**Saccharomyces cerevisiae** Esc2p Interacts with Sir2p through a Small Ubiquitin-like Modifier (SUMO)-binding Motif and Regulates Transcriptionally Silent Chromatin in a Locus-dependent Manner*1

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**Saccharomyces cerevisiae** Esc2p is a member of a conserved family of proteins that contain small ubiquitin-like modifier (SUMO)-like domains. It has been implicated in transcriptional silencing and shown to interact with the silencing protein Sir2p in a two-hybrid analysis. However, little is known about how Esc2p regulates the structure of silent chromatin. We demonstrate here that ESC2 differentially regulates silent chromatin at telomeric, rDNA, and HML loci. Specifically, ESC2 is required for efficient telomeric silencing and Sir2p association with telomeric silent chromatin and for silencing and maintenance of silent chromatin structure at rDNA. On the other hand, ESC2 negatively regulates silencing at HML and HMR and destabilizes HML silent chromatin without affecting Sir2p association with chromatin. We present evidence that Esc2p is associated with both transcriptionally silent and active loci in the genome, and the abundance of Esc2p is not correlated with the chromatin state at a particular locus. Using affinity pull-down analyses, we show that Esc2p and Sir2p interact in vivo, and recombinant Esc2p and Sir2p interact directly. Moreover, we dissect Esc2p and identify a putative SUMO-binding motif that is necessary and sufficient for interacting with Sir2p and SUMO and is required for the function of Esc2p in transcriptional silencing.

Transcriptional silencing in **Saccharomyces cerevisiae** occurs at telomeric, rDNA, **HML** (homothallic mating locus left), and **HMR** (homothallic mating locus right) loci and is mediated by a special silent chromatin akin to metazoan heterochromatin (1). Silent chromatin is associated with histone hypoacetylation, and the NAD+−dependent protein deacetylase Sir2p is essential for its formation (2–4). Sir2p associates with Sir3p and Sir4p to form the Sir complex that promotes silencing at the telomeric and **HM** loci, and it binds Net1p and Cdc14p to form the RENT complex responsible for rDNA silencing (5–8). The establishment of silent chromatin at telomeric and **HM** loci is achieved via an initiation process that recruits the Sir complex to nucleation sequences, including telomeric repeats and silencers flanking the **HM** loci. Telomeric repeats consist of multiple Rap1p-binding sites. Rap1p, together with the Ku70-Ku80 complex that binds to chromosome ends, recruits the Sir complex to telomeres. A silencer is composed of two or three of the binding sites for origin recognition complex, Rap1p, and Abf1p. Silencer-binding factors recruit the Sir complex through a direct interaction between the origin recognition complex and Sir1p that binds to Sir4p and the binding of Rap1p to Sir3p or Sir4p. A Sir complex recruited to a silencer or telomere is believed to deacetylate histones in adjacent nucleosomes through the deacetylase activity of Sir2p (3). The deacetylated nucleosomes are thought to bind additional Sir complexes, based on the findings that the Sir complex self-interacts and preferentially binds hypoacetylated histones. Through repeated cycles of histone deacetylation and Sir complex recruitment, Sir complexes propagate along the chromatin (1). At the rDNA locus, the RENT complex is targeted to non-transcribed sequence via Fob1p and RNA polymerase I (9). There is evidence suggesting that RNA polymerase I is involved in the propagation of silent chromatin at rDNA (10), but the detailed molecular mechanism has not been resolved.

Sir2p interacts with proteins other than Sir3p, Sir4p, and Net1p. These proteins include Esc2p, Esc8p, and Mcm10p (11, 12). Of particular interest is Esc2p, which belongs to an evolutionarily conserved family of proteins that contain two small ubiquitin-like modifier (SUMO)3-like domains (13). Moreover, Esc2p, like its fission yeast ortholog Rad60, also contains putative SUMO-binding motifs (SBMs) (14, 15) (see Fig. 1). Esc2p was originally shown to be required for efficient silencing at telomeric and **HM** loci (16). Recently, it was implicated in homologous recombination repair in DNA replication and sister chromatid cohesion (15, 17, 18).

Little is known about how Esc2p contributes to transcriptionally silent chromatin structure. In this work, we demonstrate that ESC2 differentially regulates transcriptionally silent chromatin at telomeric, rDNA, and **HM** loci. We also show that a SUMO-binding motif of Esc2p is necessary and sufficient for interaction with both Sir2p and SUMO and is required for the function of Esc2p in transcriptional silencing.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. S1–S3.
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3 The abbreviations used are: SUMO, small ubiquitin-like modifier; SBM, SUMO-binding motif; ChIP, chromatin immunoprecipitation; FRT, Flp1p recombination target; GAD, Gal4p activation domain; GBD, Gal4p DNA binding domain; FOA, 5-fluoroorotic acid; MMS, methyl methanesulfonate; HA, hemagglutinin; GST, glutathione S-transferase; 3AT, 3-aminotriazole; SD, SUMO-like domain; SC, synthetic complete medium; MNase, micrococcal nuclease.
**TABLE 1**

| Yeast strains          | Genotype                                                                 | Source/Reference |
|------------------------|--------------------------------------------------------------------------|------------------|
| BY4741                 | MATa his3Δ1 leu2Δ200 met15Δ0 ura3Δ0                                       | Open Biosystems  |
| CCFY101                | MATa ade2-1 can1-100 his3-15,15 leu2-3,112 trp1-289 ura3-1 kmrΔ::TRPI  Tel VR-URA3 RDN1::ADE2-CAN1 | Ref. 21          |
| JS125                  | MATa his3Δ200 leu2Δ1 ura3-167 RDN1 (NTS2::Ty1-mIURA3)                     | Ref. 22          |
| PJE9-4α                | MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2Δ::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ | Ref. 19          |
| YHK33                  | BY4741, Tel VIII-URA3                                                   | This work        |
| YHK56                  | YHK33, esc2Δ::KanMX                                                     | This work        |
| YHK61                  | PJE9-4α, sir3Δ::NatMX                                                   | This work        |
| YLS537                 | YXB76, SIR2-myc-KanMX                                                   | This work        |
| YKA21                  | BY4741, ESC2-myc-KanMX                                                  | This work        |
| YLO55                  | PJE9-4α, sir3Δ::NatMX                                                   | This work        |
| YQY495                 | JS125, esc2Δ::NatMX                                                     | This work        |
| YQY498                 | CCFY101, esc2Δ::KanMX                                                   | This work        |
| YQY537                 | YXB10, esc2Δ::KanMX                                                     | This work        |
| YQY539                 | YXB10, esc2Δ::KanMX                                                     | This work        |
| YQY541                 | JS125, sir2Δ::KanMX                                                     | This work        |
| YQY585                 | YXB6, esc2Δ::NatMX                                                     | This work        |
| YQY587                 | YXB6, esc2Δ::NatMX                                                     | This work        |
| YQY603                 | YXB6-11, esc2Δ::NatMX                                                   | This work        |
| YQY610                 | YXB6-11, esc2Δ::NatMX                                                   | This work        |
| YQY616                 | CCFY101, SIR2-myc-KanMX                                                 | This work        |
| YQY617                 | YQY616, esc2Δ::NatMX                                                    | This work        |
| YQY618                 | YQY616, trp1Δ::KanMX                                                    | This work        |
| YSK535                 | MATa ade2-1 can1-100 his3-15,15 leu2-3,112 trp1-289 ura3-1 gal4Δ::LEU2 Aeb::3xIAsG | Ref. 23          |
| YSH563                 | PJE9-4α, sir3Δ::NatMX                                                   | Ref. 24          |
| YXX6                   | MATa ura3-52 ade2-1 lys1-1 his5-1 can1-100 [c1]::LEU2::GAL10::FLP1 E-FRT-hml::B2-FRT-I | Ref. 25          |
| YXX6                   | YXX6, sir3::URA3                                                       | Ref. 25          |
| YXX10                  | MATa ura3-52 ade2-1 lys1-1 his5-1 can1-100 [c1]::LEU2::GAL10::FLP1 FRT-hml::B1-FRT   | Ref. 26          |
| YXX10                  | YXX10, sir3::URA3                                                      | Ref. 26          |
| YXX10                  | MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1::SIR3::SUP4::HML::I-HIS3 | Ref. 27          |
| YXX76                  | MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1::SIR3::SUP4::HMRΔ::I-HIS3 | Ref. 28          |
| YYS208                 | MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2 TRP1::SIR3::SUP4::HMRΔ::I-HIS3 | Ref. 29          |

