

# Heterochromatin formation via recruitment of DNA repair proteins

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**ABSTRACT** Heterochromatin formation and nuclear organization are important in gene regulation and genome fidelity. Proteins involved in gene silencing localize to sites of damage and some DNA repair proteins localize to heterochromatin, but the biological importance of these correlations remains unclear. In this study, we examined the role of double-strand-break repair proteins in gene silencing and nuclear organization. We find that the ATM kinase Tel1 and the proteins Mre11 and Esc2 can silence a reporter gene dependent on the Sir, as well as on other repair proteins. Furthermore, these proteins aid in the localization of silenced domains to specific compartments in the nucleus. We identify two distinct mechanisms for repair protein-mediated silencing—via direct and indirect interactions with Sir proteins, as well as by tethering loci to the nuclear periphery. This study reveals previously unknown interactions between repair proteins and silencing proteins and suggests insights into the mechanism underlying genome integrity.

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## INTRODUCTION

Heterochromatin formation is a common mechanism of stable gene repression in eukaryotes and involves the formation of large chromatin domains that are inaccessible to specific proteins, resulting in repression of transcription and recombination of sequences that are present within these domains. In the budding yeast *Saccharomyces cerevisiae*, heterochromatic structures are observed at the cryptic mating-type loci *HML* and *HMR* on chromosome III, as well as in subtelomeric regions of chromosomes.

At *HML* and *HMR*, silencer elements flank the genes that are silenced, whereas the telomeric repeats function as silencers for subtelomeric heterochromatic regions. Mutational and binding studies identified the proteins that bind these sequence elements and are

necessary for silencing. These include the origin recognition complex (ORC) and the transcription factors Rap1, Abf1, and Sum1, as well as telomere-bound proteins such as Ku (Rusche *et al.*, 2003; Fabre and Spichal, 2014).

The silencer-bound proteins interact with and recruit the Sir repressor proteins to silence and compact loci. ORC interacts with Sir1, which localizes primarily at the silencers. Sir1 and Rap1 interact with and recruit the other Sir proteins (Sir2, Sir3, and Sir4; Luo *et al.*, 2002; Rusche *et al.*, 2002). Once recruited to the silenced domain, Sir2 deacetylates histone tails, enabling stable interaction of the Sir2, Sir3, and Sir4 complex to the deacetylated histone tails and thereby mediating formation of inaccessible chromatin domains (Ghidelli *et al.*, 2001; Johnson *et al.*, 2009; Martino *et al.*, 2009; Oppikofer *et al.*, 2011, 2013). Whereas the Sir proteins spread bidirectionally from the silencers, the silenced domain is restricted to specific regions of the genome by DNA sequence elements called barrier insulators. Insulators are bound by proteins that use enzymatic activities that act on histone tails to disfavor Sir protein binding or aid in the maintenance of nucleosome-free regions (Donze *et al.*, 1999; Oki and Kamakaka, 2005; Dhillon *et al.*, 2009).

Furthermore, silenced domains, including *HMR* and the telomeres, cluster together at the nuclear periphery, forming silencing foci. The 16 centromeres cluster together at a single site at the nuclear periphery adjacent to the spindle pole body (Jin *et al.*, 1998), whereas the 32 telomeres cluster at several loci at the nuclear periphery stabilized by protein factors that interact with subtelomeric

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Abbreviations used: HR, homologous recombination; NHEJ, nonhomologous end joining; ORC, origin recognition complex.

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and telomeric sequences (Hediger *et al.*, 2002; Taddei and Gasser, 2004; Therizols *et al.*, 2010; Fabre and Spichal, 2014). Telomeric clustering at the periphery is dependent on the Sir proteins, nuclear pore proteins, and Esc1 and Ku proteins (Fabre and Spichal, 2014). Of interest, whereas the clustering of *HML* and *HMR* is dependent on these factors, it is also affected by mutations in double-strand-break repair proteins (Miele *et al.*, 2009; Kirkland and Kamakaka, 2013).

DNA damage is ubiquitous, and a large number of proteins are involved in DNA repair, dependent on the nature of the damage (Ataian and Krebs, 2006; Symington and Gautier, 2011; Krejci *et al.*, 2012; Iyama and Wilson, 2013). There are two major pathways for the repair of double-stranded breaks: homologous recombination (HR) repair and nonhomologous end joining (NHEJ). A large number of the proteins that function in these two pathways have been identified, and the mechanisms by which they function in repair have been elucidated.

NHEJ involves numerous protein complexes, including the Ku complex, the MRX complex, and the DNA ligase complex (Symington and Gautier, 2011). The Ku complex binds the ends of double-strand breaks (Fisher and Zakian, 2005), which leads to recruitment of the MRX complex (Mre11/Rad50/Xrs2; Stracker and Petrini, 2011). The subsequent end-bridging activity allows the Lig4/Lif1 complex to ligate the ends (Symington and Gautier, 2011).

The HR repair pathway also uses numerous proteins in a series of steps leading to DNA repair (Lisby and Rothstein, 2009). In this pathway, upon detection of a double-strand break, the MRX complex binds the break (Kinoshita *et al.*, 2009; Stracker and Petrini 2011). In addition, chromatin-modifying proteins play important roles in HR repair. One of the first steps involves the phosphorylation of histone H2A on serine 129 ( $\gamma$ -H2A) by Tel1 (ATM) or Mec1 (ATR; Flott *et al.*, 2007; Polo and Jackson 2011). Phosphorylation of H2A aids in the recruitment of various proteins, including the resection machinery (Srs2 and Exo1). Resection is followed by homology search and recognition, strand invasion, and repair mediated by the late repair proteins (Rad51, Rad52, Rad54, and Rdh54, among others; Sugawara *et al.*, 2003; Heyer *et al.*, 2006; Keogh *et al.*, 2006; Wu 2008; Kinoshita *et al.*, 2009; Mortensen *et al.*, 2009). In addition, the chromatin remodelers INO80 and SWR, the histone acetyltransferase NuA4, and the structural maintenance of chromatin proteins are also recruited during this process (Unal *et al.*, 2004; Cortes-Ledesma *et al.*, 2007; Lin *et al.*, 2008; De Piccoli *et al.*, 2009; Bose and Gerton, 2010; Wood *et al.*, 2010).

Of interest, Sir proteins are mobilized from telomeres in response to DNA damage (Martin *et al.*, 1999; McAinsh *et al.*, 1999; Mills *et al.*, 1999), and Sir3 and Sir2 localize to double-strand breaks (Martin *et al.*, 1999; Mills *et al.*, 1999; Tamburini and Tyler 2005). In HR repair, histone acetylation at the site of damage is followed by histone deacetylation (Jazayeri *et al.*, 2004; Morrison *et al.*, 2004; Shroff *et al.*, 2004; Unal *et al.*, 2004; van Attikum *et al.*, 2004; Tamburini and Tyler 2005), and it has been suggested that deacetylation may in part be mediated by Sir2. However, it is unclear how the Sir proteins are recruited to double-strand breaks (DSBs) or which specific repair proteins play a role in this process.

In addition to Sir proteins functioning during HR-mediated DNA repair, several labs have shown that some repair proteins localize to silenced chromatin in the nucleus (d'Adda di Fagagna *et al.*, 2004). The Ku proteins interact with telomeric heterochromatin as well as with the HM loci and affect silencing at these loci (Laroche *et al.*, 1998; Fisher and Zakian, 2005; Patterson and Fox, 2008; Vandre *et al.*, 2008; Bystricky *et al.*, 2009). Histone H2A is constitutively phosphorylated at serine 129 ( $\gamma$ -H2A) in silenced chromatin in

*S. cerevisiae*, *Schizosaccharomyces pombe* (Szilard *et al.*, 2010; Kitada *et al.*, 2011; Kirkland and Kamakaka, 2013), and *Drosophila* (Andreyeva *et al.*, 2008), and the presence of this histone mark at silenced domains is dependent on the presence of Sir proteins (Kirkland and Kamakaka, 2013). The MRX complex and Tel1 localize to telomeres in competition with Rap1 and Rif1/2 (Takata *et al.*, 2004; Bianchi and Shore, 2007; Hirano and Sugimoto, 2007; Sabourin *et al.*, 2007; Hirano *et al.*, 2009; Ma and Greider, 2009), and mutations in HR repair proteins leads to loss of clustering of silenced domains; these data collectively suggest direct or indirect links between silenced domains and repair proteins (Miele *et al.*, 2009; Kirkland and Kamakaka, 2013).

To clarify the links between HR repair proteins and silencing, we asked whether DSB repair proteins have the ability to interact with the Sir proteins and affect silencing. Using reporter assays, we show that DSB repair proteins can silence a reporter gene when tethered to a silencer. Using various mutants, we identify the genetic pathway involved in DSB repair protein-mediated silencing. We also show that these repair proteins can interact with the Sir proteins and use two distinct pathways for silencing—one involving direct interactions with the Sir proteins, and the second via tethering of the locus to the nuclear periphery.

