Published in final edited form as:

DNA Repair (Amst). 2006 May 10; 5(5): 618–628. doi:10.1016/j.dnarep.2006.02.005.

Esc4 and the Control of Recombination During Replication

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Abstract

When replication forks stall during DNA synthesis, cells respond by assembling multi-protein complexes to control the various pathways that stabilize the replication machinery, repair the replication fork, and facilitate the reinitiation of processive DNA synthesis. Increasing evidence suggests that cells have evolved scaffolding proteins to orchestrate and control the assembly of these repair complexes, typified in mammalian cells by several BRCT-motif containing proteins, such as Brca1, Xrcc1, and 53BP1. In *Saccharomyces cerevisiae*, Esc4 contains six such BRCT domains and is required for the most efficient response to a variety of agents that damage DNA. We show that Esc4 interacts with several proteins involved in the repair and processing of stalled or collapsed replication forks, including the recombination protein Rad55. However, the function of Esc4 does not appear to be restricted to a Rad55-dependent process, as we observed an increase in sensitivity to the DNA alkylating agent methane methylsulfonate (MMS) in a *esc4Δ rad55Δ* mutant, as well as in double mutants of *esc4Δ* and other recombination genes, compared to the corresponding single mutants. In addition, we show that Esc4 forms multiple nuclear foci in response to treatment with MMS. Similar behavior is also observed in the absence of damage when either of the S-phase checkpoint proteins, Tof1 or Mrc1, is deleted. Thus, we propose that Esc4 associates with ssDNA of stalled forks and acts as a scaffolding protein to recruit and/or modulate the function of other proteins required to reinitiate DNA synthesis.

Keywords

Esc4; *RTT107*; DNA damage; DNA recombination; Rad55

1. Introduction

To replicate a genome, DNA replication forks not only act processively to synthesize DNA, but when needed, they may pause, back up, and/or switch templates. These replication fork gymnastics are not only important for programmed gene rearrangements, but they are also required for the replication of some intrinsically difficult to replicate sequences and for the replication of damaged DNA. However, because these processes may also result in genome instability, they must be tightly regulated. The control of these processes is often referred to as the intra-S or replication stress checkpoint, which in *Saccharomyces cerevisiae* is controlled by the kinases Mec1 and Rad53 [1–4]. When a replication fork stalls, replication protein A (RPA) binds to the exposed single-stranded DNA (ssDNA) and recruits Ddc2, which also binds Mec1 [3]. Mec1 phosphorylates Mrc1, which along with Tof1 is required to stabilize the replication fork so that synthesis may resume when the stress is alleviated [5–12]. In addition,

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phosphorylated Mrc1 plays a role in the phosphorylation of Rad53 by recruiting Rad53 to Mec1 and/or by promoting Rad53 autophosphorylation [6,7]. Once activated, Rad53 phosphorylates other proteins involved in replication and recombination [13,14], such as primase [15,16] and Rad55 [17].

The activation of the replication stress checkpoint not only stabilizes stalled replication forks, but also facilitates repair. In *Escherichia coli*, homologous recombination (HR) plays a central role in the recovery of stalled and broken replication forks [18], and a similar role is envisioned for recombination in eukaryotes [14]. Central to HR is the formation of joint molecules that provide a physical linkage between interacting DNA duplexes. In *S. cerevisiae*, these joint molecules are formed by Rad51-ssDNA nucleoprotein filaments, which catalyze the homology search and DNA strand exchange [19–21]. Assembly of Rad51-ssDNA filaments on RPA-coated ssDNA requires the action of two mediator proteins, Rad52 and the heterodimer of Rad55 and Rad57 (Rad55–57) [22–24]. Rad52 function is facilitated by the physical interaction between Rad52 and both Rad51 and RPA in addition to its preference for binding ssDNA [21], while Rad55–57 function likely draws on the physical interactions between Rad55 and both Rad51 [21] and ssDNA [24]. Rad52 is required for all types of HR in vegetative and meiotic cells. While Rad55–57 is not required for spontaneous recombination in vegetative cells, it is critical for DNA damage-induced recombination in vegetative cells and for meiotic recombination [19–21]. In addition, Rad55 is phosphorylated in response to replication fork blocks and DNA damage in a Mec1- and Rad53-dependent manner and may thus help to coordinate the different facets of the stress response [17].

In general, there is increasing evidence that cells integrate and control the various responses to DNA damage by assembling specific proteins into multiprotein repair foci. Formation of these foci is likely mediated by specialized scaffolding proteins, which themselves do not necessarily possess any catalytic activity, but which facilitate repair by coordinating the functions of different enzymes, increasing their local concentration, and/or directly stimulating their activity. Binding to the scaffolding proteins may be mediated post-translationally by protein phosphorylation. For example, Brca1 C-terminal (BRCT) motifs [25,26] are thought to be phosphopeptide-binding domains that mediate protein-protein interactions involved in fork repair and other aspects of the DNA damage response [27–29]. In mammalian cells, several mediator proteins, such as XRCC1 and 53BP1, appear to employ BRCT motifs to coordinate the activities of DNA repair and recombination proteins [30–34]. In *S. cerevisiae*, the observation that many replication stress response and HR proteins are associated in S-phase nuclear foci [35,36] suggests that scaffolding proteins may exist to coordinate the assembly of replication fork repair foci in this organism, as well.

