

Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins

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Summary

DNA repair is an essential process for preserving genome integrity in all organisms. In eukaryotes, recombinational repair is choreographed by multiprotein complexes that are organized into centers (foci). Here, we analyze the cellular response to DNA double-strand breaks (DSBs) and replication stress in *Saccharomyces cerevisiae*. The Mre11 nuclease and the ATM-related Tel1 kinase are the first proteins detected at DSBs. Next, the Rfa1 single-strand DNA binding protein relocates to the break and recruits other key checkpoint proteins. Later and only in S and G2 phase, the homologous recombination machinery assembles at the site. Unlike the response to DSBs, Mre11 and recombination proteins are not recruited to hydroxyurea-stalled replication forks unless the forks collapse. The cellular response to DSBs and DNA replication stress is likely directed by the Mre11 complex detecting and processing DNA ends in conjunction with Sae2 and by RP-A recognizing single-stranded DNA and recruiting additional checkpoint and repair proteins.

Introduction

The cellular response to DNA double-strand breaks (DSBs) involves a large number of checkpoint and repair proteins responsible for damage detection, checkpoint activation, damage repair, and postrepair resumption of the cell cycle (Game and Mortimer, 1974; Melo and Toczyski, 2002; Symington, 2002). Many of these proteins are also important for repair of other kinds of DNA damage including stalled and collapsed DNA replication forks (Chang et al., 2002; Lopes et al., 2001; Tercero and Diffley, 2001). The importance of the DSB repair pathway for preserving genomic integrity is underscored by its conservation throughout evolution (Melo and Toczyski, 2002). In the yeast *Saccharomyces cerevisiae*, the major pathway for DSB repair is homologous recombination (HR) whereas nonhomologous end joining

(NHEJ) plays only a minor role (Friedl et al., 1998). During HR, sequence information from a homologous DNA molecule is used as a template for restoring genetic information lost at the DSB.

Recombinational repair of DSBs proceeds via a number of steps and involves proteins such as replication protein A (RP-A consisting of the Rfa1-3 subunits), Rad51, Rad52, Rad54, Rdh54(Tid1), Rad55, Rad57, and Rad59 (Game and Mortimer, 1974; Symington, 2002). First, the DSB is processed to yield 3'-single-stranded ends. These ends are bound by RP-A, which is presumed to protect the DNA against degradation and inhibit the formation of secondary structures (Alani et al., 1992). The subsequent invasion of the single-stranded DNA into homologous duplex DNA is catalyzed by the RecA homolog, Rad51, and stimulated by Rad52, Rad55/Rad57, and Rad54 in vitro and in vivo (Alexeev et al., 2003; New et al., 1998; Sugawara et al., 2003; Sung, 1997a, 1997b). The invading strand primes DNA synthesis of the homologous template, ultimately restoring genetic information disrupted at the DSB. Concurrent with recombinational repair, the DNA damage checkpoint is activated to slow DNA replication and arrest cells before cell division (G2 phase) until the DNA lesion has been repaired. A functional checkpoint response requires the ATR-related Mec1 kinase and its binding partner Ddc2 (homolog of human ATRIP), as well as the Rad9 checkpoint protein, the Rad53 (CHK2) kinase, the Tel1 (ATM) kinase, the Mre11/Rad50/Xrs2 (MRX; MRN in humans) complex, the Rad24 (human RAD17)/Rfc2-5 clamp loader, and the Ddc1/Mec3/Rad17 (human RAD9/HUS1/RAD1) DNA clamp (Melo and Toczyski, 2002).

In response to DSBs, a number of DNA checkpoint and repair proteins in *S. cerevisiae* including Rad51, Rad52, Rad53, Mre11, RP-A, Ddc1, Ddc2, Rad9, and Rad24 relocate from a diffuse nuclear distribution to distinct subnuclear foci (Frei and Gasser, 2000; Gasior et al., 1998; Lisby et al., 2001; Melo et al., 2001). Similar redistribution has been observed for the homologs of these proteins in human cells (Haaf et al., 1995; Lukas et al., 2003; Maser et al., 1997). DNA damage-induced foci colocalize with DSBs and with regions of single-stranded DNA in vivo (Lisby et al., 2003b; Raderschall et al., 1999). As measured by BrdU incorporation, the foci are sites of unscheduled DNA synthesis, which is a marker of ongoing homologous recombination (Haaf et al., 1999). In *S. cerevisiae*, it was further demonstrated that these foci can act as centers of recombinational DNA repair capable of simultaneously recruiting multiple DSBs (Lisby et al., 2003b). Repair foci are also observed at a low frequency in undamaged cells, likely reflecting the recruitment of repair proteins to spontaneous DNA damage. Interestingly, these foci form preferentially in S/G2 phase suggesting that recombinational DNA repair is coupled to DNA replication (Lisby et al., 2003a). At present, it is largely unknown how the proteins that are recruited to sites of DNA damage are coordinated at the cellular level. Here, we address this question by visualizing the cellular response to DSBs and DNA repli-

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cation stress in living cells using marked DSBs and fluorescently tagged checkpoint and repair proteins.

Results

Protein Composition of DNA Repair Foci

To monitor the cellular response to DNA damage *in vivo*, we fused DNA damage checkpoint and repair proteins to either blue- or red-shifted variants of green fluorescent protein (CFP and YFP, respectively) or to monomeric red fluorescent protein (RFP) (see Supplemental Figure S1A available at <http://www.cell.com/cgi/content/full/118/6/699/DC1>). The biological functionality of the resulting fusion proteins was verified for DNA damage repair (Supplemental Figures S1B–S1D available on *Cell* website) and for checkpoint activation (Melo et al., 2001).

To investigate the protein composition of DNA repair foci, we exposed cells to ionizing irradiation and used fluorescent microscopy to analyze the colocalization of various checkpoint and repair proteins with Rad52 foci. Thirty minutes after exposure to 40 Gy of γ -irradiation, 70% of S and G2 phase cells contain Rad52 foci. According to the Poisson distribution, 86% of the cells have at least one DSB at this dose, with an overall average of two DSBs/cell in the population (Lisby et al., 2001). All of the Rad52 foci contain Rfa1 and many also contain Rad59, Rdh54, Rad51, Rad55, and Rad54 (Figures 1A–1B and data not shown). As reported previously, Rad52 foci are observed only in S and G2 cells after exposure to low doses of γ -irradiation (<100 Gy, <5 DSBs/cell) (Lisby et al., 2001, 2003a). The entire recombination machinery (Rad59, Rdh54, Rad51, Rad55, and Rad54) behaves in a similar fashion (data not shown). In contrast, Rfa1 foci are also observed in G1 cells indicating that DSBs can be processed at any phase of the cell cycle to yield 3'-single-stranded ends bound by RP-A. Since Rad52 is not recruited to Rfa1 foci in G1 cells, their physical interaction must be regulated in a cell cycle-dependent manner possibly by protein modifications or expression of S and G2 phase specific protein factors (Lisby et al., 2003a). The Rdh54 protein, which in the absence of DNA damage localizes constitutively to kinetochores, partially redistributes to Rad52 repair foci after γ -irradiation (Figure 1B). Since Rdh54 is required for adaptation, we speculate that its localization to both DNA repair foci and kinetochores may aid in communication between DNA repair and the G2 checkpoint (Lee et al., 2001).

Next, we analyzed the localization of the Mre11, Tel1, Ddc1, Rad24, Ddc2, Rad9, and Rad53 checkpoint proteins relative to Rad52 and Rfa1 foci. Interestingly, these proteins form foci at any stage of the cell cycle in response to ionizing irradiation in contrast to Rad52 foci that form only in S/G2 (Figures 1C–1F and Supplemental Figures S1E–S1F available on *Cell* website). This observation implies that these checkpoint proteins have Rad52-independent functions during DNA repair. Indeed, even during S phase when Rad52 foci can form, as many as 10%–20% of the cells with Ddc1, Rad24, Ddc2, Rad9, or Rad53 foci, and 40%–50% of the cells with Mre11 foci, do not contain a colocalized Rad52 focus. Similarly, Mre11 may also act independently of Rfa1 since 50% of the Mre11 foci form in the absence

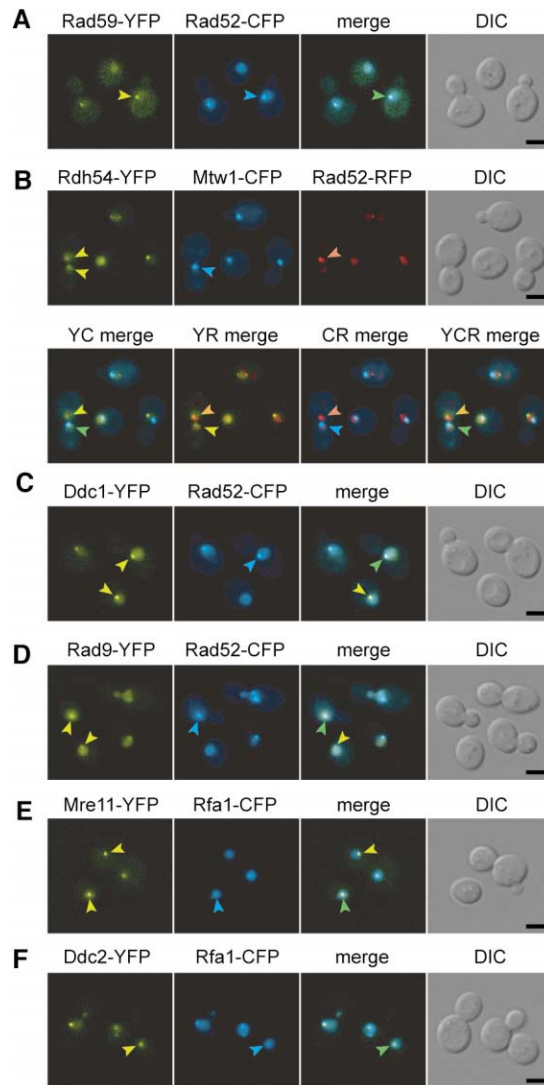


Figure 1. Colocalization of Checkpoint and Repair Foci

Focus formation was analyzed in asynchronously growing cells before (data not shown) and 30 min after exposure to 40 Gy of γ -irradiation. Arrowheads indicate selected foci. Scale bar is equal to 3 μ m.