## RESULTS

### Gal4-Mre11-mediated repression at *HMR*

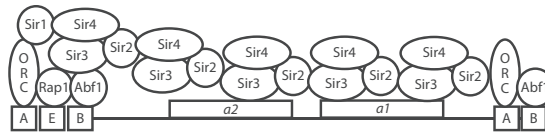
To investigate the role of DSB repair proteins in gene silencing, we initially sought the consequence of recruiting a specific repair protein to the *HMR* locus. Two silencers, *HMR-E* and *HMR-I*, flank the *a1* and *a2* genes at *HMR*. The essential *HMR-E* silencer contains binding sites for ORC, Rap1, and Abf1 proteins, and the important *HMR-I* silencer contains binding sites for ORC and Abf1. A strain in which Gal4-binding sites replace the ORC-binding sites at the two silencers is unable to recruit the Sir proteins and is unable to silence the genes present at *HMR*. Loss of silencing of the *a1* gene in an  $\alpha$  cell results in an inability of this cell to mate with an *a* cell and form diploid colonies. Expression and recruitment of Gal4-Sir1 to these modified *HMR* silencers results in the repression of the *a1* gene, which allows the  $\alpha$  haploid strain to once again mate with an *a* strain, forming diploid colonies (Chien *et al.*, 1993). This is due to the fact that the Gal4 DNA-binding domain of Gal4-Sir1 fusion enables this protein to be recruited to the modified silencer even in the absence of the ORC-binding site (Fox *et al.*, 1997). This modified silencer-containing strain can therefore be used to assay silencing after recruitment of DSB repair proteins to the *HMR* silencers. The Gal4 DNA-binding domain alone is not able to silence (Figure 1B), whereas Gal4-Sir1 is able to robustly silence the *a1* gene (Figure 1C), and these serve as negative and positive controls, respectively.

We first asked whether tethering of the fusion protein Gal4-Mre11 could aid in silencing. Recruitment of Gal4-Mre11 to the silencer was able to silence the reporter gene (Figure 2, WT panels). Comparative serial dilution assays show that Mre11 was not as robust as Gal4-Sir1 in silencing but clearly demonstrate that the repair protein Mre11 has the ability to significantly and reproducibly repress the *a1* reporter gene present at *HMR*.

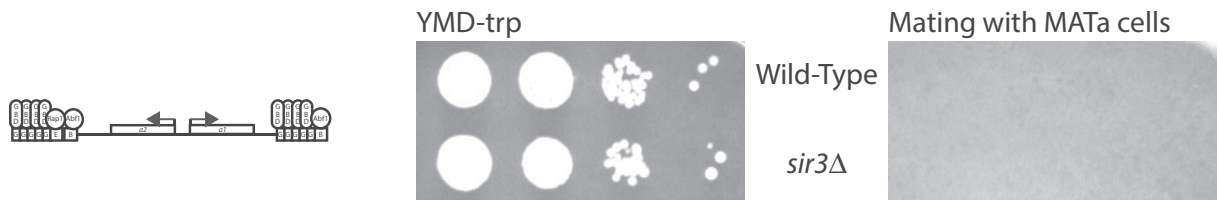
### Mre11-mediated silencing is dependent on Sir proteins

To ascertain whether Mre11-mediated repression was simply localized repression mediated by occlusion/steric hindrance as opposed to gene silencing, we asked whether Gal4-Mre11-mediated gene repression was dependent on the presence of the other Sir proteins. In a *sir3 $\Delta$*  background, neither Gal4-Sir1 nor Gal4-Mre11 was able to

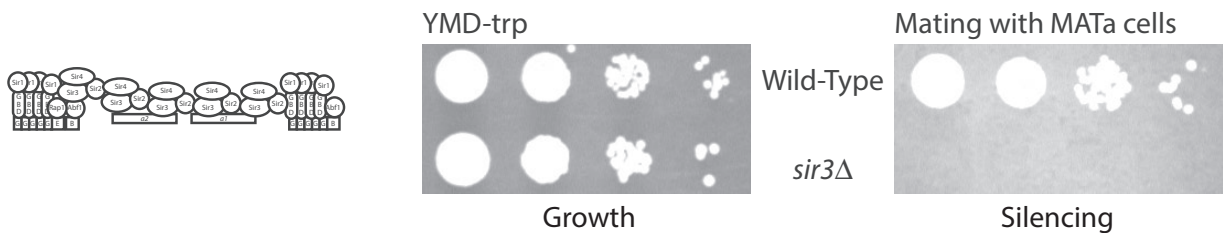
A.



B.



C.



**FIGURE 1:** (A) Schematic of the wild-type silenced locus. (B) Schematic of the modified locus used in this study and the mating assay when the Gal4 DNA-binding domain alone is expressed in the cell, resulting in no repression of the *a1* gene. (C) Schematic of the modified locus used in this study and the mating assay when Gal4-Sir1 is expressed in the cell, resulting in silencing of the *a1* gene.

silence the gene (Figure 2). These results are not specific to Sir3, since these fusion proteins were also not able to silence the reporter gene at *HMR* in a *sir2Δ* strain (unpublished data) or a *sir4Δ* strain (Figure 2). The loss of silencing in a Sir protein-deficient background demonstrates that Gal4-Mre11-dependent silencing is not due to recombination or resection of the reporter gene either. These results indicate that Mre11-mediated repression of the reporter gene was operating via a bona fide silencing pathway.

### Mre11-mediated silencing is partially dependent on Esc2 and histone H2A phosphorylation

To dissect this novel form of gene silencing, we investigated the other factors necessary for Mre11-mediated silencing. In the strain containing the modified *HMR* locus, we deleted specific genes and asked whether this affected Gal4-Mre11-mediated silencing (Figure 2A). Loss of Mre11, Rad50, and Rad51 had no effect on Gal4-Mre11-mediated silencing. Gal4-Mre11 was also able to silence the *a1* gene in the absence of the heterochromatin nuclear tethering proteins Esc1 and Ku70. However, silencing was reduced in the absence of the protein Esc2, which has been implicated in both DNA repair and gene silencing (Dhillon and Kamakaka, 2000; Cuperus and Shore, 2002; Ohya *et al.*, 2008; Mankouri *et al.*, 2009; Miele *et al.*, 2009; Sollier *et al.*, 2009; Choi *et al.*, 2010; Mimura *et al.*, 2010; Yu *et al.*, 2010; Albuquerque *et al.*, 2013).

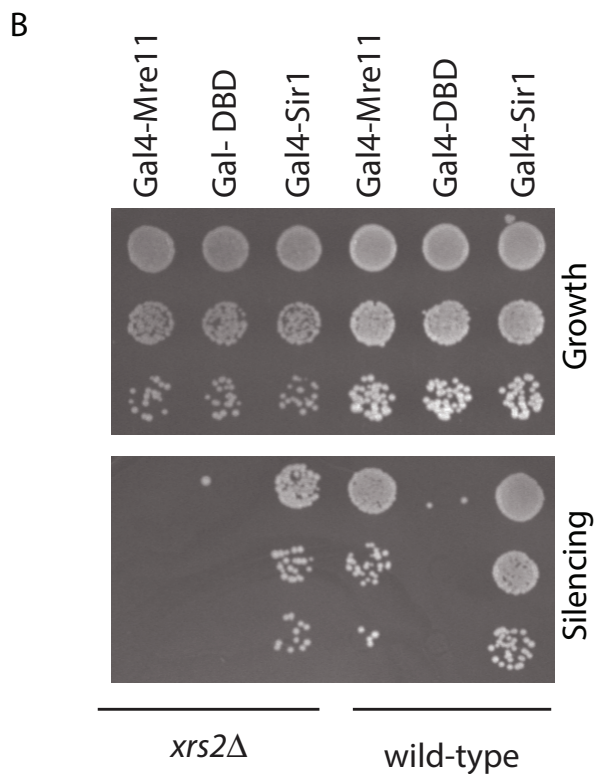
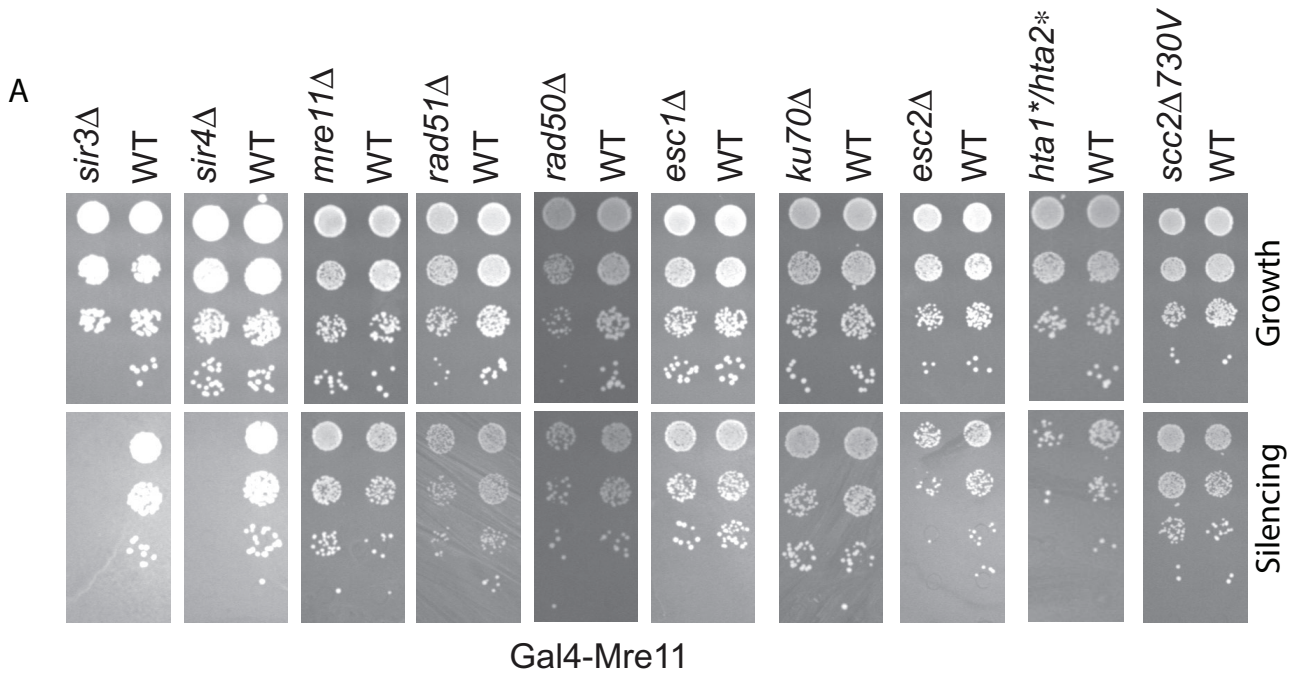
We next assayed a H2A mutant that could not be phosphorylated by the phosphoinositide 3 (PI3) kinases Mec1 and Tel1 (Downs *et al.*, 2000). Strains that cannot phosphorylate H2A at Ser-129 show a modest decrease in silencing, indicating that

