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A SUMO-Like Domain Protein, Esc2, Is Required for Genome Integrity and Sister Chromatid Cohesion in *Saccharomyces cerevisiae*

Tomoko Ohya,* Hirokazu Arai,* Yoshino Kubota,* Hideo Shinagawa*,† and Takashi Hishida*,[1]

**Research Institute for Microbial Diseases, Osaka University, Osaka, 565-0871 Japan and †BioAcademia, Ibaraki, Osaka, 565-0085 Japan*

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ABSTRACT

The *ESC2* gene encodes a protein with two tandem C-terminal SUMO-like domains and is conserved from yeasts to humans. Previous studies have implicated Esc2 in gene silencing. Here, we explore the functional significance of SUMO-like domains and describe a novel role for Esc2 in promoting genome integrity during DNA replication. This study shows that *esc2*Δ cells are modestly sensitive to hydroxyurea (HU) and defective in sister chromatid cohesion and have a reduced life span, and these effects are enhanced by deletion of the *RRM3* gene that is a Pif1-like DNA helicase. *esc2*Δ *rrm3*Δ cells also have a severe growth defect and accumulate DNA damage in late S/G_2. In contrast, *esc2*Δ does not enhance the HU sensitivity or sister chromatid cohesion defect in *mrc1*Δ cells, but rather partially suppresses both phenotypes. We also show that deletion of both Esc2 SUMO-like domains destabilizes Esc2 protein and functionally inactivates Esc2, but this phenotype is suppressed by an Esc2 variant with an authentic SUMO domain. These results suggest that Esc2 is functionally equivalent to a stable SUMO fusion protein and plays important roles in facilitating DNA replication fork progression and sister chromatid cohesion that would otherwise impede the replication fork in *rrm3*Δ cells.

POST-TRANSLATIONAL modification, including phosphorylation, ubiquitination, and other types of covalent protein modification, is an important mechanism for rapidly altering protein stability, activity, or localization (Schwartz and Hochstrasser 2003). The process/pathway of SUMOylation, which is mechanistically analogous to ubiquitination, requires a distinct group of SUMO-specific enzymes to covalently attach SUMO to its protein targets (Muller *et al.* 2001; Seeler and Dejean 2003; Johnson 2004). In contrast to ubiquitination, SUMOylation usually enhances the stability of protein targets or the formation of protein complexes and therefore plays a role in regulating multiple cellular processes, including subcellular localization, signal transduction, cell–cycle progression, and genome stability.

Saccharomyces cerevisiae ESC2 (*E*stablishment *s*ilent *c*hromatin *2*) was first identified as genes necessary for silencing at mating-type locus that, when mutated or deleted, give rise to a partial defect in gene silencing (Dhillon and Kamakaka 2000; Cuperus and Shore 2002; Andrulis *et al.* 2004). Sequence analysis showed that Esc2 includes two tandem C-terminal SUMO-like domains and an N-terminal polar low-complexity domain, and its domain architecture is conserved in fission yeast (Rad60) and humans (NIP45) (Novatchkova *et al.* 2005). Although *ESC2* is not essential for growth, *Schizosaccharomyces pombe rad60* is essential for growth and *rad60* mutants are hypersensitive to DNA damaging agents (Morishita *et al.* 2002; Boddy *et al.* 2003). The essential function of *S. pombe* Rad60 may be to regulate homologous recombination at stalled or collapsed DNA replication forks or to prevent cell cycle progression in cells with DNA damage (Miyabe *et al.* 2006; Raffa *et al.* 2006). Thus, since the functions known for Esc2 and Rad60 appear to be largely disparities, additional studies are needed to determine the functional similarities and/or differences between *S. cerevisiae* Esc2 and *S. pombe* Rad60.

Tightly bound proteins or protein complexes and aberrant DNA structures can impede progression of the replication fork during S phase. Cells utilize several mechanisms, including DNA repair, DNA damage tolrerance, and DNA damage checkpoint pathways, to overcome such impediments and resume cell cycle progression (Cox *et al.* 2000; Barbour and Xiao 2003). Recent studies in yeast show that a Pif1-like 5′ to 3′ DNA helicase called Rrm3 facilitates restart of replication forks blocked by stable protein-DNA complexes (Ivessa *et al.* 2003). Cells that lack Rrm3 grow normally and are resistant to DNA damaging agents; however DNA replication is less processive due to frequent pausing, and an intra-S-phase/DNA damage checkpoint is activated in *rrm3*Δ cells (Torres *et al.* 2004; Azvolinsky *et al.* 2006). *rrm3*Δ is also a synthetic lethal with *mrc1*Δ, a claspin-like protein that is required for S-phase checkpoint activation and to stabilize stalled

[1]*Corresponding author:* Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail: hishida@biken.osaka-u.ac.jp

replication forks (Osborn and Elledge 2003; Torres *et al.* 2004; Szyjka *et al.* 2005). The replication function, but not the checkpoint function of Mrc1 is essential in *rrm3*Δ cells, because *mrc1-AQ*, which selectively inactivates Mrc1-dependent checkpoint activation, does not inhibit cell growth in *rrm3*Δ cells (Szyjka *et al.* 2005). These results suggest that Rrm3 and Mrc1 play distinct roles in rescuing stalled DNA replication forks.

This study characterized the *in vivo* function of the SUMO-like protein Esc2. The results demonstrate that deletion of both SUMO-like domains of Esc2 destabilizes and functionally inactivates Esc2, but one SUMO or one SUMO-like domain is sufficient to restore its stability. *esc2*Δ *rrm3*Δ double mutants show a severe growth defect and have a high rate of endogenous DNA damage and a defect in sister chromatid cohesion. The Esc2 SUMO-like domain is also likely to be involved in modulating the function of Mrc1 at stalled replication forks. These data suggest that Esc2 plays important roles not only in gene silencing but also in facilitating DNA replication fork progression and sister chromatid cohesion.