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strains**

The plasmids encoding Gal4p DNA-binding domain (GBD)–fused proteins are numbered 1–20 and 1’, 2’, and 18’ (Figs. 6A, 7, and 8). Plasmid 1 is pGBDU-C1 (19). Plasmids 2–12 were made by replacing the BamHI-Sall fragment of pGBDU-C1 with PCR-amplified full-length or truncated ESC2 fragments illustrated in Fig. 6A. Plasmid 13 was made by deleting the sequence corresponding to amino acids 116–195 of Esc2p from plasmid 2 by QuikChange mutagenesis (Stratagene). Plasmid 14 was made by sequentially inserting the sequence encoding amino acids 1–115 of Esc2p as a BamHI-Sall fragment and that encoding 156–456 residues of Esc2p as a Sall-PstI fragment into pGBDU-C1. Plasmids 15–17 were similarly constructed. Plasmid 18 was derived from plasmid 2 by converting T359 and T362 in ESC2 coding sequence to A via QuikChange mutagenesis. Plasmid 19 was made by inserting the sequence encoding C-terminal residues 732–1358 of Sir4p as an EcoRI-Sall fragment into pGBDU-C1. Plasmid pGADSUMO encoding GAD-SUMO was made by inserting PCR-amplified SMT3 (yeast SUMO) coding sequence as a BamHI-Sall fragment into pGAD-C1 (19). The correctness of all PCR-amplified sequences of ESC2, SIR4, and SMT3 in the above plasmids was confirmed by DNA sequencing. Plasmids 1’, 2’, and 18’ were derived from 1, 2, and 18, respectively, by replacing their IRA3 gene with LEU2. Plasmid 20 encoding GBD-Sir1p is pR1815 from Dr. Jasper Rine. Plasmid pGADSIR2 encoding GAD-Sir2p has been described (12).

Plasmid pBG-ESC2-His-HA is the Esc2p-encoding member of a yeast plasmid library designed to express each yeast open reading frame that is under the control of a GAL1 promoter and fused on the C terminus to a tandem affinity tag that includes His6, as well as a hemagglutinin (HA) tag (20). Likewise, pBG-Cyt2-His-HA is the Cyt2p-encoding member of the library. Plasmid pHK49 encoding GST-Sir2p was made by inserting an EcoRI-SIR2-Xhol fragment into pGEX6p-1 (Amersham Biosciences). Plasmid pHK52 encoding His6-HA-Esc2p was made by inserting an Ndel-HA-ESC2-Xhol fragment into pET16b (Novagen).

Yeast strains are listed in Table 1. YHK53 was made by transforming BY4741 to Ura+ with EcoRI-Sall-digested pT1 (30). Strains carrying the esc2Δ::KanMX or esc2Δ::NatMX allele were made by transforming their corresponding parents to Geneticin- or nourseothricin-resistant with a PCR-generated fragment composed of KanMX or NatMX bracketed by 5'- and 3'-flanking sequences of ESC2 coding sequence. Strain YKA21 was made by transforming BY4741 to Geneticin-resistant with a PCR-produced fragment encoding 9-Myc linked to KanMX embedded in a sequence spanning the 3’-region of ESC2 coding sequence. YL020 was made by replacing TRP1 at the sir4Δ locus in strain YSH563 with KanMX. YQY541 was made by replacing Sir2p in JS125 with KanMX. YQY616 was made by transforming CCFY101 to Geneticin-resistant with a PCR-produced fragment encoding 9-Myc linked to KanMX embedded in a sequence spanning the 3’-region of SIR2 coding sequence. YJ537 was similarly derived from YXB76 (28). YQY618 was made by replacing the TRP1 open reading frame in YQY616 with KanMX.

**Analysis of DNA Topology**

Cells grown in YPR medium (1% yeast extract, 2% bactopeptide, and 2% raffinose) were treated with galactose (2%) for 2.5 h to induce the expression of PGAL10-FLP1. Nucleic acids
were isolated using the glass bead method and fractionated on agarose gels supplemented with 20 or 16 μg/ml chloroquine. DNA circles were detected by Southern blotting. The profiles of topoisomers were determined using the NIH Image software.

**Chromatin Immunoprecipitation (ChIP)**

ChIP was carried out as described (28). The sequences of PCR primers used in ChIP are listed in supplemental Table 1.

**Chromatin Mapping**

Chromatin mapping by micrococal nuclease (MNase) digestion and indirect end labeling was carried out as described (30). About $2 \times 10^6$ permeabilized spheroplasts were treated with MNase at 75 and 150 units/ml, respectively, at 37 °C for 5 min. Genomic (naked) DNA from untreated cells was digested with MNase at 7.5 units/ml.

**Affinity Pull-down Analyses**

Ni²⁺-NTA Affinity Pull-down—Yeast cells were first grown in synthetic medium containing 2% raffinose to an $A_{600}$ of 0.8. Galactose was added to the culture to a final concentration of 2% to induce the expression of Esc2p-His$_6$-HA or Cyt2p-His$_6$-HA. Cells were harvested, washed, and lysed by the glass bead method, and the protein concentration of the cell extract was determined. Ni²⁺-nitrilotriacetic acid-agarose gels were equilibrated in Wash Buffer (5% glycerol, 150 mM NaCl, 20 mM HEPES, pH 7.5), and proteins were eluted by boiling for 5 min in Laemmli buffer.

