Rtt107/Esc4 binds silent chromatin and DNA repair proteins using different BRCT motifs
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Abstract

Background: By screening a plasmid library for proteins that could cause silencing when targeted to the HMR locus in Saccharomyces cerevisiae, we previously reported the identification of Rtt107/Esc4 based on its ability to establish silent chromatin. In this study we aimed to determine the mechanism of Rtt107/Esc4 targeted silencing and also learn more about its biological functions.

Results: Targeted silencing by Rtt107/Esc4 was dependent on the SIR genes, which encode obligatory structural and enzymatic components of yeast silent chromatin. Based on its sequence, Rtt107/Esc4 was predicted to contain six BRCT motifs. This motif, originally identified in the human breast tumor suppressor gene BRCA1, is a protein interaction domain. The targeted silencing activity of Rtt107/Esc4 resided within the C-terminal two BRCT motifs, and this region of the protein bound to Sir3 in two-hybrid tests. Deletion of RTT107/ESC4 caused sensitivity to the DNA damaging agent MMS as well as to hydroxyurea. A two-hybrid screen showed that the N-terminal BRCT motifs of Rtt107/Esc4 bound to Slx4, a protein previously shown to be involved in DNA repair and required for viability in a strain lacking the DNA helicase Sgs1. Like SLX genes, RTT107ESC4 interacted genetically with SGS1; esc4Δ sgs1Δ mutants were viable, but exhibited a slow-growth phenotype and also a synergistic DNA repair defect.

Conclusion: Rtt107/Esc4 binds to the silencing protein Sir3 and the DNA repair protein Slx4 via different BRCT motifs, thus providing a bridge linking silent chromatin to DNA repair enzymes.

Background

Transcriptional silencing in the budding yeast Saccharomyces cerevisiae occurs at the silent mating-type loci HMR and HML, telomeres, and at the rDNA locus. At all of these silenced regions, DNA binding proteins recognize specific motifs and recruit a silencing protein complex (reviewed in [1]). HMR and HML are flanked by E and I ‘silencers.’ Each silencer has binding sites for ORC, and Rap1 or Abf1. The potent HMR-E silencer has a binding site for all three proteins. At telomeres, Rap1 also contributes a critical DNA-binding function, binding to the TG1–3 repeats.

At both the silent mating loci and at telomeres, the DNA-binding proteins recruit a Sir protein complex that can spread to silence genes at a distance (reviewed in [2]). At HMR-E, for example, this is achieved by ORC recruitment.
of Sir1 via a Sir1-Orc1 interaction [3], and Rap1 and Abf1 binding to Sir4 and Sir3 [4,5]. Sir4 and Sir3 multimerize, both with themselves and each other [4,6]. Sir4 also binds Sir2, and Sir2 plays a crucial role in the spreading of a Sir2, Sir3, Sir4 complex on chromatin by deacetylating histone H4 lysine 16. The deacetylation produces a novel product, 2′ O-acetyl-ADP-ribose [7-9]. This compound has recently been shown to produce a conformational change in Sir3 that is likely to promote spreading of the Sir complex. Sir2, Sir3 and Sir4 are essential for silencing at the HM loci and at telomeres, while Sir1 plays a prominent role in silencing at the mating type loci but not at telomeres. The Yku70/Yku80 heterodimer that binds to DNA ends plays an important role in silencing at telomeres, while being dispensable for silencing at HM loci [10].

Deletion of the HMR-E silencer leads to loss of silencing of genes at the HMR locus [11,12]. However, if heterologous DNA binding sites (such as Gal4 sites) are integrated in place of HMR-E and the strain is transformed with certain Gal4 DNA binding domain (Cterminal) – silencing protein hybrids, silencing can be restored due to targeting of Sir proteins or proteins that bind to Sir proteins to HMR-E ([13]; see also Figure 1). This so-called “targeted silencing” has been a useful tool for investigating the process of Sir protein recruitment to silenced loci [3,13-17]. We previously described a screen for proteins capable of targeted silencing at HMR [16]. In this screen many known proteins were identified, as well as several proteins not characterized at the time, which we called Esc proteins because they establish silent chromatin. One of these was Esc4 whose characterization we describe in this report. RTT107/ESC4 was also identified in screens for mutants with increased Ty transposition mobility or DNA repair defects [18-20]. For simplicity we will only use the name ESC4 in the remainder of this paper. Esc4 has been shown to be phosphorylated by Mec1 kinase on SQ/TQ motifs in response to DNA damage during S phase [21]. The phosphorylation by Mec1 has quite recently been proposed to be phosphorylated by Mec1 kinase on SQ/TQ motifs in response to DNA damage during S phase [21].