MATERIALS AND METHODS

Strains and plasmids: All yeast strains used in this study are listed in Table 1. The 2.6-kb *Pst*I–*Pst*I genomic fragment containing *ESC2* was cloned into pUC19. The *esc2* mutants described below were obtained from pUC19-ESC2 by site-directed PCR mutagenesis. *esc2*Δ*sd1* was constructed by insertion of *Spe*I sites at the 195th and 296th amino acids, digestion with *Spe*I, and ligation. *esc2*Δ*sd2* and *esc2*Δ*C* were constructed by inserting a stop codon at the 375th or 195th amino acids, respectively. *esc2*Δ*C-SUMO* and *esc2*Δ*sd2-SUMO* were constructed by inserting *Spe*I sites at the positions encoding the 195th and 375th amino acids, digesting with *Spe*I, and then ligating with the *S. cerevisiae* SUMO gene. The C-terminal four amino acids of full-length Smt3, including the Gly-Gly found at the C terminus of mature Smt3, were not included in these constructs to prevent covalent attachment of SUMO to other protein targets. Wild-type *ESC2* and *esc2* mutants were tagged with an HA cassette at the NH_2 terminus. The *Pst*I–*Pst*I fragments of *ESC2* and *esc2* mutants containing the promoter and open reading frame were cloned into pRS306 or pRS426 for genome replacement and overexpression studies, respectively. pRS306-series plasmids were digested with *Sph*I and integrated into the genome. The resultant strains were then plated onto SC plates containing 5-fluoroorotic acid to select for *ura*$^-$ cells. The *Escherichia coli* overexpression plasmids were constructed by ligating the *Nde*I–*Bam*HI fragment of *ESC2* and *esc2* mutants tagged with the HA cassette into pET3a. pRS415-GFP-RAD52 was constructed by ligating the *Sal*I–*Sal*I *RAD52* fragment of pSC52 (Hishida *et al.* 2002) into pRS415 and inserting a GFP cassette at its NH_2 terminus.

Yeast two-hybrid assay: Gal4-based Matchmaker Two-Hybrid System 3 (Clontech) was used for the yeast two-hybrid assay. The Sir2 protein was fused to the Gal4 activation domain in pGADT7 vector and the Esc2 protein and several *esc2* mutant proteins were fused to the Gal4 DNA-binding domain in pGBKT7, and expressed in *S. cerevisiae* tester strain AH109.

Preparation of yeast extracts and Western blotting: Total protein extract was prepared from 5×10^6 cells from logarithmically growing culture using the trichloroacetic acid (TCA) method described by Pellicioli *et al.* (1999). Proteins were analyzed by SDS–PAGE, transferred to PVDF membranes, and probed with anti-HA or -Myc monoclonal antibody (Roche). Detection was performed with HRP-conjugated secondary antibodies followed by treatment using the ECL advance Western blot detection kit (BD Biosciences).

Immunoprecipitation: Cell cultures ($1–2 \times 10^7$ cells/ml) were collected by centrifugation and washed once with lysis buffer (10% glycerol, 50 mm Tris-HCl at pH 7.5, 150 mm NaCl, 1 mm EDTA, 0.1% NP-40, 1 mm PMSF, Protease Inhibitor Cocktail) (Sigma). Cells were resuspended in the same volume of lysis buffer, and 2 volumes of glass beads were added. Cells were disrupted using a Bead Beater (BIOSPEC) for 1 min, four times. After centrifugation, the supernatant fraction was collected and used for immunoprecipitation. For immunoprecipitation, 40 μl of anti-HA agarose conjugate beads (Sigma) were used for 1 ml of cell lysate and the mixture was rotated for 2 hr at 4°. The beads were washed four times, resuspended in sample buffer, and boiled for 3 min. Samples were analyzed by SDS–PAGE and signals were detected by Western blot analysis (BD Biosciences).

Silencing assay: *HMR::ADE2* silencing was performed as described previously (Chi and Shore 1996; Dhillon and Kamakaka 2000). Isogenic strains were grown in YPD culture to early logarithmic phase ($2–5 \times 10^6$ cells/ml) at 30°, and serial dilutions were plated on YPD plates to obtain well-dispersed, discrete colonies. Plates were incubated at 30° for 3 days and then 4° for 7 days until colony color (red, white, pink, or sector) could be distinguished and then photographed.

Senescence analysis: Senescence was analyzed by counting the number of replicative cycles before cessation of cell division, as described previously (Kennedy *et al.* 1994).

Other materials and methods: Fluorescence-activated cell sorting (FACS), microscopy, and sister chromatid cohesion assay were performed as described previously (Hishida *et al.* 2002, 2006; Xu *et al.* 2004).

RESULTS

Construction of *esc2* truncation mutants: Esc2 has a N-terminal low complexity polar region enriched in positively and negatively charged residues and a C-terminal globular region with two SUMO-like domains, SD1 and SD2 (Figure 1). To examine the biological functions of Esc2 and the specific roles of SD1 and SD2, expression constructs for a series of domain truncation mutants of Esc2 were generated. Two domain substitution mutants were also generated, in which a SUMO domain replaced SD2 or SD1/2 of Esc2. These truncated forms of Esc2 are shown schematically in Figure 1.

Interaction between Sir2 and Esc2 truncation mutants: Previous two-hybrid studies showed that Esc2 interacts with Sir2, implicating Esc2 in gene silencing (Cuperus and Shore 2002). Here, the physical interaction between Esc2 and Sir2 was mapped by performing yeast two-hybrid assays in which the "bait" domain included truncated derivatives of Esc2 fused to the Gal4 DNA binding domain (Gal4 BD). These fusion proteins were expressed in yeast on a multi-copy plasmid from an *ADH1* promoter and detected in crude extract by Western blot analysis (data not shown). The two-hybrid assay demonstrated that Esc2ΔC lacking both SD1 and SD2 still interacts with Sir2, but Esc2ΔN lacking the N-terminal domain does not (Figure 2A),

