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Competing roles of DNA end resection and non-homologous end joining functions in the repair of replication-born double-strand breaks by sister-chromatid recombination

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ABSTRACT

While regulating the choice between homologous recombination and non-homologous end joining (NHEJ) as mechanisms of double-strand break (DSB) repair is exerted at several steps, the key step is DNA end resection, which in *Saccharomyces cerevisiae* is controlled by the MRX complex and the Sgs1 DNA helicase or the Sae2 and Exo1 nucleases. To assay the role of DNA resection in sister-chromatid recombination (SCR) as the major repair mechanism of spontaneous DSBs, we used a circular minichromosome system for the repair of replication-born DSBs by SCR in yeast. We provide evidence that MRX, particularly its Mre11 nuclease activity, and Sae2 are required for SCR-mediated repair of DSBs. The phenotype of nuclease-deficient MRX mutants is suppressed by ablation of Yku70 or overexpression of Exo1, suggesting a competition between NHEJ and resection factors for DNA ends arising during replication. In addition, we observe partially redundant roles for Sgs1 and Exo1 in SCR, with a more prominent role for Sgs1. Using human U2OS cells, we also show that the competitive nature of these reactions is likely evolutionarily conserved. These results further our understanding of the role of DNA resection in repair of replication-born DSBs revealing unanticipated differences between these events and repair of enzymatically induced DSBs.

INTRODUCTION

A number of endogenous and exogenous agents can cause interruptions in the continuity of the DNA double helix, affecting one [single-strand break (SSB)] or both DNA strands [double-strand break (DSB)] (1). SSBs constitute a common type of spontaneous DNA lesions that alone, unless accumulated in high numbers, do not pose a serious threat to cell survival or genome integrity. In contrast DSBs, although less frequent, are potentially lethal and highly genotoxic (2). As SSBs can be converted into DSBs by replication run off, they can also have deleterious consequences to the cell if they are not repaired. Indeed, this has been postulated as the main source of spontaneous DSBs and recombinogenic DNA damage in human cells (3).

In eukaryotic cells, two main mechanisms contribute to DSB repair: non-homologous end joining (NHEJ) and homologous recombination (HR). In *Saccharomyces cerevisiae*, NHEJ starts by binding of the Ku heterodimer (Yku70-Yku80) to DSB ends, which are then processed and ligated to restore the integrity of the DNA molecule, even though mutations can be a by-product of the reaction. The basic functions of NHEJ are conserved from yeast to higher eukaryotes, although in the latter, additional activities are involved. Thus, Ku70-Ku80 associates with DNA-PKcs (catalytic subunit) to form the DNA-PK complex (4–6). This initiates a signaling cascade by phosphorylating numerous substrates in the vicinity of the break that then promote recruitment or retention of other repair factors. In contrast, HR is characterized by the requirement for an intact homologous DNA sequence as the repair template, resulting in

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a transfer of genetic information toward the broken molecule (7). Four basic and evolutionarily conserved steps can be distinguished within the HR process: resection of 5′ ends of the DSB; invasion of the generated 3′ ssDNA ends into the homologous duplex DNA sequence used as repair template; DNA synthesis primed from the invading 3′ ends on the invaded template; and resolution or dissolution of the branch-generated structure.

Both HR and NHEJ safeguard genome integrity and, therefore, act as tumor-suppressing mechanisms in higher eukaryotes (8–10), even though NHEJ can also contribute to the appearance of mutations and genomic rearrangements. In the case of HR, the choice of the donor among all available homologous sequences can greatly affect the final outcome of the recombination process (11,12). While the use of ectopic repeats or the homologous chromosome (in diploid cells) can result in chromosomal rearrangements and loss of heterozygosity, the identical sister chromatid, available during the S and G2 phases of the cell cycle, ensures the accuracy of the HR process and thereby the integrity of the genome. In light of these issues, cells have to ensure that the most appropriate repair pathway and substrate are used according to a series of cellular signals responding to cell cycle stage, chromatin structure and the type of DSB.