ESC4 (also known as *RTT107*) was first identified as part of the *S. cerevisiae* DNA damage response in a screen for deletion strains that are hypersensitive to methane methylsulfonate (MMS) [37]. Based this sensitivity, the presence of six BRCT motifs, and the hyper-dependency on post-replication repair of DNA damage in its absence, we speculated that Esc4 might help recognize or repair stalled replication forks [37]. Consistent with this idea, it was then shown that Esc4 is phosphorylated in a Mec1-dependent manner and is required for the resumption of DNA synthesis after damage [38] and for normal progression through S-phase [39]. Here, we report genetic and biochemical data that suggest that Esc4 physically associates with stalled or collapsed replication forks, and helps mediate their repair. The evidence suggests that Esc4 acts as a scaffolding protein that facilitates the assembly of replication fork maintenance and repair foci.

2. Materials and Methods

2.1. Strains, Media and General Procedures

Standard protocols were used to culture yeast in rich and synthetic media [40] and to construct strains by targeted deletion [41,42]. Gene deletions were verified by PCR, and multiple clones were analyzed for phenotype. Sequences of primers used to construct deletion cassettes and verify their proper insertion are available upon request.

Strains used to determine genetic interactions are derivatives of BY4741 and the *kanMX4* null deletion strains of the *Saccharomyces* Genome Deletion Project [43] (OpenBiosystems). For phosphorylation studies, the stop codon of *ESC4* was replaced with a *13myc::kanMX6* cassette [42], generating the C-terminal fusion protein Esc4-13myc. Microscopy of Esc4 was done by replacement of the *ESC4* stop codon with a cassette encoding GFP(S65T) [42]. For a list of strains used in this work, see Table 1.

2.2. Yeast Two-Hybrid Screen

Using the LexA-based yeast two hybrid system (Origene, Rockville, MD), Esc4 bait constructs were screened against a *S. cerevisiae* genomic fragment library in pJG4-5 (kindly provided by R. Brent, Molecular Sciences Inst.) in yeast strain EGY48 carrying the reporter plasmid pSH18-34. Esc4 residues 2 to 660, followed by a GGSGG peptide linker, were cloned into pNLexA, yielding pNLexA-Esc4²⁻⁶⁶⁰. Transformation of yeast strain EGY48 containing the reporter plasmid and pNLexA-Esc4²⁻⁶⁶⁰ with the pJG4-5 library yielded ~10⁷ transformants; 3 × 10⁷ were screened to identify galactose dependent Leu⁺ clones that were also blue in the presence of X-gal. In the second screen, residues 836-1070, preceded by a GGSGG peptide linker were cloned into pEG202, yielding pEG202-Esc4⁸³⁶⁻¹⁰⁷⁰. Unlike pNLexA-Esc4²⁻⁶⁶⁰, cells containing pEG202-Esc4⁸³⁶⁻¹⁰⁷⁰ did not transform well with the library. Thus, strain EGY48 containing the pJG4-5 library (10⁸ transformants) was mated with strain RFY206 containing the reporter plasmid and pEG202-Esc4⁸³⁶⁻¹⁰⁷⁰ to identify interactors. Residues 661-835 were excluded from the screens, since constructs containing this region resulted in galactose-independent autoactivation of *lacZ* transcription. Library inserts isolated in both screens were specific for the Esc4 bait used in their isolation and did not cross-interact. The pJG4-5 library inserts were amplified by PCR and digested with *Mse*I to identify unique, positive clones. A total of 14 and 3 unique clones were identified in the pNLexA-Esc4²⁻⁶⁶⁰ and pEG202-Esc4⁸³⁶⁻¹⁰⁷⁰ screens, respectively. Plasmids were rescued from these clones into *E. coli* KC8, amplified in *E. coli* XL1-Blue, and sequenced to identify library inserts. The specificity of the interaction between Esc4 bait constructs and isolated library plasmids was confirmed against LexA alone, as well as other non-specific LexA-bait fusion constructs.

2.3. Immunoprecipitation and Immunoblotting

Esc4 was visualized by introducing a genomic C-terminal myc-tag. Growth and MMS sensitivity of the *ESC4*-13myc tagged strains were similar to *ESC4* strains, implying that Esc4 retains its cellular function in the presence of the 13myc fusion. Co-immunoprecipitation of Esc4 and Rad55 was performed using previously described methods [17] with an anti-Rad57 antibody. Cell lysates for phosphorylation assays were prepared in the presence of protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (cocktails I and II, Sigma). Lysates were normalized by Bradford assay. Visualization of Esc4-13myc by immunoblotting was done with anti-myc antibody (9E10, Santa Cruz Biotechnol.), horseradish peroxidase-conjugated mouse secondary antibody (Upstate), and ECL(+) (AP Biotech).

2.4. Sensitivity to Methane Methylsulfonate

Midlog phase cultures were normalized by cell density and serially diluted. Dilutions were plated onto rich media containing MMS and colonies were counted or photographed after three days.