TABLE 1

Strains used in this study

Strain	Genotype	Source
W1588-4A	W303-1A (*MAT***a**), *RAD5*	McDonald *et al.* (1997)
W1588-4B	W303-1B (*MAT*α), *RAD5*	McDonald *et al.* (1997)
YTO571	W1588-4A, HA-*ESC2*	This study
YTO665	W1588-4A, HA-*esc2*Δ*sd1*	This study
YTO575	W1588-4A, HA-*esc2*Δ*sd2*	This study
YTO613	W1588-4A, HA-*esc2*Δ*C*	This study
YTO708	W1588-4A, HA-*esc2*Δ*sd2-SUMO*	This study
YTO610	W1588-4A, HA-*esc2*Δ*C-SUMO*	This study
YTO13	W1588-4A, *esc2*Δ*::kanMX4*	This study
YLS586	W303-1B, *hmr*Δ*B::ADE2*	Chi and Shore (1996)
YTO549	YLS586, *esc2*Δ*::kanMX4*	This study
YTO670	YLS586, *esc2*Δ*sd1*	This study
YTO550	YLS586, *esc2*Δ*sd2*	This study
YTO731	YLS586, *esc2*Δ*N*	This study
YTO619	YLS586, *esc2*Δ*C*	This study
YTO620	YLS586, *esc2*Δ*C-SUMO*	This study
YTO642	W1588-4A, *esc2*Δ*::HIS3*	This study
YTO646	W1588-4A, *sir2*Δ*::kanMX4*	This study
YTO647	W1588-4A, *esc2*Δ*::HIS3 sir2*Δ*::kanMX4*	This study
YTO533	W1588-4A, *rrm3*Δ*::ADE2*	This study
YTO655	W1588-4A, *rrm3*Δ*::URA3*	This study
YTO739	W1588-4A, *sir2*Δ*::kanMX4 rrm3*Δ*::URA3*	This study
YTO541	W1588-4A, *esc2*Δ*::kanMX4 rrm3*Δ*::ADE2*	This study
YTO164	W1588-4A, *esc2*Δ*sd2*	This study
YTO543	W1588-4A, *esc2*Δ*sd2 rrm3*Δ*::ADE2*	This study
YTO51	W1588-4A, *rad51*Δ*::URA3*	This study
YTO693	W1588-4A, *mrc1*Δ*::kanMX4*	This study
YTO695	W1588-4A, *esc2*Δ*::HIS3 mrc1*Δ*::kanMX4*	This study
YPH1444	*MAT***a** *ade2 his3 trp1 ura3 leu2 can1 lacI-NLS-GFP::HIS3 lacO::URA3::CEN15*	Xu *et al.* (2004)
YTO536	YPH1444, *esc2*Δ*::kanMX4*	This study
YTO534	YPH1444, *rrm3*Δ*::ADE2*	This study
YTO535	YPH1444, *esc2*Δ*::kanMX4 rrm3*Δ*::ADE2*	This study
YTO540	YPH1444, *sir2*Δ*::kanMX4*	This study
YTO701	YPH1444, *mrc1*Δ*::kanMX4*	This study
YTO702	YPH1444, *esc2*Δ*::LEU2 mrc1*Δ*::kanMX4*	This study
YTO703	YPH1444, *rrm3*Δ*::ADE2 sir2*Δ*::kanMX4*	This study
TH500	W1588-4A, *RAD53-myc.kanMX4*	This study
YTO675	TH500, *esc2*Δ*::HIS3*	This study
YTO676	TH500, *esc2*Δ*sd2*	This study
YTO728	TH500, *esc2*Δ*sd2-SUMO*	This study
YTO721	TH500, *rrm3*Δ*::ADE2*	This study
YTO678	TH500, *esc2*Δ*::HIS3 rrm3*Δ*::ADE2*	This study
YTO679	TH500, *esc2*Δ*sd2 rrm3*Δ*::ADE2*	This study
YTO729	TH500, *esc2*Δ*sd2-SUMO rrm3*Δ*::ADE2*	This study
YTO740	TH500, *sir2*Δ*::HIS3*	This study
YTO741	TH500, *sir2*Δ*::HIS3 rrm3*Δ*::ADE2*	This study

indicating that the N-terminal domain of Esc2 is responsible for its interaction with Sir2.

Domains of Esc2 required for silencing at the *HMR* locus: To next determine which domains of Esc2 are required for silencing, a functional screen for *ESC2* was developed on the basis of an *ADE2* reporter gene at *HMR*. In this screen, wild-type *ESC2* silences the *ADE2* reporter at *HMR*, giving rise to red colonies, and null or partial *esc2* function yields white, pink, and/or sectored colonies (Dhillon and Kamakaka 2000; Cuperus and Shore 2002). *esc2* mutants were integrated into the genome at the *ESC2* locus under control of the native promoter and screened for function. The results indicate strong silencing of *ADE2* and mostly red colonies in cells expressing wild-type *ESC2* and truncated *esc2* lacking SD1 or SD2 (*i.e.*, *esc2*Δ*sd1* or *esc2*Δ*sd2*) (Figure 2, B and C). In contrast, expression of *esc2*Δ*N*, *esc2*Δ*C*, or *esc2*Δ led to a large increase in the fraction of pink and

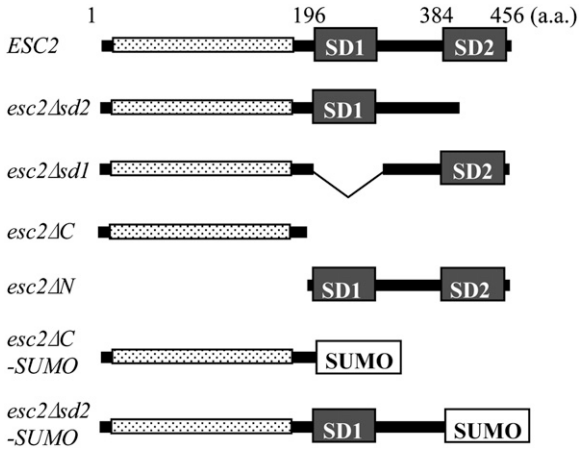

FIGURE 1.—Schematic of Esc2 truncation mutants. The domains of Esc2 are shown schematically. The N-terminal low complexity region with polar and charged residues is represented by the stippled bar; the C-terminal globular region has two SUMO-like domains, indicated as SD1 and SD2. The SUMO domain is encoded by *SMT3* gene.

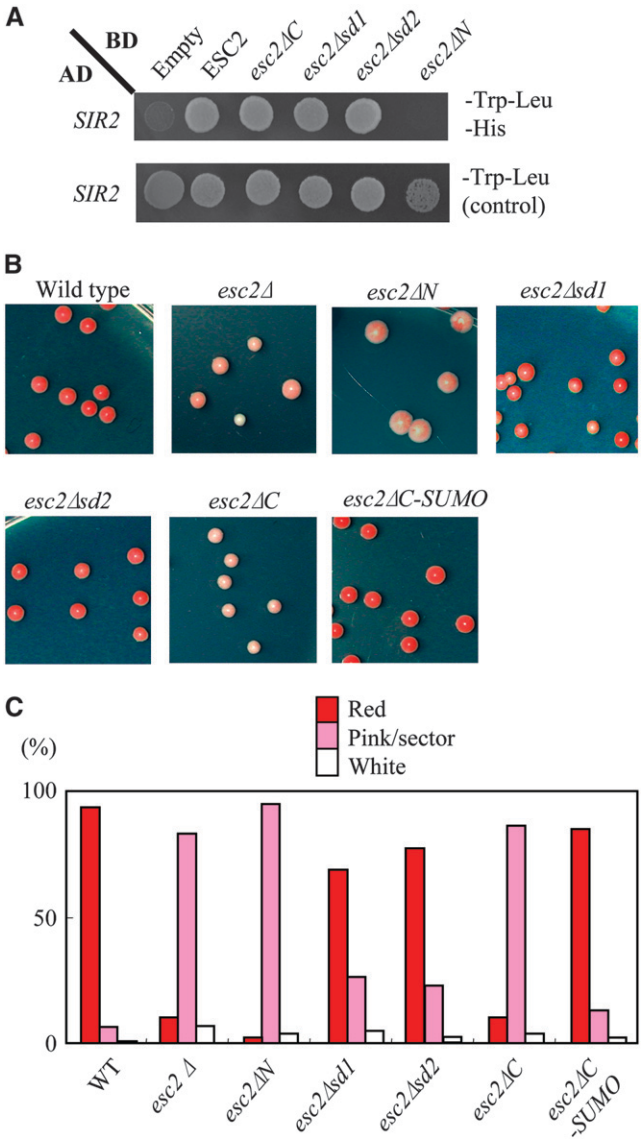

FIGURE 2.—N-terminal region of Esc2 is required for gene silencing. (A) Two-hybrid assays were conducted with wild-type Esc2 or truncated versions of Esc2 and Sir2, as described in MATERIALS AND METHODS. Positive interactions were detected by growth on SC −Trp −Leu −His plates. (B) A colorimetric assay was used to evaluate *HMR::ADE2* silencing in isogenic strains bearing either wild type, *esc2*Δ, *esc2*Δ*N*, *esc2*Δ*sd1*, *esc2*Δ*sd2*, *esc2*Δ*C*, or *esc2*Δ*C-SUMO*. Cells were grown to log phase, serially diluted, and plated on YPD plates. Plates were incubated at 30° for 3 days and at 4° for 7 days. (C) The colonies were scored as solid red (silenced), red with white sectors (reduced silencing), or white (no silencing). More than 200 individual colonies were scored for each strain.