$\gamma$ -H2A was necessary to some degree for Gal4-Mre11-mediated silencing.

The MRX complex is involved in telomere length homeostasis and double-strand break repair. Xrs2 is a member of this complex, interacts with Tel1, and is necessary for the recruitment of Tel1 to double-strand breaks (Ritchie and Petes, 2000; Tsukamoto *et al.*, 2001; Nakada *et al.*, 2003; Shima *et al.*, 2005). Because mutations in H2A affect Mre11-mediated silencing, we asked whether this silencing was dependent on Xrs2. The data show that in the absence of Xrs2, Mre11-mediated silencing was lost, but Sir1-mediated silencing was not significantly affected (Figure 2B).

### Gal4-Tel1-mediated silencing

Double-strand breaks in DNA are initially recognized and bound by the MRX complex, as well as by the PI3 kinases. After this, the kinases phosphorylate histone H2A in the vicinity of the break. The PI3 kinase Tel1 functions in the same genetic pathway as Mre11 (Ritchie and Petes, 2000), and it was shown that simply tethering a fragment of ATM was sufficient to phosphorylate H2A, whereas tethering ATR activates the DNA-damage checkpoint in an H2A-dependent manner (Bonilla *et al.*, 2008; Soutoglou and Misteli, 2008). Because Mre11-mediated silencing appeared to be dependent on H2A phosphorylation and Tel1 localizes to telomeres and has kinase-independent functions in telomere maintenance (Ma and Greider 2009), we asked whether Tel1 itself had the ability to recruit Sir proteins and silence the reporter gene at *HMR* or whether this property was unique to Mre11. We fused full-length wild-type Tel1 to the Gal4 DNA-binding domain and transformed the strain

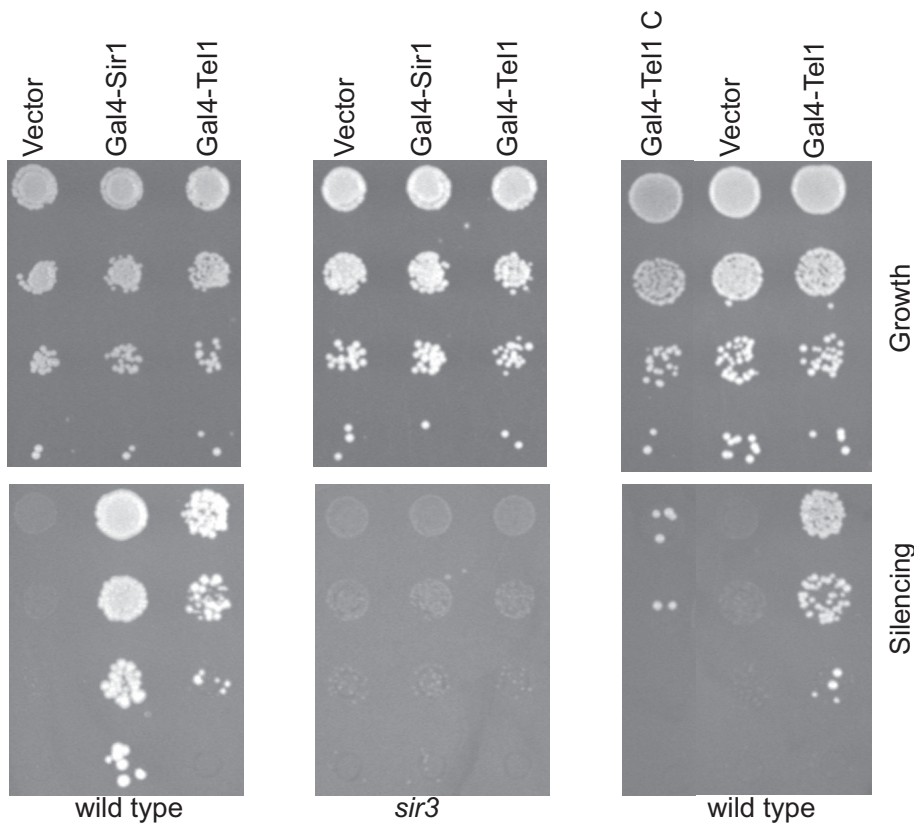


**FIGURE 2:** Gal4-Mre11-mediated silencing. (A) Gal4-Mre11-mediated silencing in wild type and various mutant derivatives of this strain. Tenfold dilutions of MAT $\alpha$  strains containing the modified *HMR* locus were spotted on a YMD-Trp plate (growth control) or a YMD plate containing a lawn (JRY19a) to assay silencing. (B) Gal4-Mre11- and Gal4-Sir1-mediated silencing in a strain lacking Xrs2.

containing the modified *HMR* locus with this fusion construct. To our surprise, we discovered that Gal4-Tel1 was able to robustly silence the *a1* reporter gene (Figure 3). We next asked whether Gal4-Tel1 was able to silence the gene in the absence of Sir proteins. Loss of

Sir3 resulted in complete loss of Gal4-Tel1-mediated silencing. This was not a Sir3-specific effect, since we saw similar complete loss of silencing in a *sir2* $\Delta$  strain (Figure 4A), suggesting that Gal4-Tel1-mediated silencing used the entire Sir complex.





**FIGURE 3:** Gal4-Tel1-mediated silencing in wild-type and *sir3Δ* strains. Tethered silencing in the wild-type strain with a C-terminal truncation of Tel1 (Gal4-Tel1 CΔ).

The kinase domain of Tel1 resides in the C-terminus of the protein (Greenwell *et al.*, 1995; Khanna *et al.*, 2001). This domain is believed to interact with and phosphorylate the numerous substrates of Tel1 (Smolka *et al.*, 2007; Chen *et al.*, 2010; Gobbin *et al.*, 2013). We truncated the fusion protein such that it lacked this kinase domain. In the absence of this domain, Gal4-Tel1 was unable to silence *HMR*, suggesting that this domain played a role in silencing (Figure 3). Protein blotting suggests that the truncated protein is expressed to approximately the same extent as wild-type Gal4-Tel1 (Supplemental Figure S1).

### Tel1-mediated silencing is dependent on Esc1/Ku, Esc2, and histone H2A phosphorylation

To better understand the factors involved in Gal4-Tel1-mediated silencing, we deleted proteins involved in DNA repair and chromatin remodeling to identify factors that are necessary for Tel1-mediated silencing. Deletions in genes involved in repair were generated in the strain containing the modified *HMR* locus. Loss of specific repair proteins, such as Rad54, Rdh54, Rad50, Rad51, Xrs2, and so on, had either no effect or very subtle effects on Tel1-mediated silencing (Figure 4). Similarly, we tested mutants in chromatin-remodeling factors, since these proteins affect DSB repair in heterochromatin (Clapier and Cairns, 2009; Sinha *et al.*, 2009; Deem *et al.*, 2012). Loss of various chromatin-remodeling factors also had no effect on Tel1-mediated silencing. However, loss of Esc1, Ku70, and Esc2 led to a significant reduction of Tel1-mediated silencing, suggesting a dependence on these proteins for Tel1-mediated silencing.

Because one of the earliest substrates of Tel1 in DNA damage repair is histone H2A, we asked whether Tel1-mediated silencing was dependent on H2A phosphorylation (Figure 4C). In a mutant

strain in which H2A cannot be phosphorylated, there was a partial loss of silencing. While there are likely additional Tel1 phosphorylated substrates involved in silencing and Tel1 may even have kinase-independent functions in silencing (Ma and Greider, 2009), this result indicates that Tel1-mediated silencing is partially dependent on H2A phosphorylation and is consistent with the demonstration that the catalytic domain of Tel1 might also be necessary.