**GST Pull-down—Escherichia coli**

BL21 cells bearing pHK52, pHK49, or pGEX6p-1 were treated with isopropyl 1-thio-D-galactopyranoside (0.5 mM) for 4 h to induce the expression of His$_6$-HA-Esc2p, GST-Sir2p, or GST, respectively. Cells were washed and resuspended in E. coli Lysis Buffer (20 mM Tris, pH 8.0, 350 mM NaCl, 1% Nonidet P-40, 10 mM dithiothreitol, and protease inhibitors) and lysed by treatment with lysozyme (0.5 mg/ml) for 30 min on ice, followed by sonication. Protein abundance was determined by Coomassie staining. Lysate containing an equivalent amount of GST or GST-Sir2p was bound to 100 μl of glutathione-Sepharose slurry (Amersham Biosciences) for 2 h at 4 °C. The resin was washed four times with 1 ml of E. coli Lysis Buffer. Lysate containing His$_6$-HA-Esc2p was added to the GST-resin and incubated for 2 h at 4 °C. The resin was washed five times with 1 ml of E. coli Lysis Buffer and eluted by boiling in Laemmli buffer. Proteins were separated by SDS-PAGE and detected by Coomassie staining (GST) or immunoblotting (His-HA-Esc2p) with an anti-HA antibody (Sigma).

**Two-hybrid Analysis**

Two-hybrid analyses in PJ69–4aα and its derivatives were carried out as described (19). Briefly, the strain was transformed to Ura$^+$ Leu$^+$ with a plasmid encoding a GBD-fused protein (URA3 marked) and a plasmid encoding a GAD-fused protein (LEU2 marked). Two independent transformants were grown in −Ura−Leu liquid to late log phase, and serial 10-fold dilutions of the cultures were spotted on −Ura−Leu, −Ura−Leu−His+3AT, and −Ura−Leu−Ade plates. The drug 3-aminotriazole (3AT) is a competitive inhibitor of His3p activity and is added to the −Ura−Leu−His media to raise the stringency of HIS3 selection.

**RESULTS**

**ESC2 Regulates Transcriptional Silencing in a Locus-dependent Manner**—Esc2p has previously been implicated in telomeric and HMR silencing (16), but it is not known whether it also plays a role in rDNA or HML silencing. We set out to examine more systematically the role of Esc2p in transcriptional silencing at different silent loci. We showed, as expected, that ESC2 deletion (esc2Δ) significantly reduced silencing of the URA3 gene integrated near the right end of chromosome V (Tel VR-URA3) or left end of chromosome VII (Tel VII-URA3) (Fig. 1B, compare with 2 and compare with 4 with 3 on 5-fluoroorotic acid (FOA); note that URA3 expression renders cells sensitive to 5-fluoroorotic acid). We demonstrated for the first time that esc2Δ also significantly reduced silencing of URA3 integrated at the rDNA array (Fig. 1B, compare with 6 with 5 on FOA). Therefore, ESC2 is required for efficient silencing at both the telomeric and rDNA loci.

On the other hand, we found that esc2Δ enhanced silencing of URA3 integrated at HMR (Fig. 1B, compare with 8 with 7 on FOA). Consistently, silencing of TRP1 inserted at HMR was also enhanced by esc2Δ (Fig. 1B, note reduced growth of strain 2 versus strain 1 on −Trp). Although the increase in silencing of URA3 or TRP1 at HMR by esc2Δ observed here was modest, it was reproducible. Moreover, we also found that esc2Δ enhanced silencing of URA3 inserted at HML (Fig. 1B, compare with 10 with 9 on FOA). Therefore, our results demonstrate that ESC2 plays a negative role in silencing at both HML and HMR. We noted that a previous study showed that silencing of ADE2 placed at HMR was reduced by esc2Δ (16), which is in contrast to our results described above. However, we did not observe a significant effect of esc2Δ on the silencing of ADE2 inserted at HMR (supplemental Fig. S1). It is formally possible that the different genetic backgrounds of the strains used in testing ADE2 silencing are the cause of the discrepancy.

We also showed that all our esc2Δ strains were more sensitive to the genotoxic agent methyl methanesulfonate (MMS) than their respective parents (Fig. 1B, compare even-numbered strains with odd-numbered ones on MMS), which is in accord with recent findings demonstrating a role for ESC2 in tolerance of genotoxic stress (15, 17, 18).

ESC2 Negatively Regulates Telomere Length—Telomere shortening is often correlated with a decrease in telomeric silencing (e.g. see Ref. 31). We investigated whether reduction of telomeric silencing by esc2Δ was accompanied by telomere shortening. We examined the length of the URA3-marked Tel VIII in strain YHK53 and its esc2Δ derivative YHK56. As shown in Fig. 1C, Tel VIII was longer in YHK56 than in YHK53. We also obtained a similar result in a pair of isogenic ESC2 and esc2Δ strains in the W303 background (data not shown). Therefore, interestingly, esc2Δ increases telomere length but reduces telomeric silencing. This, however, is not unprecedented because, for example, overexpression of DOT5 has been shown to lengthen telomerites and decrease telomeric silencing (32).
Regulation of Silent Chromatin by Esc2p

Effect of esc2Δ on Sir2p Association with Silent Loci—Given its ability to interact with Sir2p in a two-hybrid analysis (11), Esc2p could potentially regulate transcriptional silencing by influencing the association of Sir2p (or Sir complex) with silent chromatin. To test this hypothesis, we asked whether esc2Δ affected the abundance of Sir2p associated with different silent loci. We C-terminally tagged Sir2p with the Myc epitope in strains YQY616 (ESC2) and YQY617 (esc2Δ). Cells bearing Myc-tagged Sir2p exhibited the same phenotypes as their untagged counterparts, and Sir2p-Myc was expressed as determined by Western blotting (data not shown). ChIP was performed to examine the association of Sir2p-Myc with sequences in silent and active loci using an anti-Myc antibody in both YQY616 and YQY617. Three independent experiments were performed, and the data from a representative experiment are presented in Fig. 2A. The data were quantified and normalized against input control. The mean of data from all three experiments together with S.D. value is graphed (with the value for HMR in the ESC2 background taken as 1) in Fig. 2B.

As expected, in the ESC2 background, Sir2p-Myc was associated to various extents with silent loci but not active loci (Fig. 2B, compare open bars 0.5, 1.5, and 3.5, HML, HMR, and rDNA with ALD1 and ACT1). ESC2 deletion reduced the level of Sir2p-Myc associated with sequences near Tel VIR (Fig. 2B, compare open and shaded bars at 0.5, 1.5, and 3.5). The difference in Sir2p-Myc abundance at 0.5 or 3.5 is statistically significant as the p value (calculated using Student’s t test) is <0.01 or <0.03, respectively. This is consistent with the finding that esc2Δ decreases telomeric silencing (Fig. 1B). The p value for the data at 1.5 is <0.08 and therefore may not be statistically significant. We found that esc2Δ did not affect Sir2p-Myc abundance at silent HM and rDNA loci or active ALD1 and ACT1 loci (Fig. 2B).

Association of Esc2p with the Genome—It was previously shown that a GFP-Esc2p chimera was strictly localized in the nucleus (16). However, whether Esc2p is physically associated with the genome is not known. Here we tested if Esc2p could be cross-linked to various silent and active loci by ChIP. The C terminus of endogenous ESC2 in BY4741 was tagged with Myc to make strain YKA21. YKA21 was phenotypically indistinguishable from BY4741, and Esc2p-Myc was expressed as determined by Western blotting (data not shown). An anti-Myc antibody was used to perform ChIP in YKA21. The abundance of sequences from the transcriptionally silent Tel VIR (1.5 kb), HML, HMR, and rDNA loci as well as active ALD1, ACT1, and HHO1 were measured by PCR. Three independent experiments were performed, and the mean of data from all of the experiments was graphed. As shown in Fig. 2C, Esc2p-Myc appeared to associate with both the silent and active loci examined. The abundance of Esc2p associated with HML was apparently lower than that associated with the other loci tested. There did not seem to be a correlation between the abundance of Esc2p and the mode of its function (positive or negative) at a particular silent locus (Figs. 1B and 2C).