white colonies and a large decrease in the fraction of red colonies (Figure 2, B and C). Interestingly, the level of *ADE2* silencing and fraction of red colonies was very similar in cells expressing *ESC2* and *esc2*Δ*C-SUMO*, in which SD1/2 was replaced with one SUMO protein. These results suggest that the N-terminal region of Esc2 and at least one SUMO-like or SUMO domain are required for Esc2-mediated silencing at *HMR*.

The SUMO-like domain is required for Esc2 stability: The above results suggest that different domains of Esc2 are required for interacting with Sir2 and for functional silencing of *ADE2* at *HMR*. However, these data could also reflect differential levels of expression or stability of *esc2* truncation mutants. This possibility was addressed by quantifying expression of genomic *esc2* truncation mutants carrying an N-terminal HA epitope tag. The results of this analysis are shown in Figure 3. Note that wild-type HA-Esc2 had an apparent electrophoretic mobility of 85 kDa, which is larger than its predicted size of 60 kDa. Similar results were observed when HA-Esc2 was expressed in *E. coli* (data not shown) and therefore may be due to the high density of charged amino acids in the Esc2 N-terminal domain. Wild-type Esc2 and all of the Esc2 derivatives were stable and were expressed at a similar level except Esc2ΔC, whose expression was not detected in crude-cell extracts (Figure 3A) or was weakly detected with a mobility of ~45 kDa in immunoprecipitates of whole-cell extracts (Figure 3B). In contrast to Esc2ΔC, Esc2ΔC-SUMO expression was detected in both crude extract and immunoprecipitates of whole-cell extracts although the relative expression level was slightly reduced in crude extracts as compared with other HA-tagged Esc2 proteins (Figure 3, A and B). These data suggest that Esc2ΔC is intrinsically unstable and that one C-terminal SUMO or SUMO-like domain is sufficient to stabilize Esc2ΔC. This is consistent with the observation above that *esc2*Δ*C*-SUMO silenced *ADE2* at *HMR* as effectively as *ESC2*.

Esc2 is involved in cell life span: Previous studies show that *sir2*Δ cells have a shorter life span and reach senescence faster than wild-type cells (KAEBERLEIN *et al.* 1999). Here, the effect of *esc2*Δ on yeast life span was

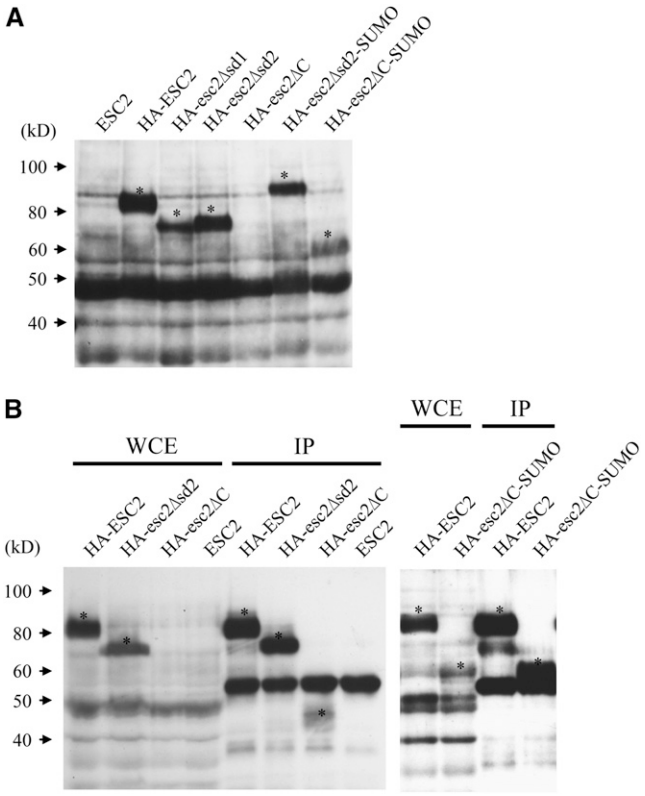

FIGURE 3.—Expression of HA-tagged Esc2 truncation mutants. (A) Protein extracts were prepared from the indicated strains. Samples were analyzed by SDS–PAGE followed by Western blotting with anti-HA antibody. (B) Crude extracts were immunoprecipitated and Western blotted with anti-HA antibody as described in MATERIALS AND METHODS. HA-tagged Esc2 proteins are indicated by an asterisk.

measured by counting the number of daughter cells generated from an individual mother cell (KENNEDY *et al.* 1994). The results show that the mean life span for *esc2*Δ cells is 9.8 generations, while the mean life span of wild-type yeast is 19.0 generations (Figure 4A), indicating that mutation of *ESC2* causes a shortening of life span relative to wild type. *sir2*Δ cells also have a short life span (mean of 13.5), but young *esc2*Δ cells have higher mortality than *sir2*Δ cells (Figure 4A). *esc2*Δ *sir2*Δ cells have an even shorter mean life span of 5.2 generations (Figure 4A), suggesting that *esc2* and *sir2* are not epistatic with respect to life span. These results suggest that Esc2, like Sir2, plays a role in yeast senescence, but that these genes act independently on life span.