Regulating the choice between HR and NHEJ is exerted at several levels, but the key one seems to be at the stage of controlling the processing of DNA ends by DNA end resection (13). While NHEJ can only occur at breaks that have undergone little or no processing, the extensive 5′ degradation of DNA ends appears to be an initial and essential step in HR. Thus, DNA resection can be considered the licensing step that switches repair from NHEJ to HR (13). As expected, cell cycle, checkpoint activation, chromatin structure or the type of DSB also control HR activation through the regulation of DNA resection. Notably, DNA end resection is a two-step mechanism. It is initiated by the endonucleolytic attack of the DNA end by the combined action of Sae2 and the Mre11-Rad50-Xrs2 (MRX) complex in budding yeast or by their counterparts CtIP and the Mre11-Rad50-Nbs1 (MRN) complex in higher eukaryotes. Following this short-range processing, resection is extended by the activities of the exonuclease Exo1 and the helicase-nuclease protein complex Sgs1-Dna2 (14,15).

The most widely accepted mechanism for the spontaneous appearance of DSBs and recombinogenic DNA damage is during DNA replication (16,17). However, most DSB repair studies have been performed with DSBs induced by mega-endonucleases. Moreover, due to the high cleavage efficiency of such systems, even if they act during or after replication, both chromatids may be simultaneously cleaved, thus limiting their potential to be used as HR repair templates. For this reason, most DSB-induced recombination systems rely on the use of ectopic and not completely homologous templates as donors of genetic information. In contrast, we have developed a new system (TINV) that has proven to be an appropriate tool for the molecular analysis of repair of replication-born DSBs (11,18). It is based on a 24-bp mini-HO cleavage site instead of the full 117-bp site that, upon HO-endonuclease induction, is cleaved in one DNA strand. The resulting SSB is then converted into a DSB during replication, but leaving one sister chromatid intact to be used as the DSB repair template during recombination. This system allows monitoring repair by SCR at a molecular level, thereby mimicking a natural scenario for the repair of replication-born DSBs, and has proven useful for the identification of new roles in SCR for factors that did not have effects in standard analyses (19).

Here, we reasoned that resection should be especially important for the repair of DSBs generated during replication, a stage in which HR, and in particular SCR, needs to be favored. The TINV system was used to explore this possibility in *S. cerevisiae* cells. We provide evidence for the involvement of the MRX complex, particularly of its Mre11-associated nuclease activity, and Sae2 in SCR-mediated repair of replication-born DSBs. We show that the phenotype of nuclease-deficient MRX mutants can be suppressed by ablation of Yku70 or overexpression of the exonuclease Exo1, which suggests a competition of NHEJ and resection factors for DNA ends accumulating during replication. In addition, we observe partially redundant roles for Sgs1 and Exo1 in SCR, but with a more prominent role for the Sgs1 helicase. We discuss these data in regard to repair mechanisms for replication-born DSBs and how these may differ from those employed at enzymatically induced DSBs, particularly at the early steps of DNA end resection. We show that the competitive nature of these reactions is likely conserved in human cells.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains used in this article are listed in Table 1. All strains (named WS) are in the W303 genetic background, as indicated. Plasmid pRS316TINV containing a 24-bp mini-HO site at the EcoRI internal site of *LEU2* was described earlier (20).