2.5. Subcellular Localization of Esc4

The diploid Esc4-GFP strain was treated with ethidium bromide to create a ρ^0 variant and facilitate nuclear staining [47]; localization of Esc4 was not affected by loss of mitochondria. Cells were grown at room temperature (21 °C) in synthetic complete media to allow for optimal GFP chromophore formation [48] and processed as described [49] with minor modifications. Cultures were grown to early log phase and MMS was added. After 90 m, cells were treated with 5% sodium thiosulfate, washed twice in fresh media, and mounted in 1.5% low-melt agarose. Images were acquired with a CoolSnapHQ cooled CCD camera (Roper Scientific) mounted on a Nikon E600 microscope with a Plan-Apochromat 100 $\times$, 1.4 N.A. objective, using filter sets for GFP (λ_{ex} =470; λ_{em} =525) and 4'-6-diamino-2-phenylindole (DAPI, λ_{ex} =360; λ_{em} =460), and the acquisition software IP LAB 3.9.2 (Scanalytics). Images of ~300 cells were scored for morphology and nuclear foci. The percentage of cells containing ≥ 3 nuclear foci was calculated for each cell morphology (unbudded, budded, double budded), and the average of three independent determinations is reported.

2.6. SUP4 Recombination Assay

Methods for the determination of deletion frequency between the five, direct and inverted repeat δ sequences at the *SUP4* locus in strain J599 and its derivatives were based on reported procedures [44–46]. J599 contains the *SUP4-o* allele linked to a selectable *URA3* marker, as well as the two ochre suppressible mutations, *ade2-1* and *can1-100*. Cells were grown to 5×10^7 cells/mL, washed with saline (9 g/L), and plated onto YPD to determine the total number of viable cells, and onto synthetic media lacking Arg containing 5 μ g/mL adenine, 60 μ g/mL canavanine, and 1 g/L 5-fluoroorotic acid (FOA) to detect deletion events. The red, FOA resistant, canavanine resistant colonies arising on the selective plates after 5 d were counted and attributed to recombination processes that led to a simultaneous loss of *SUP4-o* and *URA3*. The frequency of marker loss was calculated from the average number of colonies observed among the eight to twenty-four independent cultures of each strain per experiment; reported values are the average of three experiments. Representative clones from each experiment were assayed by PCR to determine whether deletion occurred via recombination between the direct or inverted repeats [46].

3. Results and Discussion

Replication forks stall during DNA synthesis for a variety of reasons, and different mechanisms have evolved to ensure their stabilization and reactivation [18]. The absence of such mechanisms results in chromosomal instability. *ESC4* was first identified in a screen for genes involved in the formation of silent chromatin (R. Sternglanz, personal communication and ref. [50]) and a screen for genes involved in the regulation of retrotransposon mobility [51]. It has six BRCT domains, and weak sequence homology to the *S. pombe* protein Brc1, a protein required for chromosome stability [52] that appears to function with Slx1 to initiate recombinational repair [53]. We originally identified *ESC4* in a genome-wide screen for genes involved in the tolerance of MMS-induced damage [37]. Our initial characterization identified a significant increase in the MMS sensitivity of an *esc4* Δ strain upon deletion of *RAD18*. However, epistasis analysis with genes involved in nucleotide excision repair (*rad14* Δ), recombination repair (*rad52* δ), and cell cycle control (*rad9* Δ , *rad24* Δ) indicated that *ESC4* function is independent of these processes [37]. A second genome wide screen for MMS-sensitive mutants also identified *esc4* Δ and demonstrated that the absence of *ESC4* leads to a

defect in S-phase progression [39]. Further studies indicated that Esc4 is a nuclear protein that is phosphorylated in a Mec1-dependent manner and involved in the resumption of DNA replication after damage [38,54]. Finally, Esc4 phosphorylation by Mec1 was shown to depend on Slx4, but not Slx1 [55]. These observations prompted us to further investigate the role that Esc4 might play in stabilizing and/or repairing stalled or collapsed replication forks.

3.1. Esc4 interacts with proteins involved in the repair of damaged DNA, including the recombination protein Rad55

We first conducted a yeast two hybrid screen to identify any potential interactions between Esc4 and proteins involved in the damage response. A genomic fragment library was employed to ensure that the screen would not be biased against genes that require induction by DNA damage. The sequence of Esc4 is distinguished by four well-defined BRCT motifs in the N-terminal portion of the Esc4 sequence, and at least one BRCT motif in the C-terminal portion that is readily identified by sequence homology, although two motifs have been reported in the literature [25] (Figure 1). Therefore, screens were performed with two different Esc4 bait constructs encompassing either the N-terminal (residues 2–660) or C-terminal (residues 836–1070) domain.

Library inserts that were independently isolated at least three times are shown in Figure 1. It is immediately apparent that the N-terminal domain of Esc4 is predicted to interact with a number of different proteins that are involved in DNA processing or repair, including Rad55, Mms22, Tof1, and Sgs1. A complex containing Esc4 and Mms22 was identified previously by immunoaffinity precipitation with Mms22-Flag [56]. Mms22 forms nuclear foci both spontaneously and in response to the topoisomerase II poison, etoposide, and *MMS22* is also epistatic with *ESC4* in response to etoposide [57]. DSBs resulting from etoposide treatment are predicted to be repaired by the single-strand annealing mechanism of HR, but while the frequency of this repair is lower in a *mms22Δ* mutant, cells containing double deletions of *MMS22* and the other genes involved in HR are more sensitive than the single mutants [57]. It has been suggested that Mms22 processes stalled replication forks and prepares them for repair by other pathways, and Esc4 may have a similar function.