Here we demonstrate that the C-terminal two BRCT motifs of Esc4 bind to Sir3 and are sufficient for SIR-dependent targeted silencing at HMR. Furthermore, the N-terminal four BRCT motifs in Esc4 bind to Sls4, thus linking this DNA repair protein to silent chromatin.

Results

**Esc4 establishes targeted silencing when targeted to HMR**

In a screen described previously, we identified Esc4 as a protein that could restore silencing when targeted to an HMR locus harboring a deletion of the HMR-E silencer [16]. Targeting of proteins to HMR was mediated by the binding of a Gal4 DNA binding domain (Cterminal)-hybrid protein to a Gal4 DNA binding site (G) that replaced the HMR-E silencer (Figure 1). Silencing was assessed using a URA3 reporter gene integrated at the HMR locus.
**Targeted silencing by Esc4 is SIR-dependent**

In order to understand how Esc4 promoted silencing when targeted to the HMR locus, we tested silencing by G_{BD}-Esc4 in various strains. First, targeted silencing by G_{BD}-Esc4 was compared to G_{BD} alone or to the potent targeted silencing factor G_{BD}-Esc1 in the strain in which the screen was performed, which has the entire HMR-E silencer deleted and replaced with a Gal4 DNA binding site (HMR-E aeb::G). The fraction of cells silenced by G_{BD}-Esc4 was not as great as by G_{BD}-Esc1 but, nevertheless, significant targeted silencing at HMR was observed despite the complete absence of the HMR-E silencer (Figure 2, HMR-E aeb::G). When targeted silencing by G_{BD}-Esc4 was assessed in strains harboring deletions of just the E and B sites (HMR-E aeb::G) or the A and E sites (HMR-E aeb::G) of the HMR-E silencer, silencing was increased roughly 50 to 100-fold, as expected for strains with at least one element of the natural silencer (Figure 2).

To test whether targeted silencing by G_{BD}-Esc4 was SIR-dependent, it was tested in strains deleted for the SIR2, SIR3 or SIR4 genes. As seen in Figure 2, silencing by Esc4 required each of these SIR genes. This was expected since targeted silencing by other proteins has been shown to be SIR-dependent in every case examined previously [14,16]. It thus seemed likely that Esc4 caused silencing by recruiting the Sir protein complex.

**Saccharomyces Esc4 proteins contain six BRCT motifs and are homologous to S. pombe Brc1**

Esc4 contains several copies of the BRCT motif, originally identified in the human breast cancer susceptibility gene,
The presence of these motifs in Esc4 encoded by yeast ORF YHR154w has been observed previously by database searches [48,49], although there has been disagreement as to how many BRCTs exist in this protein, with some reports suggesting six BRCTs [49,50] and others only four [21,22,48]. Our own analyses including use of Pfam [51,52] confirms the first five putative BRCTs in Esc4, and it seems likely that residues 935–1049, which are BRCT-like with a conserved W or Y residue near the C-terminus, also form a domain that folds into a BRCT type structure as previously reported [49] (Figure 3). Furthermore, analysis of a homolog of Esc4 from S. pombe, Brc1, using Pfam shows six BRCTs in this homologous protein, with the last two again being the most alternative (E-values of $4.1 \times 10^{-5}$ and $5.8 \times 10^{-2}$ for the fifth and sixth, respectively, compared to values ranging from $2.8 \times 10^{-6}$ to $1.7 \times 10^{-14}$ for the first four BRCTs), further supporting a conserved total of six motifs in these homologs (Figure 3).