(A) Rad52 and Rad59 foci in strain W3457-4D.

(B) Rad52, Rdh54, and Mtw1 foci in strain W5019-15C. Pairwise combinations and the triple merge are shown.

(C) Rad52 and Ddc1 foci in strain W3848-4A.

(D) Rad52 and Rad9 foci in strain W4658-3C.

(E) Mre11 and Rfa1 foci in strain W4809-1B.

(F) Ddc2 and Rfa1 foci in strain W3924-11B. See Supplemental Table S1 (available on *Cell* website) for strains.

of an Rfa1 focus (Figure 1E). In contrast, Ddc1, Rad24, and Ddc2 foci always contain Rfa1. Furthermore, as measured by the normalized fluorescent intensities of Ddc1-CFP and Rad24-YFP, approximately 4-fold more molecules of Ddc1 than Rad24 are recruited to sites of DNA damage (Supplemental Figure S1G available on *Cell* website). This *in vivo* observation is consistent with *in vitro* experiments showing that each Rad24/Rfc2-5 complex associates with and loads multiple Ddc1/Rad17/Mec3 complexes onto damaged DNA (Majka and

Burgers, 2003). The frequent colocalization of checkpoint and repair proteins with Rad52 foci suggests that, along with Rad52, these proteins are recruited to DSBs. This was confirmed for Mre11, Rfa1, Ddc1, and Ddc2 by showing that they localize to an endonuclease-induced DSB (see below and data not shown). Taken together, the simultaneous presence of multiple checkpoint and recombination proteins at damaged sites suggests that DSB repair is a concerted process rather than a one by one association/dissociation of each protein sub-complex.

Genetic Dissection of DNA Repair Foci

To test the interdependencies of repair and checkpoint proteins during their recruitment to sites of DNA damage, we performed a genetic analysis of focus formation. After the induction of DNA damage, the localization of each fluorescently tagged protein was scored in a series of gene disruption backgrounds. Interestingly, Rad52 foci form efficiently in *rad59Δ*, *rad51Δ*, *rdh54Δ*, *rad55Δ*, and *rad54Δ* strains but not in cells lacking Rfa1 (Figure 2A), suggesting that Rad52 is recruited to foci via its physical interaction with Rfa1 (Hays et al., 1998). Similar analyses show that recruitment of Rad59 to foci depends on Rad52 but not on Rdh54, Rad51, Rad55, or Rad54. Moreover, Rad59 fails to properly localize to the nucleus in *rad52Δ* cells, implying that Rad59 is transported to the nucleus in a complex with Rad52 (Figure 2E). Nuclear transport was not affected for any other protein in the disruption strains. Recruitment of Rdh54 to foci depends on Rad52 and Rad51, while its localization to kinetochores does not (data not shown). Rad51 depends on Rad52 for localization to foci but not on Rdh54, Rad59, Rad55, or Rad54, suggesting that Rad51 is recruited via its physical interaction with Rad52 (data not shown) (Hays et al., 1995; Shinohara et al., 1992). Rad55 focus formation is dependent on Rad51 and Rad52 but not on Rdh54, Rad59, or Rad54 (Figure 2B). Thus, Rad55 is likely recruited to foci via its physical interaction with Rad51 (Hays et al., 1995). Finally, Rad54 is dependent on Rad51, Rad52, and Rad55 for localization to foci but not on Rdh54 or Rad59 (Figure 2B). Although Rad54 interacts directly with Rad51, this interaction is insufficient for recruitment of Rad54 to Rad51 foci in the absence of Rad55 (Clever et al., 1997; Jiang et al., 1996; Krejci et al., 2001). Interestingly, Rad54 can localize to the kinetochore in an *rdh54Δ* strain but not in wild-type cells implying that it can substitute for the function of Rdh54 at kinetochores (data not shown). The possibility of overlapping functions for Rdh54 and Rad54 proteins has previously been proposed from genetic studies (Klein, 1997; Shinohara et al., 1997). In summary, the genetic requirements for focus formation shows that the entire recombination machinery is recruited to sites of DNA damage by Rad52 via its interaction with RP-A, thus explaining the severe DSB repair defects of *rad52Δ* cells.

The hierarchy described above shows that recombination proteins are recruited to repair foci via a network of interactions with Rfa1 (Figure 2B). Therefore, we tested the interdependencies between Rfa1 and a number of checkpoint proteins during focus formation and found that Mre11 and Tel1 foci form independently of

Rfa1 (Figure 2C and data not shown). This observation suggests that the Mre11 complex and Tel1 are recruited to DSBs before 5'-resection exposes single-stranded DNA at the break site. Interestingly, Rad24, Ddc1, and Ddc2 fail to form foci in the absence of Rfa1 implying that they are recruited to sites of DSB repair by RP-A (Figure 2D and Supplemental Figures S2A–S2B available on Cell website) (Gavin et al., 2002; Hays et al., 1998; Kim and Brill, 2001). This notion has previously been suggested by an inability of a checkpoint defective *rfa1-t11* mutant to recruit Ddc1 and Ddc2 to single-stranded DNA as measured by chromatin immunoprecipitation (Zou and Elledge, 2003a; Zou et al., 2003b). A similar dependency on RP-A has been observed for the recruitment of ATR/ATRIP in *Xenopus* extracts and in mammalian cells (Costanzo et al., 2003; Zou and Elledge, 2003a). Furthermore, Ddc1 foci are completely abolished in *mec3Δ* and *rad24Δ* cells (Figure 2D and data not shown). These observations suggest that Mec3 is necessary for the stability of the Ddc1/Rad17/Mec3 clamp and are also consistent with in vivo and in vitro studies showing that the clamp is loaded onto DNA at sites of damage by the Rad24/Rfc2-5 complex (Majka and Burgers, 2003; Melo et al., 2001). Conversely, Rad24 foci form efficiently in the absence of Mec3 suggesting that the Rad24-containing RFC complex recognizes DNA damage independently of the Ddc1/Rad17/Mec3 complex (data not shown). Rad9 and Rad53 focus formation is abolished in *mec1Δ tel1Δ sml1Δ* kinase-deficient cells, but only partially affected in either single mutant (Figure 2D). Interestingly, Rad9 is recruited to DSBs in the absence of Rad53 indicating that Rad9-Rad53 complex formation (Gilbert et al., 2001) is not required for damage recognition by Rad9. On the other hand, Rad53 focus formation requires Rad9, although its activation is only partially dependent on Rad9 (de la Torre-Ruiz et al., 1998). Tel1 foci are strictly dependent on each of the subunits of the MRX complex but independent of all other proteins tested (Figure 2D), providing an explanation for the checkpoint defects of *mre11Δ* mutants (D'Amours and Jackson, 2001; Grenon et al., 2001; Usui et al., 2001). Finally, Mre11 foci are completely dependent on Rad50 whereas only faint foci are formed in an *xrs2Δ* strain (Figure 2F), consistent with Rad50 and Mre11 recognizing DNA damage and Xrs2 playing a regulatory role in the MRX complex (D'Amours and Jackson, 2001; Trujillo et al., 2003).