Rad55 forms an obligate heterodimer with Rad57 and like Esc4 is phosphorylated in a Mec1-dependent manner in response to DNA damage [17,38]. To test whether Esc4 binds *in vivo* to Rad55 in its physiological relevant form, the Rad55–57 heterodimer, we used anti-Rad57 antibodies to immunoprecipitate the Rad55–Rad57 complex and detected Esc4-myc in these immunoprecipitates (Figure 2). The reverse experiment, immunoprecipitation of Esc4 and detection of Rad55–57, is not feasible because Esc4 is a relatively abundant protein, whereas the cellular concentration of Rad55–57 is extremely low [17]. The interaction between Rad55–57 and Esc4 did not depend on MMS treatment, indicating that the damage dependent phosphorylation of Esc4 and/or Rad55 is not required for complex formation. Rad55 is also constitutively phosphorylated at its C-terminal region (VIB and WDH, unpublished result), which may contribute to its recognition by Esc4. The physical interaction between Esc4 and Rad55 suggests a role for Esc4 in HR. The Rad55–57 heterodimer orchestrates the assembly of the Rad51 filament on RPA-coated ssDNA, and it is possible that Esc4 also functions at this early step of recombination.

3.2. Esc4 function is critical in the absence of different types of homologous recombination

Given the interaction between Esc4 and Rad55, we next investigated the genetic relationship between *ESC4* and genes involved in HR. HR is mediated by the *RAD52* group of genes [19–21]. We and others previously reported that an *esc4Δ rad52Δ* mutant exhibits a small, but reproducible increase in MMS sensitivity compared to *esc4Δ* and *rad52Δ* single mutants, implying that Esc4 function is at least partially independent of Rad52 [37,38]. *RAD51*,

RAD54, *RAD55*, and *RAD57* form a *RAD52* subgroup that mediates the single-strand invasion pathway of HR [19–21]. We observed a significant increase in MMS sensitivity with deletion of *ESC4* in *rad51Δ*, *rad54Δ*, and *rad55Δ* strains (Figure 3). No growth defects were observed in the absence of damage. *RAD59* encodes a protein that along with Rad52 catalyzes the single-stranded annealing (SSA) pathway of HR [19–21]. The *esc4Δ rad59Δ* double mutant was significantly more sensitive to MMS than would be predicted based on the sensitivity of the single mutants (Figure 3), and we conclude that Esc4 function is at least partially independent of both the single-strand invasion and SSA pathways of DNA damage repair.

MRE11, *RAD50*, and *XRS2* form another subgroup of the *RAD52* group [19–21]. Deletion of *ESC4* in the single mutants, *mre11Δ*, *rad50Δ*, and *xrs2Δ*, yielded viable cells with slight growth defects and synergistic sensitivity to MMS (Figure 4A). Because *esc4Δ* was previously identified in a high-throughput screen for genes synthetically lethal with *rad50Δ* [58], we also sporulated heterozygous double mutants of *esc4Δ* and *mre11Δ*, *rad50Δ*, and *xrs2Δ* to specifically evaluate whether any of the resulting double mutants is inviable. In each case we observed a growth defect in the double mutant spores (Figure 4B). In contrast, normal, viable spore progeny were observed in *esc4Δ/esc4Δ* diploids, as well as heterozygous mutants of *esc4Δ* and *rad52Δ* or *rad55Δ* (data not shown). We conclude that Esc4 functions in damage resistance pathways that are at least partially independent of the Mre11-Rad50-Xrs2 complex.

3.3. Esc4 is Critical in the Absence of Proteins that Process Stalled Replication Forks and Recombination Intermediates

In addition to the *RAD52* group genes, repair of stalled replication forks also requires the actions of specialized, context-specific enzymes including nucleases, DNA helicases, and topoisomerases. For example, *RAD27* encodes a protein with 5'–3' exonuclease and flap exonuclease activity; deletion of *RAD27* renders cells particularly reliant on recombination to repair ssDNA gaps formed during lagging strand synthesis [59]. We observed that deletion of *ESC4* and *RAD27* leads to a slight growth defect on rich media and a dramatic increase in sensitivity to MMS compared to either single deletion mutant (Figure 5A). This suggests that Esc4 facilitates recombinational repair of ssDNA.

esc4Δ was isolated previously in a screen for genes synthetically lethal with mutations in the DNA helicases, *SRS2* and *SGS1*, as well as in the gene encoding the type I topoisomerase Top3 [58,60], which can function in concert with Sgs1. Thus, *ESC4* appears to be required when Srs2, Sgs1, and Top3 are absent. Interestingly, Sgs1 is one of the proteins predicted to bind Esc4 based on the above described two-hybrid screen. While this physical interaction remains to be confirmed in independent assays, it may reflect a more intimate role of Esc4 in the Sgs1-Top3 pathway.

In addition, several nucleases involved in processing stalled replication forks or recombination intermediates have been identified by their functional overlap with Sgs1/Top3. These include the Mms4-Mus81 and Slx1–Slx4 endonucleases [61–64]. Deletion of *ESC4* in haploid mutants of *mms4Δ* or *mus81Δ* had no significant effects on growth, but resulted in a significant increase in sensitivity to MMS relative to the single mutants (Figure 5B). In contrast, deletion of *SLX1* or *SLX4* did not increase the MMS sensitivity of the *esc4Δ* mutant (Figure 5C–D). While the lack of sensitivity of the *slx1Δ* strain precludes a definitive conclusion regarding its genetic relationship to *esc4Δ*, the similar MMS sensitivity of the *slx4Δ esc4Δ* and *esc4Δ* mutants suggests that the corresponding proteins function in the same pathway. Slx1–Slx4 is a DNA structure-selective DNA endonuclease that has been shown to be required to resolve replication intermediates specifically in the rDNA of budding yeast [63,64]. It has been proposed that the Slx1–Slx4 heterodimer functions to cleave stalled or converging replication forks resulting in SSA. In addition, it has recently been reported that Slx4 interacts physically with Esc4 and promotes the phosphorylation of Esc4 by Mec1, independently of Slx1 [55]. The epistasis

between *esc4Δ* and *slx4Δ* suggests that the corresponding proteins function in the same pathway, possibly involving rDNA replication. This function may explain why the *rad52Δ esc4Δ* double mutant is slightly more sensitive than the *rad52Δ* single mutant, as SSA in rDNA has been shown to be Rad52-independent [63,64].