Additional roles for Esc2 in yeast: Rrm3 is a member of the Pif1 family of DNA helicases, a family that is highly conserved from yeasts to humans (BOULE and ZAKIAN 2006) and its helicase activity is required for efficient replication past specific, particularly stable chromatin-associated complexes (TORRES *et al.* 2004; AZVOLINSKY *et al.* 2006). This study and previous studies have shown that *esc2*Δ *rrm3*Δ double mutants have a severe growth defect (Figure 5A) (TONG *et al.* 2004). In contrast to *esc2*Δ *rrm3*Δ cells, we found that *sir2*Δ *rrm3*Δ cells have no growth defect (Figure 5B), suggesting that severe growth defect of *esc2*Δ *rrm3*Δ cells is not due to a silencing defect. Moreover, the *esc2*Δ *rrm3*Δ double mutant has a significant reduction in mean life span (2.3 generations) compared to either single mutant (Figure 4B). The majority of *esc2*Δ *rrm3*Δ cells cease cell division as large-budded cells in G_2 at the end of their life span, whereas the terminal phenotype of wild-type or *esc2*Δ *sir2*Δ cells are usually as unbudded G_1 cells (data not shown). In addition, a comparison of the mortality rates of the early generations (~10 generations) shows that *esc2*Δ *rrm3*Δ cells have a significantly increased mortality compared to *esc2*Δ cells, even though *rrm3*Δ cells have similar mortality rates as wild type (Figure 4B). In contrast, *sir2*Δ mutation has no apparent effect on the mortality rates of the early generations of *esc2*Δ cells (Figure 4A). Thus, the synergistic reduction in the life span of *esc2*Δ *rrm3*Δ mutants supports the notion that Esc2 have an additional function other than its silencing function.

Cell cycle progression in *esc2*Δ *rrm3*Δ cells was evaluated by analyzing the DNA content of early logarithmic phase asynchronous cultures grown at 30°. The results indicated a normal cell cycle progression in wild-type and *esc2*Δ cells, a modest increase in *rrm3*Δ cells with 2C DNA content, and significant accumulation of *esc2*Δ *rrm3*Δ cells with 2C DNA content (Figure 5C). Furthermore, the fraction of large-budded cells was ~40, 40, 49, and 70% in wild-type, *esc2*Δ, *rrm3*Δ, and *esc*Δ *rrm3*Δ cells, respectively (Figure 5D). Most of the large-budded *esc2*Δ *rrm3*Δ cells had one nucleus at the single bud neck (Figure 5D). These results suggest that cell cycle progression through S/G_2 is delayed in *esc2*Δ *rrm3*Δ cells.

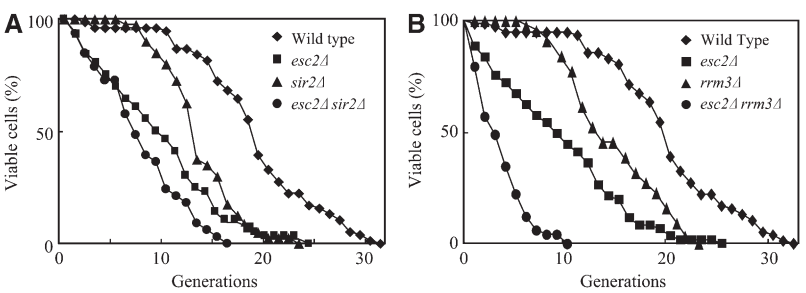

FIGURE 4.—*esc2*Δ cells show the reduced life span. (A) Life span was measured for wild-type, *esc2*Δ *sir2*Δ, and *esc2*Δ *sir2*Δ cells. Mean number of replicative cycles is as follows: Wild type, 19.0; *esc2*Δ, 9.8; *sir2*Δ, 13.5; *esc2*Δ *sir2*Δ, 5.2. (B) Life span was measured for wild-type, *esc2*Δ *rrm3*Δ, and *esc2*Δ *rrm3*Δ cells. The data of wild-type and *esc2*Δ cells are the same as in Figure 4A. Mean number of replicative cycles is as follows: Wild type, 19.0; *esc2*Δ, 9.8; *rrm3*Δ, 13.5; *esc2*Δ *rrm3*Δ, 2.3.

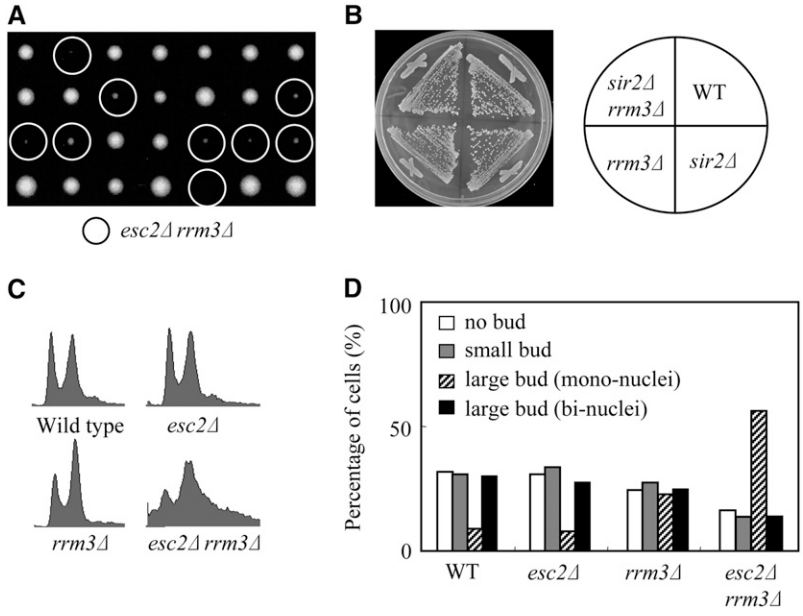

FIGURE 5.—Cells lacking Esc2 and Rrm3 exhibit a severe growth defect. (A) Tetrads of heterozygous diploid cells formed by the crosses indicated were dissected and grown on YPD at 30° for 3 days. (B) Yeast strains of indicated genotypes were streaked out on YPD plate and grown at 30° for 3 days. (C) S/G_2 cell cycle delay of *esc2*Δ *rrm3*Δ cells. FACS analysis of the DNA content of log-phase cells. Cells were grown to early logarithmic phase at 30° and DNA content of asynchronous cultures of the indicated strains was measured by FACS. (D) Mutants were grown to early logarithmic phase and stained with DAPI. Cells were analyzed microscopically for the presence of single cells, small-budded cells, and large-budded (mononucleated or binucleated) cells.

Accumulation of DNA damage and activation of the S/G_2 checkpoint in *esc2*Δ *rrm3*Δ cells: Cells with defects in S-phase progression are often hypersensitive to HU, which inhibits DNA replication. Thus, the relative sensitivity of *esc2*Δ, *rrm3*Δ, and *esc2*Δ *rrm3*Δ cells to HU was evaluated. The results showed that *esc2*Δ and *rrm3*Δ cells were modestly sensitive to HU, but *rrm3*Δ *esc2*Δ cells were hypersensitive to HU (Figure 6A). Rad53 phosphorylation was also evaluated as a marker for induction of the DNA damage/replication checkpoint. An electrophoretic mobility shift assay showed that a low level of slow migrating phosphorylated Rad53 accumulates in *rrm3*Δ cells as reported previously (TORRES *et al.* 2004), but a higher level of hyperphosphorylated Rad53 accumulates in *rrm3*Δ *esc2*Δ cells (Figure 6B), suggesting a higher-than-normal level of spontaneous DNA damage in these cells. In addition, slow-migrating hyperphosphorylated Rad53 was not detected in *rrm3*Δ *sir2*Δ cells (Figure 6B). These data suggest that Esc2 is involved in tolerating spontaneous DNA replication problems such as stalled replication forks.

