 in a *SIR4*-dependent manner. This demonstrates that natural replication stress can also cooperate with silencer elements to establish SIR-dependent silencing.

Consequently, we checked whether Sir proteins were enriched at *tDNA* by analyzing the high-resolution genome-wide binding site maps of Sir3 and Sir4 published by Sperling and Grunstein (2009). Averaging Sir3 and Sir4 occupancy at tRNA genes revealed a narrow peak of both Sir3 and Sir4 (Fig. 4E). More generally, we tested whether natural replication stress sites were associated with the SIR complex at a genome-wide scale. This was achieved by averaging Sir3 and Sir4 occupancy (Sperling and Grunstein 2009) at replication pause sites detected by genome-wide mapping of loci enriched for the catalytic subunit of the DNA replication polymerase ε, Pol2 (Azvolinsky et al. 2009). Interestingly, Sir4 but not Sir3 showed a weak enrichment around the Pol2 peaks detected in wild-type cells (Supplemental Fig. S4D). In contrast, both Sir3 and Sir4 were clearly enriched in wild-type cells at Pol2 peaks detected in an *rrm3* mutant (Fig. 4F), in which pause sites corresponding to stable protein–DNA complexes are exacerbated (Azvolinsky et al. 2009). Thus, Sir3 and Sir4 enrichments appear as general features of replication pause sites.

Together, our data indicate that arrays of protein tightly bound to DNA, such as LacI and TetR, or the stable association of the RNA polymerase III transcription initiation factor complex (TFIIIC) at tRNA genes induce the recruitment of silencing factors. Although weak, this SIR recruitment can cooperate with silencers or telomeric repeats if located in the vicinity (<8 kb), leading to silencing and association of the locus with the nuclear periphery (Supplemental Fig. S5).

Stable DNA–protein complexes are known to induce replication fork pausing and possibly replication stress. Intriguingly, several previous studies have shown that damaged chromatin is recruited to the nuclear periphery (Gartenberg 2009). However, the *lacO*/LacI-induced relocalization to the nuclear periphery does not appear to be the consequence of persistent DNA damage, but rather to result from Sir4 recruitment (Fig. 3; Supplemental Fig. S3).

Our data suggest that SIR proteins are recruited to stable DNA–protein complexes as a consequence of the replication stress occurring at these sites. Supporting the role of DNA replication in SIR recruitment, a tightly bound *lacO* array, integrated at an internal locus bearing an ectopic copy of the *HML* silencers, is recruited to the nuclear periphery only in S phase in a *cac1* mutant in which silent chromatin is unstable (Supplemental Fig. S6A,B).

Interestingly, replication has been proposed to be required to establish silencing in *S. cerevisiae* (Miller and Nasmyth 1984). Although this notion has been challenged (Kirchmaier and Rine 2001; Li et al. 2001), it is noteworthy that Sir proteins are recruited at *HM* silencers, telomeric regions, and tRNA genes that are sites of transient replication fork pausing (Ivessa et al. 2003). At these sites, Rrm3 helps the replication fork to pass through the DNA–protein complexes formed by Rap1, Orc1, and Abf1, even in the absence of Sir proteins (Ivessa et al. 2003). Importantly, we showed that a tRNA gene, which represents a natural replication pause site, also promotes SIR-mediated silencing when inserted close to an ectopic copy of the *HML* silencers. However, in normal cells, massive and stable SIR recruitment occurs only at sites harboring combinations of DNA–protein complexes that have affinity for

components of the SIR complex (i.e., Rap1, Abf1, and Orc1). It is thus possible that replication fork pausing imposed by these complexes contributes to SIR complex recruitment at these sites, where they are then maintained by the affinity of these proteins for Sir3 or Sir4. Consistent with our hypothesis, SIR-dependent silencing of cryptic mating type loci in *Kluyveromyces lactis*, another yeast closely related to *S. cerevisiae*, requires a different set of DNA-binding proteins, including a yet unknown protein, Reb1 and Ume6 (Sjostrand et al. 2002; Barsoum et al. 2010). Interestingly, direct interaction between these two latter proteins and the SIR complex could not be shown so far (Barsoum et al. 2010), suggesting that any protein tightly bound to DNA can help recruit the SIR complex.