As we reported previously, spontaneous Rad52 foci form in a low percentage of cells even in the absence of exogenous DNA damage (Lisby et al., 2001). Similarly, a low frequency of spontaneous foci is observed for all of the proteins examined in this study, likely reflecting the recruitment of the checkpoint and repair machinery to sites of spontaneous DNA damage (Gasior et al., 2001; Lisby et al., 2001). The same genetic interdependencies of focus formation described for γ -ray induced foci were observed for spontaneous foci (Figure 2) suggesting that many steps in the processing of spontaneous lesions are similar to those for radiation-induced damage. In summary, the genetic dissection of checkpoint and repair foci indicates that the assembly of DNA repair centers is directed by a cascade of protein-protein interactions triggered by the MRX complex recog-

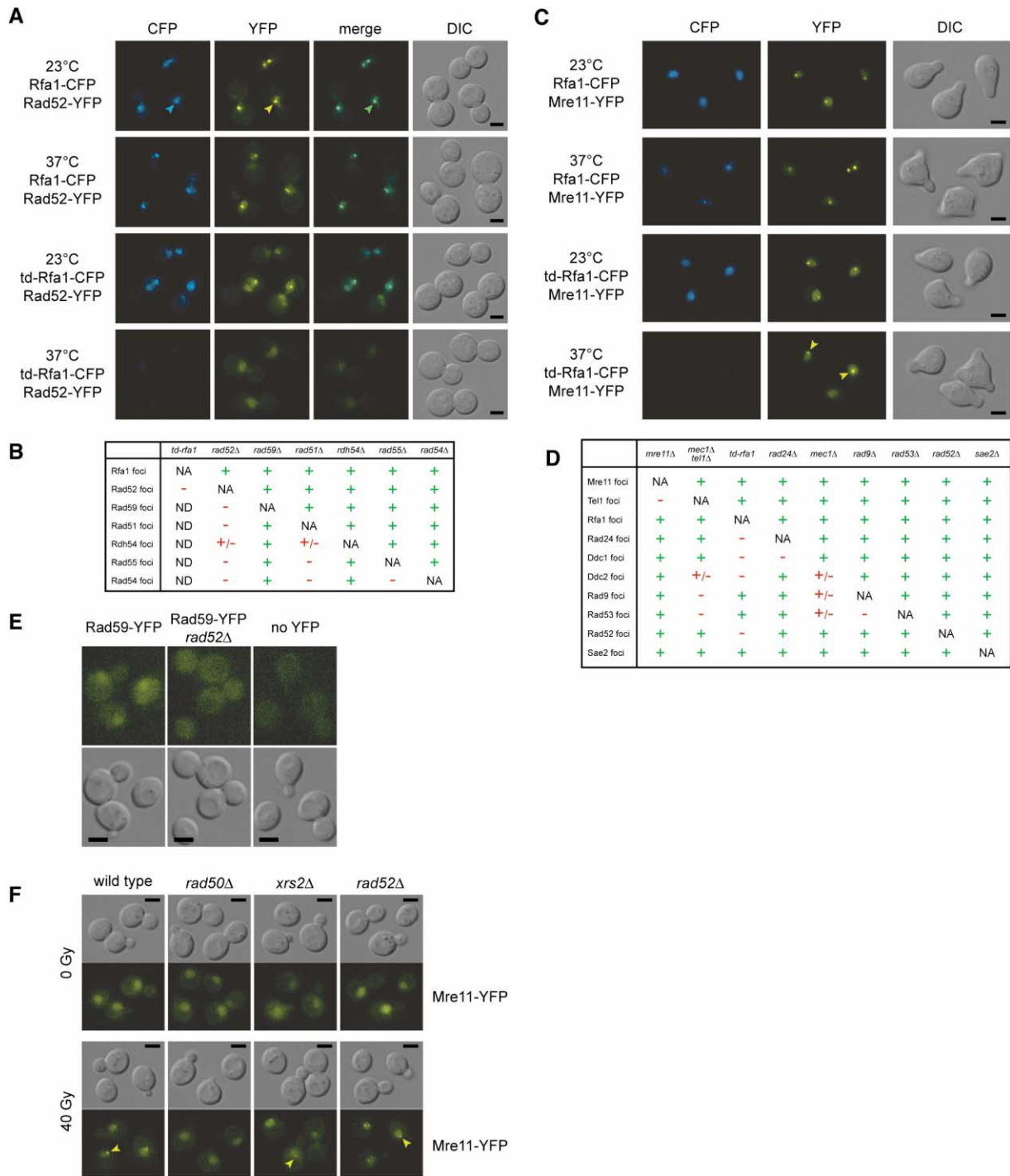


Figure 2. Genetic Requirements for Focus Formation

The localization of checkpoint and repair proteins was determined in various single and double-mutant genetic backgrounds before and after exposure to γ -irradiation (40 Gy). Arrowheads indicate selected foci. Scale bar is equal to 3 μ m.

(A) Rad52 foci require Rfa1. Strains W4637-12D (*RFA1-CFP RAD52-YFP*) and W3783-16A (*td-RFA1-CFP RAD52-YFP*). Cells were arrested in G2 at 23°C prior to elimination of degen-tagged Rfa1 by shifting to 37°C as detailed in the Experimental Procedures.

(B, D) Genetic requirements for spontaneous and γ -ray induced repair and checkpoint foci. The ability of proteins to form foci in exponentially growing cells in SC medium was determined. +, competent in focus formation. -, no foci formed. NA, not applicable. ND, not determined. +/-, few and faint foci observed.

(C) Mre11 foci form independently of Rfa1. Mre11 foci form independently of Rfa1. Strains W4809-1B (*RFA1-CFP MRE11-YFP*) and W4770-10B (*td-RFA1-CFP MRE11-YFP*). Cells were arrested in G1 at 23°C prior to elimination of Rfa1.

(E) Nuclear localization of Rad59 requires Rad52. Localization of Rad59-YFP was examined in wild-type (W3457-12C) and *rad52Δ* (W3837-6C) strains using DAPI staining (data not shown) and an untagged strain (W3749-1A) as a reference.

(F) Mre11 focus formation. No Mre11 foci were observed in the *rad50Δ* strain (W3483-2B). In contrast, the *xrs2Δ* strain (W4025-6A) formed Mre11 foci although these were less bright than those observed in the wild-type (W3483-10A) and in the *rad52Δ* strains (W3797-2D).

nizing DSB ends and by RP-A binding to single-stranded DNA (see below).

Temporal Analysis of DNA Repair Foci

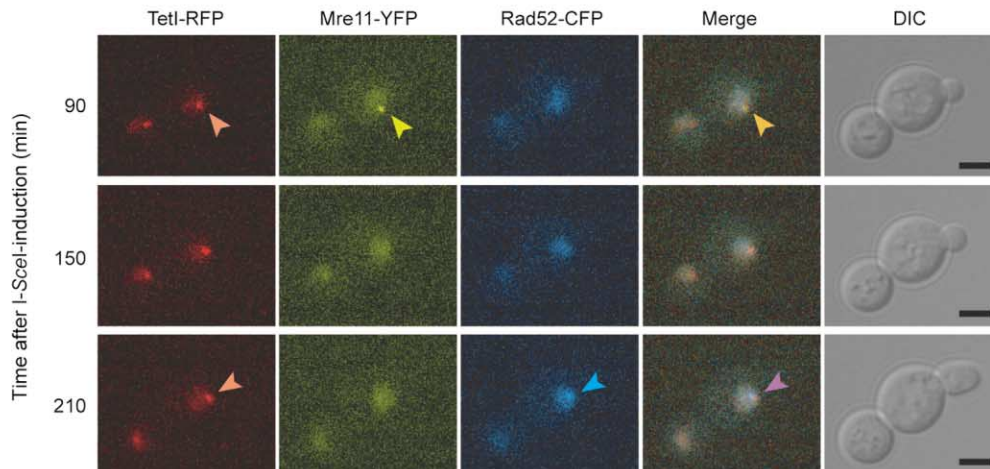
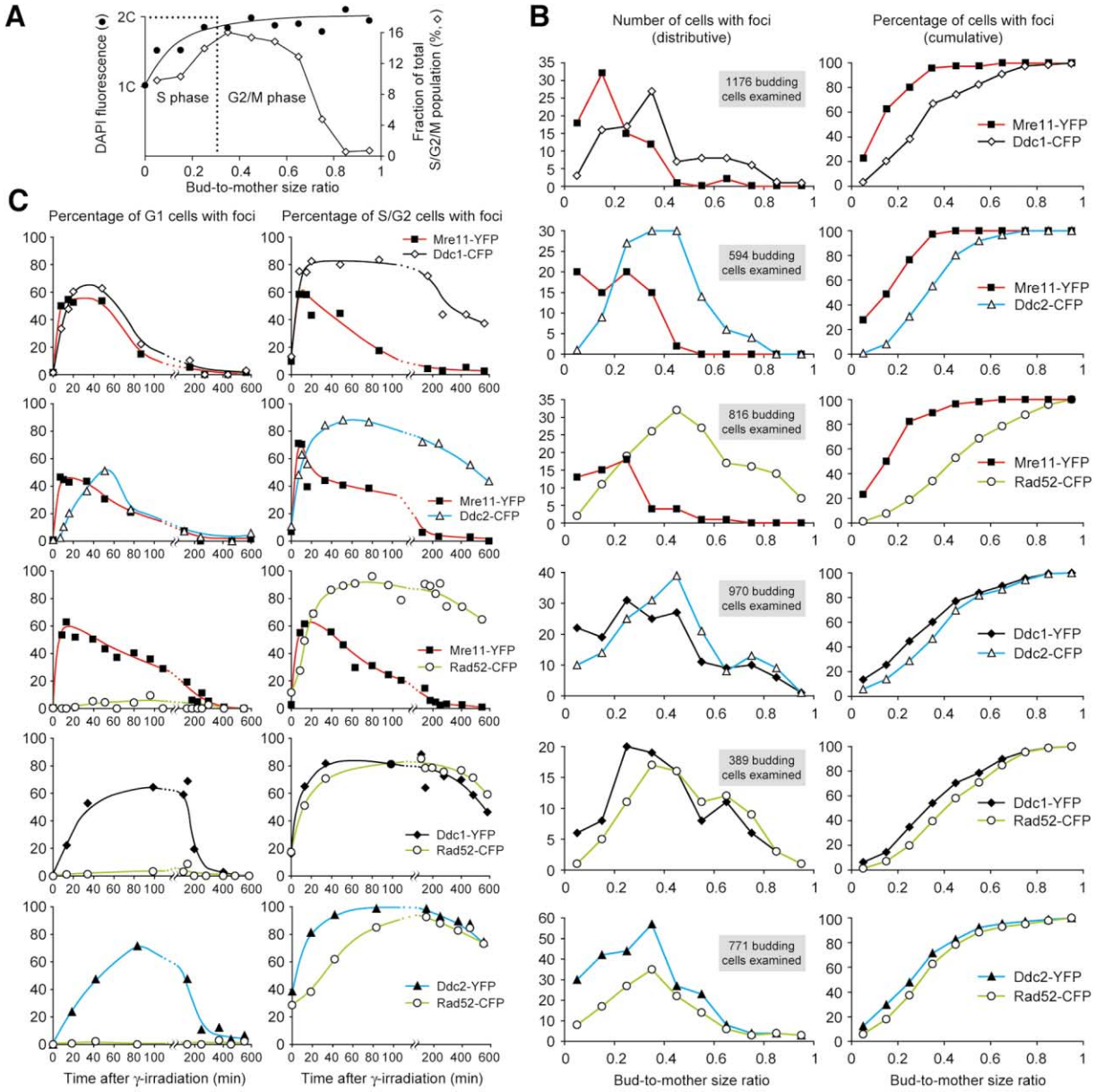
The genetic analysis of DNA repair foci indicates that proteins are recruited to sites of DNA damage in a distinct order. To examine this order in more detail, we conducted a temporal analysis of focus formation. First, cells expressing pairwise combinations of Mre11, Ddc1, Ddc2, and Rad52 fused to either CFP or YFP were inspected for spontaneous foci in S phase. Whenever a focus was observed, the size of the cell and its bud was measured and the bud-to-mother size ratio was used as an indicator of progression through S phase (Figure 3A). This analysis reveals that Mre11 is the earliest protein to form foci as cells enter S phase. Mre11 focus formation is followed by recruitment of Ddc1, then Ddc2 and, only later in S phase, by Rad52 (Figures 3A–3B). As Rad52 is recruited to repair foci, Mre11 dissociates suggesting that the MRX complex has an early function in processing DNA lesions that precedes the recruitment of recombination proteins. In contrast, Ddc1 and Ddc2 colocalize with Rad52 foci as cells progress into G2 implying that the DNA damage checkpoint remains activated until the recombination machinery disengages (Figure 3B).