The Rad53 activation suggests that *esc2*Δ *rrm3*Δ cells accumulate spontaneous DNA damage. To test this possibility, we examined GFP-Rad52 foci formation in *esc2*Δ *rrm3*Δ cells because Rad52 forms discrete foci at sites of DNA damage in replicating cells (LISBY *et al.*

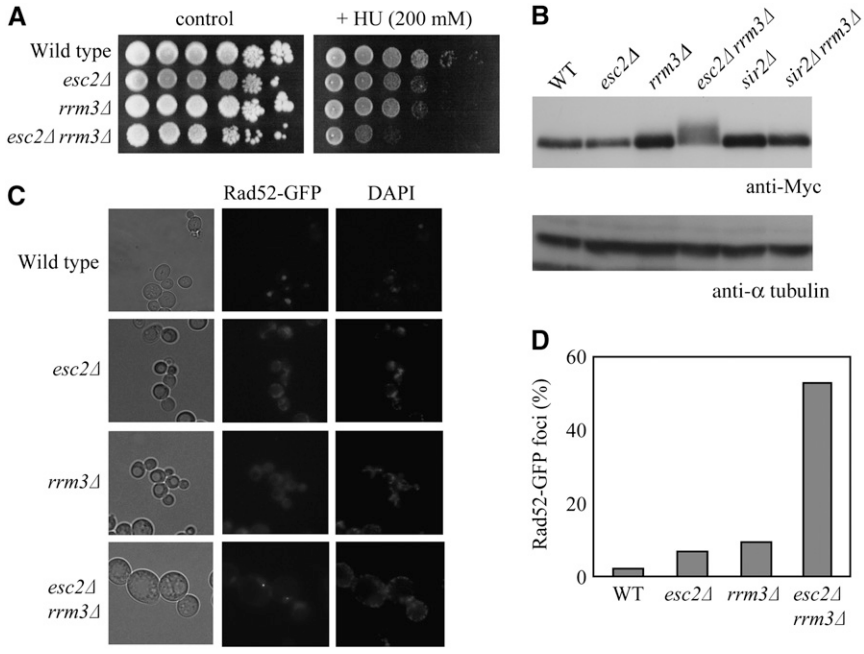

FIGURE 6.—Constitutive hyperphosphorylation of Rad53 and accumulation GFP-Rad52 foci in *esc2*Δ *rrm3*Δ cells. (A) *esc2*Δ *rrm3*Δ cells are sensitive to HU. Serial dilutions of the indicated strains were spotted onto selective media containing 200 mM HU and incubated at 30° for 4 days. (B) Phosphorylation of Rad53 increases in *esc2*Δ *rrm3*Δ cells. Cells were grown to early logarithmic phase (2–5 × 10^6 cells/ml). The indicated strains were harvested and protein extracts were prepared. Rad53 protein phosphorylation was analyzed by 6% SDS–PAGE followed by Western blotting using anti-Myc antibody. α-Tubulin was used as a loading control. (C) The number of GFP-Rad52 foci increases in *esc2*Δ *rrm3*Δ cells. Wild-type, *esc2*Δ *rrm3*Δ, and *esc2*Δ *rrm3*Δ cells containing pGFP-RAD52 were grown to early logarithmic phase and then examined by fluorescence microscopy. (D) The number of GFP-RAD52 foci were counted. At least 100 cells were examined for each strain.

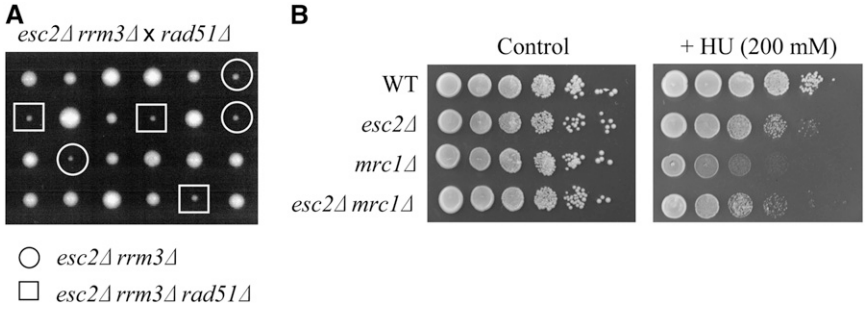

FIGURE 7.—*ESC2* interacts genetically with *MRC1* (A) The *rad51*Δ mutation does not suppress the growth defect of *esc2*Δ *rrm3*Δ cells. Tetrads of diploid cells formed by the crosses indicated were dissected and grown on YPD at 30° for 3 days. (B) *esc2* mutation is epistatic to *mrc1* mutation with regard to HU sensitivity. Serial dilutions of the indicated strains were spotted onto selective media containing the 200 mM HU and incubated at 30° for 3 days.

2001), and these foci can be readily visualized by fluorescence microscopy in cells expressing GFP-Rad52. GFP-Rad52 functions normally *in vivo*, since it fully complements the repair deficiency of a *RAD52* deletion strain (data not shown). In a logarithmically growing culture, very few Rad52 foci are visible in wild type. Mutations of *ESC2* or *RRM3* cause a slight increase of spontaneous Rad52 foci (Figure 6, C and D). However, a significant number of Rad52 foci are detected in *esc2*Δ *rrm3*Δ cells (Figure 6, C and D), and most of the foci occur in large-budded cells with a single nucleus, while only a few Rad52 foci occur in unbudded cells (data not shown). These results indicate that *esc2*Δ *rrm3*Δ cells accumulate DNA damage in S/G_2 and spontaneously activate the DNA damage checkpoint.

***ESC2* interacts genetically with *MRC1*:** Previous studies have shown that the synthetic lethality of *rrm3*Δ *sgs1*Δ and *rrm3*Δ *srs2*Δ cells is suppressed by deletion of *RAD51* or *RAD52* (SCHMIDT and KOLODNER 2004; TORRES *et al.* 2004), suggesting that toxic recombination intermediates accumulate and cause cell death in these cells. However, a different mechanism may lead to poor growth in *esc2*Δ *rrm3*Δ cells, because deletion of *RAD51* does not suppress their severe growth defect (Figure 7A). Like *esc2*Δ *rrm3*Δ cells, deletion of *RAD51* also fails to suppress the synthetic lethality of *mrc1*Δ *rrm3*Δ mutants (TORRES *et al.* 2004). We, therefore, examined the genetic interaction between *esc2*Δ and *mrc1*Δ. Interestingly, *esc2*Δ *mrc1*Δ double mutants are not severely defective for growth, and deletion of *ESC2* does not exacerbate the HU sensitivity of *mrc1*Δ cells, but rather suppresses it to the same level as *esc2*Δ cells (Figure 7B). These results suggest that *ESC2* interacts genetically with *MRC1* and Esc2 may modulate Mrc1 function(s) during DNA replication.

