In the yeast *Saccharomyces cerevisiae*, the two silent mating-type loci and subtelomeric regions are subjected to a well-characterized form of gene silencing. Establishment of silencing involves the formation of a distinct chromatin state that is refractory to transcription. This structure is established by the action of silent information regulator proteins (Sir2, Sir3, and Sir4) that bind to nucleosomes and initiate the deacetylation of multiple lysine residues in histones H3 and H4. Sir2 protein is a conserved histone deacetylase that is critical for mating-type and telomeric silencing, as well as a Sir3/4-independent form of silencing observed within the ribosomal DNA (rDNA) repeat locus. We report here that sumoylation plays an important role in regulating gene silencing. We show that increased dosage of SIZ2, a SUMO (small ubiquitin-related modifier) ligase, is antagonistic to gene silencing and that this effect is enhanced by mutation of ESC1, whose product is involved in tethering telomeres to the nuclear periphery. We present evidence indicating that an elevated SIZ2 dosage causes reduced binding of Sir2 protein to telomeres. These data support the idea that sumoylation of specific substrates at the nuclear periphery regulates the availability of Sir2 protein at telomeres.

Eukaryotic chromosomes have distinct euchromatin and heterochromatin regions. Whereas euchromatin contains the bulk of the expressed regions of the genome, heterochromatin consists of transcriptionally silenced regions. The special chromatin modifications and the molecular processes that contribute to and maintain these states have been studied extensively using several model systems, including budding yeast, fission yeast, and *Drosophila* (22, 43, 54). In the budding yeast *Saccharomyces cerevisiae*, transcriptionally silenced chromatin is found at HML and HMR (HM loci), the two silent mating-type loci, at the telomeres, and at the ribosomal DNA (rDNA). Transcriptional repression at HM loci is initiated by silencer elements E and I, which flank the two loci. The HMR-E silencer, which is most well studied, consists of about 150 bp of DNA that contains binding sites for three different sequence-specific DNA-binding proteins: origin recognition complex (ORC), Rap1, and Abf1. These silencer-bound proteins recognize and recruit four silent information regulator (SIR1 to -4) proteins that bind to chromatin. Our current understanding is that multiple interactions between silencer-binding proteins, SIR proteins, and nucleosomes establish and maintain silenced chromatin (8, 18).

Sir1 is specifically required for the establishment of silent chromatin at HM loci through direct interactions with Orc1 and Sir4 (4, 53). Sir4 and Sir3 proteins are recruited through interactions with Rap1 and are stabilized by interactions among them (36). Sir4 also recruits Sir2 protein, an evolutionarily conserved NAD-dependent histone deacetylase that removes acetyl groups from histone tails in adjacent nucleosomes. Because Sir3 protein binds with higher affinity to a histone H4 N-terminal tail in which K16 is deacetylated (24), one current model proposes that SIR complexes spread to adjacent deacetylated nucleosomes, recruiting more Sir2 protein and thus initiating a cycle of deacetylation and nucleosome binding by the SIR complex (reviewed in reference 43). Silencing at telomeres appears to follow a similar mechanism, apart from some differences in the recruitment of SIR proteins. The truncated telomeres used in most studies of telomere position effect (TPE) do not engage Sir1 in the Sir2/3/4 recruitment process and also display unstable silencing. Instead, telomeres use the Yku70/80 heterodimer to recruit Sir proteins (specifically interacting with Sir4), together with Rap1, which binds with high affinity to the telomere repeat sequences at all chromosome ends (29, 35). Interestingly, silencing can be improved by the artificial tethering of Sir1 (11) at truncated telomeres, and Sir1 is required for silencing at native telomeres (40). Silencing at both telomeres and HM loci is not promoter specific but does appear to be competed by strong activators (3, 59). Furthermore, boundary elements counteract the spreading of silent chromatin into euchromatin sites (16).

A different form of gene silencing has been described that affects RNA polymerase II (RNAPII) promoters found naturally (or more often placed artificially) within the rDNA locus. This form of transcriptional silencing requires the Sir2 deacetylase but is independent of Sir1, Sir3, and Sir4, suggesting a mechanism different from the one that operates at HM loci and telomeres (6, 45, 46).

SUMO (small ubiquitin-related modifier, encoded by SMT3 in yeast) is an ~100-amino-acid protein, structurally similar to ubiquitin, that is added posttranslationally to proteins (34, 38, 55, 62, 64). Sumoylation is a dynamic and reversible process. SUMO modification of proteins alters their protein-protein interactions, protein-DNA interactions, subcellular localization, or susceptibility to ubiquitination. Sumoylation regulates a variety of cellular...
processes, including DNA replication and repair, transcription, chromosome segregation, intracellular transport, and apoptotic signaling. Several targets of SUMO have been identified through genome-wide screens, but the functional significance of most targets awaits elucidation (14, 23, 39, 56, 66). However, a critical role of sumoylation has been demonstrated for several proteins, including PCNA, Rad18, Rad52, and p53 (5, 26, 44, 57).