We next tested the order of recruitment to repair foci induced by γ -irradiation (Figure 3C). The percentage of cells with Mre11 foci peaks at the first time-point (6 min after irradiation) and subsequently decreases. In contrast, the percentage of cells with Ddc1, Ddc2, and Rad52 foci peaks later and these foci persist for several hours. Thus, the order of focus formation after exposure to γ -irradiation is the same as that observed for spontaneous foci. In both cases, the inferred order of recruitment of proteins is based on “snapshots in time”. As a confirmation of these results, we performed time-lapse microscopy of cells containing a specific inducible DSB and pairwise combinations of Rad52 with Mre11 or Ddc2 (Figure 3D and Supplemental Figures S2C and S3A available on *Cell* website). In these experiments, the exact same order of recruitment was observed further demonstrating that checkpoint and repair proteins are sequentially recruited to sites of DNA damage. Interestingly, the time-lapse of Mre11 and Rad52 recruitment to an I-SceI cut site revealed that, in most cases (18/21), Mre11 dissociates from the DSB before Rad52 is recruited. The few cells (3/21) where Mre11 and Rad52 foci simultaneously localize to the break site may reflect instances where the two ends of the DSB are at different stages of processing. Similarly, the higher degree (approximately 50%) of colocalization of Mre11 and Rad52 foci observed after 40 Gy of γ -irradiation (approximately 2 DSBs per cell) is likely due to colocalization of DSBs in repair centers that are at different stages of processing. In fact, when the γ -ray dose was reduced to 20 Gy (mean of 1 DSB per cell) and monitored by time-lapse microscopy, the occasional (2/22) overlap of Mre11 and Rad52 foci was observed no more often than with a single I-SceI cut site (Supplemental Figure S3B available on *Cell* website). Thus, the time-lapse experiments corroborate the time-course results and indicate further that the localization of Mre11 and Rad52 to damaged DNA is mutually exclusive during DSB repair.

If the sequential assembly of proteins into foci is correlated with function, then disruption of proteins that appear later should not affect the time of recruitment of early proteins such as Mre11. To examine this idea, the kinetics of Mre11 foci in response to γ -irradiation was determined in various mutant genetic backgrounds. As predicted, the time of recruitment of Mre11 into foci was largely unaffected in *rad24* Δ , *mec1* Δ *smf1* Δ , and *rad52* Δ deletion strains (Figure 4A). However, the Mre11 foci persisted to some extent in these mutants, probably reflecting a failure to effectively repair DNA damage. The delay in the disassembly of Mre11 foci is particularly dramatic in *sae2* Δ and *rad50S* mutant cells (Figure 4A), which were previously identified by their defects in processing of Spo11-induced DSBs during meiosis (see below) (Keeney and Kleckner, 1995).

Next, the kinetics of Rad52 focus formation were determined in several mutants that lack proteins recruited earlier than Rad52. Unfortunately, deletion of *MRE11*, the first protein recruited to foci, causes high levels of spontaneous Rad52 foci probably due to telomeric instability making it difficult to interpret the kinetics of further induction by γ -irradiation (Figure 4B) (Moreau et al., 1999; Nugent et al., 1998). However, it should be noted that a substantial number of G1 cells contain Rad52 foci in the *mre11* Δ strain. This is likely due to a partial checkpoint defect of *mre11* Δ cells that allows progression of Rad52 focus-containing cells from G2 into G1 (D'Amours and Jackson, 2001; Grenon et al., 2001). Similarly, deletion of another checkpoint gene, *RAD24*, also results in an increase of G1 cells with Rad52 foci. Furthermore, the *RAD24* deletion does not affect the kinetics of Rad52 focus assembly after DNA damage (Figure 4B) showing that it is not required for timely recruitment of Rad52. The assembly of Rad52 foci was next examined in a *sae2* Δ strain, which causes the persistence of Mre11 foci (see above). Interestingly, the absence of Sae2 delays the appearance of Rad52 foci indicating that it plays a transitional role in the dissociation of Mre11 from, and the recruitment of Rad52 to, repair foci (Figure 4B). Time-lapse microscopy of a single DSB induced by either the I-SceI endonuclease or γ -irradiation confirmed that Mre11 foci persist in a *sae2* Δ and that the subsequent recruitment of Rad52 is correspondingly delayed (Figure 5A and Supplemental Figures S3C–S3D available on *Cell* website). The view that Sae2 acts at the transition from Mre11 function to Rad52 function is supported by the observation that the Sae2 foci appear at the precise time when Mre11 foci disassemble and Rad52 foci form (Figures 4A, 4C–4D). Along with Mre11, Tel1 foci also persist for up to 3 hr in *sae2* Δ cells (data not shown). The delayed dissociation of the Tel1 checkpoint kinase from DSBs may account for *sae2* Δ suppression of checkpoint defects in a *mec1* Δ mutant (Grenon et al., 2001; Usui et al., 2001).

To investigate further the role of Sae2, we compared the *sae2* Δ phenotype to that of *mre11-D56N* and *mre11-H125N* nuclease-deficient cells (Moreau et al., 1999). It has previously been demonstrated that *sae2-1* and certain *mre11* mutants are unable to resect Spo11-induced DSBs during meiosis (Keeney and Kleckner, 1995; Moreau et al., 1999). Moreover, these mutants also share a defect in processing of hairpin-capped DSBs, which are substrates for the Mre11 nuclease in wild-type cells



(Lobachev et al., 2002; Trujillo and Sung, 2001). When Mre11-H125N and Rad52 foci are monitored by time-lapse microscopy after induction of a DSB by either the I-SceI endonuclease or by γ -irradiation, Mre11-H125N foci persist and the subsequent recruitment of Rad52 is delayed comparable to *sae2* Δ cells (Figures 5A–5B and Supplemental Figures S3E–S3F available on Cell website). This result suggests that, as in meiotic cells, Sae2 is required for Mre11 nuclease activity in mitotic cells. Interestingly, the duration of Mre11 foci in *sae2* Δ and *mre11-H125N* cells is significantly longer with a γ -ray-induced DSB than with an I-SceI-induced DSB (Figure 5A). This likely reflects the ability of an alternative nuclease(s) to substitute for Mre11-H125N in resection of the “clean” 5'-phosphate ends of an I-SceI-induced DSB whereas many γ -ray-induced DSBs likely contain aberrant “dirty” structures that are not easily processed by nucleases other than Mre11 (Moreau et al., 2001). Finally, Sae2 foci persist in *mre11-D56N* and *mre11-H125N* cells after γ -irradiation, suggesting that Sae2 remains associated with DSBs along with Mre11 in nuclease-deficient cells (Figure 5C).