In all, the data suggests that Esc4 functions with Slx4, and perhaps the Sgs1-Top3 complex, and plays an important role in recognizing and/or repairing ssDNA gaps at stalled or collapsed replication forks.

3.4. Esc4 is Phosphorylated in a Mec1- and Rad53-Dependent Manner

To investigate the phosphorylation of Esc4, we treated *ESC4-13myc* cells with MMS and subjected the cell lysates to immunoblotting (Figure 6). The abundance of Esc4 in the cell was sufficient for direct detection in whole cell lysates. Treatment with MMS led to a clear retardation of Esc4 over time to a slower migrating band (Figure 6A). Incubation of cell lysates with phosphatase converted the Esc4 band to a faster migrating species (compare Figure 6B lanes 2 and 3). These results are consistent with phosphorylation of Esc4 in response to MMS-induced DNA damage and agree with previously reported results based on visualizing Esc4 with an anti-phospho-SQ/TQ antibody [38].

We investigated whether the gel shift was dependent on a specific protein kinase in the known DNA damage checkpoint kinase cascade. Deletion of *MEC1*, the main effector of the damage-response kinase cascade, fully abolished the observed shift (Figure 6C), as observed in a previous study [38]. Deletion of *RAD53* led to a reproducible decrease in the intensity of the band corresponding to phosphorylated Esc4 (Figure 6D, lanes 3 and 4), although interpretation of this data is complicated by the apparent decrease also observed in the intensity of the band corresponding to the unmodified protein. Deletion of the downstream kinases *DUN1* or *CHK1* had no effect on the band migration, and thus they do not appear to play a role in Esc4 phosphorylation. Deletion of *RAD51*, *RAD54*, *RAD55*, *MRC1*, or *TOF1* also had no effect (Figure 6D – 6F). We propose that after binding to the ssDNA of a stalled replication fork, Esc4 is phosphorylated by Mec1 and to a lesser, but significant extent by Rad53.

3.5. Esc4 forms Foci Spontaneously and in Response to DNA Damage, possibly by recognizing ssDNA at stalled replication forks

To examine the subnuclear localization of Esc4 we constructed a homozygous diploid *ESC4-GFP(S65T)* strain. The Esc4-GFP fusion did not increase the MMS sensitivity of the cells and thus the protein was presumed to retain its function. During asynchronous growth, the Esc4-GFP signal was coincident with the nuclear DAPI signal, as also reported in a high-throughput assay [54]. Esc4-GFP was found in one of three nuclear distributions (Figure 7A). Nearly all of the G1 (unbudded) and G2/M (large-budded) cells displayed even, diffuse nuclear localization without any observable foci, and treatment with MMS had little effect on these cells. In contrast, we observed 1 or 2 foci in about half of the untreated S phase (small budded) cells, typically located at the edge of the nucleus, and diffuse localization in the other half. Treatment with MMS increased the number of foci in S phase cells, such that ~60% of them contained multiple, nuclear foci (Figure 7B). Similar behavior was also observed upon treatment with HU (Figure 7C). The change in Esc4-GFP localization from an even distribution throughout the nucleus, to distinct foci in S-phase cells, suggests a role for Esc4 in a DNA replication process. In addition, the apparent increase in the number of foci upon treatment with MMS supports a role for Esc4 in the stabilization and/or repair of stalled replication forks.

To determine whether the foci observed in undamaged S-phase cells could be the result of stalled replication in the rDNA, we examined Esc4-GFP in the presence of plasmid expressing Nop1-RFP (kindly provided by R. Rothstein). Nop1-RFP was clearly visible within the

nucleus, indicative of the rDNA (Figure 7D). At least a fraction of the Esc4 foci appeared to be coincident with Nop1, suggesting that Esc4 may associate with rDNA. This is consistent with the genetic interactions between *ESC4* and *SLX4*, discussed above. However, foci were also apparent that clearly did not co-localize with Nop1 suggesting that the Esc4 foci are not limited to the rDNA. Thus, we propose that in undamaged cells, Esc4 foci may be located in the rDNA where they are involved in the resolution of converging replication forks, as well as outside the rDNA, where they may be associated with stalled DNA replication forks. Esc4 foci in undamaged cells may also be storage reservoirs of protein, unassociated with any DNA.