The conjugation of SUMO to target proteins, similarly to ubiquitin, is achieved by the action, in series, of three enzymes involved in activation (E1), conjugation (E2), and ligation (E3) reactions. SUMO is synthesized as a precursor and is processed at the C terminus by an isopeptidase (Ulp1) to reveal a di-glycine motif that is used for conjugation. The mature SUMO is activated in an ATP-dependent manner by the E1 enzyme (a heterodimer of Aos1 and Uba2 in yeast) followed by conjugation to the E2 enzyme (Ubc9) and finally ligation to the epsilon amino group of a lysine residue in the target protein by E3 ligases. The S. cerevisiae genome encodes a single SUMO (Smt3), E1 (Aos1/Uba2), and E2 (Ubc9) but has at least four known E3 ligase genes, Sir2, Siz2, Mms21, and Zip3. Mammals have several SUMO variants and E3 ligases (reviewed in reference 34). There are also two known isopeptidase genes in yeast, Ulp1 and Ulp2, whose products are required for both SUMO processing and cleaving SUMO from target proteins (desumoylation). In yeast, only Ulp1 is required for processing precursor SUMO to its mature form, although both Ulp1 and Ulp2 are involved in desumoylating target proteins. Sumoylation is therefore rapid and transient and is controlled by regulated activity of sumoylating and desumoylating enzymes.

In the context of transcriptional regulation, sumoylation has been shown to promote both repression and activation through diverse mechanisms (19, 28, 31, 37). In this work, we report a connection between sumoylation and heterochromatin establishment in yeast. We show that an elevated dosage of a gene coding for a specific SUMO ligase, Siz2, disrupts silencing in yeast. This effect is exacerbated by a mutation in Siz2 gene along with its promoter, and mating assays. Gal4 binding domain (Gbd) fusion proteins with Rap1 and Sir4 were described previously (7, 33). Gbd-Yif1 is a gift from R. Wotton and Shore, aEB (12a) Cuperus et al., 2000 (12a) Wotton and Shore, 1997 (56a) Chien et al., 1993 (11) Ferreira et al., 2011 (17)

### MATERIALS AND METHODS

#### Strains and plasmids

All strains used in this study are isogenic with W303. The genotypes of the strains used are described in Table 1. Manipulations in the yeast strains, such as gene knockouts or protein tagging, were carried out by PCR-based homologous recombination (32) and were confirmed by Southern blotting. All mutations described here are full-length gene (open reading frame [ORF]) replacements. The epitope-tagged strains were confirmed to be functional by telomere length and mating assays. Gal4 binding domain (Gbd) fusion proteins with Rap1 and Sir4 were described previously (7, 33). Gbd-Yif1 is a gift from R. Sternaglmanz (1). The multicopy Siz2 plasmid was constructed by digesting D4 genomic library plasmid with Xhol. The full-length Siz2 gene along with its promoter region was thus isolated and ligated into Sall-cut Yeplac181 (Ckm6), a 2µm vector that contains the LEU2 selectable marker. The full-length Mms21 gene along with its promoter was amplified by PCR and cloned into BamHI- and Sall-digested yEplac181 vector. Full-length Siz2 along with its promoter was subcloned from pT-23 (51) into yEplac181 by digestion with PstI and Sall. Quantification of silencing levels was carried out by plating out cells from at least three independent transformants on control plates (Sc-Leu) and test plates (Sc-Leu Trp or Sc-Leu with 5-fluoroorotic acid [5-FOA] added) and computing the average of fraction repressed or derepressed. Site-directed mutagenesis was performed using Stratagene QuikChange kit and confirmed by DNA sequencing.

#### mRNA analyses

Total RNA was prepared from logarithmically growing cells (optical density at 600 nm [OD600] of 1) using a RiboPure-Yeast kit (Ambion) and treated with RNase free DNase. Two micrograms of RNA was taken, and cDNAs were prepared using a high-capacity reverse kit (Ambion) and treated with RNase free DNase. Two micrograms of normalized cDNA were subjected to reverse transcription reaction (Applied Biosystems) and used as the template for the real-time PCR using Sybr green PCR master mix (Applied Biosystems). The primers were designed and PCR was performed as described before (13, 61). Quantification was performed using the comparative threshold cycle (Ct) method. Graphs were plotted from the average of three experiments, and error bars show standard deviations.

#### Analysis of sumoylation

Enrichment of sumoylated proteins was done essentially as described in reference 9. Briefly, myc-tagged strains containing 8×His-tagged SUMO were grown to an OD600 of 1, pelleted, and frozen in liquid nitrogen. Equal amount of cells was thawed in 1 ml of 8 M guanidinium hydrochloride, and an equal amount of protein from this sample was incubated with nickel beads. Beads were washed thoroughly and boiled in SDS buffer and then separated by SDS-PAGE. Western blots were probed with anti-myc antibody. The myc-tagged strains without 8×His-tagged SUMO were used as controls to ensure specificity of the