Physical analysis of replication-induced DSBs and SCR

SCR assays were carried out essentially as described earlier (18). Briefly, cells carrying pRS316-TINV were grown to mid-log phase in SC-Ura 3% glycerol 2% lactate; then, galactose (2%) was added to induce HO expression. Next, the required glucose (2%) was added to stop HO induction. Samples were collected at various time points and the DNA was purified, digested with SpeI–XhoI, and analyzed by Southern using Hybond N+ (GE Healthcare) membranes. A ^{32}P-labeled 0.22-kb *LEU2* probe was obtained by PCR using the primers 5′-GTTCCACTTCCAGATGAGGC-3′ and 5′-TTAGCA AATTGTGGCTTGA-3′. Bands were quantified in a Fuji FLA-5100. SCR values represent the percentage of 4.7 kb band versus the total plasmid DNA signal in each lane. Percentages of DSBs are calculated by the sum of 1.4 and 2.4 kb bands versus the total plasmid DNA signal in each lane. Average and standard deviation of at least three independent experiments were performed in each case.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
WS	*MAT***a**-*inc trp1-1 ura3-1 ade2-1 his3-11,15 can1-100 ade3::GAL-HO leu2::SFA1 rad5-G535R*	(18)
WS-M11	WS *mre11Δ::KanMX4*	(18)
WS-R50	WS *rad50Δ::KanMX4*	(52)
WS-X2	WS *xrs2Δ::KanMX4*	This study
WS-M125	WS *mre11-125N*	This study
WS-SAE2	WS *sae2Δ::KanMX4*	(22)
WS-K70	WS *yku70Δ::KanMX4*	This study
WS-DN4	WS *dnl4Δ::HphMX4*	This study
WS-K70M125	WS *yku70Δ::KanMX4 mre11-H125N*[a]	This study
WS-SG1	WS *sgs1Δ:: KanMX4*	This study
WS-EX1	WS *exo1Δ::KanMX4*	This study
WS-SGEX	WS *exo1Δ::KanMX4 sgs1Δ::KanMX4*	This study

[a]*mre11-nd* is the *mre11-H125N* mutation.

Cell culture and shRNA-mediated depletion

U2OS cells stably transfected with an shRNA targeted against the 3′ UTR region of CtIP (TRCN0000005403, Sigma), or a scrambled sequence as a control, were obtained by lentiviral infection of the shRNA construct and further selection with puromycin. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (BioSera), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) supplemented with 0.5 mg/ml puromycin (1 μg/ml, Sigma).

Immunofluorescence microscopy

For Replication Protein A (RPA) focus detection, U2OS cells were treated with 10 Gy of ionizing radiation (IR) and collected 1 h afterwards. Following pre-extraction for 5 min on ice (25 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% Triton X-100), cells were fixed with 4% paraformaldehyde (w/v) in PBS for 15 min. Coverslips were washed three times with PBS and then co-immunostained with antibodies against γH2AX (Cell Signaling Technology) and RPA32 (Lab Vision Corp.). For detection, Alexa Fluor 488- (green) and 594-(red) conjugated secondary antibodies were used (Molecular Probes, Paisley, UK). Samples were visualized with a Leica inverted microscope by sequential scanning of the emission channels.

Immunoblotting

Extracts were prepared in Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8), proteins were resolved by SDS–PAGE and transferred to nitrocellulose followed by immunoblotting. R. Baer (Columbia University) provided the mouse monoclonal antibodies raised against the CtIP C-terminus. Other antibodies were from Sigma (Tubulin) and Abcam (phosphor DNA-PKcs).

HR/NHEJ assay

Equal amounts of plasmid pRS316-LEU2 uncut or linearized with StuI–NcoI were used to transform yeast strains. Transformation efficiency (TE) after selection in SC-Leu was calculated for each condition. The percentage of DSBs repaired for each strain was calculated with the following formula: 100 × TE (linearized)/TE (uncut). To calculate the HR:NHEJ ratio, for each strain studied between 85 and 125 colonies transformed with the linearized plasmid was examined by gel electrophoresis of PCR products generated with the GCGGGTGTTGG CGGGTGTCG and ATAAGGGATTTTGCCGATTT primers.

RESULTS

SCR depends on Mre11 and Sae2 nuclease activity

As shown in Figure 1A, Southern analysis of cells harboring the TINV system allows physical detection of replication-dependent DSB induction. While the 3.8-kb fragment corresponds to the intact plasmid, 1.4 and 2.4 kb fragments arise after HO cleavage. Moreover, a 4.7-kb fragment is specific of repair by SCR, which also results in the appearance of a 2.9-kb fragment that is ruled out for analysis as it can also arise by additional recombination mechanisms (11). As mentioned earlier, the MRX complex plays key roles in the initial processing steps of DSB ends. Although its role in HR *in vivo* is not easily detected (MRX mutants do not display diminished frequency of mitotic heteroallelic recombination), we have previously reported that *MRE11* inactivation significantly reduces DSB-induced SCR (18). Here, we extended this study to all components of the MRX complex (Figure 1B). Each of the three null mutations, *mre11Δ, rad50Δ* and *xrs2Δ* stabilize the DSBs after HO endonuclease induction, consistent with the role of the MRX complex in DSB end processing. Indeed, as can be seen in Supplementary Figure S1, the amount of intact HO-induced DSBs in wild-type cells decreased with a fast kinetics following HO repression (DSBs were at 5% of the initial levels after 12 h of glucose addition). Instead, DSBs disappeared with a slower rate in *mre11Δ* cells (DSBs at 12 h were at ∼50% of initial levels). In addition, SCR was significantly reduced to similar levels in the *MRE11*, *RAD50* and *XRS2*-null mutants (Figure 1B), indicating that the three MRX subunits are equally relevant for SCR.