Role of ESC2 in Maintaining Silent Chromatin Structure at rDNA—Our finding that esc2Δ reduced silencing but did not alter Sir2p association with rDNA could be explained by assuming that esc2Δ induced other changes in rDNA chromatin. We tested this hypothesis by directly mapping chromatin at rDNA with MNase digestion followed by indirect end labeling. In strain JS125, a Ty1-URA3 construct was integrated at NTS2 (nontranscribed spacer 2) in the rDNA array (Fig. 3, top) (22). We mapped chromatin around the 5‘-flanking region of URA3 in JS125 as well as its sirΔ and esc2Δ derivatives. As shown in Fig. 3, sirΔ induced changes in a ~1.5-kb region spanning part of the URA3 coding sequence and its 5‘-flanking sequence (Fig. 3, compare lanes 3 and 4 with lanes 1 and 2; note that the relative intensities of bands a, c, and d are lower in lanes 3 and 4, but that of e is higher). This is in line with the fact that rDNA silent chromatin is SIR2-dependent (22). The profile of MNase-sensitive sites in the esc2Δ strain was different from that in wild
Regulation of Silent Chromatin by Esc2p

ESC2 Negatively Regulates the Stability of Silent Chromatin at HML—The enhancement of silencing at HMR and HML by esc2Δ (Fig. 1B) was not accompanied by an increase in the level of Sir2p associated with these loci (Fig. 2B) and might have resulted from changes in the structure and/or dynamics of silent chromatin. We tested this hypothesis by using a DNA topology-based assay to examine silent chromatin structure at HML in esc2Δ versus ESC2 strains. As the topology of eukaryotic DNA reflects the density and conformation of nucleosomes, the degree of negative supercoiling of chromosomal DNA can be used as a measure of the state of chromatin. We have previously developed a method to examine the topology of DNA at a particular locus by exciting the region of interest as a minichromosome circle via site-specific recombination in vivo and isolating the DNA circle whose supercoiling can be determined by gel electrophoresis in the presence of a DNA intercalator (Fig. 4A) (25).

We and others previously found that DNA circles excised from HML and HMR were more negatively supercoiled when these loci were silenced than when they were derepressed (25, 27, 33). We further showed that HML circles bearing the silencers maintained their high degree of negative supercoiling indefinitely during cell growth, whereas circles lacking silencers lost their high supercoiling as cells progressed through the cell cycle, which suggests that the structure of silent chromatin dissociated from silencers is subject to disruption by cell cycle dependent events (Fig. 4, B and C, top) (25). To test if ESC2 plays a role in silent chromatin structure at HML, we deleted it from strain YXB10 in which the modified HML locus, including the E and I silencers, was bracketed by two copies of Flp1p.

FIGURE 3. Effect of esc2Δ on silent chromatin structure at rDNA. Top, rDNA array in JS125 (ESC2 SIR2), YQY541 (sir2Δ), YQY495 (esc2Δ), and YQY618 (trp1Δ). The gene encoding the 35S precursor rRNA is indicated by a black arrow. NTS, nontranscribed spacer. In each strain, a Ty1-mURA3 silencing reporter was integrated at one of the nontranscribed spacers. Arrowhead, long terminal repeats of Ty1. The URA3 coding region is indicated by an open arrow. Bottom, mapping chromatins around the URA3 promoter, DNA isolated from MNase-treated chromatin in each strain was digested with AlwNI and then subjected to gel electrophoresis and Southern blotting using a probe abutting the AlwNI site within URA3. N, naked genomic DNA.

FIGURE 2. Esc2p is associated with the genome and regulates Sir2p association with silent loci. A, effect of esc2Δ on Sir2p association with chromatin. The abundance of the indicated sequences in YQY616 (ESC2 SIR2-Myc) or YQY616 (esc2Δ SIR2-Myc) was measured by PCR before (input) and after chromatin IP (α-Sir2p-Myc) with an α-Myc antibody. The gel picture of PCR products from one of three independent experiments is shown. The data were quantified and plotted in 8. The value for HMR in ESC2 cells was taken as 1. No Ab, samples from mock ChIP without using antibody. C, ChIP analysis of Esc2p association with the genome. Three independent ChIP experiments were performed with an α-Myc antibody in YKA21 carrying ESC2-Myc. Data from one experiment are shown on the left. Data were quantified and plotted on the right. The value for ACT1 was taken as 1.

type (WT) strain and that in the sir2Δ strain, suggesting that esc2Δ alters silent chromatin structure. For example, esc2Δ reduced the intensities of bands b and e while increasing the intensities of c and d (Fig. 3, compare lanes 5 and 6 with lanes 1 and 2). As a negative control, we showed that deletion of TRP1 that is not involved in rDNA silencing had no effect on chromatin structure at rDNA (Fig. 3; note that the profiles of MNase-sensitive sites in lanes 7 and 8 are similar to that in lanes 1 and 2). Therefore, esc2Δ induces changes in chromatin structure upstream of URA3, which correlates with a reduction in URA3 silencing at the rDNA locus (Fig. 1B).
Regulation of Silent Chromatin by Esc2p

**A**

ESC2 negatively regulates the stability of silent chromatin at HML. A, strategy for examining the topology of chromosomal DNA. Two FRT sequences (filled arrows) are inserted to flank the chromosome region of interest. Recombination between FRTs by the site-specific recombinase Flp1p excises the region as a minichromosome circle. After deproteinization, the topology of the DNA circle can be examined by electrophoresis in the presence of a DNA intercalator. Filled circles, nucleosomes. B and C, effect of esc2Δ on the supercoiling of HML DNA. Top, strategy for examining the topology of HML DNA with or without silencers. Two FRT sites are inserted in direct orientation at HML to flank a region that includes (B) or excludes (C) the silencers. Recombination between the FRTs leads to the excision of a minichromosome that has captured HML silent chromatin (labeled $SIR^+$). Cell cycle progression would convert the silenced $SIR^+$ circle free of silencers into an active circle (labeled $sir^-$) but does not affect the $SIR^-$ circles associated with silencers (25). The filled and shaded circles indicate nucleosomes in silent and active chromatin, respectively. Middle, cells of the indicated strains were grown in YPR to early log phase, at which time galactose was added. After 2.5 h of incubation, DNA was isolated and subjected to gel electrophoresis in the presence of 20 μg/ml chloroquine. Under this condition, more negatively supercoiled circles migrate more slowly. The positions of nicked (N) and linear (L) circles are indicated. Samples in lanes 1–7 were from YXB10, YQY537, YXB10s, YQY539, YXB6, YQY585, and YXB6s, respectively. Topoisomers of the HML circle associated with silent or active chromatin are labeled as $SIR^+$ or $sir^-$, respectively. A densitometer scan of each lane is shown at the bottom. The distribution center of topoisomers in each sample is indicated by a dot. The asterisk indicates a gel artifact. D, deletion of ESC2 enhanced the stability of HML silent chromatin. Top, scheme of the experiment. Silencer-free HML circles were excised from ESC2 (YXB6) or esc2Δ (YQY585) cells arrested in G1 phase by α-factor treatment. The purpose of exciting HML circles from non-cycling cells (arrested in G1) instead of asynchronously growing cells is to prevent the disruption of silent chromatin on the circle by cell cycle progression during the 2.5-h induction of circle excision. The supercoiling of the HML circles were then followed through subsequent cell growth in YPD. DNA samples taken at 0, 1, 2, 4, and 5 h after release from G1 arrest were subjected to electrophoresis in the presence of 16 μg/ml chloroquine.