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### Table 1: List of the yeast strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| KRY2   | W303-1A (leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535) MATa | R. Rothstein (52a) |
| KRY3   | W303-1B (leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535) MATa | R. Rothstein (52a) |
| KRY12  | adh4::URA3-TelVIII MATa | Gotschling et al. (21) |
| KRY33  | SIR4-13×mcy::KanMX MATa | This study |
| KRY109 | SIR4-13×mcy::KanMx (diploid) | This study |
| KRY211 | ESC1-13×mcy::KanMx MATa | This study |
| KRY213 | YSB35, except esc1::KanMx | This study |
| KRY218 | YSB35, except yku80::KanMx | This study |
| KRY223 | YSB35, except yku70::KanMx | This study |
| KRY294 | KRY33, except esc1::TRP1 | This study |
| KRY295 | esc1::TRP1 SIR4-13×mcy (diploid) | This study |
| KRY341 | KRY12, except esc1::KanMx | This study |
| KRY354 | YD126, except esc1::KanMx | This study |
| KRY485 | RDN1::mU3A3-HIS3 MATa | This study |
| KRY459 | KRY458, except esc1::KanMx | This study |
| KRY737 | SIR4-13×Myc::KanMx×8×His SMT3-TRP1 mata | This study |
| KRY697 | YKU80-13×Myc::KanMx-His SMT3-TRP1 mata | This study |
| KRY705 | SIR3-13×Myc::His3×8×His SMT3-TRP1 mata | This study |
| KRY734 | YKU70-9×Myc::TRP1 8×His SMT3-TRP1 mata | This study |
| GCS55  | RDN1::mU3A3-HIS3 hmr A::TRP1 adh4::AED2-4TelVIII MATa | Cuperus et al., 2000 |
| YDW126 | hmr ΔAEB::TRP1 MATa | Wotton and Shore, 1997 (56a) |
| YSB35  | hmr ΔAEB::3×UASg TRP1 MATa | Chien et al., 1993 (11) |
| GAS347 | 8×His SMT3-TRP1 mata | Ferreira et al., 2011 (17) |

*All strains used in this study were isogenic to W303.*
SUMO enrichment, and slower-moving bands were never detected in these strains. Comparisons of slower-moving sumoylated proteins were made between strains containing empty vector and those containing SIZ2 plasmid.

**Fluorescence microscopy.** Immunofluorescence was done as described previously (20, 35). Briefly, cells grown in appropriate media were fixed with paraformaldehyde, spheroplasted, and spotted on multiwell slides. They were further permeabilized with methanol and acetone and then blocked with 1% ovalbumin in phosphate-buffered saline (PBS). Cells were incubated with appropriate primary antibody dilutions overnight at 4°C and then washed thoroughly and incubated with the recommended dilutions of fluorescently labeled secondary antibody. After washes, slides were mounted in mounting medium containing DAPI (4',6-diamidino-2-phenylindole) and then viewed and photographed in a Leica (AOBS) TCS SP5 system and analyzed using LAS AF software version 2.2.0.

**ChIP.** At least three independent chromatin immunoprecipitation (ChIP) experiments were done by growing KRY109 and KRY295 transformed cells with empty vector or SIZ2 in 50 ml of Sc-Leu minimal medium until an OD_600 of 1.5 to 1.0, as described in reference 60. Briefly, cells were cross-linked with 1.4 ml of 37% formaldehyde, and cross-links were quenched with 3.4 ml of 2 M glycine. Then cells were pelleted and washed with ice-cold Tris-buffered saline (TBS). Cells were lysed in 400 μl of ice-cold lysis buffer with protease inhibitor (0.1% deoxycholic acid, 1 mM EDTA, 50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1% Triton X-100) by addition of an equal volume of glass beads and vortexing at maximum speed for 10 min at 4°C. Lysate was sonicated in a Biorupter sonifier to shear the chromatin to an average length of 500 to 800 bp. The samples were then clarified, and the supernatant was precleared by adding a 30-μl aliquot of protein A-Sepharose beads (Amersham Biosciences). Sample was centrifuged at 7,500 rpm for 5 min at 4°C, and the supernatant was aliquoted equally into two tubes. At this point, 50 μl of sample was taken in a fresh tube and used as input DNA. Primary antibody against the Myc epitope (Abcam) or Sir2 protein was added to the sample, and the sample was incubated overnight at 4°C with constant rotation. Thirty microliters of protein A-Sepharose beads was added to the chromatin-antibody mixture and incubated for 2 h at 4°C with constant rotation. Protein A-Sepharose beads were washed with 1 ml of each lysis buffer, lysis-500 buffer, LiCl-detergent solution (0.5% deoxycholic acid, 1 mM EDTA, 250 mM NaCl, 0.5% NP-50, 10 mM Tris-Cl [pH 8.0]), and TBS buffer. Chromatin immunoprecipitate was eluted first with 100 μl of 1% SDS in Tris-EDTA (TE: 10 mM Tris-Cl [pH 8.0], 1 mM EDTA [pH 8.0]) and then with 150 μl of 0.67% SDS in TE buffer by incubation at 65°C for 10 min. DNA from bound and unbound chromatin (input sample) was purified with phenol-chloroform-isooamyl alcohol extraction and ethanol precipitation. A total of 150 ng of DNA sample from ChIP experiments was analyzed by real-time PCR using Sybr green master mix (Applied Biosystems) according to the manufacturer’s instructions on an Applied Biosystems 7500 HT fast real-time PCR system. The primers used are located on the end of chromosome VI-R and reported in reference 60. Relative quantification of immunoprecipitated DNA was done based on the comparative Ct value method using sequence detection software. Statistical significance of differences in binding of Sir proteins in the presence and absence of Sir2 was evaluated by performing a Mann-Whitney U test. The P values reported were obtained using data from at least four independent experiments.