***esc2*Δ cells have a defect in sister chromatid cohesion:** Mrc1 is known to be required for sister chromatid cohesion (XU *et al.* 2004), raising the possibility that the interaction between Esc2 and Mrc1 could modulate this function of Mrc1. Here, a quantitative assay for chromatid cohesion was performed in strain background containing a *lac* operator array and expressing LacI-GFP (XU *et al.* 2004); lac operator repeats are integrated near the centromere of chromosome XV in the yeast genome. The results showed that *esc2*Δ cells have a modest defect in sister chromatid cohesion, while *mrc1*Δ cells have a more severe defect in chromatid cohesion (Figure 8). Interestingly, the sister chromatid cohesion defect of *mrc1*Δ is partially suppressed by *esc2*Δ. In contrast, esc2Δ *rrm3*Δ cells but not *sir2*Δ *rrm3*Δ cells have a severe defect in sister chromatid cohesion, even though *rrm3*Δ cells do not have a defect in sister chromatid cohesion (Figure 8), indicating that the severe sister chromatid cohesion defect of *esc2*Δ *rrm3*Δ cells is not due to a silencing defect. These data suggest that Esc2 and Mrc1 act cooperatively during sister chromatid cohesion and that Rrm3 might play a cryptic role in sister chromatid cohesion in the absence of Esc2.

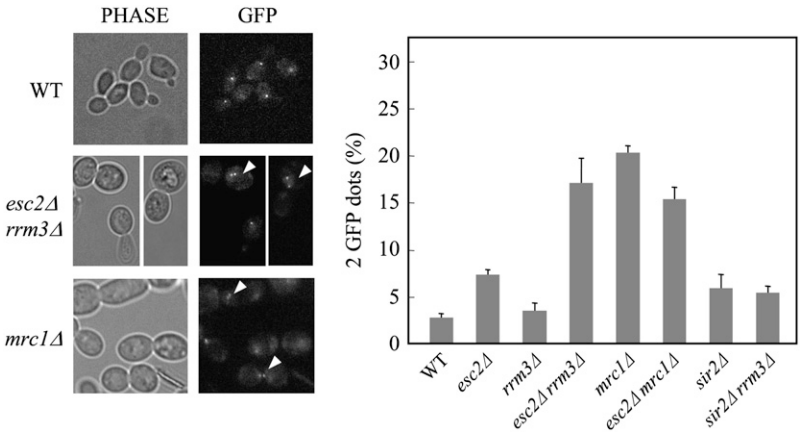

FIGURE 8.—Esc2 is required for sister chromatid cohesion. Sister chromatid cohesion assays. Sister chromatid cohesion was analyzed in the indicated strains, counting at least 300 cells for each genotype. One GFP dot was observed in normal G_2/M phase cells. When sister chromatid cohesion is defective, the proportion of cells with two GFP dots increases. The results represent the average of three independent measurements.

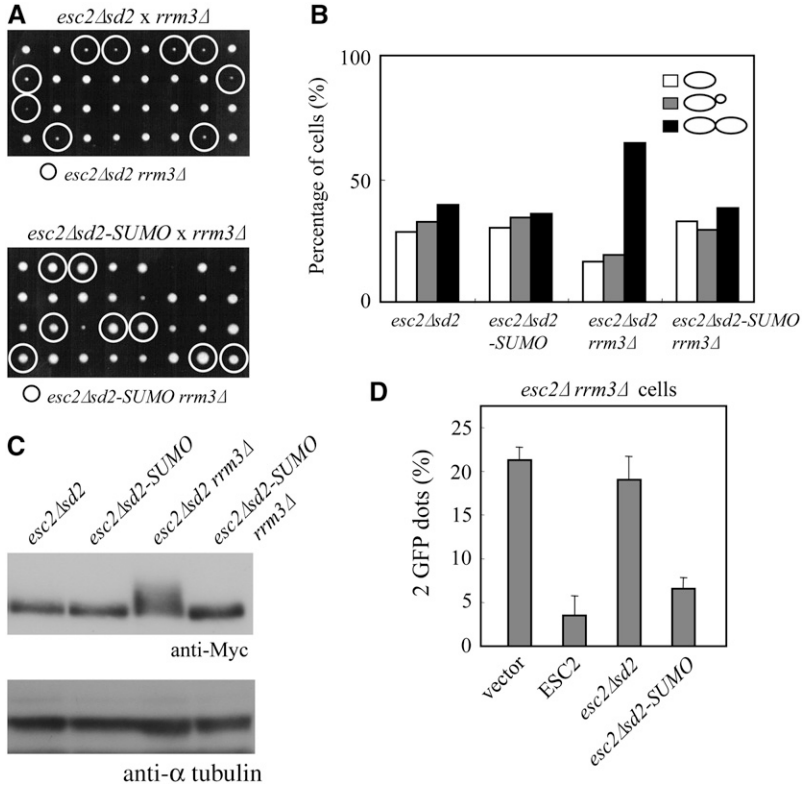

FIGURE 9.—SUMO can functionally substitute for the SUMO-like domain of ESC2. (A) Tetrads of heterozygous diploid cells formed by the crosses indicated were dissected and grown on YPD at 30° for 3 days. (B) Mutants were grown to early logarithmic phase and analyzed microscopically for the presence of single cells, small-budded cells, and large-budded cells. (C) *esc2*Δ*sd2-SUMO* suppresses the hyperphosphorylation of Rad53 in *rrm3*Δ cells. Cells were treated as described in the legend for Figure 6B. (D) Sister chromatid cohesion defect in *esc2*Δ*sd2 rrm3*Δ cells. *esc2*Δ *rrm3*Δ cells were transformed with pRS415 (vector), pESC2, pESC2Δsd2, and pESC2Δsd2-SUMO. Cells were assayed as described in the legend for Figure 8.

SUMO-like domain is required for S-phase progression in *rrm3*Δ cells: The role of the SUMO-like domain of Esc2 during DNA replication-associated stress was examined by comparing cell growth in *esc2*Δ*sd2 rrm3*Δ and *esc2*Δ *rrm3*Δ cells. The results demonstrated an equally severe growth defect in *esc2*Δ*sd2 rrm3*Δ and *esc2*Δ *rrm3*Δ, and that normal growth was restored in *esc2*Δ*sd2-SUMO rrm3*Δ cells (Figure 9A). We also confirmed that cell cycle delay in late S/G_2, aberrant phosphorylation of Rad53 and a defect in sister chromatid cohesion occur in *esc2*Δ*sd2 rrm3*Δ cells, as demonstrated above in *esc2*Δ *rrm3*Δ cells (Figure 9, B–D). In addition, *esc2*Δ*sd2-SUMO* restored the normal cell cycle progression to wild-type levels and suppressed the aberrant checkpoint activation (Figure 9, B and C). In addition, *esc2*Δ*sd2-SUMO*, but not *esc2*Δ*sd2*, complemented the defect in sister chromatid cohesion in *esc2*Δ *rrm3*Δ cells (Figure 9D). Thus, SD2 of Esc2 appears to be essential for normal growth and sister chromatid cohesion in *rrm3*Δ cells and be functionally substituted by the authentic SUMO.