The six BRCT motifs of Esc4 protein exist as a set of four tandem motifs at the N-terminus of the protein and two more at the C-terminus (Figure 3, yellow lines). Although there are proteins from various budding yeasts with compelling sequence similarity to S. cerevisiae Esc4 (e.g. Ashbya gossipii, Kluyveromyces lactis, and Candida glabrata, as shown in Figure 3), currently the only obvious non-budding yeast homolog candidate is Brc1 from the evolutionarily distant fission yeast Schizosaccha-
**Saccharomyces cerevisiae** Esc4 has six BRCT motifs that are conserved in both sequence and domain architecture among yeast homologs. The six BRCT motifs in Esc4 are indicated with brackets and yellow color and were described previously [49]. The motifs in Esc4 are shown as they align with a subset of Esc4 homologs: three from other budding yeasts *Ashbya gossypii*, *Kluyveromyces lactis* and *Candida glabrata* and one from the evolutionarily distant fission yeast *Schizosaccharomyces pombe* Brc1. The highly conserved aromatic residue, a hallmark of BRCT motifs, is marked above the alignment with a red asterisk. Blue color indicates similarity with the predominant residue at the position in the alignment, with darkness correlated with greater similarity as calculated by ClustalW software and displayed with Jalview alignment editor’s BLOSUM62 score viewing option [67, 68].

### Figure 3
**Saccharomyces cerevisiae** Esc4 has six BRCT motifs that are conserved in both sequence and domain architecture among yeast homologs. The six BRCT motifs in Esc4 are indicated with brackets and yellow color and were described previously [49]. The motifs in Esc4 are shown as they align with a subset of Esc4 homologs: three from other budding yeasts *Ashbya gossypii*, *Kluyveromyces lactis* and *Candida glabrata* and one from the evolutionarily distant fission yeast *Schizosaccharomyces pombe* Brc1. The highly conserved aromatic residue, a hallmark of BRCT motifs, is marked above the alignment with a red asterisk. Blue color indicates similarity with the predominant residue at the position in the alignment, with darkness correlated with greater similarity as calculated by ClustalW software and displayed with Jalview alignment editor’s BLOSUM62 score viewing option [67, 68].

The C-terminal two BRCT motifs of Esc4 are sufficient for targeted silencing

The Esc4 hybrid protein isolated in our targeted silencing screen was full-length [16] and therefore contained all six predicted BRCT motifs. To determine which part of Esc4 and which BRCT motifs were responsible for the SIR-dependent targeted silencing, we constructed three GBD hybrids: one to the N-terminal four BRCT motifs (GBD-Esc4N, aa 1–480), one to the linker between the N- and C-terminal sets of motifs (GBD-Esc4L, aa 480–836), and one to the C-terminal two BRCT motifs (GBD-Esc4C, aa 818–878).
These constructs were tested for targeted silencing in a strain harboring deletions of the E and B sites (HMR-E Aeb::C) and in a sir2A derivative of that strain (Figure 4; see Table 1 for strain information). Significant targeted silencing was observed by GAD-Esc4C, although it was not as much as with full-length Esc4. The observed silencing by GAD-Esc4C was Sir1-dependent, as observed for the full-length protein (Figure 2). No significant silencing was seen with Esc4N or with the linker region, Esc4L (Figure 4).

