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© 2015 Kirkland, Peterson, Still, Brueggeman, et al. 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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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.

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...
and telomeric sequences (Hедiger et al., 2002; Тadcde i 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 HM и 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-strand 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 (Kinoshiba 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 (γ-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; Kinoshiba 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; McAnish 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 (Laroché 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 (γ-H2A) in silenced chromatin in S. cerevisiae, Schizosaccharomyces pombe (Szlard 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 a 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 a 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 sir3Δ background, neither Gal4-Sir1 nor Gal4-Mre11 was able to
s 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; Cupero and Shore, 2002; Ohya et al., 2008; Mankoun 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 (P13) kinases Mec1 and Tel1 (Downs et al., 2000). Strains that cannot phosphorylate H2A at Ser-129 show a modest decrease in silencing, indicating that γ-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 P13 kinases. After this, the kinases phosphorylate histone H2A in the vicinity of the break. The P13 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 telomers 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...
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 sir2Δ strain (Figure 4A), suggesting that Gal4-Tel1–mediated silencing used the entire Sir complex.

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 sir2Δ strain (Figure 4A), suggesting that Gal4-Tel1–mediated silencing used the entire Sir complex.
expressed to approximately the same extent as wild-type Gal4-Tel1 (Figure 3). Protein blotting suggests that the truncated protein is HMR, suggesting that this domain played a role in silencing. In the absence of this domain, Gal4-Tel1 was unable to silence dependence on these proteins for Tel1-mediated silencing. However, loss of Esc1, Ku70, and Esc2 led to a significant reduction of Tel1-mediated silencing, suggesting a role in 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 γ-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 γ-H2A was used as a negative control. Analysis of the α1 gene at the modified HMR locus shows clear enrichment of γ-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 γ-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 γ-H2A at the native HMR locus (Kirkland and Kamakaka, 2013).

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.

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
Esc2-mediated silencing. These results 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
that there are likely to be two HR repair protein–mediated silencing pathways—one dependent on Esc1/Ku70 and the other on Esc2. 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.

FIGURE 5: ChIP mapping of γH2A 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. Our 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 Lac–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 sir3A 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-4), 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, 2009). DSBs in yeast cells also result in partial dissociation of Sir proteins from telomeres, which is dependent on Fli3 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 α1 and α2 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;
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
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 7: Silencing of ADE2 at the modified HMR locus by various fusion proteins.](image)

Gal4-DBD  Gal4-Sir1  Gal4-Mre11

Gal4-Tel1  Gal4-Esc2  Gal4-Esc1
ment of Sir2, Sir3, and Sir4 to the silenced HML and HMR domains is through silencer-bound Sir1. Although it is possible that DSB repair proteins bind silencers and help recruit Sir proteins, we

**FIGURE 8:** Two-hybrid analysis of interactions between Sir proteins and repair proteins. (A) A wild-type strain was transformed with two different 2μ plasmids to coexpress Gal4-DBD fusions and Gal4-AD fusions. Growth on YMD plates lacking histidine was used to monitor interactions. (B) Two-hybrid analysis in strains lacking Sir proteins, using Gal4-Tel1 as bait.

binds directly to the silencers or localizes to the silenced domain indirectly via interactions with the Sir proteins is not known. We favor the idea, however, that the primary pathway for the recruit-
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; 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 γ-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 γ-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 γ-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 γ-H2A and affect silencing. It is intriguing, however, that γ-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; Szlard 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 2μ 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 (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 OD600. 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 ADE2 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 OD600 ≤ 0.6. Cells were rinsed in YMD + AA before imaging and placed on YMD + AA, 1.5% agarose plates. Colonies were allowed to grow at 30°C for 48 h and then transferred to 4°C for color development.

**TABLE 1: Plasmids used in this study.**

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

Experimental details and micrographs were performed using an Olympus IX70 inverted wide-field microscope with DeltaVision precise stage (Applied Precision) using a CoolSNAP HQ2 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×/1.4 oil objective was used. Acquisition software softWoRx 3.7.1 was used for image acquisition and analysis. All images were taken at 25°C. Cropping of images was performed in Photoshop (Adobe).
TABLE 2: Strains used in this study.

| Strain         | Description                                                                 |
|----------------|-----------------------------------------------------------------------------|
| ROY 5008       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ swr1α::NatMX          |
| ROY 5010       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rad16α::KanMX        |
| ROY 5012       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rad5α::KanMX         |
| ROY 5014       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rad54α::KanMX        |
| ROY 5016       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ rsc2α::KanMX         |
| ROY 5018       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ chd1α::KanMX         |
| ROY 5022       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ sfr5α::KanMX         |
| ROY 5024       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ isw1α::KanMX         |
| ROY 5030       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ ises5α::KanMX         |
| ROY 5020       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ fun30α::KanMX         |
| ROY 5024       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ isw2α::KanMX         |
| ROY 5034       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ sir2α::KanMX         |
| ROY 5036       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ ssc2α::KanMX         |
| ROY 5038       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ xrs2α::LEU2           |
| ROY 5049       | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ xrs2α::LEU2           |
| ROY 5315*      | MATα HMR::5xGEB-a1-B5xG his3 ura3 leu2 trp1 ADE+ LYS+ mph1α::KanMX         |
| ROY 5378       | MATα HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc2α::KanMX       |
| ROY 5379       | MATα HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 esc2α::KanMX       |
| ROY 5380       | MATα HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 mre11α::KanMX      |
| ROY 5381       | MATα HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 mre11α::KanMX      |
| ROY 5382       | MATα HMR::5xGEB-a1-B5xG LYS2 ade2-1 his3 trp1 leu2 ura3 rad51α::KanMX      |

*Not isogenic with W303.

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 χ² test (Ruben et al., 2011).

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Supplemental Materials
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Supplementary Figure 1

Supplementary figure 1

Protein blot with anti-HA antibodies to analyze expression levels of Gal4-HA-Tel1 and Gal4-HA-Tel1-CΔ.