### Tethering Tel1 induces H2A phosphorylation at *HMR*

Both Gal4-Mre11 and Gal4-Tel1 are able to silence *HMR* in an H2A-dependent manner, suggesting that these proteins mediate this phenotype via phosphorylation of H2A at *HMR*. We tested this model by directly measuring H2A phosphorylation at S129 using quantitative chromatin immunoprecipitation (ChIP). Yeast cells with the modified silencers were transformed with Gal4-Tel1, Gal4-Mre11, Gal4-Sir1, or Gal4 DNA-binding domain (DBD) alone and grown under selection before cross-linking and immunoprecipitation with antibodies against  $\gamma$ -H2A. Quantitative ChIP was used to determine the levels of this histone modification at *HMR* (Figure 5). A probe in the *ACT1* gene that lacks  $\gamma$ -H2A was used as a negative control. Analysis of the *a1*

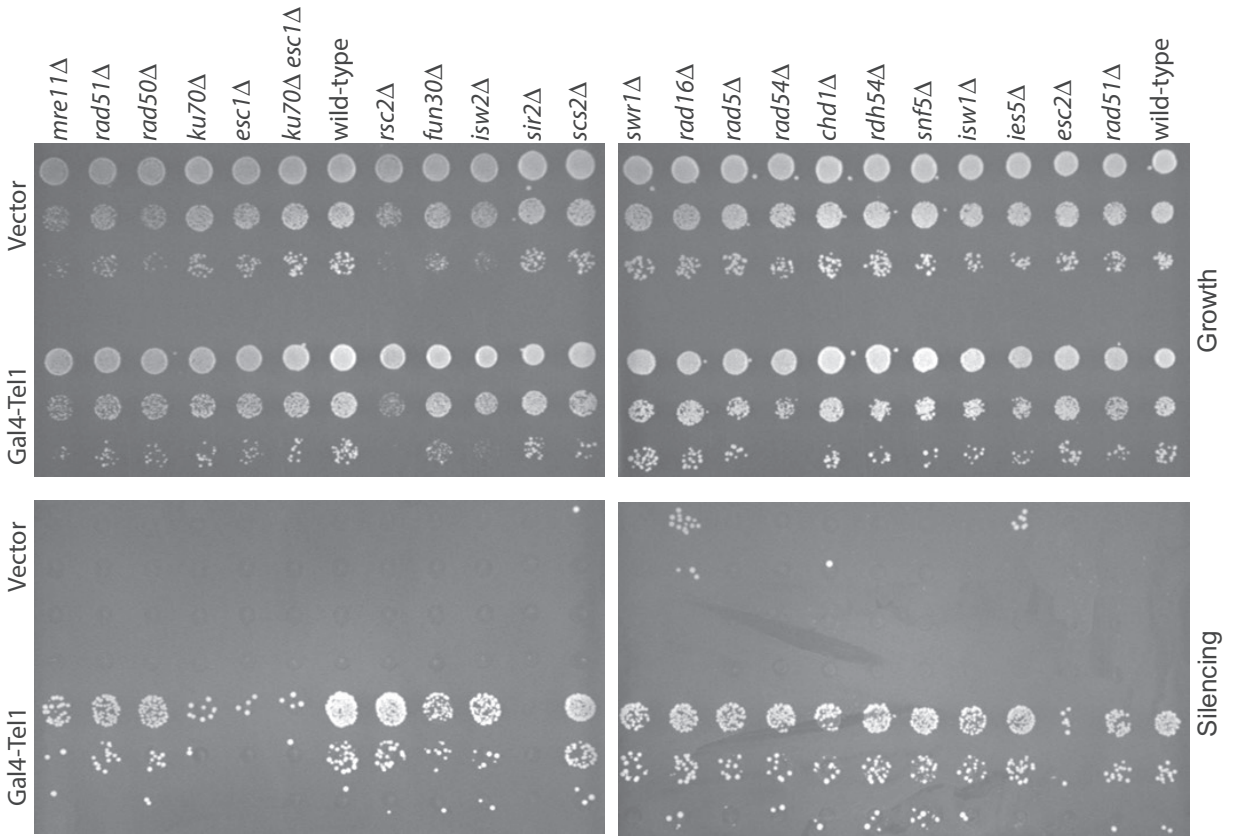
gene at the modified *HMR* locus shows clear enrichment of  $\gamma$ -H2A in the strain containing Gal4-Tel1, Gal4-Mre11, and Gal4-Sir1 but not in the strain lacking these fusion proteins. Furthermore, a probe immediately outside the *HMR-E* silencer is also enriched for  $\gamma$ -H2A in these strains. This suggests that the histone modification at *HMR* is dependent on Tel1 and Mre11 and furthermore that it spreads in both directions from the recruitment site and is not just found between the modified silencers, consistent with the spread of Sir3 and  $\gamma$ -H2A at the native *HMR* locus (Kirkland and Kamakaka, 2013).

### Gal4-Esc2-dependent silencing is independent of Esc1

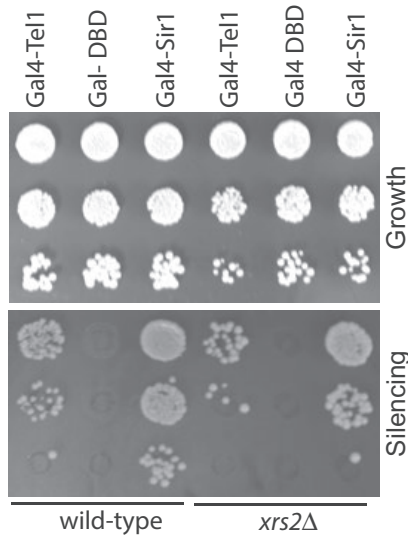
Both Mre11 and Tel1 required wild-type Esc2 in order to silence robustly the reporter at *HMR*. Overexpression of Esc2 silences reporter genes in the presence of a mutant Sir1 (Dhillon and Kamakaka, 2000), and Esc2 has been shown to function in DNA repair (Mankouri *et al.*, 2009; Sollier *et al.*, 2009). We therefore wished to know whether Gal4-Esc2 was able to silence genes and whether silencing was dependent on other repair proteins (Figure 6A). Consistent with previous data, Gal4-Esc2 is able to silence the reporter gene at *HMR*. Whereas Gal4-Esc2-mediated silencing was entirely dependent on Sir2 and Sir3, loss of Mre11, Rad51, Esc1, or Ku70 had no effect on Esc2-mediated silencing.

Mph1 and Esc2 interact genetically in repair, and whereas Esc2 plays a role in the resolution of replication-coupled recombination intermediates, Mph1 promotes the formation of these intermediates when replication forks encounter DNA damage (Mankouri *et al.*, 2009; Sollier *et al.*, 2009; Choi *et al.*, 2010). We therefore tested whether Esc2-mediated silencing was altered in the absence of Mph1. Our data show that loss of Mph1 had no effect on

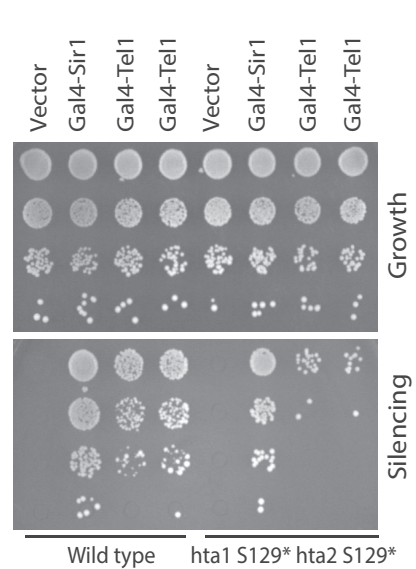
A



B



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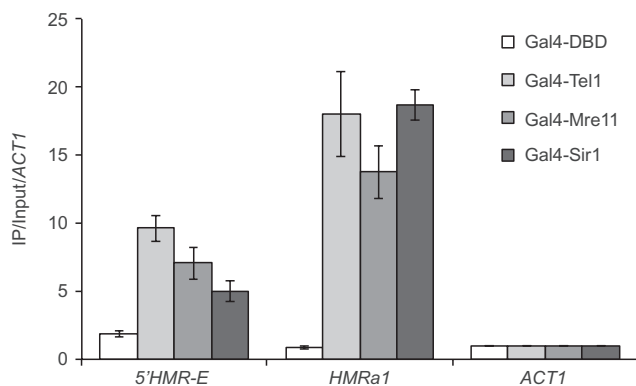
**FIGURE 4:** Gal4-Tel1–mediated silencing. (A) Gal4-Tel1–mediated silencing in wild-type and various mutant derivatives of this strain. (B) Gal4-Tel1– and Gal4-Sir1–mediated silencing in a strain lacking Xrs2. (C) Gal4-Tel1–mediated silencing in a strain unable to phosphorylate H2A at serine 129.

Esc2-mediated silencing. These result collectively suggest that Esc2 likely functions downstream of Mre11 and Tel1 in silencing.