In summary, the temporal analyses show that Mre11, Tel1, Rad9, Rad53, Rfa1, Rad24, Ddc1, and Ddc2 are recruited early to sites of DNA damage irrespective of the cell cycle phase. In contrast, recombination proteins (Rad52, Rad59, Rdh54, Rad51, Rad55, and Rad54) appear later and only in S and G2 phase. In addition, Mre11 and Tel1 dissociate from foci as recombination proteins are recruited with Sae2 acting as a cofactor during this replacement.

The Response to DNA Replication Stress

Spontaneous checkpoint and repair foci are observed primarily in S phase implying that DNA lesions are being recognized and repaired during replication. Although the nature of these spontaneous lesions is unknown, their timing suggests that they are due to stalled or collapsed replication forks. Some proteins are found at stalled replication forks (Ddc2) (Katou et al., 2003) and others are required to stabilize stalled forks (Ddc2 and Rad53) (Desany et al., 1998; Lopes et al., 2001; Tercero and Diffley, 2001) or for slowing S phase in response to damage (such as Ddc1) (Paulovich et al., 1997). Therefore, cells were examined for focus formation for these and other checkpoint and repair proteins after treatment with hydroxyurea (HU), which stalls DNA replication by depleting dNTP pools (Lopes et al., 2001; Reichard, 1988). After 30 min in HU, most cells contain multiple Rfa1, Ddc1, Ddc2, and Rad53 foci (Figures 6A–6B and

data not shown), consistent with the notion that these proteins are recruited to stalled forks. It should be noted that, in general, these foci are less bright than those induced by γ -irradiation, suggesting that less single-stranded DNA is exposed at stalled-replication forks than at DSBs. We also examined Mre11 and Rad52, since *mre11* Δ and *rad52* Δ mutants are extremely sensitive to HU. Surprisingly, neither protein forms foci after HU treatment, indicating that they are not recruited to stalled replication forks. However, these proteins do form foci after prolonged exposure to HU (16 hr, data not shown). Thus, the sensitivity of *mre11* Δ and *rad52* Δ mutants to HU is likely due to DNA lesions arising as cells eventually progress through mitosis with partially replicated chromosomes (Allen et al., 1994; D'Amours and Jackson, 2001). Interestingly, spontaneous Rad52 foci are almost completely eliminated by HU, implying that either Rad52 is actively excluded from stalled replication forks or the structures it recognizes are not exposed at stalled forks (Figure 6A).

It has been demonstrated previously that stalled replication forks collapse irreversibly in cells lacking Mec1 or Rad53 (Lopes et al., 2001; Tercero and Diffley, 2001). In such mutant cells, Rad52 foci form rapidly after treatment with HU (Figure 6C). Similarly, Mre11 focus formation is induced by HU in *mec1* Δ cells (Figure 6C). These results demonstrate that although Mre11 and Rad52 are not recruited to HU-stalled replication forks in wild-type cells, these two proteins are recruited efficiently to collapsed forks (e.g., in *mec1* Δ or *rad53* Δ mutants). We interpret the HU experiments to imply that Rfa1, Ddc1, Ddc2, and Rad53 are recruited to single-stranded DNA at stalled replication forks to help stabilize them. In the event of replication fork collapse, DNA ends are exposed, triggering the recruitment of Mre11 and Rad52 for processing and recombinational restart of the replication fork, respectively.

Discussion

The cellular responses to DSBs and to replication stress involve many of the same protein factors and here we demonstrate that this set of proteins is choreographed in a distinct fashion depending on the nature of the DNA lesion. For DSBs, Mre11 is the first protein detected at the break site. The MRX complex is required for the recruitment of the Tel1 kinase and it is likely that these proteins play a role in the early decision between HR (Game and Mortimer, 1974) and NHEJ (Boulton and Jackson, 1998). Indeed, genetic studies have described

Figure 3. Order of Recruitment of Mre11, Ddc1, Ddc2, and Rad52 to Sites of DNA Damage

(A) Correlation between DNA replication and bud-to-mother size ratio in a wild-type strain (W3749-1A). Five hundred sixty seven budding cells were examined. Strains W3434-14C (*MRE11-YFP RAD52-CFP*), W3838-14A (*MRE11-YFP DDC1-CFP*), W3839-8D (*MRE11-YFP DDC2-CFP*), W3848-4A (*DDC1-YFP RAD52-CFP*), W3849-15C (*DDC2-YFP RAD52-CFP*), and W4208-12A (*DDC1-YFP DDC2-CFP*) were used in the experiments shown in (B) and (C).

(B) Recruitment of Mre11, Ddc1, Ddc2, and Rad52 to spontaneous foci. To the right are the cumulative representations of each distribution (Padmore et al., 1991). For each strain, the distribution of bud sizes in the population is similar to that displayed in (A).

(C) Kinetics of Mre11, Ddc1, Ddc2, and Rad52 focus formation after γ -irradiation (40 Gy). Based on cellular morphology, cells were grouped into G1 (unbudded) and S/G2 (budded).

(D) Time-lapse of Mre11 and Rad52 recruitment to an irreparable I-SceI-induced DSB. Selected time-points after addition of galactose are shown of strain W4761-2A. Rad52 is never recruited to the I-SceI cut site before Mre11 and in most time-lapse experiments (18/21) the Mre11 focus disassembles before recruitment of Rad52. Arrowheads indicate selected foci. Scale bar is equal to 3 μ m.

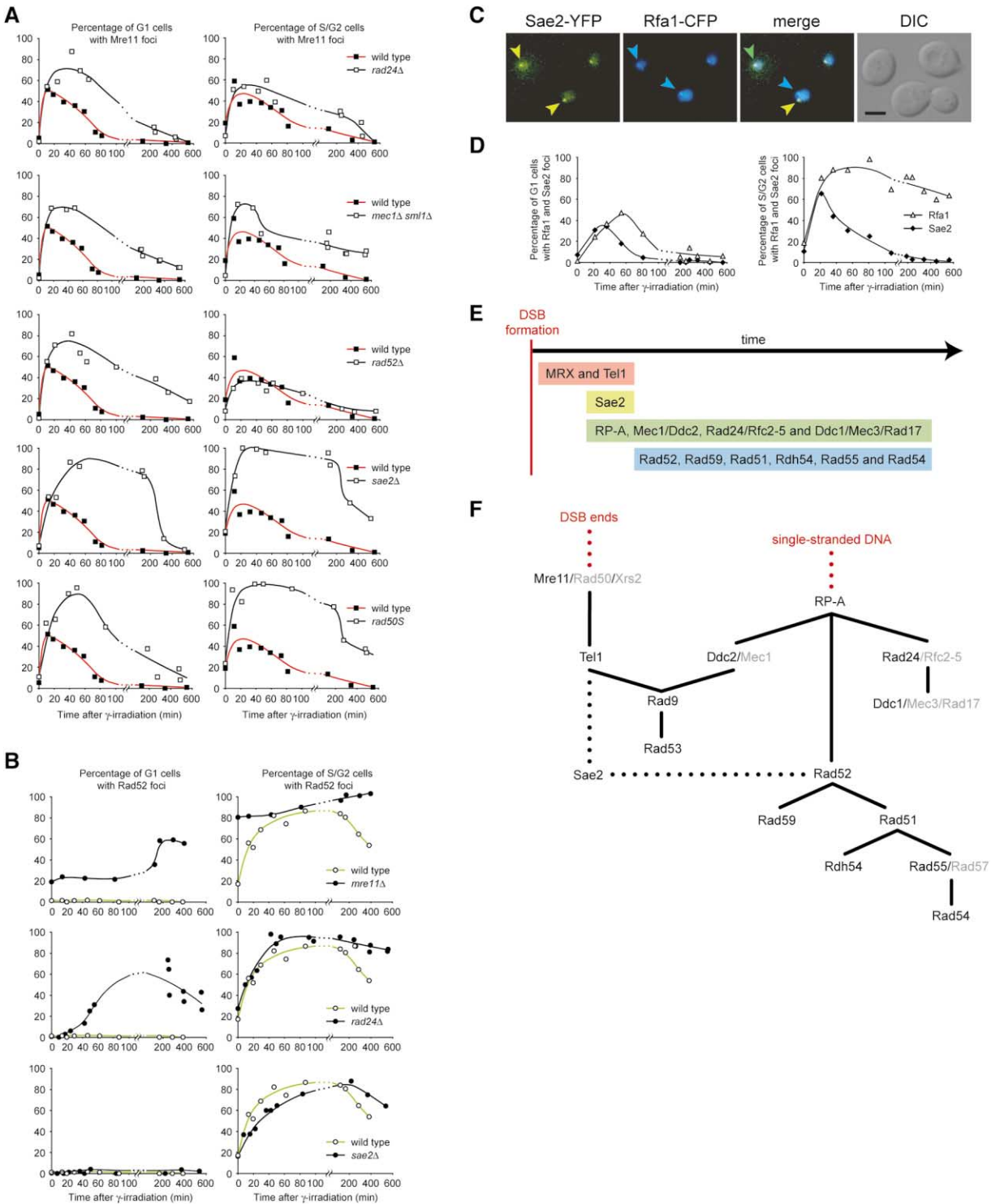


Figure 4. Choreography of Checkpoint Activation and DNA Repair

(A) Kinetics of Mre11 focus formation after 40 Gy of γ -irradiation.