**RESULTS**

**Genetic screen for factors that disrupt Sir protein localization at the nuclear periphery.** Previous studies have shown that mutations in HMR locus silencer elements (ORC, Rap1, or Abf1 binding sites) that abolish repression can be bypassed by tethering of Sir proteins to the deleted silencers in the form of Gal4 DNA-binding domain (Gbd) fusions (11, 33). Remarkably, silencer mutations can also be bypassed by tethering of the locus to the nuclear periphery, through overexpression of several different Golgi apparatus/endoplasmic reticulum (ER) proteins, such as Yif1, Yip1, and Yip3, fused to Gbd (1). Targeted silencing by these Gbd fusion proteins is also Sir dependent and requires at least one functional silencer element at the HMR-E silencer. It was hypothesized that these proteins establish silencing by placing the defective HMR locus in the milieu of Sir proteins, which normally accumulate at the nuclear periphery.

If silencing by these Gbd fusion proteins is dependent on Sir protein accumulation at the periphery, then loss of Sir proteins at the periphery should lead to loss of silencing by these proteins. We tested this in a yku70 mutant background where peripheral localization of Sir proteins is abolished (29). YSB35, a yeast strain that has the E and B elements of the HMR-E silencer replaced with a a gene replaced (YSB35) and with yku70 (KRY223) strains. Cultures were grown, and 5 μl of 10-fold serial dilutions of each of these was spotted on SC-His (total cells) and SC-His Trp plates (fraction derepressed) to assess the effects of these plasmids on silencing of TRP1 locus. The plates were incubated for 2 days at 30°C. (B) Targeted silencing at HMR locus with elevated SIZ2 dosage. YSB35 was transformed with Gbd or Gbd-Yif1 along with the genomic library plasmid (D4) or SIZ2 in the 2μm vector. Silencing assays were performed as described above.

![FIG 1 Gbd-Yif1-mediated silencing targeted silencing is affected by elevated SIZ2 dosage.](ec.asm.org)
for transformants that showed robust growth on medium lacking tryptophan. We isolated a plasmid from one candidate transformant (named D4) that reproducibly conferred a modest level of derepression of the \textit{TRP1} marker (Fig. 1B). Sequencing of D4 revealed that it contained the chromosomal region encompassing \textit{ISN1}, \textit{SIZ2}, \textit{PUP1}, \textit{SME1}, and \textit{PET123}. In order to identify which of these genes is responsible for derepression, we separated them. We first tested if \textit{SIZ2} was responsible for derepression. Indeed, we found that \textit{SIZ2} alone on a high-copy-number plasmid abrogated the targeted silencing by Gbd-Yif1 to the same level as plasmid D4 (Fig. 1B).

Elevated gene dosage of \textit{SIZ2} causes loss of gene silencing. Transcriptional silencing at \textit{HM} loci and telomeres (referred to hereafter as telomere position effect, or TPE) is established and maintained by very similar mechanisms. Therefore, in order to confirm and extend the observations that elevated \textit{SIZ2} dosage disrupts targeted silencing by Gbd-Yif1, we tested its effect on TPE. KRY12, which contains a modified telomere on the left arm of chromosome VII (TelVIIL), with a \textit{URA3} gene inserted adjacent to telomere repeats, was used to assay TPE (21). Silencing of the \textit{URA3} gene allows strains to grow on plates containing the drug 5-fluoroorotic acid (5-FOA). As shown in Fig. 2A, when the \textit{SIZ2}-containing plasmid was introduced (row 2), growth on 5-FOA was reduced significantly, suggesting that elevated \textit{SIZ2} dosage affects silencing at telomeres as well.

\textit{Siz2} belongs to a conserved class of SP-ring-containing E3-like SUMO ligases (25, 27, 51). Four SUMO ligases have been identified in yeast: \textit{Siz1}, \textit{Siz2}, \textit{Mms21}, and \textit{Zip3}. \textit{Siz1} and \textit{Siz2} account for most of the protein sumoylation observed \textit{in vivo} (41). \textit{Mms21} sumoylates several proteins involved in chromosome stability (65), and \textit{Zip3} is a meiosis-specific E3 ligase (10). We tested if elevated gene dosage of any of the other mitotic E3 ligases also had the same effect as \textit{SIZ2} or if the effects were specific to \textit{SIZ2}. The \textit{SIZ1} and \textit{MMS21} genes were cloned in a multicopy vector, transformed into KRY12, and assayed for TPE. As shown in Fig. 2A, expression of \textit{SIZ1} and \textit{MMS21} had marginal effects on TPE, as indicated by good growth on plates containing 5-FOA, whereas the \textit{SIZ2} plasmid strongly reduced TPE, as indicated by poor growth on 5-FOA plates. These observations show that TPE loss is largely \textit{SIZ2} specific and suggest that a substrate unique to \textit{Siz2} might be affected. They also rule out any global effects due to elevated dosage of SUMO ligases in general.