The C-terminal BRCT motifs of Esc4 interact with Sir3
Because the C-terminal two BRCT motifs of Esc4 gave targeted silencing, we suspected that this region of the protein was binding to a silencing protein to recruit the Sir complex. Using a LexA-Esc4C hybrid and the two-hybrid reporter strain L40 [54], we tested for two-hybrid interactions with several Gal4 activation domain (GAD)-silencing protein constructs, including Sir1, Sir2, Sir3, Sir4 and Rap1 [3,17,55]. A strong interaction (i.e., the lacZ reporter gene generated blue color from X-gal visible in 15 minutes) with GAD-Sir3 (aa 252–978) was identified, as well as a weaker interaction with GAD-Sir4 (aa 839–1358) (Table 2). None was detected with Sir1, Sir2 or Rap1. Because the GAD-Sir4 (aa 839–1358) hybrid contained the region known to bind to Sir3 [7], we hypothesized that LexA-Esc4 was binding to GAD-Sir4 via a bridge of endogenous Sir3. To test this, we used a derivative of strain L40 harboring a sir3A mutation and examined the LexA-Esc4 interaction with GAD-Sir4. In this case, the interaction with GAD-Sir4 was no longer observed, whereas the interaction with GAD-Sir3 and an unrelated two-hybrid control interaction were unaffected (Table 2). When a sir4A derivative of L40 was used, no change in the LexA-Esc4 interactions with GAD-Sir3 or GAD-Sir4 was observed, further supporting the idea that Esc4 requires Sir3 to interact with Sir4, and not vice versa. Taken together, the targeted silencing data strongly suggest an interaction (probably a direct one) between the C-terminal BRCT motifs and the silencing protein Sir3.

The N-terminal four BRCT motifs of Esc4 bind to Slx4
The above results indicated that Esc4 caused SIR-dependent targeted silencing primarily by binding to Sir3 through its C-terminal two BRCT motifs. In addition, Esc4 also contains four BRCT motifs at its N-terminus and these BRCT motifs are more similar to those found in various proteins from diverse eukaryotes (Figure 3, and ref. [49]). In order to identify proteins that bind to these N-terminal BRCT motifs, a two-hybrid screen was performed using a LexA-Esc4N (aa 1–480) fusion protein. Strikingly, from screening ~2 × 10^7 library plasmids expressing GAD protein hybrids, thirteen clones containing in-frame fusions of GAD to Slx4 were identified (Table 3). In-frame GAD fusions to the six different positions in Slx4 were isolated (see Table 3) and all contained at least the C-terminal half of the protein (residues 383–748). The Esc4N-Slx4C two-hybrid interaction was a very strong one and was specific. Also, Slx4 did not show an interaction with Esc4C. Thus, the N-terminal BRCT motifs of Esc4 are not only necessary for binding Slx4, as reported while this manuscript was in preparation [22], but are indeed sufficient for this interaction in vivo. Furthermore, our two-hybrid data show that the region of Slx4 sufficient for Esc4 binding resides in its C-terminus.

Genetic and phenotypic analysis of ESC4
A heterozygous diploid strain with a complete deletion of ESC4 was constructed and dissected to generate a null mutant haploid. This esc4Δ mutant grew normally and also mated with normal efficiency (suggesting no gross defect in HM silencing). Furthermore, when an esc4Δ mutation was introduced into a strain with a telomere reporter gene, no telomeric silencing defect was seen (data not shown). Thus, although Esc4 binds to Sir3, Esc4 does not appear to be a protein required for Sir protein-mediated silencing.

The BRCT motif was originally identified in the human BRCA1 tumor suppressor protein [23]. BRCA1 functions in DNA repair and DNA damage-sensing in cell cycle checkpoints (reviewed in [56]). As shown in Figure 5, strains deleted for ESC4 grew significantly less well than wild type on medium containing either MMS or HU. This result confirms reports that have since been published [18,21,22,46], as does our observation that esc4Δ mutants are not sensitive to ultraviolet radiation [18,21] (data not shown).

As shown in Figure 5, like esc4Δ mutants, slx4Δ mutants were sensitive to 0.032% MMS. Furthermore, the esc4Δ slx4Δ double mutant did not exhibit a greater MMS sensitivity than either single mutant (Figure 5A and data not shown), suggesting that they cooperate in providing resistance to MMS. Another group reported that an esc4Δ slx4Δ double mutant was more sensitive than either single mutant but the difference was very slight [22]. In contrast, an esc4Δ, but not a slx4Δ mutant, was significantly HU-sensitive, and the double mutant was no more sensitive than the esc4Δ strain. Thus, Esc4 appears to act independently of Slx4 in providing resistance to HU.