**Gal4-Esc1–dependent silencing is independent of Esc2**

Esc1 interacts with Sir4 and tethers silenced loci to the nuclear periphery (Taddei *et al.*, 2005). Whereas Mre11-mediated silencing

was independent of Esc1, Tel1-mediated silencing was partially dependent on Esc1 and Ku70. We therefore asked whether Gal4-Esc1 was sufficient for silencing the reporter gene, consistent with previous results (Andrulis *et al.*, 2002, 2004). Gal4-Esc1 was able to silence *HMR* to similar levels as Esc2 and Mre11. We investigated Esc1 further and determined that Gal4-Esc1–dependent



**FIGURE 5:** ChIP mapping of  $\gamma$ -H2A at the modified *HMR* locus. ChIP was performed in a strain with the modified *HMR* locus expressing either the Gal4 DNA-binding domain alone or Gal4-Sir1, Gal4-Mre11, or Gal4-Tel1. Data are presented as the mean enrichment of IP/input further normalized to an *ACT1* amplicon for four IPs from two independent cross-links. Error bars are SD from the mean.

repression was also independent of Mre11, Rad51, or Esc2 but dependent on the Sir proteins (Figure 6C). These data suggest that there are likely to be two HR repair protein-mediated silencing pathways—one dependent on Esc1/Ku70 and the other on Esc2.

#### Gal4-Esc2-mediated silencing is stably inherited

The partial silencing at *HMR* by Mre11, Tel1, and Esc2 could reflect either intermediate levels of expression of the reporter genes in all cells in a colony or a population in which some cells expressed the gene and others did not (Kamakaka, 1997). To distinguish between these two models, we generated a strain with the *ADE2* gene under the control of the modified *HMR* silencers. Yeast colonies lacking *ADE2* activity are red, and colonies where the *ADE2* gene is active are white. If derepression of *HMR::ADE2* were partial in all cells, then the colony color would convert from red to pink. In contrast, if the derepression occurred in only a fraction of the cells and the transcription states were mitotically stable, then there should be a mixture of colonies—some red, some white, and some that exhibit red and white sectors.

In the presence of the Gal4 DNA-binding domain alone, the test strain formed only white colonies, indicating that the *ADE2* gene was active. Expression of Gal4-Sir1 repressed the *ADE2* gene at *HMR*, resulting in mostly red colonies with some white colonies (Figure 7). Expression of Gal4-Esc2 and Gal4-Esc1 resulted in colonies that were red with white sectors. These data support a binary mode of silencing by Gal4-Esc2 at *HMR* and indicated that transcription states of *ADE2* at *HMR*, once established by Gal4-Esc2, were stably inherited for several generations. On the other hand, Gal4-Tel1 generated primarily white/light pink colonies, and Gal4-Mre11 colonies were primarily white, suggesting repression that was unstable and prone to frequent changes.

#### Mre11 and Tel1 interact with Sir proteins

Our data suggest that HR repair proteins have the ability to silence when tethered to the *HMR* silencer. We next wished to know whether this silencing was due to direct interactions between the HR proteins and the Sir proteins. To test whether HR repair proteins interacted with the Sir proteins, we performed a two-hybrid analysis (Figure 8A). Gal4-Mre11, Gal4-Tel1, and Gal4-Esc2 were assayed for interaction with Sir2, Sir3, Sir4, and Esc2 that were fused to the activation

domain of Gal4. The two-hybrid analysis showed that whereas the Gal4 DNA-binding domain alone was not able to interact with any of the proteins fused to the activation domain, Gal4-Sir1 interacted specifically with Sir4 and Gal4-Esc2 interacted with Sir2; these results are consistent with previous data (Chien et al., 1991; Cuperus and Shore, 2002). Surprisingly, Gal4-Mre11 showed a weak interaction with Sir2. Of great interest, Gal4-Tel1 interacted robustly with Sir3 and Sir4 and weakly with Esc2, suggesting that Tel1-mediated silencing may be via direct interactions with the Sir proteins. These interactions appear to be direct, since loss of the other Sir proteins did not dramatically alter the interactions (Figure 8B).

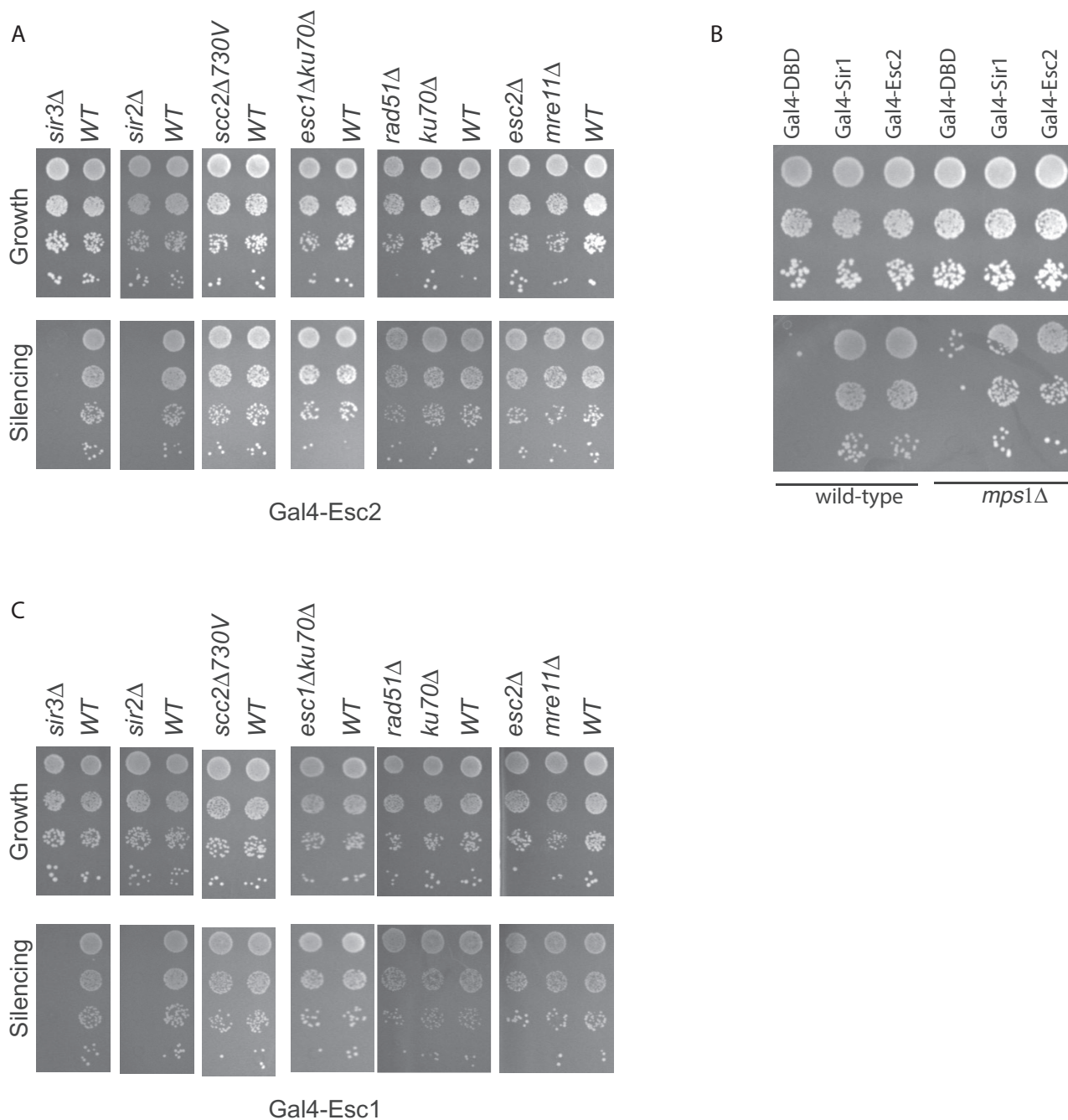
#### Peripheral tethering of *HMR* by DSB-repair proteins

One of the characteristic features of silencing is the clustering of these domains at the nuclear periphery, which aids in the efficiency of silencing and is dependent on Esc1 and Ku proteins (Taddei et al., 2009, 2010). We therefore inquired whether silencing mediated by Gal4-Mre11, Gal4-Tel1, and Gal4-Esc2 resulted in the silenced locus moving to the nuclear periphery and whether this movement was dependent on the Sir proteins. We inserted a LacO array adjacent to the modified *HMR* locus. This strain contained a LacI-green fluorescent protein (GFP) fusion protein that binds the array, thus marking the *HMR* locus. Furthermore, we marked the nuclear periphery by expressing HDEL-lac-mCherry fusion protein (Madrid et al., 2006; Ruben et al., 2011). The shortest distance between *HMR* and the nuclear periphery was measured, as was the diameter of the nucleus through the *HMR* focus. This enabled us to divide the nucleus into two zones of equal surface area—one internal and one peripheral—and we assigned the *HMR* locus to one of these zones (Figure 9).