(B) Kinetics of Rad52 focus formation after 40 Gy of γ -irradiation.

(C) Colocalization of Sae2 and Rfa1 foci after 40 Gy of γ -irradiation. Arrowheads indicate selected foci. Scale bar is equal to 3 μ m.

(D) Kinetics of Sae2 and Rfa1 focus formation after 40 Gy of γ -irradiation.

(E) Schematic time-line for association of proteins at a DSB.

(F) Summary of genetic interdependencies for focus formation. Solid lines indicate absolute requirements. Dotted lines indicate regulatory functions. Names of visualized proteins in black and names of those that have not been visualized in gray.

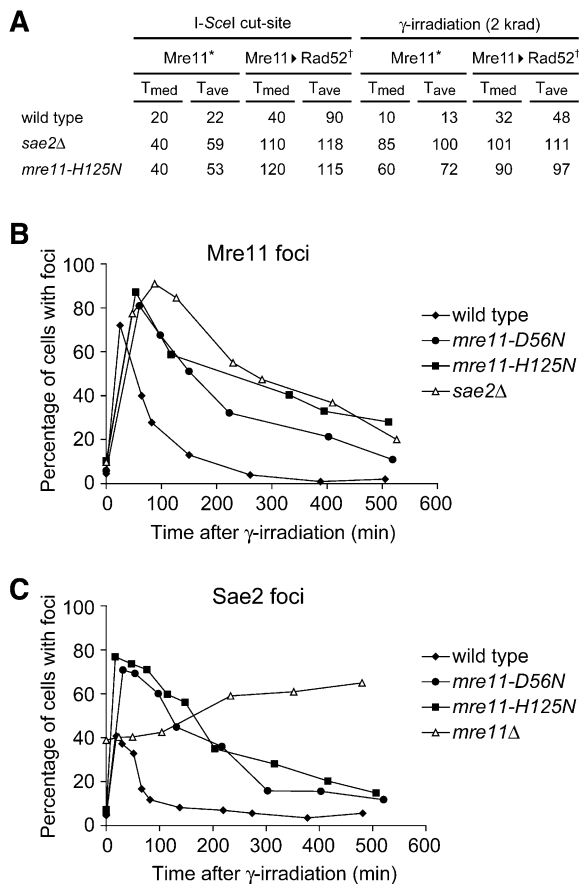


Figure 5. Kinetics of Mre11, Rad52, and Sae2 Foci
(A) Summary of time-lapse microscopy of Mre11 and Rad52 foci after I-SceI cleavage or γ -irradiation. *, median (T_{med}) and average (T_{ave}) duration of Mre11 foci. †, time from Mre11 appearance to Rad52 recruitment. See Supplemental Figure S3 (available on Cell website) for details.
(B) Kinetics of Mre11 focus formation after 40 Gy of γ -irradiation. Strains W3483-10A (*MRE11-YFP*), W3750-1B (*MRE11-YFP sae2* Δ), W5549-4A (*mre11-D56N-YFP*), and W5536-3A (*mre11-H125N-YFP*) were used in the experiment
(C) Kinetics of Sae2 focus formation after 40 Gy of γ -irradiation. Strains W4249-5C (*SAE2-YFP*), W4249-6B (*SAE2-YFP mre11* Δ), W5579-15B (*SAE2-YFP mre11-D56N*), and W5578-2B (*SAE2-YFP mre11-H125N*) were used in the experiment.

a competitive relationship between HR and NHEJ (Frank-Vaillant and Marcand, 2002). In this context, it is interesting to note that *TEL1* is a negative regulator of NHEJ (Chan and Blackburn, 2003). The interdependence between Mre11 and Tel1 is also found in mammalian cells, where Mre11 is required for activation of ATM, the *TEL1* homolog (Carson et al., 2003; Lee and Paull, 2004; Uziel et al., 2003). Although Ku80 has been shown to be associated with DSBs by chromatin immunoprecipitation (Martin et al., 1999), we did not microscopically detect a focus at DSBs (data not shown) suggesting that only a few molecules of Ku80/Ku70 are sufficient to channel DSBs into NHEJ. Among the large number of mutants tested, only deletion of Rad50 and Xrs2, the other two subunits of the MRX complex, interferes with the assembly of Mre11 foci, strongly suggesting that the MRX complex is the earliest sensor of DSBs by

binding directly to DNA ends (de Jager et al., 2001) (illustrated in Figure 7).

Next, irrespective of the phase of the cell cycle, Mre11 and Tel1 are followed by Rfa1, which in turn is required for recruiting the checkpoint proteins, Rad24, Ddc1, and Ddc2, implying that RP-A mediates the checkpoint response to single-stranded DNA as has also been suggested by other studies in yeast as well as in *Xenopus* and humans (Costanzo et al., 2003; Zou et al., 2002, 2003b; Zou and Elledge, 2003a). Strikingly, the MRX-Tel1 and RP-A-Ddc2/Mec1 pathways are independent and redundant for activating the Rad9 and Rad53 checkpoint transducers in response to ionizing radiation, implying that these pathways respond to distinct DNA structures, namely DNA ends and single-stranded DNA, respectively. Similar complementarity for Tel1 and Mec1 was recently reported for telomere association (Takata et al., 2004). Although Rad53 foci are not formed in the absence of Rad9, a partially intact Rad53 checkpoint is observed in *rad9* Δ cells indicating that an accumulation of Rad53 at repair foci is not an absolute requirement for its activation (de la Torre-Ruiz et al., 1998). In fact, Rad53 foci are faint, suggesting that it is more loosely associated with DSB sites, which has been shown to be important for the checkpoint function of CHK2, the Rad53 homolog in mammalian cells (Lukas et al., 2003; Melo et al., 2001). Interestingly, Rfa1 foci form at all phases of the cell cycle (although they are less bright in G1), indicating that DNA ends can be processed by 5' resection any time in preparation for homologous recombination.

Finally, the recruitment of Rad52 and Rad51 also depends on Rfa1 and occurs only after Mre11 and Tel1 foci have disassembled, which may explain why Mre11 and Rad51 foci rarely colocalize in mammalian cells (Maser et al., 1997; Nelms et al., 1998). The occasional colocalization of Mre11 and Rad52 foci in yeast and mammalian cells may reflect different stages of repair when multiple DSBs are recruited to a single repair center (Aten et al., 2004; Lisby et al., 2003b). Furthermore, the recombination proteins form foci only after cells have entered S phase possibly to delay recombination until DNA replication. The entire recombination machinery (Rad51, Rad54, Rdh54, Rad55, and Rad59) depends on Rad52 for recruitment to sites of DNA damage, which is consistent with the severe phenotype of *rad52* Δ mutants in budding yeast. The weak *rad52* Δ phenotype in mammalian cells may be due in part to its redundancy with XRCC3, a Rad51 paralogue, for recruiting recombination proteins to sites of DNA damage (Benson et al., 1998; Fujimori et al., 2001; Rijkers et al., 1998).

The DNA damage checkpoint and repair machinery likely evolved to deal with DNA replication stress such as stalled or collapsed replication forks. Surprisingly, only a subset of the proteins that are recruited to DSBs are also recruited to hydroxyurea-stalled replication forks (e.g., Rfa1, Ddc1, Ddc2, and Rad53). The recruitment of Rfa1 indicates that single-stranded DNA becomes exposed at stalled replication forks as previously demonstrated by electron microscopy (Sogo et al., 2002). Interestingly, Mre11 is not recruited, suggesting that free DNA ends are not exposed at stalled replication forks. Furthermore, the absence of Rad52 at stalled forks implies that DNA synthesis can resume without