recombination target (FRT), the recognition site for the site-specific recombinase Flp1p (Fig. 4B, top). Induction by galactose of a $P_{GAL}$-FLP1 gene resident elsewhere in the genome would lead to the expression of Flp1p and recombination between the FRT sites, resulting in the excision of HML as a closed minichromosome circle. After being deproteinized, the supercoiling of the circle can be examined by gel electrophoresis in the presence of the DNA intercalator chloroquine (Fig. 4B, lane 1). Deletion of $SIR3$, which abolishes HML silencing, reduced the negative supercoiling of HML circle by a linking number change ($ΔLk$) of 7 (Fig. 4B, compare the centers of topoisomer distributions in lanes 3 and 1; note that more negatively supercoiled topoisomers migrate more slowly under the conditions used). We found that esc2Δ did not affect the negative supercoiling of HML DNA in either the $SIR^+$ or $sir^-$ background (Fig. 4B, compare 2 with 1 and compare 4 with 3), suggesting that ESC2 does not play a key role in the steady state structure of HML silent chromatin that is associated with silencers.

We next examined whether esc2Δ affected the stability and dynamics of HML silent chromatin that was dissociated from the silencers. ESC2 was deleted from strain YXB6 in which the modified HML locus excluding silencers was bracketed by two FRTs, making strain YQY587 (Fig. 4C, top). Strains YXB6 and YQY587 were grown to log phase before being treated with galactose for 2.5 h to induce the excision of the HML circle.
During this 2.5-h incubation, the cells continued to grow, and a portion of the circles in YXB6 cells lost their silenced state, as evidenced by the appearance of a population of topoisomerase that resembled those in the sir2Δ strain (Fig. 4C, compare lanes 5 and 7). We found that esc2Δ markedly reduced the percentage of sir− topoisomers in HML circles (Fig. 4C, compare 6 and 5).

The above results suggest that esc2Δ enhances the stability of silent chromatin at HML so that it is more resistant to disruption. To test this notion further, we examined the kinetics of the conversion of silent chromatin on a silencer-free HML circle to active chromatin during cell cycle progression in strains YXB6 (ESC2) and YQY587 (esc2Δ). This was achieved by first excising the HML circles and then examining the topology of these circles during subsequent cell growth. To prevent cell cycle-induced disruption of silent chromatin within the 2.5-h galactose treatment for circle excision, we excised the circles from cells synchronized in G1 (by α factor treatment) instead of asynchronously logarithmically growing cells (Fig. 4D, lanes 1 and 2, note the absence of sir− circles). Cells were then shifted to fresh YPD medium without α factor, which inhibited pGAL-FLP1 and allowed cell cycle progression to resume (Fig. 4D, top). The fate of the HML circles was followed during further cell growth. In ESC2 wild type YXB6 cells, as expected, the proportion of topoisomers with high negative supercoiling (SIR+) characteristic of silent chromatin gradually decreased, whereas that with lower negative supercoiling (sir−) characteristic of active chromatin increased as a function of growth time (Fig. 4D, compare 3, 5, 7, and 9 with 1 and 11). The rate of conversion of the HML circles from the SIR+ to sir− state was significantly reduced by esc2Δ (Fig. 4D, compare lanes 4, 6, 8, and 10 with 3, 5, 7, and 9, respectively). These results indicate that ESC2 negatively regulates the stability of silent chromatin at HML, which is in accord with our finding that esc2Δ enhances HML silencing (Fig. 1B). As a negative control, we have previously shown that deletion of HHO1 or certain mutations of histone H4 did not affect the stability of silent chromatin at HML (34, 35).

A Putative SUMO-binding Motif of Esc2p Is Necessary and Sufficient for Interacting with Sir2p and SUMO—Esc2p has previously been shown to interact with Sir2p in a two-hybrid analysis (11). Here we used affinity pull-down to confirm the interaction between Esc2p and Sir2p in cells expressing Sir2p that is C-terminally tagged with the Myc epitope and Esc2p that is C-terminally tagged with the His6-HA-Protein A tandem purification tag (referred to here as His-HA for convenience). We showed that affinity pull-down of Esc2p-His-HA with nickel resin also brought down Sir2p-Myc (Fig. 5A, lane 2) but not untagged Sir2p (Fig. 5A, lane 3’). As a negative control, we showed that affinity pull-down of Cyt2p-His-HA did not bring down Sir2p-Myc (Fig. 5A, lane 4’; note that Cyt2p is cytochrome c1, heme lyase that is not linked to silencing or Sir2p), demonstrating that neither Cyt2p nor the His-HA tag alone interacts with Sir2p-Myc. These results further establish an in vivo interaction between Esc2p and Sir2p.

It has been shown that the N-terminal 195 residues of Esc2p are necessary and sufficient for its two-hybrid interaction with Sir2p (18). To gain insights into the mechanism of Esc2p-Sir2p interaction, we attempted to define the minimum sequence of Esc2p responsible for interacting with Sir2p. We made plasmids encoding a series of Esc2p fragments fused to the GBD (Fig. 6A, left). We showed that these fusion proteins were expressed by Western blotting using an anti-GBD antibody (Fig. 6B). The abilities of these Esc2p fragments to interact with GAD-Sir2p (Gal4p activation domain fused to Sir2p) were tested in the two-hybrid reporter strain P69−4a, which bears GAL1-HIS3 and GAL7-ADE2 in its genome (19). GAL-HIS3 is generally easier to activate than GAL7-ADE2; hence, HIS3 and ADE2 expressions are considered indicators of moderate and strong two-hybrid interactions, respectively (19).

Consistent with previous studies, we found that both full-length Esc2p and its N-terminal 1−195 sequence strongly interacted with Sir2p (Fig. 6A, robust growth of strains 2 and 3 on both −Ura−Leu−His+3AT and −Ura−Leu−Ade media), whereas its C-terminal 196−456 sequence did not interact with Sir2p (Fig. 6A, no growth of strain 6 on −Ura−Leu−His+3AT or −Ura−Leu−Ade), Esc2p(116−456) lacking the N-terminal 115 residues still interacted with Sir2p, albeit with a slightly lower efficiency (Fig. 6A, robust growth of strain 5 on −Ura−Leu−His+3AT but limited growth on −Ura−Leu−Ade). The above results suggest that the 116−195 sequence of Esc2p is responsible for binding Sir2p. In support of this, deleting 116−195 from Esc2p abolished its ability to interact with Sir2p (Fig. 6A, lack of growth for strain 13 on −Ura−Leu−His+3AT and −Ura−Leu−Ade), and Esc2p(116−195) per se interacted with Sir2p (Fig. 6A, 8). These results demonstrated that the 80-residue 116−195 region of Esc2p was necessary and sufficient for interacting with Sir2p. We dissected Esc2p(116−195) further and found that its N-terminal half, Esc2p(116−155), was neces-