Interestingly, the foci are not dependent on the presence of *MEC1*, *RAD55* or *MMS22* (data not shown), suggesting that neither phosphorylation nor the proposed interactions with Rad55 and Mms22 control foci formation. To determine whether Esc4 might bind the ssDNA associated with stalled replication forks, we examined the behavior of Esc4 in *mrc1Δ* and *tof1Δ* strains (Figure 7D and 7E). In the absence of either Mrc1 or Tof1, unconstrained replisome progression results in physical separation of the replication proteins from the replication fork and the accumulation of ssDNA at an otherwise undamaged replication fork [5]. While the number of Esc4 foci in *mrc1Δ* and *tof1Δ* strains in the presence of MMS was identical to that observed in wild-type cells, the number of foci in these deletion strains was also greatly elevated in the absence of MMS treatment. The large increase in the number of spontaneous Esc4 foci observed in the *mrc1Δ* and *tof1Δ* strains, relative to their wild-type counterparts, suggests that Esc4 binds stalled replication forks that accrue ssDNA.

3.6. Deletion of *ESC4* Affects Recombination at the *SUP4* Locus

To investigate the role of Esc4 in recombination, we characterized recombination among the five δ sequences at the *SUP4* locus [45,46] (Table 2). The δ sequences are derived from the long terminal repeats of the yeast Ty transposon, are 71 to 97% homologous, and are arranged in both direct and inverted orientations around the *SUP4-o* gene. The *SUP4* locus may be difficult to replicate due to the δ repeats, as well as natural pause sites. While recombination at this locus may be repaired by error-free means, such as sister chromatid exchange [45,65], synaptic misalignment during recombination or an SSA mechanism may result in deletion of the *SUP4-o* gene [46].

We found that wild-type cells exhibit a low occurrence of deletions, on the order of just a few events for every 10^7 cells, with recombination between direct and inverted repeats contributing about equally. Conversely, cells lacking Rad51 or Rad55 exhibit a measurable increase in deletion events. Nearly 100% of the deletions observed in the *rad51Δ* and *rad55Δ* mutants occur by recombination between direct repeats, in agreement with the previous conclusion that deletion of *RAD51* or *RAD55* increases SSA [46]. Qualitatively, these results agree with previous reported values [46]; discrepancies in the magnitude of the observed frequencies are attributed to differences in the method of calculating the recombination events. We found that deletion of *ESC4* has little effect on deletion frequency, but does result in a bias for recombination between direct repeats. Deletion of *ESC4* in a *rad51Δ* or *rad55Δ* mutant, restores recombination between inverted repeats to a modest level, with a small reduction in overall frequency. Thus, Esc4 appears to affect recombination between the long terminal repeats present at *SUP4* in a complex manner that depends on the presence of other recombination proteins. The data suggest that Esc4 is not a core recombination factor but rather an accessory component, consistent with the idea that Esc4 is a scaffolding protein.

3.7. The role of Esc4 in the *S. cerevisiae* DNA damage response

The physical, genetic, and biochemical data suggest that Esc4 associates with stalled or converging replication forks and helps to mediate their stabilization and repair. We suggest that Esc4 functions as a scaffolding protein that helps coordinate the various functions of

different repair proteins. This is consistent with the physical interactions with Rad55 and Mms22, as well as the other predicted interactions with Sgs1 and Tof1. The genetic data suggests that while Esc4 is required for fork repair, its activities are not limited to a single process. In addition, the epistasis between *esc4Δ* and *slx4Δ* [63,64] and the localization of Esc4 to rDNA in undamaged cells suggests that these activities include the repair of forks that collapse within the rDNA repeats.

Elegant cytological work has suggested the presence of multi-protein complexes that localize multiple DNA lesions to a single repair center. In addition, DNA replication in prokaryotes and eukaryotes is organized in replication centers, where multiple forks are serviced by an overabundance of essential and non-essential replication factors [66,67]. In mammalian cells, several BRCT-containing proteins appear to function as scaffolds that help to organize such multi-protein complexes to provide an optimal disposition of repair and possibly replication factors [34]. The importance of these scaffolding proteins is not manifest in obvious replication defects or a strong sensitivity to DNA damage in their absence. Rather the absence or mutation of such scaffolding proteins is strongly correlated with disease, such as cancer, suggesting that these proteins make critical contributions to genome stability through the subtle control of the various recombinational outcomes at stalled replication forks. We envision a similar role for Esc4.

Acknowledgments

We thank R. Kolodner, R. Rothstein, R. Brent, and M. Longtine for the generous gifts of materials. This work was supported by the National Institutes of Health (R01 GM068569 to FER and R01 CA92276 to WDH) and the Susan G. Komen Breast Cancer Research Foundation (PDF02-1450 to FER).

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Figure 1.

Protein interactions with Esc4 identified by yeast two hybrid screening. (A) Schematic representation of Esc4; BRCT motifs are shown as black boxes. (B) The bait construct expressed by pNLexA-Esc4²⁻⁶⁶⁰ and the identified interactions. (C) The bait construct expressed by pEG202-Esc4⁸³⁶⁻¹⁰⁷⁰ and the identified interaction. Regions corresponding to library inserts are shown as hatched boxes and the number of clones isolated for each insert is given in parentheses.

Figure 2.

Esc4 co-immunoprecipitates with the Rad55–57 heterodimer. Protein extracts were prepared from control and MMS-treated cells and subjected to immunoprecipitation with anti-Rad57 antibody (lanes 1–4). Experiments lacking the anti-Rad57 antibody were also performed (lanes 5 and 6). Immunoprecipitates were analyzed for the presence of Rad55 and Esc4 with anti-Rad55 and anti-Myc antibodies, respectively. In cells lacking myc-tagged Esc4, only Rad55 is detected (lanes 1 and 2), consistent with the presence of the Rad55–57 heterodimer. In *ESC4-13myc* cells, both Rad55 and Esc4-13myc are detected (compare lanes 3 and 4 with lanes 5 and 6).