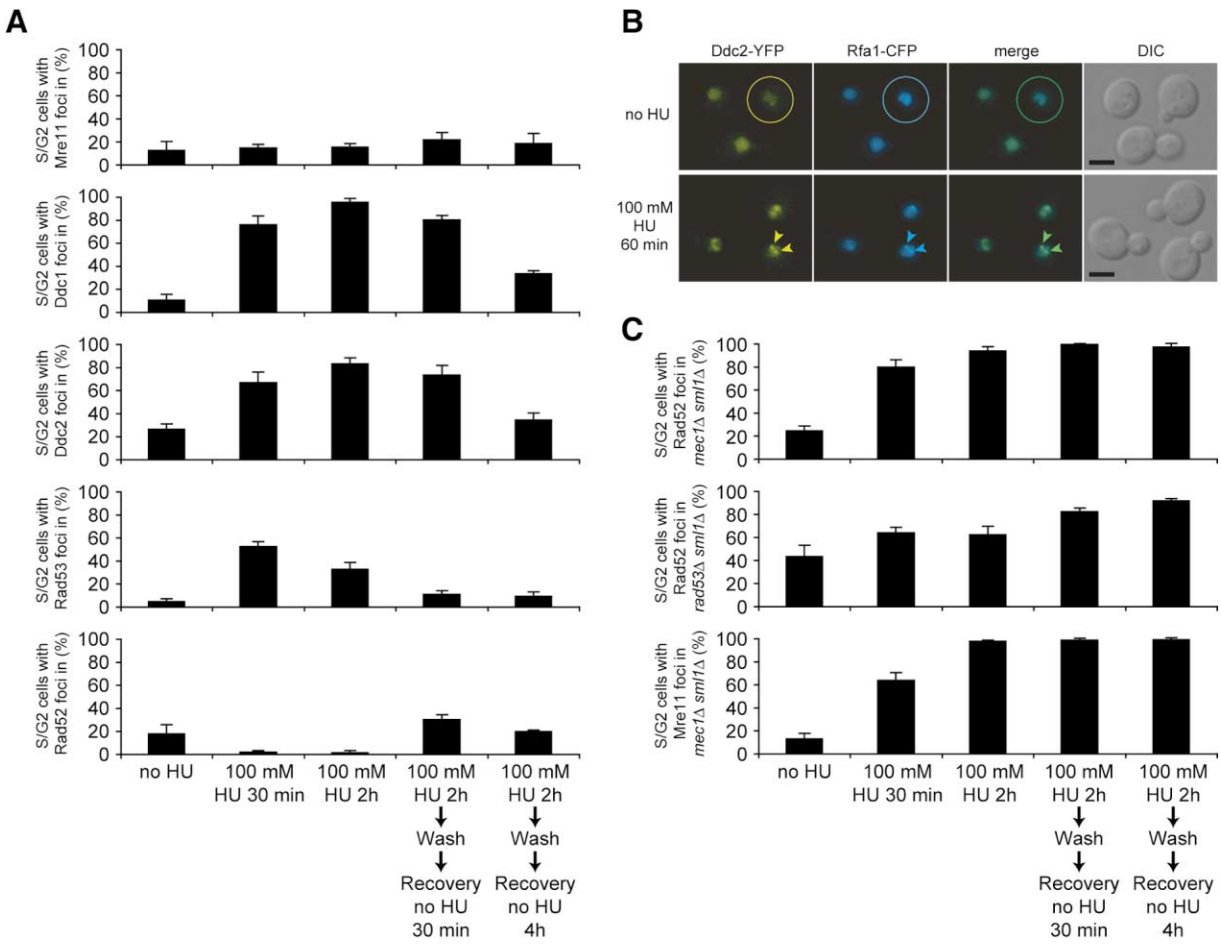


Figure 6. Induction of Checkpoint and Repair Foci by Stalled or Collapsed Replication Forks

The percentage of cells with foci was determined after treatment with 100 mM HU for 30 min and 2 hr. The cells were then washed twice in SC medium without HU, resuspended in fresh SC medium and allowed to recover for 30 min and 4 hr before reexamination for foci.

(A) HU-induced focus formation in wild-type cells (stalled replication forks). The percentage of cells with Mre11-YFP (W3483-10A), Ddc1-YFP (W3923-12B), Ddc2-YFP (W3792-4B), Rad53-YFP (W4826-13A), or Rad52-YFP (W3749-14C) foci is plotted for each of the indicated treatments with HU.

(B) Colocalization of HU-induced Rfa1 and Ddc2 foci. Representative localization of Rfa1 and Ddc2 before and after treatment with HU for 60 min in strain W3924-11B (*DDC2-YFP RFA1-CFP*). Arrowheads indicate selected foci. Circle marks undamaged S phase cell with speckled localization of Rfa1 and Ddc2. Scale bar is equal to 3 μ m. In the absence of HU, Rfa1 and Ddc2 displays a speckled localization in S phase cells. This localization partially overlaps with Mcm2, which marks origins of replication. This result is consistent with the role of RP-A in DNA replication and suggests that low amounts of the Mec1/Ddc2 complex are permanently associated with the replication apparatus (data not shown).

(C) HU-induced focus formation in mutant cells (collapsed replication forks). The percentage of cells with Rad52-YFP foci in *mec1Δ smf1Δ* (W4125-5B) and *rad53Δ smf1Δ* (W4774-16B) is plotted. The percentage of cells with Mre11-YFP foci in *mec1Δ smf1Δ* cells (W4211-11C) is plotted.

the need for homologous recombination. In contrast, when replication forks collapse, e.g., after treating *mec1Δ* or *rad53Δ* cells with HU, then Mre11 and Rad52 are recruited efficiently into foci indicating that collapsed forks expose DNA ends that are recognized and bound by these proteins (Van Dyck et al., 1999). Although the HU-induced Rad52 foci observed in *mec1Δ* cells may be due to DSBs generated during mitotic exit, it is unlikely since foci form efficiently when mitotic exit is prevented by addition of the microtubule destabilizing drug nocodazole together with HU (Supplemental Figure S4 available on Cell website). However, the fact that, in a recent study, no recruitment of Rad51 or Rad52 to a specific DNA origin of replication (*ARS305*) in HU-treated *rad53* cells could be detected (Lucca et al.,

2004), may reflect differences in the experimental strategies or specific features of the *ARS305* locus.

Once recruited to a collapsed fork, recombination proteins likely help to restart DNA synthesis and facilitate lesion bypass. Ddc1 foci also form in response to HU, suggesting that the Ddc1/Mec3/Rad17 complex is involved in stabilizing stalled replication forks. However, in their absence, replication forks do not collapse since Rad52 foci are not induced (data not shown). We suspect that spontaneous Rad52 foci are due to replication fork collapse or true DSBs, since Rad52 foci form in cells with collapsed replication forks (HU-treated *mec1Δ smf1Δ* cells) but not in cells with stalled replication forks (HU-treated wild-type cells). Moreover, replication fork stalling in wild-type cells must be rare, since we observe

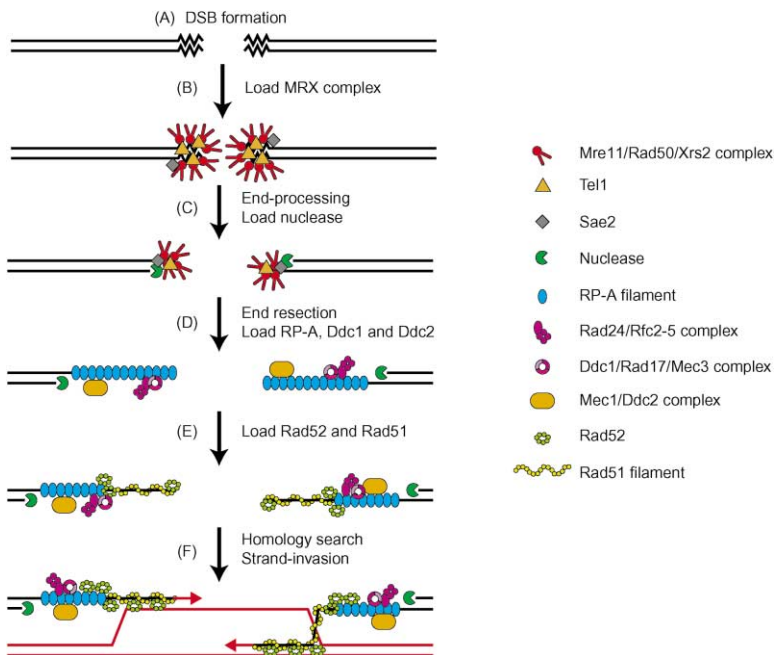


Figure 7. Model for Recruitment of Checkpoint and Recombination Proteins to a DSB (A–B) DSB formation and recruitment of the MRX complex, Tel1, and Sae2. (C) The MRX complex initially processes the DSB ends and dissociates as 5′-3′ resection is initiated by an unknown nuclease(s). (D) Single-stranded DNA generated by resection is bound by RP-A, which recruits the Ddc2/Mec1, Rad24/Rfc2-5, and Ddc1/Rad17/Mec3 complexes. (E–F) Only after cells have entered S phase is Rad52 recruited to DSB ends to catalyze the displacement of RP-A by Rad51, which is a prerequisite for strand invasion.

no increase in the frequency of spontaneous Rad52 foci in cells where stalled forks would be converted to collapsed forks, e.g., *mec1Δ sml1Δ* cells (Figure 6, no HU; Tercero and Diffley, 2001).