To extend this observation further, we tested silencing at the \textit{HMR} locus directly, using a strain containing a weakened silencer (\textit{HMR-\DeltaR}) linked to the \textit{TRP1} reporter that had been shown previously to be a sensitive indicator of silencing at \textit{HMR} (49). This strain (YDW126) was transformed with either empty vector or vector containing one of the three ligase genes and subjected to the silencing assay as described for panel A. An extra copy of \textit{SIR2} was introduced as a positive control for silencing.

\textbf{FIG 2} Elevated \textit{SIZ2} reduces silencing at telomeres and \textit{HMR} but not rDNA. KRY12 and YDW 126 were transformed with empty vector (–), \textit{SIZ2}, \textit{SIZ1}, or \textit{MMS21} in a 2μm plasmid. In the top panel, 10-fold serially diluted KRY12-derived cells were plated on Sc-Leu for growth control and Sc-Ura and 5-FOA to assess TPE. In the lower panel, YDW126 transformants were plated on Sc-Leu Trp to assess repression of \textit{TRP1} locus. (C) Effect of elevated doses of E3 ligases on silencing at the rDNA locus. KRY458 (rDNA::\textit{mURA3-HIS3}) was transformed with high-copy \textit{SIZ1} or \textit{SIZ2} or \textit{MMS21} plasmids and subjected to the silencing assay as described for panel A. An extra copy of \textit{SIR2} was introduced as a positive control for silencing.
dosage interferes with gene silencing at both telomeres and the HMR locus.

Elevated SIZ2 gene dosage does not reduce silencing at rDNA locus. In Saccharomyces cerevisiae, apart from telomeres and HM loci, the other predominant silent region, with respect to RNAP II-driven transcription, is the rDNA locus. Silencer protein Sir2 is a NAD-dependent deacetylase involved in silencing telomeres, HM loci and the rDNA locus. However, the mechanism involved in establishing and maintaining silent chromatin at the rDNA locus is different from that of telomeres and HM loci and is dependent on Sir2 but not Sir3 or Sir4. Sir2 protein is sequestered by Net1p to the RENT complex, which is required for rDNA silencing (48). Since our results show that elevated dosage of SIZ2 reduces silencing both at telomeres and HMR, we asked whether SIZ2 also affects silencing at the rDNA locus. To test this, we introduced the three E3 ligases (SIZ2, SIZ1, and MMS21) or an extra copy of SIR2 into KRY458 (rDNA::mURA3-HIS3) and subjected them to silencing assay on 5-FOA medium (Fig. 2C). The silencing phenotypes of the three ligases were indistinguishable from that of the vector-alone control, showing that elevated SIZ2 dosage has no detectable effect on rDNA silencing, although the extra copy of SIR2 improves silencing, as expected.

escl mutation exacerbates gene silencing defects caused by elevated SIZ2 gene dosage. In order to gain insights into the molecular pathways involved in this process, we tested the effect of elevated SIZ2 dosage in different mutant backgrounds. We generated yku70, yku80, and escl deletion mutations in tethered silencing reporter strain YSB35. These mutations were chosen because the corresponding proteins have been shown to be involved in anchoring telomeres to the nuclear periphery (29, 30). Gbd, Gbd-Sir4, and Gbd-Yif1 were transformed into these strains. We also tested Gbd-Sir4-mediated silencing because Gbd-Yif1-mediated silencing is compromised in these strains due to reduced anchoring of telomeres to the nuclear periphery (our unpublished observations and Fig. 1). In the escl strain, when only empty vector is cotransformed, Gbd-Rap1, Gbd-Sir4, and Gbd-Yif1 establish strong silencing (Fig. 3A; see the wild-type [WT] control in Fig. S1 in the supplemental material). However, upon cotransformation with a high-copy SIZ2-containing plasmid, all hybrids, including Gbd-Rap1 and Gbd-Sir4, were unable to establish silencing, as seen by the increased growth on plates lacking trytophan. In the yku70 and yku80 mutants, though, Gbd-Sir4-mediated silencing was hardly affected by increased SIZ2 gene dosage (Fig. 3B). The same results were obtained with Gbd-Rap1 and Gbd-Sir3 (data not shown).

As targeted silencing by all of the hybrids was reduced in escl mutants, we tested the effect of elevated dosage of the other SUMO ligases in escl mutants on gene silencing. We deleted the ESl1 gene in KRY12 and transformed this strain with either empty vector or vector containing any one of the SUMO ligases. The indicated mutants were transformed with Gbd or Gbd-Sir4 along with either empty vector or SIZ2, and serial dilutions were spotted on Sc-His-Leu for growth control and Sc-His-Leu Trp for testing repression.