Figure 3.

Deletion of *ESC4* results in a small increase in the MMS sensitivity of a *rad52Δ* mutant and a significant increases the MMS sensitivity of *rad51Δ*, *rad54Δ*, *rad55Δ*, and *rad59Δ* mutants. Ten-fold serial dilutions of logarithmically growing cultures were plated on YPD and YPD containing 0.004% MMS (*rad51Δ*, *rad52Δ*, *rad54Δ*, and *rad55Δ*) or 0.007% MMS (*rad59Δ*).

Figure 4.

Genetic interactions between *ESC4* and *MRE11*, *RAD50*, or *XRS2*. (A) Strains were grown and diluted as described in Figure 3 and plated on YPD and YPD containing 0.0025% MMS. (B) Five tetrads are shown from each of the crosses indicated. Double mutants are indicated by arrows. Tetrads from crosses with wild type (BY4742) and *mre11Δ*, *rad50Δ*, and *xrs2Δ* single mutants yielded four viable spores (data not shown).

Figure 5. Genetic interactions between *ESC4* and *RAD27*, *MUS81*, *MMS4*, *SLX1*, and *SLX4*. (A–B) Deletion of *ESC4* synergistically increases the MMS sensitivity of *rad27*, *mus81Δ*, and *mms4Δ* mutants. Cells were plated as in Figure 3 on 0.003% MMS (*rad27*), or 0.005% MMS (*mus81* and *mms4*). Deletion of *ESC4* and *SLX1* or *SLX4* is epistatic in response to MMS. (C) Cells were plated as in Figure 3 on 0.018% MMS. (D) Logarithmically growing cells were plated on YPD containing the amount of MMS indicated. The number of colonies appearing on plates lacking MMS was defined as 100% survival. Survival curves represent the average of at least three independent determinations.

Figure 6.

Esc4 is phosphorylated in response to MMS. (A) Modification of Esc4 with increasing exposure to 0.1% MMS. An asynchronous culture of strain FR467 was grown to early log phase and treated with MMS; cells were removed at the times indicated and processed as described (see Methods). (B) Cells were grown as in (A), but lysed in buffer without phosphatase inhibitors. Lysate from untreated cells is shown in lane 1. Lysate from cells exposed to 0.1% MMS for 90 minutes is shown in lanes 2 and 3. In lane 3, 22 μ g of lysate was treated with 600 units of λ protein phosphatase (New England Biolabs) for 20 min at 30 $^{\circ}$ C prior to analysis. (C) Phosphorylation of Esc4 is Mec1-dependent. Strains FR672 and FR673 cells were grown as in (A) with or without 0.1% MMS for 90 minutes. (D) Phosphorylation of Esc4 is mildly affected by deletion of *RAD53*, but not other, downstream, damage checkpoint functions. Strains FR713, FR716, FR718, and FR715 were treated as in (C). (E) Phosphorylation of Esc4 is not dependent on the presence of recombination mediator proteins. Strains FR674, K37-3, K37-7, and K37-15 were treated as in (C). (F) Phosphorylation of Esc4 is not dependent on either Mrc1 or Tof1. Strains FR674, FR1007, and FR1009 were treated as in (C). Arrows on the right side of each panel indicate the different forms of Esc4. Lanes contain 3 μ g of total cell lysate, separated on 5% SDS-PAGE.

Figure 7.

Esc4 forms S-phase specific foci in response to MMS. (A) Esc4-GFP(S65T) exhibits three types of nuclear localization: diffuse (*upper*), 1–2 foci at the edge of the nucleus (*middle*), or ≥ 3 distinct foci (*lower*). (B) Treatment of wild-type cells with MMS increases the percentage of budded cells that contain ≥ 3 foci. (C) Nuclear foci are also observed in S-phase cells in response to treatment with 100 mM HU for 1 hr. (D) Foci observed in the nucleus of undamaged S-phase cells may be localized to the rDNA. rDNA is visualized with Nop1-RFP (red); nucleus is stained with DAPI (blue). In cells lacking either *MRC1* (E) or *TOF1* (F), a significant number of budded cells contain foci in the absence of MMS.

Table 1

Strains used in this work.