In an otherwise wild-type background, expressing just the Gal4 DNA-binding domain resulted in the modified *HMR* locus residing in the peripheral zone 53.1% of the time and in the internal zone 46.9% of the time. When Gal4-Sir1 or Gal4-Mre11 was expressed, the *HMR* locus shifted to the peripheral zone 82.7% and 80.4% of the time, respectively. Both of these shifts were statistically significant ( $p = 5.4e^{-7}$  and  $9.6e^{-8}$ , respectively). In a *sir3Δ* background, Gal4-Sir1 still localized the modified *HMR* to the periphery, even though the locus was not silenced (zone 1, 79.7%;  $p = 3.6e^{-6}$ ), revealing a novel role for Sir1 in tethering *HMR* to the nuclear periphery independent of Sir3. In the absence of Sir3, Gal4-Mre11 was no longer able to localize the *HMR* locus to the periphery (zone 1, 64.0%;  $p = 0.10$ ). Gal4-Tel1 also shifted the locus to the nuclear periphery (78.4%), as did Gal4-Esc2 (74.8%). However, Gal4-Tel1- and Gal4-Esc2-mediated localization to the periphery was not altered in the absence of Sir3, unlike that of Gal4-Mre11.

#### DISCUSSION

Repair of chromosomal breaks is essential for cell survival, and there are numerous pathways involved in the repair of breaks (Lisby and Rothstein, 2009). There are some reports of linkages between repair pathways and silencing proteins. The Ku protein plays a role in both DSB repair and silencing (Fisher and Zakian, 2005). DSBs in yeast cells also result in partial dissociation of Sir proteins from telomeres, which is dependent on PI3 kinases, although the importance of this dissociation is unclear (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). Although initially the Sir proteins were believed to play a direct role in NHEJ repair (Tsukamoto et al., 1997), later studies suggested an indirect role for these proteins in this pathway of repair via their function in silencing the *a1* and  $\alpha 2$  genes at *HMR* and *HML* (Astrom et al., 1999; Lee et al., 1999). On the other hand, histone acetylation is an early step during HR repair (Jazayeri et al., 2004; Morrison et al., 2004; Shroff et al., 2004; Unal et al., 2004;



**FIGURE 6:** Gal4-Esc2- and Gal4-Esc1-mediated silencing. (A) Gal4-Esc2-mediated silencing in wild type and various mutant derivatives of this strain. (B) Gal4-Esc2- and Gal4-Sir1-mediated silencing in wild type and a strain lacking Mph1. (C) Gal4-Esc1 mediated silencing in wild type and various mutant derivatives of this strain.

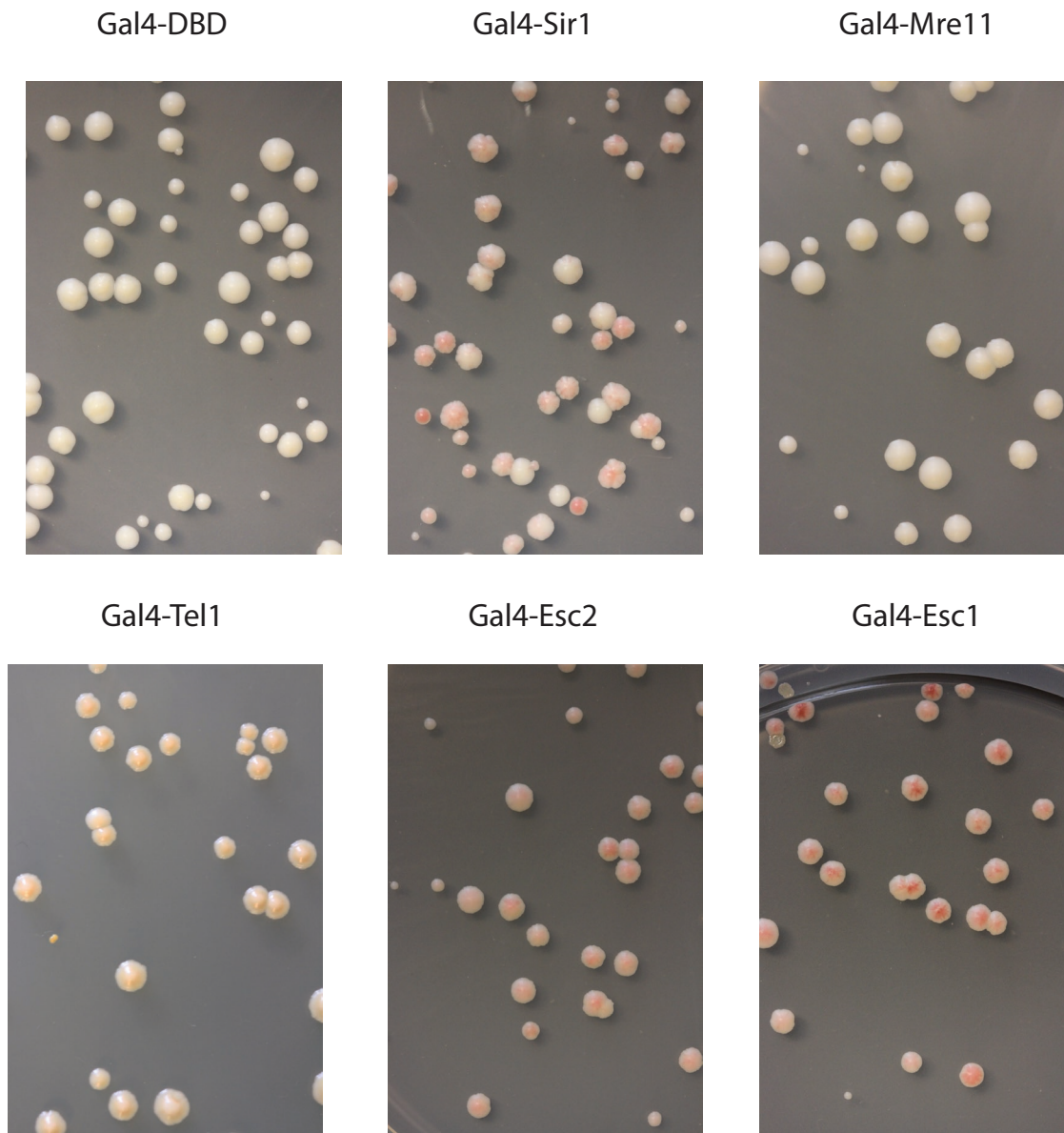
van Attikum *et al.*, 2004; Lin *et al.*, 2008), and histone deacetylation occurs during the later stages of repair (Tamburini and Tyler, 2005), although the function of the Sir proteins in these processes is not clear. We now show that specific repair proteins have the ability to interact with the Sir proteins. Tel1 interacts strongly with Sir4 and Sir3 and weakly with Esc2, which in turn interacts robustly with Sir2. Furthermore, tethering Tel1 to a defective silencer enables silencing of the gene at that locus, and this silencing is dependent on the Sir proteins, demonstrating that Tel1 has the ability to directly or indirectly recruit these proteins to DNA. Data also show that Sir4 is a substrate of Tel1 enzymatic activity (Chen *et al.*, 2010), suggesting that the interaction between these two proteins might be direct. It

therefore appears that the localization of Tel1 to sites of damage could potentially result in the recruitment of Sir proteins to these sites, although additional experiments will be necessary to test this model.

Mre11 is one of the first proteins to be recruited to a double-strand break. We have now shown that Mre11 has the ability to recruit the Sir proteins to a modified silencer. Given the weak interaction between Mre11 and Sir proteins, we favor a model in which Mre11 recruits the Sir proteins indirectly via its interactions with other proteins, most likely Tel1.

Esc2 contains two tandem SUMO-like domains that are necessary for its interactions with Sir proteins (Dhillon and Kamakaka, 2000; Cuperus and Shore, 2002; Yu *et al.*, 2010). Recent results show