DISCUSSION

This study explored the functional significance of the SUMO-like domains of Esc2. We demonstrated that truncated Esc2ΔC protein, which lacks SD1/2, interacts with Sir2 in a yeast two-hybrid assay, but is defective for silencing a reporter gene at *HMR*. However, the silencing defect of *esc2*Δ*C* is probably due to the fact that it is expressed at a very low level and appears to be unstable in yeast cells, even though it is soluble and relatively stable in *E. coli* (data not shown). Fusion of one SUMO domain to the C terminus of *esc2*Δ*C* (*esc2*Δ*C-SUMO*) increases the steady state protein level *in vivo* and restores functional gene silencing at *HMR*. These results suggest that the N-terminal domain of Esc2 interacts with Sir2 and plays a role in gene silencing and that one SUMO or SUMO-like domain is at least required for the stability of Esc2 *in vivo*. It should be noted that the Esc2ΔC protein is not aberrantly folded because the Esc2ΔC protein still interacts with Sir2 when overproduced and exists as a soluble protein when expressed in *E. coli*. Therefore, the SUMO-like domains might act as the stabilizer in yeast to protect degradation. The previous study has shown that the SUMO-like domain of fission yeast Rad60, an ortholog of *ESC2*, is required to form a homodimer through the interaction with putative SUMO-binding motifs of its own (RAFFA *et al.* 2006). Therefore, the inability of Esc2ΔC to form a homodimer might affect its stability *in vivo*. Alternatively, a protein complex formation with other partners and/or subnuclear localization of Esc2 via the SUMO-like domains might contribute to the stability of Esc2.

This study also demonstrated that *esc2* mutants have a shorter life span than wild-type or *sir2*Δ cells, that *esc2*Δ *sir2*Δ double mutants have a shorter life span than *esc2*Δ or *sir2*Δ single mutants, and that *esc2*Δ appears to differentially increase mortality in young cells (as indicated by the shape of the curve shown in Figure 4).

Previous studies in *sgs1* mutants suggested that high mortality in young *sgs1* cells (the early part of the curve) reflected an increased frequency of DNA damage-induced cell cycle arrest, while mortality of older cells (the later part of the curve) reflected age-related cell death (McVey *et al.* 2001). Therefore, these results suggest that Esc2 and Sir2 may act independently of life span, and it is possible that the effect of *esc2*Δ on life span reflects increased genome instability during DNA replication, as discussed below.

Mutations in *ESC2* caused a severe defect in cell growth when combined with *rrm3*Δ, whereas the mutation in *SIR2* did not, suggesting that the severe growth defect of *esc2*Δ *rrm3*Δ cells is not due to a silencing defect. FACS and morphological analysis showed that *esc2*Δ *rrm3*Δ cells fail to progress normally through the cell cycle and accumulate in late S/G_2 primarily as large-budded cells with a single nucleus. In addition, Rad53 is constitutively hyperphosphorylated in *rrm3*Δ *esc2*Δ cells, and the number of Rad52 foci is significantly higher than in wild-type, *rrm3*Δ, and *esc2*Δ cells in the absence of exogenous DNA damage. These data suggest that spontaneous DNA damage accumulates in *rrm3*Δ *esc2*Δ cells, causing constitutive activation of a DNA damage checkpoint. *esc2*Δ cells also have a defect in sister chromatid cohesion, which is greatly enhanced by *rrm3*Δ. This defect is fully complemented by expressing wild-type *ESC2* but not by *esc2*Δ*sd2*. *esc2*Δ*sd2* is expressed at a similar level as wild-type *ESC2* and is proficient in gene silencing. Thus, it appears that SD2 may play a role in replication fork progression and/or the rescue of stalled replication forks in the absence of Rrm3. In this regard, Esc2 may be at least partially redundant with Rrm3 in facilitating replication fork progression. In particular, in cells lacking Esc2, Rrm3 may prevent breakage of stalled replication forks, suppress activation of the S-phase checkpoint response, and promote sister chromatid cohesion.

Defects in homologous recombination suppress the severe growth defect of *sgs1*Δ *rrm3*Δ and *srs2*Δ *rrm3*Δ cells (Schmidt and Kolodner 2004; Torres *et al.* 2004). This result can be explained as follows: in *rrm3* mutants, stalled or broken replication forks are substrates for Rad51-dependent homologous recombination, which generates toxic recombination intermediates in *sgs1* or *srs2* cells. However, the growth defect of *esc2*Δ *rrm3*Δ cells is not suppressed by *rad51*Δ, suggesting that there is a defect upstream of Rad51-dependent homologous recombination. Like *esc2*Δ *rrm3*Δ cells, the *mrc1*Δ *rrm3*Δ lethality is also not suppressed by *rad51*Δ (Torres *et al.* 2004). In addition, *mrc1*Δ mutants have a defect in sister chromatid cohesion and have a partial loss of silencing at *HMR* or telomeric loci (Hu *et al.* 2001; Xu *et al.* 2004). Given the phenotypic similarities between *esc2*Δ and *mrc1*Δ, it is likely that Esc2 and Mrc1 are epistatic. This is consistent with the observation that *esc2*Δ does not enhance the HU-hypersensitivity and defect in sister chromatid cohesion in *mrc1*Δ cells, but rather partially suppresses both phenotypes. These data suggest that Esc2 might modulate the replication function of Mrc1 in the presence of replication stress. Thus, deregulation of Mrc1 function might explain the partial defect in sister chromatid cohesion in *esc2*Δ mutants and the growth defect of *esc2*Δ *rrm3*Δ double mutants.

Our data suggest that Esc2 plays important roles not only in gene silencing but also in facilitating DNA replication fork progression and sister chromatid cohesion. Although there is currently no evidence for a direct interaction between Esc2 and Mrc1, it remains possible that Esc2 may interact with Mrc1 transiently or modulate the replication function of Mrc1 indirectly through its interactions with other proteins involved in replication fork progression. Furthermore, our findings unveiled a novel function for Rrm3 in sister chromatid cohesion and yeast life span. Additional studies to more precisely elucidate the roles of Esc2, Rrm3, and Mrc1 during replication fork progression will provide insight into the mechanisms of crosstalk between proteins involved in DNA replication, sister chromatid cohesion, transcriptional silencing, and aging.

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