Strain	Genotype	Reference
EGY48	<i>MATa trp1 his3 ura3 leu2 :: 6 LexAop-LEU2</i>	Origene
RFY206	<i>MATa trp1 Δ :: hisG his3 Δ 200 ura3-52 lys2 Δ 201 leu2-3</i>	Origene
BY4741 ¹	<i>MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0</i>	ATCC
BY4742 ¹	<i>MATa his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0</i>	ATCC
FR184	BY4741 with <i>esc4Δ::LEU2</i>	This study
FR366	BY4742 with <i>esc4Δ::LEU2</i>	This study
FR409	BY4741 with <i>esc4Δ::kanMX4 rad51Δ::LEU2</i>	This study
FR414	BY4741 with <i>esc4Δ::kanMX4 rad54Δ::LEU2</i>	This study
FR311	BY4741 with <i>esc4Δ::kanMX4 rad55Δ::LEU2</i>	This study
FR532	BY4741 with <i>xrs2Δ::kanMX4 esc4Δ::LEU2</i>	This study
FR568	BY4741 with <i>rad50Δ::kanMX4 esc4Δ::LEU2</i>	This study
FR180	BY4741 with <i>mre11Δ::kanMX4 esc4Δ::LEU2</i>	This study
FR528	BY4741 with <i>rad27Δ::kan esc4Δ::LEU2</i>	This study
FR901	BY4741 with <i>mus81Δ::kanMX4 esc4Δ::LEU</i>	This study
FR896	BY4741 with <i>mms4::kanMX4 esc4Δ::LEU</i>	This study
FR1005	BY4741 with <i>slx1Δ::kanMX4 esc4Δ::LEU</i>	This study
FR313	BY4741 with <i>slxΔ::kanMX4 esc4Δ::LEU</i>	This study
FR350	BY4741 with <i>ESC4-GFP(S65T)::HISMx6</i>	This study
FR616	BY4742 with <i>ESC4-GFP(S65T)::HISMx6</i>	This study
FR1027	FR350 × FR616p-	This study
FR1080-51	FR350 with <i>tof1Δ::LEU2</i>	This study
FR1080-35	FR616 with <i>tof1Δ::LEU2</i>	This study
FR1080	FR1080-51 × FR1080-35	This study
FR1082-6	FR350 with <i>mrc1Δ::LEU2</i>	This study
FR1082-23	FR616 with <i>mrc1Δ::LEU2</i>	This study
FR1082	FR1082-6 × FR1082-23	This study
FR1036	FR1027 containing pWJ1322 expressing Nopl-RFP	This study
W1588-4C ²	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	[46]
FR467	W1588-4C with <i>ESC4-13myc::kanMX6</i>	This study
RDKY3615 ¹	<i>MATa ura3-52 leu2 Δ 1 tyr1 Δ 63 his3 Δ 200 lys2 Δ Bgl hom3-10 ade2 Δ 1 ade8 hxt13 :: URA3</i>	[3]
RDKY3735	RDKY3615 with <i>mec1Δ::HIS3 sml1Δ::KAN</i>	[3]
FR672	RDKY3615 with <i>ESC4-13myc::HISMx6</i>	This study
FR673	RDKY3735 with <i>ESC4-13mycΔHISMx6</i>	This study
WDHY1800 ²	<i>MATa leu2-3,112 his3-11,15 ura3-1 trp1-1 can1-100 met17-s ADE2 RAD5 TRP1 :: GAP-RNR1</i>	[68]
WDHY1817	WDHY1800 with <i>rad53Δ::HIS3</i>	[68]
WDHY1827	WDHY1800 with <i>dun1Δ::HIS3</i>	[68]
WDHY1819	WDHY1800 with <i>chk1Δ::HIS3</i>	[68]
FR713	WDHY1800 with <i>ESC4-13myc::kanMX6</i>	This study
FR716	WDHY1817 with <i>ESC4-13myc::kanMX6</i>	This study

Strain	Genotype	Reference
FR718	WDHY1827 with <i>ESC4-13myc::kanMX6</i>	This study
FR715	WDHY1819 with <i>ESC4-13myc::kanMX6</i>	This study
FR674	BY4741 with <i>ESC4-13myc::HISMx6</i>	This study
K37-3	FR674 with <i>rad51Δ::LEU2</i>	This study
K37-7	FR674 with <i>rad54Δ::LEU2</i>	This study
K37-15	FR674 with <i>rad55Δ::LEU2</i>	This study
FR1007	BY4741 with <i>mrc1Δ::kanMX4 ESC4-13myc::HISMx6</i>	This study
FR1009	BY4741 with <i>tof1Δ::kanMX4 ESC4-13myc::HISMx6</i>	This study
J599 ³	<i>MATa SUP4-o :: URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura 3-1 rad 5-535</i>	[46]
FR280	J599 with <i>esc4Δ::LEU2</i>	This study
FR500	J599 with <i>rad51Δ::HIS3MX6</i>	This study
FR481	J599 with <i>rad55Δ::HIS3MX6</i>	This study
FR502	J599 with <i>esc4Δ::LEU2 rad51Δ::HIS3MX6</i>	This study
FR483	J599 with <i>esc4Δ::LEU2 rad55Δ::HISMx6</i>	This study
FR1007	BY4741 <i>mrc1Δ::kanMX4 ESC4-13Myc::HISMx6</i>	This study
FR1009	BY4741 <i>tof1Δ::kanMX4 ESC4-13Myc::HISMx6</i>	This study

¹ Strain background S288C.

² Strain background W303 containing the corrected *RAD5* allele.

³ Strain background W303 with *rad5-535*.

Table 2

Deletion at the SUP4 locus.

Genotype	Deletion frequency ($\times 10^{-7}$)	Deletion Class		Number of clones assayed
		Frequency of GC ($\times 10^{-7}$) ^a	Frequency of SSA ($\times 10^{-7}$) ^a	
WT	12 $\pm$ 14	7	5	52
<i>esc4</i> Δ	7 $\pm$ 4	2	5	48
<i>rad51</i> Δ	248 $\pm$ 6	30	218	33
<i>rad55</i> Δ	162 $\pm$ 13	6	156	56
<i>rad51</i> Δ <i>esc4</i> Δ	139 $\pm$ 40	32	107	30
<i>rad55</i> Δ <i>esc4</i> Δ	86 $\pm$ 19	11	75	55

^aDeletion class frequencies were calculated from the fraction of gene conversion (GC) or single-strand annealing (SSA) observed in the total number of clones assayed multiplied by the deletion frequency.