**FIGURE 7:** Silencing of *ADE2* at the modified *HMR* locus by various fusion proteins.

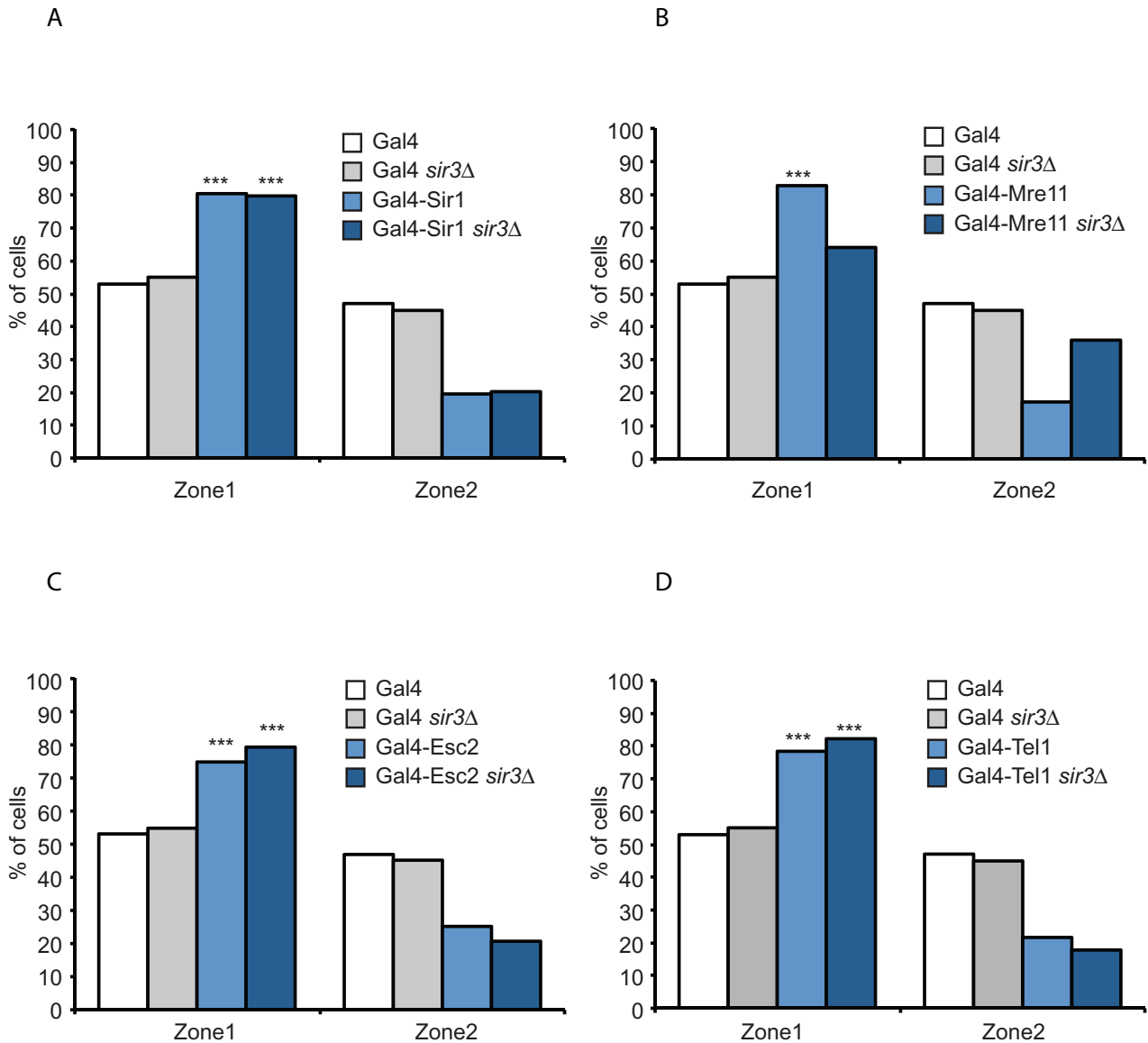
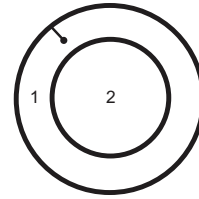
that Esc2 functions with Mms21 in HR repair by preventing the accumulation of recombination intermediates that are generated by Mph1, Mms2, and the SHU complex (Mankouri *et al.*, 2009; Sollier *et al.*, 2009; Choi *et al.*, 2010; Mimura *et al.*, 2010; Albuquerque *et al.*, 2013). The demonstration that both Tel1- and Mre11-mediated silencing is partially dependent on Esc2 and that Esc2 interacts robustly with Sir2 led us to propose that Esc2 is another molecular link between repair proteins and silencing.

Double-strand breaks that are rapidly repaired using the HR repair pathway do not elicit a robust checkpoint response, and the breaks are usually repaired in the interior of the yeast nucleus. However, breaks that are repaired with slower kinetics or ones that were not repairable are recruited to the nuclear periphery in an Mps3-dependent (Oza *et al.*, 2009) and Nup84-dependent (Therizols *et al.*, 2006; Nagai *et al.*, 2008) manner requiring robust checkpoint signaling. In addition, breaks that occur in silenced heterochromatin also need to be tethered to the nuclear pore to be repaired (Therizols *et al.*, 2006). It has been suggested that

tethering to the periphery enables telomere-bound complexes to aid in repair (Bennett *et al.*, 2001). It is therefore possible that release of Sir proteins from telomeres upon DNA damage and their recruitment to break sites via direct and indirect interactions with Tel1, Mre11, and Esc2 help target these loci to the nuclear periphery if the break is not repaired rapidly. Consistent with this model is the observation that targeting Nup84 to the *HMR* locus results in the targeting of this locus to the periphery and its silencing in a Sir-dependent manner (Ruben *et al.*, 2011), although it is unclear whether this Nup84-mediated silencing is dependent on Esc1 and/or Esc2 or other repair proteins.

Mutants in HR repair proteins lead to a reduction in the clustering of *HML* and *HMR* (Kirkland and Kamakaka, 2013). Whereas mutants in Mre11 have no noticeable defects in silencing at native *HML* or *HMR*, mutants in Ku70 and Esc2 do have small defects in silencing at these loci, and Ku70 localizes to the native *HML* and *HMR* domains in wild-type cells (Dhillon and Kamakaka, 2000; Patterson and Fox, 2008; Vandre *et al.*, 2008). Whether Ku70





**FIGURE 9:** Localization of the modified *HMR* locus in the yeast nucleus. A schematic of a yeast nucleus with two zones of equal surface area and a fluorescent locus are shown in the line diagram. (A–D) Graphs of the percentage of cells in each of the two zones in various strains. The data for Gal4 DNA-binding domain alone are shown simply for ease of comparison. \*\*\* $p < 0.001$  by  $\chi^2$  test.

favor the alternative possibility that the presence of chromatin-bound Sir proteins at these loci recruits the repair proteins (such as Ku and Tel1) to these domains, and the loss of these repair proteins results in effects such as disruption of *HML-HMR* long-range clustering in the nucleus with only subtle effects on silencing.

The role of  $\gamma$ -H2A in gene silencing is intriguing and puzzling. The location of the residue in histone H2A, close to the C-terminus, places this site close to the entry and exit sites of DNA in the nucleosome. Although it was originally proposed that phosphorylation of

H2A might facilitate chromatin decondensation (Downs *et al.*, 2000), subsequent studies showed that this was not the case (Fink *et al.*, 2007). Proteins containing FHA and BRCT domains, such as INO80, SWR1, and the PI3 kinases, recognize phosphorylated H2A (Kinner *et al.*, 2008). Among the yeast proteins that possess BRCT domains is Rap1, and although Rap1 is a sequence-specific factor, it also binds silenced chromatin and spreads along the domain in a sequence-independent manner, although the mechanism by which this occurs is unknown (Lieb *et al.*, 2001; Valenzuela *et al.*, 2008;

Ozaydin and Rine, 2010; Zhang *et al.*, 2011). One possible scenario could involve interactions between  $\gamma$ -H2A present in silenced chromatin and Rap1, which could then further aid in silencing. Phosphorylation of H2A is also necessary for the binding of cohesins to silenced chromatin and the clustering of HML with HMR (Kirkland and Kamakaka, 2013). Thus  $\gamma$ -H2A could alternatively affect silencing via cohesin-mediated clustering of heterochromatic domains. Obviously these are not mutually exclusive scenarios, and other proteins could also interact with  $\gamma$ -H2A and affect silencing. It is intriguing, however, that  $\gamma$ -H2A localizes to silenced chromatin in the distantly related yeast *S. cerevisiae* and *S. pombe* as well as in *Drosophila* (Andreyeva *et al.*, 2008; Rozenzhak *et al.*, 2010; Szilard *et al.*, 2010; Kitada *et al.*, 2011; Kirkland and Kamakaka, 2013), and there are suggestions of a similar distribution of this modification in vertebrates (Fernandez-Capetillo *et al.*, 2003; Ichijima *et al.*, 2005; Shechter *et al.*, 2009).

In conclusion, our data collectively suggest that different DSB repair proteins can interact with the Sir proteins, and these repair proteins, when recruited to sites in the genome, can induce the recruitment of Sir proteins and concomitant silencing at these sites via distinct mechanisms. Mre11 most likely recruits Sir proteins indirectly, and this recruitment of Sir proteins at specific sites in the genome then targets the bound locus to the nuclear periphery. This explains the observation that Mre11-mediated silencing is not altered in the absence of Esc1 and Ku, but is consistent with the demonstration that movement of the Gal4-Mre11-silenced locus to the periphery is dependent on the Sir proteins. On the other hand, Tel1 silences via two distinct mechanisms. It directly interacts with the Sir proteins, as shown by the two-hybrid analysis and the fact that Sir4 is a substrate of this kinase. It also affects silencing via the phosphorylation of H2A. This is evidenced by the observation that cells lacking this histone modification are not able to robustly silence. Finally, Tel1 has the ability to target the locus to the nuclear periphery independent of the Sir proteins. This targeting to a compartment rich in Sir proteins would further favor the formation of silenced chromatin.

It should also be borne in mind that one function of heterochromatin is to reduce recombination of repetitive DNA. The function of Esc2 is the suppression of chromosomal recombination, and it is entirely possible that this function is mediated in part via its ability to bind/recruit Sir2 and the subsequent targeting of Sir protein-bound loci to the nuclear periphery, resulting in the suppression of recombination, as well as allowing the healing of persistent breaks via alternative repair machineries or de novo telomere formation.

## MATERIALS AND METHODS

### Yeast strains and plasmids

All yeast strains used in this study are in the W303 background except the strains used for the two-hybrid analysis (see Tables 1 and 2). Most gene deletions were performed by transformation and subsequent replacement of the open reading frame with a KanMx cassette. KanMx cassettes were amplified using PCR with primers flanking the deletion using genomic DNA from the deletion collection as template. Strains containing gene deletions marked by auxotrophic markers were built by crossing the test strain (TM47/JKY383) to previously published strains.