Because Esc4 bound to Slx4 and because the mutant was sensitive to MMS, this suggested that Esc4 might function in the same pathway as Slx4. SLX4 was first identified in a screen for genes required for viability of yeast cells deleted for SGS1 [39]. Therefore, we tested if esc4Δ was also synthetically lethal with sgs1. To do this, an esc4Δ mutant was crossed with an sgs1Δ mutant, the diploid was sporulated and dissected and meiotic progeny were analyzed. Hap-
loid esc\(4\Delta\) sgs1\(\Delta\) cells were viable, but were noticeably slower-growing that either single mutant (e.g., see Figure 5, YPD plate). While this work was in progress, this genetic interaction was also observed in genome-wide studies [41,46].

An sgs1\(\Delta\) mutant showed sensitivity to both MMS and HU (Figure 5), as expected based on previously published results. An asf1\(\Delta\) mutant was used as a control and displayed sensitivity to both DNA damaging chemicals, as expected [57,58]. Interestingly, an esc\(4\Delta\) sgs1\(\Delta\) mutant displayed MMS and HU sensitivity that was much more pronounced than that of either single mutant (Figure 5A and 5B). The enhanced sensitivity of this double mutant (10,000-fold on 0.014% MMS plates) seemed to be due to a synergistic repair defect and not entirely due to the growth defect (10-fold difference in colony number on YPD control plates) that was also observed in the esc\(4\Delta\) sgs1\(\Delta\) strain.

**Discussion**

By screening a library of factors that could function in place of the HMR-E silencer when targeted to DNA, we identified Esc4 for its ability to establish silent chromatin [16]. Protein sequence analysis showed that Esc4 protein contains six BRCT motifs; four are found in tandem at the amino-terminus and two more are at the carboxy-terminus. The entire Esc4 protein was present in the hybrid identified in the targeted silencing screen. Since targeted silencing by Esc4 at HMR was found to be SIR-dependent, it seemed likely that some region within Esc4 was attracting a silencing protein complex to DNA. We tested subsets of the BRCT motifs, as well as the linker between them, for targeted silencing at HMR. These experiments demonstrated that the C-terminal two BRCTs caused targeted silencing that was nearly as strong as with full-length Esc4. Because silencing by this pair of BRCTs of Esc4 was also SIR-dependent, it seemed very likely that this region was recruiting a Sir protein when tethered to DNA. Therefore, we tested the C-terminal BRCT motifs for interactions with known silencing proteins by two-hybrid analysis. We identified a specific interaction with Sir3 (aa 252–978). We conclude that binding of Sir3 by Esc4 is likely to be responsible for the SIR-dependent targeted silencing activity.

In some cases BRCT motifs have been shown to bind to phosphorylated serine residues. Specifically, they have been shown to bind to phosphopeptides with the following consensus: pSxxF [32]. Interestingly, Sir3 has an SxxF

![Figure 4](http://www.biomedcentral.com/1471-2199/7/40)
sequence (aa 583–586) within the Esc4-interacting region that we describe here (aa 252–978) and, furthermore, Sir3 protein has been shown to be phosphorylated [59,60], suggesting that an Esc4 BRCT motif or perhaps the combination of the two in the C-terminus may bind to phospho-Sir3. However, not all proteins bound by BRCT motifs have the SxxF motif [61], so the precise BRCT-interacting region of Sir3 could be elsewhere.

In addition to binding Sir3 via C-terminal tandem BRCT motifs, Esc4 also binds to Sxl4 via four tandem N-terminal BRCTs, as we have shown here by two-hybrid screening. This two-hybrid result demonstrates that these four BRCTs are sufficient for binding Sxl4 and agrees with a recent report showing that the N-terminal BRCT motifs are required for this interaction [22]. It seems quite possible that Esc4 could bind Sir3 and Sxl4 concurrently, given that these nuclear proteins' binding sites within Esc4 map to BRCT clusters separated by a long linker. Sxl4 has been shown to heterodimerize with the endonuclease Sxl1 to cleave DNA containing 5'-flap structures, such as in stalled replication forks, to facilitate their repair [62]. Thus, Esc4 binds the silencing protein Sir3 and also to Sxl4, an important DNA repair complex component. Esc4 may play a role in facilitating repair of aberrant DNA structures, perhaps specifically within silent chromatin.