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**FIGURE 5.** Esc2p directly interacts with Sir2p. **A**, Esc2p and Sir2p interaction in vivo. Proteins isolated from strain YJS537+ with (+Esc2p-His-HA) or without (−Esc2p-His-HA) the pBG-ESC2-His-HA plasmid were added to N’-nitroliotric acid resin. The resin was then washed and eluted by boiling. The input (lanes 1 and 2) and eluate (lanes 1’ and 2’) protein samples were subjected to SDS-PAGE and Western blotting. The blot was then separately probed with the α-Myc and α-HA antibodies. Only the relevant parts of the blots are shown. A similar experiment was done with YXB76 with untagged Esc2p-His-HA and Sir2p and were the same as those in lanes 2 and 2’, respectively. **B**, interaction between recombinant Esc2p and Sir2p. Recombinant GST-Sir2p or GST was pulled down by glutathione-Sepharose beads with bacteria lysate containing His-HA-Esc2p. After SDS-PAGE, GST-Sir2p and GST were detected by Coomassie staining, and His-HA-Esc2p was detected by immunoblotting with α-HA antibody.
sary and sufficient for interacting with Sir2p (Fig. 6A, 9 and 14). Finally, we showed that Esc2p-(116–135) interacted with Sir2p, and Esc2p deleted for 116–135 failed to interact with Sir2p (Fig. 6A, 11 and 16). Therefore, we have identified the 20-residue 116–135 sequence of Esc2p as necessary and sufficient for interacting with Sir2p.

It is interesting that the 116–135 sequence of Esc2p (116MKESVVEINSSEDLDEDKN135) mainly contains SBM1, one of its four SUMO-binding motifs. SBM1 (119VVEINSSEDLDED132) fits perfectly a SUMO-binding consensus sequence consisting of a hydrophobic core (V/I)(V/I)X(V/I) (where X can represent any residue) (VVEI in Esc2p) flanked by an acidic patch (ESDLDED) (36, 37). Although it was previously shown that Esc2p interacted with SUMO (Smt3p) in a two-hybrid analysis (15), the contributions of its SUMO-binding motifs, SBM1 through SBM4, to SUMO-binding has not been determined. To address this question, we tested the abilities of a group of Esc2p fragments to interact with SUMO in two-hybrid assays. As expected, full-length Esc2p interacted with SUMO (Fig. 6C, growth of strain 2 on -Ura -Leu -His + 3AT). Interestingly, Esc2p-(1–195) interacted with SUMO more strongly than intact Esc2p (Fig. 6C, note that growth of strain 2 on -Ura -Leu -His + 3AT). On the other hand, Esc2p-(196–456) did not interact with SUMO (Fig. 6C, 6). Therefore, the C terminus of Esc2p containing SBM2 through SBM4 and the two SUMO-like domains is not required for interacting with SUMO. In fact, this domain apparently had an inhibitory effect on SUMO interaction (Fig. 6C, compare 3

FIGURE 6. Esc2p interacts with Sir2p and SUMO through a SUMO binding motif. A, two-hybrid analyses of interactions between GAD-Sir2p and GBD-fused Esc2p alleles. The Esc2p alleles tested are shown on the left. The symbols used here are similar to those in Fig. 1A. Note that of the four SBMs of Esc2p, only SBM1 is indicated for clarity. Growth of the host P69-4x on -Ura -Leu -His + 3AT medium indicates positive interaction, and that on -Ura -Leu -Ade indicates strong interaction (19). Two independent transformants were tested for each two-hybrid analysis. B, Western blotting analysis of GBD-fused Esc2p alleles. Equal amounts of protein extracts from P69-4x containing individual plasmids 1–18 (A) were run on SDS-PAGE and probed with an anti-GBD antiserum. C, Esc2p-(116–135) is necessary and sufficient for SUMO-binding. Growth phenotypes of PJ69-4x bearing the indicated plasmids are shown. The four SBMs in Esc2p are indicated by diamonds. D, Esc2p-Sir2p interaction is independent of SIR3 and SIR4. Growth phenotypes of YLO65 (sir3Δ sir4Δ) bearing the indicated plasmids are shown.
and 2). It is possible that the SUMO-like domains (SD1 and SD2) interacted with the SBMs, thereby preventing the latter from binding to SUMO. We further demonstrated that Esc2p-(116–135), which contains SBM1, interacted with SUMO almost as efficiently as intact Esc2p, and Esc2p deleted for 116–135 failed to interact with SUMO (Fig. 6C, 11 and 16). Therefore, the 116–135 sequence of Esc2p is necessary and sufficient for binding SUMO.

To test if SBM1 was required for the ability of Esc2p to bind SUMO and/or Sir2p, we changed the two conserved valine residues in the essential hydrophobic core of SBM1 to alanine (\((20_{\text{VVE}}^{123} \rightarrow 20_{\text{AAE}}^{123})\)). We found that the mutant Esc2p(V120A, V121A) failed to interact with SUMO or Sir2p (Fig. 6, A and C, compare 18 with 2). Therefore, SBM1 of Esc2p is required for binding to both SUMO and Sir2p.

The above results raised the intriguing possibility that Esc2p binds to SUMOylated Sir2p via its SBM1. However, previous global analyses of protein SUMOylation in yeast failed to establish Sir2p as a SUMOylated protein (38, 39). On the other hand, there is evidence that Sir3p and Sir4p are SUMOylated (38, 39). Because Sir2p interacts with Sir3p and Sir4p, it is possible that Esc2p interacts with SUMOylated Sir3p or Sir4p, which in turn binds Sir2p. This contention predicts that Esc2p-Sir2p interaction is dependent on Sir3p or Sir4p. However, we found that the abilities of intact and truncated Esc2p alleles to bind Sir2p in two-hybrid assays were not abolished by sir3Δ sir4Δ (Fig. 6D), demonstrating that Esc2p could interact with Sir2p independently of Sir3p and Sir4p. In fact, sir3Δ sir4Δ enhanced the interaction between Sir2p and Esc2p-(116–456) or Esc2p-(116–195) as evidenced by the more robust growth of strains 5 and 8 on −Ura−Leu−Ade in the sir3Δ sir4Δ strain (Fig. 6D) than in the Sir+ strain (Fig. 6A). It is possible that Sir3p or Sir4p competes with GBD-Esc2p for binding to GAD-Sir2p in a two-hybrid analysis.

Recombinant Esc2p and Sir2p Interact Directly—To address whether protein SUMOylation is involved in the interaction between Esc2p and Sir2p, we asked if recombinant Esc2p and Sir2p could still interact. Using the GST pull-down assay, we showed that GST-Sir2p, but not GST, expressed in E. coli was able to interact directly with His-HA-tagged Esc2p that was also made in E. coli (Fig. 5B). Because E. coli lacks protein SUMOylation machinery, recombinant GST-Sir2p and His-HA-Esc2p must not be SUMOylated. The fact that GST-Sir2p interacts with His-HA-Esc2p demonstrates that SUMOylation is not required for Esc2p-Sir2p interaction in vitro, despite the fact that it is SBM1 of Esc2p that mediates the interaction with Sir2p in the two-hybrid assay (Fig. 6A).