### Serial dilution mating assays

Strains were transformed with 2 $\mu$  plasmids containing Gal4 DBD (1–147) or GBD fused in-frame to a protein of interest and selected on yeast minimal dextrose (YMD) –uracil (YMD-U) or –tryptophan (YMD-T), depending on the selection marker of the plasmids. After 2 d, multiple colonies were picked and grown overnight at 30°C

Plasmid	Description	Source
pRO1000	Gal4 (1-147) 2 $\mu$ URA3	
pRO990	Gal4-Tel1 $\Delta$ 2 $\mu$ URA3	
pRO1022	Gal4-AD-SIR4 2 $\mu$ LEU2	
GLC370	Gal4-AD-SIR2 2 $\mu$ LEU2	D. Shore (University of Geneva)
PM875	Gal4-AD-SIR3 2 $\mu$ LEU2	D. Shore
pRO998	Gal4-AD-ESC2 2 $\mu$ LEU2	
pAct2.2	Gal4-AD 2 $\mu$ LEU2	
pRO83	Gal4-Sir1 2 $\mu$ URA3	
pRO963	Gal4-Tel1 2 $\mu$ URA3	
pJR1112	2 $\mu$ URA3	
pRO1001	Gal4-Mre11 2 $\mu$ TRP1	
pRO1003	Gal4-Esc1 2 $\mu$ TRP1	
pRO1005	Gal4-Sir1 2 $\mu$ TRP1	
pRO1044	Gal4-Esc2 2 $\mu$ TRP1	

TABLE 1: Plasmids used in this study.

(unless otherwise noted) in YMD-U or YMD-T liquid medium. Mating lawn strains were grown overnight in YPD liquid medium at 30°C. They were taken at OD<sub>600</sub>. For spot assays, 1 OD of mating lawn was plated on YMD plates. Tester strains were diluted to 2 OD/ml and further serially diluted 1:10 (2, 0.2, 0.02, and 0.002 OD/ml). Tester strains were spotted onto YMD mating lawns and YMD-T or YMD-U plates for growth and plasmid retention controls.

### Data presentation

Experiments were always performed with a WT strain (TM47) and GBD-only plasmid (negative control) and GBD-Sir1 plasmid (positive control) simultaneously. All experiments were performed a minimum of three independent times by two independent researchers. In some figures the order of strains was changed using image-editing software or representative images are a combination of multiple plates or experiments. In these cases, the figure is given with white line(s) to clearly show this fact.

### ADE2 silencing assay

Strains were built in which the ADE2 gene (under the *a1* promoter) replaces the *a1* coding region. These strains were transformed as described and plated on selection plates containing –uracil or –tryptophan. Colonies were allowed to grow at 30°C for 48 h and then transferred to 4°C for color development.

### Microscopy

Microscopy was performed on live cells for all experiments. Cells were grown exponentially in YMD plus amino acids (AA; Leu, Ura, Trp, Lys, Ade, His) to an OD<sub>600</sub> of  $\leq 0.6$ . Cells were rinsed in YMD + AA before imaging and placed on YMD + AA, 1.5% agarose patches on slides, covered, and imaged. Images were acquired on an Olympus xi70 inverted wide-field microscope with DeltaVision precise stage (Applied Precision) using a CoolSNAP HQ<sup>2</sup> camera (Photometrics). Optical image stacks of 20 images were acquired with a step size of 200 nm for 400–500 ms in the appropriate wavelength channel. A 100 $\times$ /1.4 oil objective was used. Acquisition software softWoRx3.7.1 was used for image acquisition and analysis. All images were taken at 25°C. Cropping of images was performed in Photoshop (Adobe).



Strain	Description	Strain	Description
ROY 5008	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ swr1 $\Delta$ ::KanMx	ROY 5383	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 hta1S129* hta2S129*
ROY 5010	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+rad16 $\Delta$ ::KanMx	ROY 5384	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 hta1S129* hta2S129*
ROY 5012	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rad5 $\Delta$ ::KanMx	ROY 5385	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS ade2-1 his3 trp1 leu2 ura3 sir4 $\Delta$ ::KanMx
ROY 5014	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rad54 $\Delta$ ::KanMx	ROY 5386	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 rad50 $\Delta$ ::KanMx
ROY 5016	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+rsc2 $\Delta$ ::KanMx	ROY 5387	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 ku70 $\Delta$ ::HYG
ROY 5018	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ chd1 $\Delta$ ::KanMx	ROY 5388	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc1::KanMx ku70 $\Delta$ ::HYG
ROY 5022	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rdh54 $\Delta$ ::KanMx	ROY 5389	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 ku70 $\Delta$ ::HYG
ROY 5026	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ snf5 $\Delta$ ::KanMx	ROY 5390	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc1 $\Delta$ ::KanMx
ROY 5028	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ isw1 $\Delta$ ::KanMx	ROY 5391	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc1 $\Delta$ ::KanMx ku70 $\Delta$ ::HYG
ROY 5030	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ ies5 $\Delta$ ::KanMx	ROY 5392	MAT $\alpha$ HMR::5xGEB-a1-B5xG ADE+ LYS+ his3 trp1 leu2 ura3
ROY 5020	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ fun30 $\Delta$ ::KanMx	ROY 5393	MAT $\alpha$ HMR::5xGEB-a1-B5xG ADE+ lys2 his3 trp1 leu2 ura3 sir1 $\Delta$ ::HIS3
ROY 5024	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ isw2 $\Delta$ ::KanMx	ROY 5394	MAT $\alpha$ HMR::5xGEB-a1-B5xG ADE+ LYS+ his3 trp1 leu2 ura3 sir3 $\Delta$ ::LEU2
ROY 5034	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ sir2 $\Delta$ ::KanMx	ROY 5146*	MATa trp1-901 leu2-3112 ura3-52 gal4 $\Delta$ gal80 $\Delta$ GAL2p-ADE2 GAL1p-HIS3 met $\Delta$ ::GAL1p-LacZ
ROY 5036	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ scs2 $\Delta$ ::KanMx	ROY 5324*	MATa trp1-901 leu2-3112 ura3-52 gal4 $\Delta$ gal80 $\Delta$ GAL2p-ADE2 GAL1p-HIS3 met $\Delta$ ::GAL1p-LacZ sir2 $\Delta$ ::KanMx
ROY 5348	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ xrs2 $\Delta$ ::LEU2	ROY 5327*	MATa trp1-901 leu2-3112 ura3-52 gal4 $\Delta$ gal80 $\Delta$ GAL2p-ADE2 GAL1p-HIS3 met $\Delta$ ::GAL1p-LacZ sir3 $\Delta$ ::TRP1
ROY 5349	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ xrs2 $\Delta$ ::LEU2	ROY 5332*	MATa trp1-901 leu2-3112 ura3-52 gal4 $\Delta$ gal80 $\Delta$ GAL2p-ADE2 GAL1p-HIS3 met $\Delta$ ::GAL1p-LacZ sir4 $\Delta$ ::KanMx
ROY 5351*	MAT $\alpha$ HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ mph1 $\Delta$ ::KanMx	ROY 5395	MAT $\alpha$ HMR::5xGEB-a1-B5xG LacO(64x) GIT1::LEU2 Lacl-GFP::ADE2 LYS2 YIP-Lac-HDELdsRED::NatMX
ROY 5378	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc2 $\Delta$ ::KanMx	ROY 5396	MAT $\alpha$ HMR::5xGEB-a1-B5xG Laco(64x) GIT1::LEU2 Lacl-GFP::ADE2 LYS2 YIP-Lac-HDELdsRED::NatMX sir3 $\Delta$ ::HIS3
ROY 5379	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc2 $\Delta$ ::KanMx	ROY 5397	MAT $\alpha$ HMR::5xGEB-ADE2p-ADE2-B5xG ade2-1 LYS2 his3 ura3 trp1 leu2
ROY 5380	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 mre11 $\Delta$ ::KanMx	JRY19a	MATa can1 his4-519 leu2-3112 trp1 ura3-52
ROY 5381	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 mre11 $\Delta$ ::KanMx	JRY19@	MAT $\alpha$ . can1 his4-519 leu2-3112 trp1 ura3-52
ROY 5382	MAT $\alpha$ HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 rad51 $\Delta$ ::KanMx		

\*Not isogenic with W303.

TABLE 2: Strains used in this study.

For zone analysis, 200-nm optical slices were taken on live cells, and only the 10 middle planes of the nucleus were assayed. Images were acquired in the GFP and mCherry channels. The position of the GFP focus in relation to the HDEL-dsRed marked nuclear envelope was determined by first identifying the plane bearing the brightest

GFP-LacI focus and then determining the position of the GFP foci in one of two concentric nuclear zones of equal surface area. Three independent trials were performed for each strain, and strains were scored in a blind manner by measuring the distance between the GFP spot (array) and the nuclear membrane (s2p) and the diameter

of the nucleus (p2p) in nanometers. A ratio (s2p/p2p) × 2 was calculated and used to designate two zones—the peripheral zone (zone 1) or the interior zone (zone 2)—of approximately equal surface area (*B*) or volume (*D*) as previously described. The *p* values were determined by  $\chi^2$  test (Ruben *et al.*, 2011).

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