FIG 3  escl mutation exacerbates silencing defects of elevated SIZ2 dosage. (A) Targeted silencing in the escl mutation containing an elevated gene dosage of SIZ2. KRY213 was cotransformed with plasmids expressing either Gbd or one of the Gbd fusion proteins (Rap1, Sir4, or Yif1) and empty vector (−) or SIZ2 on the 2μm plasmid. The transformants were tested for silencing of TRP1 as in Fig. 1. (B) Targeted silencing in yku70/80 mutants containing an elevated gene dosage of SIZ2. The indicated mutants were transformed with Gbd or Gbd-Sir4 along with either empty vector or SIZ2, and serial dilutions were spotted on Sc-His-Leu for growth control and Sc-His-Leu Trp for testing repression.
extracted from cells expressing either vector alone or any one of the three SUMO ligases. We found that all three genes were over-expressed in strains containing elevated doses; the ethidium-stained agarose gel serves as a loading control (see Fig. S2 in the supplemental material). We then tested if the increased dosage of SIZ2 altered the SUMO proteome by performing Western blots using anti-SUMO antibodies. We found that many proteins showed increased sumoylation with elevated SIZ2 dosage, further indicating that increased dosages of SIZ2 altered the sumoylation status of many proteins (see Fig. S3 in the supplemental material). We then tested if this catalytic activity of Siz2 was essential for the phenotypes observed directly by mutating the catalytic domain of Siz2. Siz2 belongs to the class of SP-RING domain-containing enzymes and has the conserved cysteine and histidine (C379 and H377) residues in the catalytic site. As other SUMO ligases with these data further firmly establish that elevated dosage leads to derepression of native silenced loci as well.

**Elevated SIZ2 dosage leads to derepression of native silenced loci.** Our results strongly suggest that increased SIZ2 dosage disrupts silencing, as measured by growth assays. Recently, two reports have suggested that the 5-FOA growth assays may not be true indicators of silencing (42, 52). We therefore tested the silencing of a native subtelomeric gene, yFR057w, previously shown to be silenced in a Sir protein-dependent manner (61). We isolated RNA from wild-type and esc1 mutant cells transformed with either empty vector or SIZ2, and performed quantitative reverse transcriptase PCR (RT-PCR). As shown in Fig. 6A and B, the RT-PCR data correlate with the growth-based assays reported above. Cells transformed with empty vector do not show derepression, whereas as expected, the sir2 mutant shows complete derepression. In wild-type cells, elevated SIZ2 dosage leads to a 4-fold increase in yFR057w, while all other plasmids show no effect. In esc1 deletion, SIZ2 shows a large increase in derepression and similar to our observation in growth assays, SIZ1 too shows a small increase in derepression. We also tested the repression at the wild-type silent mating-type loci in wild-type (data not shown) and esc1 mutant cells transformed with the same set of plasmids (Fig. 6C). There was no detectable increase in derepression in wild-type cells; however, in esc1 mutant cells, SIZ2 overexpression showed elevated derepression. This increase appears modest in comparison to the growth assays, because unlike in growth assays where we have used a weakened silencer that is more sensitive to perturbations, this assay was done on the strong wild-type silencer. Thus, these data further firmly establish that elevated SIZ2 dosage leads to derepression of silencing at the native loci as well.

**Elevated SIZ2 gene dosage increases sumoylation of proteins involved in silencing.** As our previous experiments strongly suggested elevated SIZ2 dosage increased sumoylation of target proteins, we directly tested this. Previous whole-proteome studies have shown that Yku70, Yku80, Sir4, and Sir3 are sumoylated. We tested if the sumoylation of these proteins was affected upon elevated SIZ2 dosage. All four proteins were C-terminally tagged with myc epitopes and 8×His-tagged SUMO was substituted in the place of endogenous SUMO (9). Each of these strains was transformed with either empty vector or SIZ2, and total protein was extracted and enriched for sumoylated proteins using a nickel column as reported earlier (9). We detected the myc epitope-tagged proteins using an anti-myc antibody. We found all four proteins showed slower-migrating bands in the SUMO-enriched proteome. Interestingly, we found either additional or increased intensity of some of the slower-moving bands upon increased SIZ2 dosage compared to vector alone in the tested proteins, albeit to different degrees (Fig. 7). This effect is most prominent for Sir3 and Sir4, where the upper (presumably sumoylated) band intensity increases to the level of intensity of the lower band upon increased SIZ2 dosage, whereas in the presence

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**FIG 5** Catalytically active Siz2 is essential for inducing silencing defects. The KRY12 (WT), KRY341 (esc1::KANMX), and KRY354 strains were transformed with either empty vector (−), SIZ2, or CH-SIZ2 on the 2µm plasmid. The silencing assay for telomeres (A) and HMR (B) was done as described in the legend to Fig. 2A.
of vector alone, the upper band intensity is less than the intensity of the lower band. These data provide evidence that sumoylation of multiple proteins involved in silencing is increased upon elevated SIZ2 dosage.