The assembly of checkpoint and repair foci appears to be largely governed by a network of protein-protein interactions rather than each of the involved proteins binding to the DNA lesion independently. For example, the MRX complex directs the recruitment of the Tel1 checkpoint kinase via the interaction between Tel1 and Xrs2 (Nakada et al., 2003). The recruitment of Mre11 to damage is strictly dependent on Rad50 but only partially dependent on Xrs2 in mitotic cells. In contrast, during meiosis, *XRS2*, but not *RAD50*, is required to recruit Mre11 to Spo11-induced DSBs showing that the three components of the MRX complex have different interdependencies during mitotic and meiotic DSB repair (Borde et al., 2004). After single-strand tails are formed and coated with RP-A, the Rad24/Rfc2-5 complex is recruited via the interaction between Rfa1 and Rfc4 (Kim and Brill, 2001). Next, the Rad24/Rfc2-5 complex interacts with and loads the PCNA-like complex, Ddc1/Rad17/Mec3 (Majka and Burgers, 2003). The recruitment of Ddc2 to repair foci may be explained by the direct association of the Ddc2/Mec1 complex with RP-A (Kim and Brill, 2003). Recombination is likely initiated when Rad52 is recruited by interacting directly with all three subunits of RP-A (Hays et al., 1998). Rad52 also interacts with Rad59 and Rad51 to subsequently engage these recombination proteins in DSB repair (Davis and Symington, 2001; Hays et al., 1995). The dependency of Rad51 focus formation on Rad52 is in contrast to previous reports of immunohistochemically detectable Rad51 foci in a *rad52Δ* strain (Gasior et al., 2001). However, our results are in agreement with chromatin immunoprecipitation experiments at an induced DSB (Sugawara et al., 2003). In the next step in this process, Rad51 interacts with Rhd54, Rad55, and Rad54 to recruit these

proteins to the repair center (Hays et al., 1995; Jiang et al., 1996; Petukhova et al., 2000). The requirement for Rad55 in Rad54 focus formation suggests that Rad55-dependent assembly of a Rad51 nucleoprotein filament is a prerequisite for Rad54 recruitment (Clever et al., 1997; Jiang et al., 1996; Krejci et al., 2001).

Some of the checkpoint and repair proteins are recruited in a cell cycle or DNA damage-dependent manner. For example, Rad52 interacts with RP-A, but is only recruited to foci during S and G2 likely restricting recombinational repair to these phases of the cell cycle. Furthermore, Rfa1 is recruited to both stalled and collapsed replication forks while Rad52 is recruited only to collapsed forks. Another example is Rad9, whose appearance in foci depends on the Mec1 and Tel1 checkpoint kinases after DNA damage activation, suggesting that Rad9 interacts with proteins at sites of DNA damage in a phosphodependent manner as that described for the interaction of BRCA1 with BACH1 in mammalian cells (Manke et al., 2003; Yu et al., 2003). By analogy to the phosphorylation-dependent binding of BRCA1 to BACH1 and 53BP1 in mammalian cells (Manke et al., 2003; Yu et al., 2003), it is likely that Rad9 is recruited to DSBs via binding of its BRCT domain to phosphoepitopes generated by Tel1 and/or Mec1 at the break site. Once Rad9 is activated by Mec1- and/or Tel1-dependent phosphorylation, Rad9 recruits Rad53 to DSBs.

During focus assembly/disassembly, Sae2 foci appear at the time when Mre11 and Tel1 dissociate from, and RP-A is recruited to, sites of DNA damage. The timing and transient nature of Sae2 foci suggest that Sae2 plays a role in the transition from the initial damage recognition to the 5′-3′ resection and repair by downstream factors. The effect of a *sae2Δ* deletion, which delays both disassembly of Mre11 foci and the subsequently recruitment of recombination proteins supports this notion. The similar phenotypes conferred by *sae2Δ*

and *rad50S* could be explained by a failure to recruit Sae2 to sites of DNA damage in the *rad50S* mutant. However, Sae2 foci are not disrupted in *mre11Δ* or *rad50S* mutants or in any of the other mutant genetic backgrounds that we tested (Figure 2 and a quadruple *mec1Δ tel1Δ sml1Δ mre11Δ* mutant, data not shown), suggesting that either Sae2 can interact directly with damaged DNA or it can be recruited to repair foci via multiple redundant interactions with other potentially unidentified proteins. Interestingly, nuclease-deficient *mre11-D56N* and *mre11-H125N* cells display kinetics of Mre11 and Rad52 foci similar to that of *sae2Δ* cells, suggesting that Sae2 is required for Mre11 nuclease activity *in vivo*. Furthermore, this finding suggests that the transition from initial DNA damage recognition and checkpoint activation by the MRX complex to recombinational repair by the Rad52 pathway is governed by the DNA structures exposed at the DSB site.

It has previously been shown that the *mre11-H125N* nuclease mutation has little if any effect on the rate of resection of an *HO* endonuclease-induced DSB (Moreau et al., 1999). However, using an I-SceI-induced DSB with a structure similar to the *HO*-induced DSB, we observe a small but significant delay of the disassembly of Mre11 foci and the subsequent recruitment of Rad52 compared to wild-type (Figure 5A). Perhaps the delay observed with the “clean” break of the I-SceI endonuclease reflects the occasional need to process and remove covalently attached proteins such as topoisomerases that can bind close to DNA lesions (Andersen et al., 1991). Such covalent intermediates would require removal by Mre11 similar to that seen for Spo11 intermediates during meiosis (Keeney and Kleckner, 1995). Moreover, when cells are faced with a γ -ray-induced DSB, the delay is increased approximately 2-fold, which is consistent with the intermediate sensitivity to γ -irradiation displayed by *mre11-H125N* mutants (Moreau et al., 1999). The increased delay likely reflects the requirement for Mre11 to act as a structure specific endonuclease on aberrant DSB ends generated by γ -irradiation (Lobachev et al., 2002; Trujillo and Sung, 2001). Based on the time-lapse analyses of Mre11 foci, we conclude that Mre11 is associated with a DSB only transiently in wild-type cells (approximately 10–20 min). However, resection of DSBs can proceed for several hours until repair occurs (Lee et al., 1998). Moreover, *mre11* null mutants have a more severe defect in DSB resection than the nuclease mutant (Lee et al., 1998; Moreau et al., 1999). Taken together, these findings suggest that Mre11 may be involved in recruiting another nuclease to act at later stages of resection and that the primary role of Mre11 is in checkpoint activation and initiation of resection at ends containing aberrant DNA structures.

We find Rdh54 constitutively localized at kinetochores in mitotic cells. After DNA damage, it is also found at repair foci and its recruitment depends on the recombination proteins Rad51 and Rad52. In addition, genetic studies suggest that Rdh54 and its homolog, Rad54, have overlapping functions in homologous recombination (Klein, 1997). It is noteworthy that the Rad51 and Rad52 recombination proteins are not found at kinetochores and that Rdh54 localizes to kinetochores even in the absence of recombination proteins. We speculate that the localization of Rdh54 to kinetochores reflects

its function during adaptation (Lee et al., 2001) by aiding in the communication between the DNA damage and spindle checkpoints.

This study constitutes an extensive genetic and cell biological study of proteins associated with DNA repair foci. As such, it provides insights into the spatiotemporal organization of checkpoint proteins at repair centers/foci in relation to recombination proteins. However, it remains to be established how this organization reflects the complex biochemical DNA processes that occur in the microenvironment of a DNA lesion. The organization of DSB repair into centers may provide a number of advantages to the cell. First, foci facilitate a 50-fold higher concentration of proteins at the DNA lesion, which may be essential for certain biochemical steps to proceed. At the same time, focus formation creates a lower concentration of repair proteins elsewhere in the nucleus preventing their untimely action on undamaged substrates, e.g., replication forks (Lisby et al., 2003b). Second, the colocalization of checkpoint and repair proteins at repair centers allow for coordination of cell cycle progression with repair status especially when encountering multiple DNA lesions. Third, the recruitment of DSB ends to a single repair center may be important for the rejoining of ends that have become separated during repair thus reducing chromosome loss, aberrant telomere addition, and other illegitimate recombination events (Lisby et al., 2003a).

Experimental Procedures

γ -Irradiation and Hydroxyurea Treatment

The γ -ray sensitivity of strains was determined by growing cultures in YPD to mid-log phase at 23°C. An appropriate number of cells were plated on YPD plates and exposed to different doses of γ -rays using a Gammacell-220 ⁶⁰Co irradiator (Atomic Energy of Canada). Cells analyzed by microscopy were pregrown in YPD at 23°C until OD₆₀₀ reached 0.2. At this point, the liquid cultures were exposed to defined doses of irradiation and aliquots of the cultures were processed immediately for imaging. DNA replication stress was induced by incubation with 100 mM hydroxyurea (Sigma-Aldrich, St. Louis, MO).

Yeast Live Cell Imaging and Fluorescent Microscopy

Microscopy was performed essentially as described previously (Lisby et al., 2003b). See Supplemental Data (available on *Cell* website) for details.

Degradation of Rfa1 Protein

The requirement for Rfa1 was analyzed by inducing ubiquitin-dependent degradation of Rfa1 using an Ub-DHFR^{ts}-HA-Rfa1-AAAAAA AAG-CFP (td-Rfa1-CFP) construct. Due to the essential function of Rfa1 in DNA replication, cells were first synchronized either in G1 by 2 μ g/ml α -factor or in G2 by 5 μ g/ml nocodazole for 3 hr at 23°C and then shifted to 37°C for 2 hr to induced degradation of Rfa1 before exposure to 100 Gy. Efficient degradation was confirmed by examining cells for residual CFP fluorescence. After degradation, the total nuclear fluorescence of td-Rfa1-CFP was reduced below detection in most cells. These cells did not form Ddc1, Ddc2, or Rad52 foci. In the few cells that had a low level (5%–10%) of residual td-Rfa1-CFP fluorescence, faint foci could be observed (data not shown). Cells expressing either Rfa1-CFP or the conditional td-Rfa1-CFP were grown in SC or SC-Ura medium, respectively.

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