Targeted Silencing Promoted by Esc2p Is Mediated by SBM1—Transcriptional silencing at HMR or HML is mediated by the E and I silencers (40, 41). The HMR-E silencer is composed of binding sites for ORC, Rap1p, and Abf1p that serve to recruit the Sir proteins (1). Deletion of any two or more of these sites abolishes HMR silencing. The requirement of the silencer elements for silencing can be bypassed by directly targeting Sir proteins to HMR-E (23, 42–44). For example, GBD-Sir1p could restore the silencing function of a defective HMR-E silencer whose Rap1p and Abf1p sites were replaced by Gal4p-binding sites (UASg) (23). It was previously shown that Esc2p was able to elicit such targeted silencing at HMR (45).

Here we set out to identify the minimum domain of Esc2p required for targeted silencing. Strain YSB35 has TRP1 inserted at HMR, where the Rap1p and Abf1p sites of HMR-E were replaced by three copies of UASg (Fig. 7, top). The mutant HMR-E (designated Aeb::G) in YSB35 was not able to silence TRP1 (Fig. 6, robust growth of strain 1 on −Ura−Trp) (23). As expected, expression of Esc2p, Sir1p, or the C terminus (amino
 Regulation of Silent Chromatin by Esc2p

FIGURE 8. The SBM1 of Esc2p is required for telomeric silencing but not cellular tolerance of genotoxic stress. Shown are growth phenotypes of strain YHK56 (esc2Δ) bearing plasmids 1′, 2′, or 18′ on −Leu, −Leu+FOA, and −Leu+MMS media. The Tel VII-URA3 construct in YHK56 is shown at the top.

acids 732–1358) of Sir4p fused to GBD restored TRP1 silencing (Fig. 7, lack of growth of 2, 19, and 20 on −Ura −Trp). We tested whether the GBD-fused Esc2p fragments used earlier in our two-hybrid assay were also able to restore TRP1 silencing. We found that Esc2p-(1–195) and Esc2p-(116–456) supported targeted silencing, whereas Esc2p-(1–115) did not (Fig. 7, 3–5), which suggests that the domain of Esc2p responsible for targeted silencing lies between residues 116 and 195. In support of this, we showed that Esc2p-(116–195) had robust targeted silencing activity, and Esc2p deleted for 116–195 lost the activity (Fig. 7, 8 and 13). Therefore, the 116–195 sequence of Esc2p is necessary and sufficient for targeted silencing. We further dissected the 116–195 fragment and found that its N-terminal half (residues 116–155) was necessary and sufficient for targeted silencing (Fig. 7, 9 and 14). Finally, we showed that Esc2p-(116–135) still had significant silencing activity, and Esc2p lacking 116–135 failed to support targeted silencing (Fig. 7, 11 and 16). Therefore, we have identified the 20-residue 116–135 sequence of Esc2p containing SBM1 as the domain responsible for targeted silencing. Moreover, we showed that mutating SBM1 of Esc2p greatly reduced its targeted silencing activity (Fig. 7, compare 18 with 2). Comparing Figs. 6A and 7, we noted a strict correlation between the Sir2p-binding and targeted silencing activities of the Esc2p alleles, which supports the notion that Esc2p induces targeted silencing by recruiting Sir2p.

The Function of Esc2p in Transcriptional Silencing Is Dependent on SBM1—To directly test whether SBM1 is necessary for the function of Esc2p in transcriptional silencing, we examined the effect of disrupting SBM1 by the V120A, V121A mutation on telomeric silencing. Plasmids 1′, 2′, and 18′, encoding GBD, GBD-Esc2p, and GBD-Esc2p(V120A, V121A), were transformed into the esc2Δ strain YHK56 that bears Tel VII-URA3 (Fig. 8, top). YHK56 expressing GBD had low URA3 silencing and was sensitive to MMS due to the lack of ESC2 (Fig. 8; note that cells bearing plasmid 1′ grew poorly on −Leu + FOA and −Leu + MMS). Expression of GBD-Esc2p restored URA3 silencing and tolerance of MMS (Fig. 8, compare 2′ and 1′), indicating that ectopically expressed GBD-Esc2p is able to suppress esc2Δ. On the other hand, GBD-Esc2p(V120A, V121A) rendered cells resistant to MMS but failed to restored URA3 silencing (Fig. 8, compare 18′ and 2′). Therefore, SBM1 is necessary for the role of Esc2p in telomeric silencing but not its role in cellular tolerance of genotoxic stress.

TABLE 2 Locus-specific effects of esc2Δ on different silent loci

| Silent locus | Effect on silencing | Effect on silent chromatin structure |
|-------------|---------------------|-------------------------------------|
| Telomere    | Reduced             | Reduced Sir2p association           |
| rDNA        | Reduced             | Altered chromatin structure, no change in Sir2p association |
| HML and HMR | Increased           | Increased stability, no change in Sir2p association |

DISCUSSION

Esc2p Differentially Modulates Transcriptionally Silent Chromatin at Telomeric, rDNA, and HM Loci—Esc2p is an interesting protein that contains two SUMO-like domains and four putative SUMO-binding motifs (Fig. 1A). It has been implicated in transcriptional silencing and DNA repair during replication (15–18), but the mechanisms of its actions, especially the roles of its SUMO domains and SBMs, have not been resolved. We show in this report that ESC2 is required for efficient telomeric and rDNA silencing but plays an inhibitory role in HM silencing, as summarized in Table 2. Silencing is mediated by silent chromatin that is associated with the Sir complex (at telomeric and HM loci) or RENT complex (at rDNA) (1). The mechanisms underlying the establishment and maintenance of silent chromatin at telomeric, rDNA, and HM loci are similar in principle but different in molecular details, which provides the basis for locus-specific regulation.

In accordance of its positive role in telomeric silencing, we demonstrated that ESC2 was involved in maintaining Sir2p association with chromatin near telomeres. It is interesting that ESC2 also negatively regulates telomere length. However, the lengths of yeast telomeres are maintained by a mechanism that is not dependent on telomeric silencing (46). Therefore, esc2Δ-induced telomeric shortening may or may not have a causal relationship with esc2Δ-induced reduction in telomeric silencing. In other words, ESC2 may have separate roles in telomere length maintenance and telomeric silencing. Known telomere length regulators include the telomerase complex; telomere-associated factors, such as Rap1p; DNA replication factors, such as Elg1p; and DNA repair factors (47, 48). Some of these factors also regulate telomeric silencing. Because Esc2p is involved in DNA damage repair during replication, it may regulate telomere length through a replication-mediated pathway, which is similar to negative regulation of telomere length by Elg1p (49).

Silencing at the rDNA locus is mediated by the RENT complex consisting of Sir2p, Net1p, and Cdc14p. We found that ESC2 is required for efficient silencing and the maintenance of silent chromatin structure at rDNA. Through interacting with Sir2p, Esc2p may play an auxiliary role in the formation and/or the stability of the RENT complex and/or its function in silent chromatin at the rDNA locus.