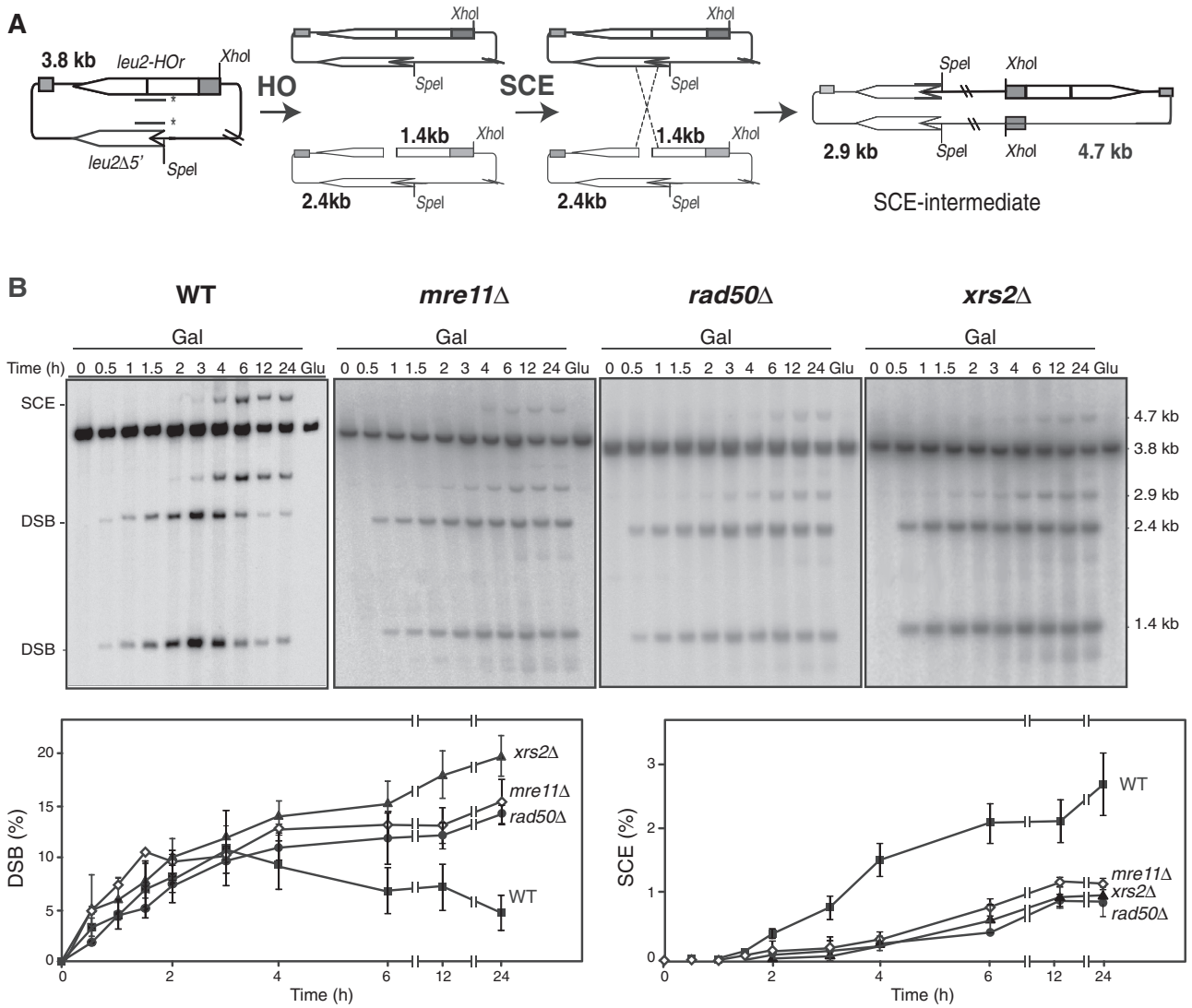

Figure 1. Role of the MRX complex in DSB repair by SCR. **(A)** Schematic representation of the physical assay used to monitor repair by SCR of an HO-induced DSB in the centromeric plasmid pRS316-TINV carrying the TINV system. Fragments generated after XhoI–SpeI digestion and detected by the *LEU2* probe (line with asterisk) are indicated with their corresponding sizes. **(B)** Kinetic analysis of DSB formation and SCR products in *mre11*Δ, *rad50*Δ and *xrs2*Δ mutants. A representative Southern, with the relevant bands indicated, and the quantification of DSBs and SCR related to total plasmid DNA is shown. Additional short bands are observed in the MRX mutants, possibly resulting from degradation of the 1.4 and 2.4 kb DSB bands. Average and standard deviation (bars) of at least three independent experiments are shown for each time point and genotype.

Various functional activities have been proposed for the MRX complex in the processing of DNA ends, including a direct role of Mre11 nuclease activity in initiating resection. To test the contribution of this specific activity to the defect observed in SCR, we analyzed the effect of the nuclease-dead *mre11-H125N* mutation, from now on referred as *mre11-nd*, which abolishes Mre11 nuclease activity but maintains the stability of the complex (21). Strikingly, no significant differences were found in the kinetics of DSB induction between wild-type cells and those bearing the *mre11-nd* mutation and, in stark contrast to *MRE11*-null cells, DSBs did not accumulate in *mre11-nd* cells (Figure 2). Nevertheless, essentially identical kinetics and overall levels of SCR-product formation were obtained for *mre11*Δ and *mre11-nd*, with SCR being significantly and equivalently reduced in both mutants as compared with wild-type cells. Therefore, we conclude that the endonuclease activity of Mre11 is important for SCR. Given the similarity of phenotypes between *sae2*Δ and nuclease-deficient MRX mutants, and the proposed role for Sae2 in controlling resection (22–26), we analyzed the effect of *sae2*Δ on SCR in our assays. As seen in Figure 2, DSBs accumulated in *sae2*Δ cells to the same levels and with similar kinetics as in *mre11-nd cells*, and as expected, the effect of *sae2*Δ on the repair of replication-born DSBs by SCR was similar to the effect of cells bearing the nuclease-defective *mre11-nd* mutation.

Exo1 overexpression or Yku70 loss suppresses MRX nuclease deficiency

Exo1 overexpression has been reported to partially suppress DSB-repair defects associated with deficiencies