Table 2: Yeast strains used in this study

| Strain | Genotype | Reference or Source |
|--------|----------|---------------------|
| W303-1a | MATa leu2–3,112 ura3-1 his3–11,15 trp1–1 ade2–1 can1–100 | R. Rothstein |
| W303-1b | MATc version of W303-1a | R. Rothstein |
| W303 | Diploid from W303-1a × W303-1b | This study |
| YDZ5 | W303 MATa/MATc esc4Δ:his5+/ESC4 | This study |
| YDZ174 | W303 MATa/MATc esc4Δ:his5*/esc4Δ:his5* | This study |
| YDZ3 | W303-1a esc4Δ:his5+ | This study |
| YDZ4 | W303-1b esc4Δ:his5+ | This study |
| YEA78 | W303-1b hmr::URA3 HMR-E (aeb::G) gal4::LEU2 | This study |
| YEA76 | YEA78 but HMR-E (aeb::G) | This study |
| YEA77 | YEA78 but HMR-E (aeb::G) | This study |
| YAM7 | YEA76 sir2Δ:kanMX6 | This study |
| YEA118 | YEA76 but GAL4 sir3::LEU2 | This study |
| YKAB17 | YEA76 sir4Δ:kanMX6 | This laboratory |
| YDS631 | Y303-1b adh4::URA3-(C1–3A)Δ | [13] |
| YDZ22 | YDS631 esc4Δ:his5+ | This study |
| YAM1 | W303-1a sxf4Δ:his5+ | This study |
| YAM2 | W303-1b sxf4Δ:his5+ | This study |
| YAM3 | W303-1a sxf4Δ:kanMX6 | This study |
| YAM4 | W303-1b sxf4Δ:kanMX6 | This study |
| YAM13 | W303-1b sgs1Δ:kanMX6 | This study |
| YDZ243 | W303-1a sgs1Δ:kanMX6 esc4Δ:his5+ | This study |
| YAM6 | W303-1a sgs1Δ:kanMX6 esc4Δ:his5+ | This study |
| YASS01 | W303-1a sgs1Δ:his5+ | This laboratory |
| L40 | MATa his3::200 trp1–901 leu2–3,112 ade2-1 URA3::(lexAop)8-lacZ LYS2::(lexAop)4-HIS3 | [54] |
| YR3 | L40 sir3Δ:kanMX6 | This study |
| YJL103 | L40 sir4Δ:kanMX6 | This study |

Table 2: Esc4 binds to Sir3 in the two-hybrid system

| LexA hybrid | GAD hybrid | L40 | L40 Δ sir3 | L40 Δ sir4 |
|-------------|------------|-----|------------|------------|
| LexA-Esc4 (818–1070) | GAD-Sir3 (252–978) | ++ | ++ | ++ |
| LexA-Esc4 (818–1070) | GAD-Sir4 (839–1358) | + | - | + |
| LexA-Esc4 (818–1070) | GAD vector | - | - | - |
| LexA-Esc4 (1–480) | GAD-Slx4 (383–748) | +++ | +++ | +++ |

* Three +++ signs means an easily visible blue color from the lacZ reporter gene was visible within 15 minutes, whereas two ++ signs means it was visible within 30 minutes and one + sign means it was visible between 40 minutes and two hours.
Esc4 is a Mec1 kinase target [21] and this phosphorylation is required for its repair function. It is possible that phosphorylation of Esc4 by Mec1, which occurs just N-terminal to the Sir3-binding BRCTs, regulates association with Sir3 or other factors required for its ability to repair particular chromosomal loci in S phase.