Elevated SIZ2 gene dosage does not cause delocalization of telomeric proteins. Having established that ESC1 deletion exacerbates the silencing defects caused by elevated SIZ2 dosage, we investigated the mechanism through which these effects are manifested. Because SUMO modification of proteins can alter their subcellular localization or stability, we asked whether Sir2 or Sir4 localization is affected by elevated SIZ2 dosage. Wild-type and esc1 derivatives of a strain expressing Sir4-Myc from the endogenous SIR4 locus were used for these experiments, and both Sir2 and Sir4 were localized by indirect immunofluorescence (20). Figure 8 shows the localization of Sir4 protein in both wild-type and esc1 strains with and without elevated SIZ2 dosage. The nuclear rim was stained with antibodies to Nsp1 protein, a component of the nuclear pore complex. As expected, Sir4 was found concentrated in 4 to 6 foci per nucleus, representing the telomeres, in wild-type cells. However, we could detect no differences in Sir4 protein localization upon elevated SIZ2 dosage in the wild type or in esc1 mutants. These results show that the loss in silencing is not due to loss of Sir4 protein localization. Sir2 protein localization in the same strains was examined using antibodies to Sir2 protein. As shown in Fig. 9, Sir2 shows a prominent nucleolar localization (red, arc-like staining), with weaker telomeric spots visible in some cells. This localization was not detectably altered in esc1 mutants or upon elevated SIZ2 dosage. We also confirmed by Western blot analysis that stability of these proteins was not affected by SIZ2 overexpression in either wild-type or esc1 strains (see Fig. S4 in the supplemental material).

Elevated gene dosage of SIZ2 affects Sir2 localization at telomeres. As immunofluorescence techniques can detect only major changes in localization, we assessed the association of Sir4 and Sir2 proteins with the telomeres through the more sensitive and quantitative ChIP technique (47, 60), using PCR primers to detect unique sequences from 0.5 kb to 5 kb internal to chromosome VI-R (60). The same immunoprecipitated DNA was also used to amplify a euchromatic internal gene, SPS2, where Sir proteins are not expected to bind. This served as an internal control to assess the amount of DNA precipitated due to nonspecific interactions (Fig. 10A). As expected, both Sir2 and Sir4 were highly enriched at the very ends of the telomere and decreased rapidly toward the centromere. Telomere association of Sir4 and Sir2 was largely unaffected by elevated SIZ2 gene dosage in wild-type strains (see Fig. S5 in the supplemental material). These data are consistent with the immunofluorescence data and indicate that Sir4/Sir2 protein association with telomeres is at most only very mildly affected by elevated SIZ2 dosage in wild-type cells.

However, when the esc1 strains were tested for Sir4/Sir2 protein localization, we detected some differences. As shown in Fig. 10A, the association of Sir4 was reduced slightly at internal sites of

FIG 6 Transcription of native silent loci. Transcription of yFR057w or HMRa1 was quantified by reverse transcription and real-time PCR analysis for wild-type cells (A) and esc1 mutants (B and C) containing the indicated 2μm plasmids (x axis). Signals were normalized against the control ACT1 amplification. Results represent an average of three experiments, and error bars denote standard deviations.

FIG 7 Sumoylation of target proteins by Siz2. The 8×His-tagged SUMO strains containing myc-epitope-tagged Yku70, Yku80, Sir3, or Sir4 were transformed with empty vector or 2μm SIZ2 plasmid, and total sumoylated proteins were enriched on a nickel column, separated by SDS-PAGE, and detected by anti-myc antibody. lane 1 contains whole-cell extracts, lane 2 contains protein from cells transformed with vector, and lane 3 contains protein from cells transformed with 2μm SIZ2. Slower-moving bands are visible only upon enrichment on the nickel column. On the right, the myc-tagged protein detected is indicated.
2.5 kb and 3.5 kb from the telomere, although not detectably affected at the most proximal site tested. On the other hand, Sir2 protein association with the telomeric chromatin was reduced by half upon overexpression of Siz2 at all sites examined, including the most proximal site. These differences (at most 2-fold) are likely undetectable by the immunofluorescence method we have used. However, this reduction in binding of Sir2 protein is statistically significant, as $P$ values at all sites were <0.05 (Fig. 10B). As the maximum derepression due to elevated SIZ2 dosage was seen in the esc1Δ HMRΔa strains, we also tested the association of Sir2 and Sir4 with the HMR locus. As shown in Fig. 10C, there was a significant reduction in association of both proteins with this locus. Therefore, it appears that increased doses of SIZ2 lead to a reduction of Sir2 protein association at the telomeres and both Sir2 and Sir4 protein at the HMR locus.

**DISCUSSION**

Elevated SIZ2 gene dosage disrupts silencing at telomeres and the HMR locus. Here we show that sumoylation influences gene silencing at telomeres and HMR loci in *S. cerevisiae*. Elevated gene dosage of SIZ2, which encodes an E3 SUMO ligase, reduces silencing at both the loci. The catalytic activity of Siz2 is essential for this phenotype, strongly suggesting that it results from sumoylation of one or more Siz2 substrates. Increasing gene dosage of the two other known mitotic SUMO ligases in yeast (Siz1 and Mms21) has a much weaker effect on silencing, implying that an Siz2-specific substrate may be involved. However, it is possible that under the conditions described here, Siz1 and especially Mms21, which is known to function in a complex with Smc5/6, do not confer the same amount of increased sumoylation activity as Siz2 and are therefore unable to influence gene silencing.