In contrast to its positive functions in telomeric and rDNA silencing, ESC2 has an inhibitory effect on silencing at HML and HMR. It is puzzling that ESC2 plays opposite roles in telomeric and HM silencing. This argues against the possibility that Esc2p regulates silencing by simply influencing the stability of the Sir complex that is an integral part of both telomeric and
HM silent chromatin. In line with this, we showed that Sir2p-Sir4p interaction is not affected by esc2Δ (supplemental Fig. S2). On the other hand, the mechanism of initiation of the formation of silent chromatin at telomeres is different from that at HM loci. Ku70-Ku80 and ORC are specifically involved in recruiting Sir complex to telomeric and HM loci, respectively. Deletion of ESC2 reduces the abundance of Sir2p at telomeric silent chromatin but not at the HM loci. It is possible that Esc2p is specifically required for the recruitment of the Sir complex to telomeres by Ku70-Ku80.

How does Esc2p inhibit HM silencing without affecting the association of Sir complex with these loci? We found that ESC2 negatively regulates the stability of silent chromatin at HML so that the Sir-dependent chromatin dissociated from silenters is more sensitive to disruption. We suggest that Esc2p-binding inhibits the function of SIR complex in maintaining silent chromatin. It is possible that this inhibitory function of Esc2p is linked to its role in cell cycle progression. We have previously shown that silent chromatin at HML is subject to disruption by cell cycle progression (25), which is consistent with the report that disturbing cell cycle progression enhances transcriptional silencing (50). It is possible that Esc2p is involved in a cell cycle-dependent process that induces modification and impairment of the SIR complex or silent chromatin. Because Esc2p is involved in mediating intra-S phase DNA damage checkpoint signaling and facilitating the progression of DNA replication in S phase (15, 17, 18), Esc2p may serve as a mediator between cell cycle signals and SIR complex in silent chromatin. Deletion of ESC2 would abrogate the connection between cell cycle and SIR complex, thereby reducing the disruptive effect of cell cycle progression on HM silent chromatin.

Although the above proposal explains why esc2Δ strengthens silent chromatin at HML, it is inconsistent with the finding that esc2Δ decreases telomeric silencing that is also dependent on the SIR complex. We think Esc2p also positively regulates telomeric silencing through a telomere-specific mechanism, such as the recruitment of SIR complex via the Ku70-Ku80 complex boud to the ends of chromosomes. Specifically, we propose that through binding Sir2p, Esc2p facilitates the interaction between the SIR complex and Ku70-Ku80. As such, esc2Δ hampers the recruitment of SIR complex to telomeres by Ku70-Ku80, which prevails over esc2Δ-induced enhancement of SIR function, leading to a net decrease in telomeric silencing.

In summary, our working model of the roles of Esc2p in transcriptional silencing posits that Esc2p negatively regulates the activity of SIR complex and positively regulates RENT function. In addition, Esc2p specifically facilitates the recruitment of SIR complex to telomeres by the Ku70-Ku80 complex. All of these functions of Esc2p in silencing are mediated by Esc2p-Sir2p interaction.

Esc2p Interacts with Sir2p and SLMO through a SLMO Binding Motif—Sir2p is an evolutionarily conserved protein deacetylase that plays important roles in transcriptional silencing and life span. It is known that Sir2p physically interacts with a number of proteins in yeast, including Sir4p, Ntz1p, Esc8p, and Mcm10p (6, 11, 12, 51). Sir2p-Sir4p and Sir2p-Ntz1p interactions are key to the formation of the Sir and RENT silencing complexes, respectively. How Sir2p interacts with any of its partners has not been revealed before. Sir2p was previously shown to interact with Esc2p in a two-hybrid assay (11). In this report, we demonstrated that Sir2p and Esc2p physically interacted in vivo in an affinity pull-down experiment (Fig. 5A). Using the two-hybrid assay, we showed that the 116–135 amino acid peptide of Esc2p containing its SBM1 (the N-terminal putative SUMO-binding motif) was necessary and sufficient for its interactions with both Sir2p and SUMO, and mutating SBM1 abolished these interactions and the ability of Esc2p to function in silencing. These results raise the possibility that Esc2p binds to SUMOylated Sir2p via SBM1. However, there is no definitive evidence for or against Sir2p SUMOylation (38, 39). Alternatively, Esc2p may bind Sir2p through a SUMOylated protein that interacts with Sir2p. Sir3p and Sir4p have been identified as SUMOylated proteins in genome-wide screens (38, 39). However, we showed that Sir2p-Esc2p interaction is SIR3- and SIR4-independent. Moreover, using a GST-pull-down assay, we found that recombinant Esc2p and Sir2p could directly interact (Fig. 5B). Therefore, Esc2p-Sir2p interaction does not require protein SUMOylation under the in vitro assay conditions used. This is despite the fact that the SBM1 of Esc2p is necessary and sufficient for interaction.

Esc2p SBM1 (119VVEI7535NSESDDLDED-132) fits perfectly the SUMO-binding consensus sequence composed of a hydrophobic core (V/I)V/I)(V/I)X(V/I) (VVEI) flanked by an acidic patch (ESDDLDED) (36). Such motifs exist in well characterized SUMO-binding proteins, such as RanBP2/Nup358 (52). The hydrophobic core and acidic residues of exemplary SBMs have been shown to interact with conserved hydrophobic and basic residues in SUMO, respectively (37, 53). Esc2p and its fission yeast ortholog Rad60 are founding members of the RENi family of proteins that contain SUMO-like domains (SDs) (13). Rad60, like Esc2p, also contains SBMs (14). There is evidence that the SDs and SBMs in Rad60 interact to mediate homodimerization (14). By analogy, SDs and SBMs in Esc2p might also mediate its self-association via SD-SBM interactions. However, intra- or intermolecular SD-SBM interactions of Esc2p (if they exist) are probably not involved in Esc2p-Sir2p interaction because we showed that Esc2p deleted for both of its SDs still interacted with Sir2p (Fig. 6A), and this two-hybrid interaction persisted in an esc2Δ background (supplemental Fig. S2).

It is possible that Esc2p directly interacts with Sir2p (that is not modified by SUMO) through its SBM1. The hydrophobic and acidic residues of SBM1 may interact with certain hydrophobic and basic residues in Sir2p. Along this line, the putative α-helix formed by the SBM1-containing Esc2p (116–133) has a patch of hydrophobic residues on one side and a patch of acidic residues on another side (supplemental Fig. S3), which bears resemblance to the amphipathic α-helix structure of the activation domain of a typical acidic activator (54). Because the amphipathic structure in an activator is usually responsible for binding co-activators (e.g. see Ref. 55), it is possible that the putative amphipathic α-helix of SBM1 interacts with Sir2p in a similar manner. Given that Sir2p and SUMO both can interact with Esc2p through SBM1, they may compete for Esc2p interaction. Esc2p may be directed to the silencing pathway through its interaction with Sir2p and to other activities, such as DNA repair, through interacting with other (SUMOylated) factors.
Although Esc2p interacts with Sir2p, it is not specifically enriched at any of the transcriptionally silent loci (Fig. 2B). It is possible that Esc2p only interacts with Sir2p molecules not associated with chromatin. In accord with this notion, we found that the interaction between Esc2p and Sir2p is enhanced by sirΔ3 sir4Δ (Fig. 6D) (data not shown). Association with “free” Sir2p may enable Esc2p to modulate the Sir2p pool in the cell, thereby influencing transcriptionally silent chromatin at different loci.

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