We analyzed Esc4 protein alignments (such as that shown in Figure 3) for evidence of conserved regions in the protein other than BRCT motifs. One region of interest was the SQ/TQ motifs between amino acids 743 and 807, which were shown to be important for function in DNA repair [21]. We did not find that these motifs were well-

| LexA hybrid         | GAD hybrid         | Number of isolates |
|---------------------|--------------------|--------------------|
| LexA-Esc4 (1–480)   | GAD-Slx4 (166–748) | 1                  |
| LexA-Esc4 (1–480)   | GAD-Slx4 (194–748) | 2                  |
| LexA-Esc4 (1–480)   | GAD-Slx4 (260–748) | 1                  |
| LexA-Esc4 (1–480)   | GAD-Slx4 (280–748) | 4                  |
| LexA-Esc4 (1–480)   | GAD-Slx4 (368–748) | 3                  |
| LexA-Esc4 (1–480)   | GAD-Slx4 (383–748) | 2                  |

Table 3: The amino-terminal BRCT motifs of Esc4 bind to Slx4

Figure 5
Genetic evidence that ESC4 and SLX4 function together in a parallel pathway to SGS1 for repair of damaged DNA. Strains were grown in YPD medium to saturation and then ten-fold serial dilutions were spotted onto YPD, YPD + HU (100 mM), or YPD + MMS (0.032% in part A and 0.014% in part B) media. Cells were incubated at 30°C for 2 days (SC and SC + MMS) or 3 days (SC + HU) before being photographed. WT, wild type. An asf1Δ mutant was used as a positive control for MMS and HU sensitivity.
conserved, suggesting that the specific site of phosphorylation is not particularly critical in proteins with otherwise similar overall BRCT domain architecture (i.e., [BRCT]4-linker-[BRCT]2 arrangement). This may be because of some differences in Esc4 functions in diverse yeasts or may suggest that flexibility is tolerated in positioning of the phosphorylation sites, and therefore the exact relative location of kinase target sites has not been constrained during evolution.

Future structural and genome sequencing studies are likely to unveil similarities and differences between multi-BRCT domain containing proteins. Whether these proteins play largely protein-scaffolding roles or also contain intrinsic enzymatic properties will be interesting to discover.

Conclusion
We have shown that Esc4 caused targeted silencing when tethered at a weakened HMR locus. The targeted silencing activity was primarily due to the C-terminal two tandem BRCT motifs in Esc4, which bound to Sir3, probably through a direct interaction. This interaction led to the recruitment of the Sir complex and hence caused targeted silencing. The N-terminal BRCT domains were sufficient for binding to Slx4, which functions with Esc4 in DNA repair. Thus, the nuclear Esc4 protein uses its six BRCT motifs to connect diverse proteins involved in DNA repair and silent chromatin.

Methods
Targeted silencing
Esc4 was identified in a targeted silencing screen that has been described previously [16]. Briefly, a Gal4 DNA binding domain (G_brd) library was screened for hybrid proteins capable of establishing silencing of a URA3 reporter gene integrated in place of mating-type genes at an HMR locus that had the HMR-E silencer replaced by a Gal4 DNA binding site (G) (see Figure 1A). A full length C_brd-Esc4 (1–1070) clone, aeb15, was identified as being capable of establishing targeted silencing of hmr::URA3, causing resistance to 5-fluororotic acid (5-FOA). This C_brd-Esc4 clone was subsequently transformed into strain YEA76 (HMR-E Aeb::G hmr::URA3 gal4::LEU2) and YEA77 (HMR-E aeb::G hmr::URA3 gal4::LEU2) (see Table 1 and Yeast Strain section for details) and tested for targeted silencing. To test SIR-dependence of targeted silencing by Esc4 at HMR, shown in Figure 4, targeted silencing in strain YEA76 (SIR+) was compared with that in sir mutant derivatives YAM7 (sir2Δ), YEA 118 (sir3Δ), and YKAB17 (sir4Δ).

For the targeted silencing experiments shown in Figures 2 and 4, assays were carried out as follows: strains were transformed with plasmids expressing the appropriate G_brd hybrid protein, grown at 30°C for two days in SC-Trp medium (to select for the G_brd plasmid), serially diluted ten-fold five times and spotted on SC-Trp + 5-FOA plates (to assay for silencing at HMR) or on SC-Trp control plates.