Although the detailed mechanism leading to the recruitment of silencing factors remains to be deciphered, it may well be conserved in other species. Indeed, previous work in *Arabidopsis thaliana* showed that transgenic repeats frequently associate with pericentric heterochromatin, and that this frequency is further increased for LacI-bound *lacO* arrays compared with unbound arrays (Pecinka et al. 2005). Furthermore, in a human cell line, similar to the situation in yeast, HP1α localization at *lacO* arrays is triggered by tight binding of LacI but not LacI** (P Beuzer, M Dubarry, A Taddei, and G Almouzni, unpubl.). Thus, the recruitment of silencing factors at arrays of protein tightly bound to DNA likely reflects a widespread phenomenon. Importantly, LacI-bound arrays were also shown to induce replication fork stalling in human cells as in bacteria (Possoz et al. 2006; Jegou et al. 2009). Links between replication and transcriptional repression have been reported recurrently in many different species. However, it remained unclear how general replication factors could trigger silencing factors at specific sites. Here we propose that replication stress contributes to the establishment of silencing at natural sites in eukaryotic genomes, possibly contributing to genome integrity by preventing collision between the replication and transcription machineries.

Our results also have important technical bearings. Operator-based gene-tagging systems have been used broadly over the last decade, and have proven very useful for following the dynamics of individual loci or for purifying associated complexes. Our data uncover that, in some instances, using such systems could introduce a significant bias on both the localization and the expression status of the tagged locus. Furthermore, we characterized a LacI variant that does not introduce this bias. This variant will be helpful to both revisit previous observations and study chromatin dynamics in the future.

Materials and methods

Media and growth conditions

Yeast cells were grown in either YPD-rich medium (yeast extract–peptone–dextrose; Difco and Carlo Erba Reagent) or synthetic medium (YNBAS from MP Biomedicals), supplemented with 2% glucose (w/v) and the appropriate complete supplement mixture (CSM from BIO101) as in Ruault et al. (2011). The selection for operator arrays was maintained by growing cells on synthetic medium lacking tryptophan.

Strains and plasmids

Genotypes of the strains used in this study are described in Supplemental Table S1. Details of strains and plasmid constructions are provided in the Supplemental Material.

Silencing assays

For the *ADE2* color colony assay, cells were plated on synthetic medium lacking tryptophan (SC-TRP) for 3 d at 30°C and then shifted for roughly 3 d to 4°C to allow the pink color to develop. Strains presented in a given figure panel were always cropped from a picture of the same plate to allow proper comparison.

The expression of the *URA3* reporter at *HMR* and telomere VI-R was monitored by a spot assay (Gottschling et al. 1990) on YPD-rich medium or 5-FOA medium as in Ruault et al. (2011).

Microscopy

For in vivo position analysis, a Metamorph-driven inverted Nikon TE2000 microscope was used to capture 21 image stacks of 0.2-μm step size. The zonal position of the GFP spot was determined on one focal section as described in Hediger et al. (2002) on cells grown on SC-TRP plate for 20 h.

Quantitative transcript analysis

Total RNA was isolated from yeast cells using RNeasy kit (Qiagen) and then DNase-treated (Qiagen) to remove contaminating DNA. First strand cDNA was prepared from 0.5 μg of RNA and random hexamers using SuperScript III (Invitrogen) for 1 h at 42°C in 50 μL. Primer sequences are provided in Supplemental Table S2. Detailed procedure of qPCR analyses can be found in the Supplemental Material. Values were normalized by *ACT1* expression levels.

ChIP

ChIP was adapted from Ruault et al. (2011) using a polyclonal antibody anti-Sir3 (Ruault et al. 2011) or monoclonal anti-GFP (Roche). The signal from a region was normalized to that from the *OGG1* control locus in immunoprecipitated and input DNA samples. Detailed procedures of ChIP and qPCR analyses can be found in the Supplemental Material.

ChIP-on-chip analyses

The position of DNA Pol2 peaks in wild-type and *rrm3Δ* strains were retrieved from Azvolinsky et al. (2009). Data of Sir3- and Sir4-binding enrichment for the wild-type strain were retrieved from Sperling and Grunstein (2009). The enrichment profiles were computed on both sides of each DNA Pol2 peak center in 50-base-pair adjacent windows.

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