*esc1* exacerbates the silencing defect of elevated SIZ2 gene dosage. Significantly, we found that the silencing defect induced by increased SIZ2 dosage is enhanced by deletion of ESC1. Esc1 is a large coiled-coil protein associated with the inner nuclear membrane that was discovered in a screen for proteins that establish silencing when targeted to a defective HMR locus (2). *esc1* mutants

**FIG 8** Elevated SIZ2 does not visibly alter Sir4p localization. Immunolocalization of Sir4 protein was done in the WT and *esc1* mutants containing either empty plasmid (columns 1 and 3) or 2 μm SIZ2 (columns 2 and 4). Anti-myc antibody (red) was used to stain Sir4 protein, and the spots represent clustered telomeres. Columns 1 and 2 are wild-type cells, and columns 3 and 4 are *esc1* cells. Antibodies to Nsp1 (green) stain the nuclear pore complex, indicating that the spots seen are nuclear. Bars equal 5 μm.

**FIG 9** Elevated SIZ2 gene dosage does not visibly alter localization of Sir2 protein. Immunolocalization of Sir2 protein was done in the WT and *esc1* mutants containing either empty plasmid (columns 1 and 3) or 2 μm SIZ2 (columns 2 and 4). Anti-Sir2 antibody (red) was used to stain Sir2 protein and shows prominent nucleolar and few telomeric spots. Columns 1 and 2 are wild-type cells, and columns 3 and 4 are *esc1* cells. Antibodies to Nsp1 (green) stain the nuclear pore complex, indicating that the spots seen are nuclear. Bars equal 5 μm.
binding to telomeres, particularly in the absence of Esc1. Binding of both proteins is reduced at the HMR-E silencer. This suggests that Siz2 overexpression influences gene silencing by interfering with Sir2 and perhaps Sir4 association with heterochromatin. Two recent reports also link silencing to sumoylation via Sir2 (13, 63). Deletion of the SUMO isopeptidase gene ULP2 leads to a loss of telomere silencing, although its effect on HMR was not reported (13). Furthermore, deletion of SLX5, which leads to increased overall sumoylation, also reduced gene silencing. This work also indicated that the effects of ULP2 and SLX5 mutation are linked to Sir2, further supporting our observation that SUMO homeostasis is important for gene silencing and, more specifically, for the function of Sir2. Ulp2 is localized throughout the nucleus, whereas Ulp1 is mostly at the nuclear periphery, and this localization is dependent on Esc1. Based on our data, we predict that elevated SIZ2 dosage causes increased sumoylation of target proteins at the nuclear periphery that leads to a loss in silencing. Esc2, a protein that physically interacts with Sir2 (12) and has SUMO-like motifs, influences gene silencing in a locus-dependent manner (15, 63). As Esc2 interacts with both Sir2 and SUMO and also influences the association of Sir2 protein with telomeric heterochromatin, it could potentially be one of the targets for Siz2-mediated silencing defects.

When this article was in preparation, two reports appeared which showed that Siz2 sumoylates multiple substrates at the nuclear periphery and telomeres, including Yku70/80, Rap1, Cdc13, and Sir4. The anchoring of telomeres to the nuclear periphery was shown to be dependent on sumoylation of Yku70/80. Other possible substrates, such as Esc1, Sir3, and Sir2, which are also known to be sumoylated (23, 56), were not tested. Relevant to the work presented here, these studies show that Siz2 functions at the nuclear periphery and has multiple substrates. Second, Siz2 affects telomere organization and function through modification of these substrates. Although these studies were focused on the role of Siz2 in telomerase function and did not study silencing, our work suggests that sumoylation by Siz2 also influences heterochromatin at telomeres, by decreasing the association of Sir2 with these loci. Our studies show that several proteins could be potential targets, and identification of the substrate(s) of Siz2 that influences gene-silencing properties by specifically mutating each of the multiple potential sumoylation sites in these proteins will be important to understand the mechanistic basis of how Sir protein association and heterochromatin establishment are influenced by SUMO metabolism. We favor the idea that sumoylation on one or more targets either directly or indirectly influences the Sir2 association with the heterochromatin.

Interestingly, although TPE and HMR silencing are reduced upon Siz2 overexpression, rDNA silencing is not detectably affected under these conditions. This finding is somewhat puzzling, since Siz2 overexpression caused reduced binding of Sir2 at telomeres, and Sir2 is the one Sir protein that is also required for rDNA silencing. One explanation for this apparent discrepancy is that Siz2 overexpression affects Sir2 exclusively (or most strongly) at the nuclear periphery, with nucleolar pools of Sir2 remaining largely unaffected. This is partly supported by the report described above, which showed that telomeres present in the nuclear interior were not subjected to SUMO-dependent inhibition of telomerase and were elongated efficiently (17). These studies indirectly support the idea that relevant Sir2-specific targets are present at the nuclear periphery and hence only those functions
are likely to be significantly altered by perturbing Sir2 function. We imagine that the normal sumoylating activity of Sir2 would with the desumoylating activity of Ulp1 at the nuclear periphery acts to maintain a balance of sumoylation/desumoylation that supports the localization of Sir2/Sir4 to these silent regions. This balance is perturbed in an escl strain due to delocalization of Ulp1. In conclusion, our studies provide a new perspective for investigating the molecular mechanisms that control the distribution of Sir2 protein within the nucleus and specifically point to a possible role for sumoylation in this process.

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