Plasmids
Plasmid aeb15, expressing C_brd-Esc4(1–1070) was isolated in the targeted silencing screen. This plasmid was recovered from a library based on pGBT9.C (ADH1_UAS-G_brd TRP1, CEN4/ARS1; see Acknowledgements). To generate plasmids for use in the two-hybrid system, ESC4 sequences were amplified from genomic DNA and subcloned into plasmid pSST91, a derivative of pBlM116 that contains the ADE2 gene ([63]; see also [64]). Plasmids pAM2 (LexA-Esc4N (a.a. 1–480) and pAM7 (LexA-Esc4C (a.a. 818–1070) were used for two-hybrid experiments. To test for LexA-Esc4C binding to GAD-Sir3 and GAD-Sir4, plasmid pEDA195, GAD-Sir3(252–978), and pCTC36, GAD-Sir4(839–1358), were used. pCTC36 expresses the same region of SIR4 as plasmid pCTC18 [55] except that the Sir4 hybrid is expressed from pGAD-R. pEDA195 was constructed by cloning a PstI fragment of the SIR3 gene into vector pGAD424. Plasmid pAM2, expressing LexA-Esc4(1–480), and a clone expressing GAD-Slx4(383–748) isolated in the two-hybrid screen were used as a positive control in two-hybrid experiments summarized in Table 3.

Yeast strain construction
All strains are listed in Table 1. To make slx4Δ, slx4Δ, sir2Δ, sir4Δ, and sgs1Δ mutants, PCR primers with 5’ homology to sequences flanking these ORFs and 3’ homology to sequences in a plasmids harboring selectable marker genes were used for PCR, generating targeting cassettes that were transformed into yeast as has been previously described [65]. esc4Δ::his5+ mutants were generated by the same method, but using a different plasmid as a template for PCR (gift of N. Dean, Stony Brook University). Strain YEA76 and its derivatives are derived from strain YSB35 [13].

Two-hybrid screening and direct tests
Screening was performed essentially as described [54,63]. Plasmid pAM2, which expresses LexA-Esc4(1–480), (ADE2, TRP1, 2μ) was co-transformed with approximately 1 μg of GAD library (LEU2, 2μ [66]) into strain L40 [54], which contains LexA binding sites upstream of both the HIS3 gene and the LacZ gene. The following specificity tests were performed: (1) His+ candidates were assayed for lacZ expression both after curing candidates of either the LexA or the GAD plasmid, (2) GAD hybrids were tested for interactions with nonspecific LexA hybrids (e.g. LexA-lamin) by mating candidates cured of the bait plasmid to LexA-containing AMR70 [54], and (3) the
GAD hybrid plasmids which passed the aforementioned tests were subsequently retransformed into L40 along the original bait and tested for LacZ expression.

Two-hybrid tests done using the C-terminal BRCT motifs in Esc4 were performed by co-transforming L40 with plasmid pAM7, expressing LexA-Esc4(818–1070), and various GAD hybrids to silencing proteins, such as Sir1, Sir2, Sir3, Sir4, and Rap1. To test whether Sir3 bridged binding of LexA-Esc4(818–1070) to Sir4, a sir3Δ::kanMX6 derivative of L40 was generated (see yeast strains section for details), strain YRJ3, and the interaction was then retested in this strain. The control sir4Δ-derivative of L40 used was strain YJL03.

**DNA damage sensitivity tests**

Cultures were grown in YPD medium ~18 h at 30°C and then serially diluted ten-fold, five times before being spotted onto plates containing YPD medium with MMS, HU, or no chemical. Cells were then incubated at 30°C and, in the case of MMS media, the plates were wrapped in aluminum foil.

**Authors’ contributions**

RS and DCZ initiated and oversaw the course of the study, designed the experiments and wrote the paper. DCZ did the targeted silencing tests, protein sequence alignments and made the figures. ASRM and DCZ performed the two-hybrid screening and direct two-hybrid tests. JJC and RAI did some of the genetic analyses. All authors have read and approved this manuscript.

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