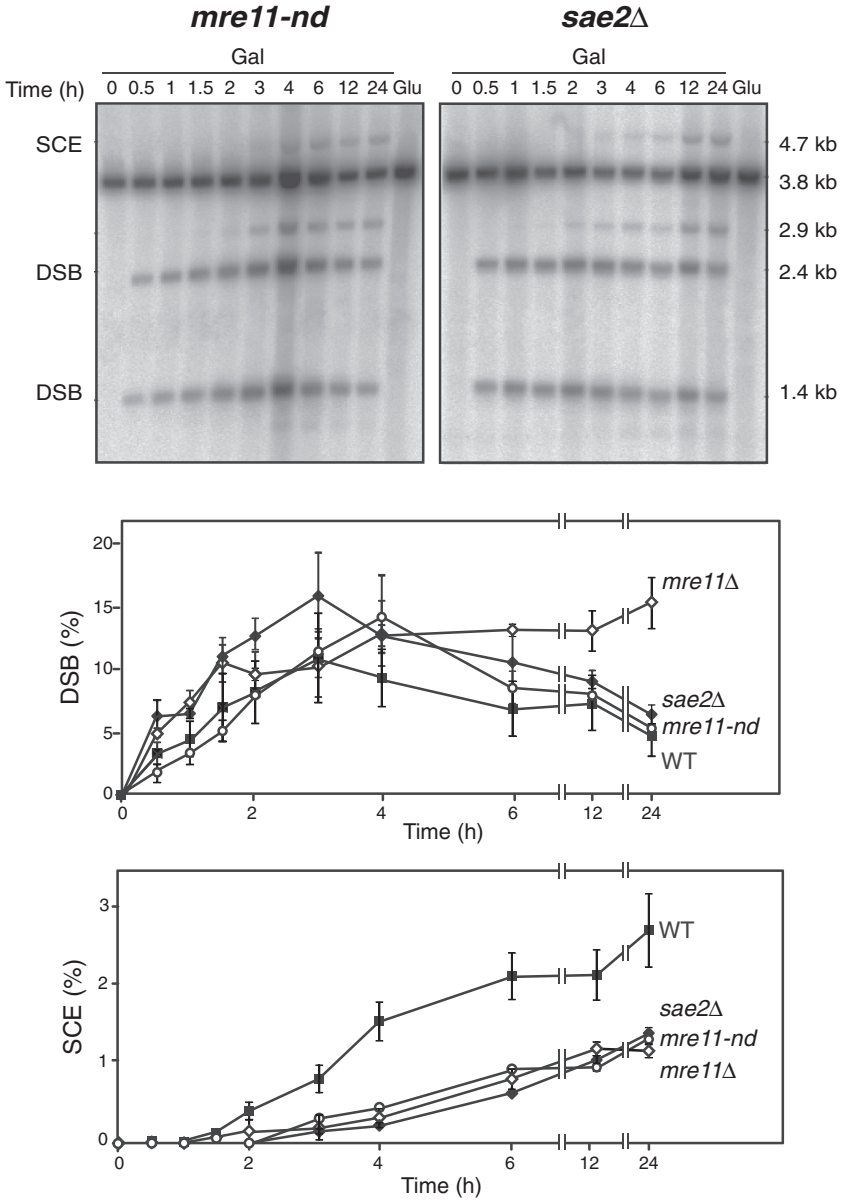

Figure 2. Analysis of the Mre11 and Sae2 nuclease activity involvement in SCR. Kinetic analysis of DSB induction and repair by SCR using the TINV system in isogenic *mre11*Δ, *mre11-nd* (nuclease-dead) and *sae2*Δ mutants. The same data of WT and *mre11*Δ of Figure 1 are plotted to facilitate comparison of results. Other details as in Figure 1.

in Mre11 nuclease activity (27). We therefore used the TINV system to test whether this was also the case for SCR induced during replication as a way to evaluate the relevance of this activity in SCR. Notably, while SCR in *mre11-nd* cells was restored to wild-type levels by Exo1 overexpression, no suppression was observed by overexpressing Exo1 in *mre11*Δ cells (Figure 3). These results suggest that, for DSBs generated during replication, Exo1 can compensate for the absence of Mre11 nuclease activity but its action on DSB ends requires the physical presence of the MRX complex.

It has been shown that impairment of NHEJ can facilitate DNA-end resection and favor HR (28,29). To evaluate the relevance of NHEJ in the repair of replication-born DSBs, we analyzed SCR levels in NHEJ-deficient *yku70*Δ cells. As shown in Figure 4A, DSBs accumulated with similar kinetics and at similar levels in wild type and *yku70*Δ strains. By contrast, SCR was modestly increased in *yku70*Δ cells, suggesting that a minor but significant fraction of replication-born DSBs are repaired via NHEJ in wild-type cells. It should be noted that DSBs produced in G1 and left unrepaired in NHEJ-deficient cells cannot result in SCR because after replication both sister chromatids are broken. In contrast, a deletion mutant of DNA ligase 4 (*dnl4*Δ) showed essentially the same SCR kinetics as the wild type (Figure 5), indicating that the effect observed in *yku70*Δ is not a general feature of NHEJ mutants. Interestingly, we found that *yku70*Δ was epistatic over *mre11-nd* in SCR product formation; it suppressed the defect caused by

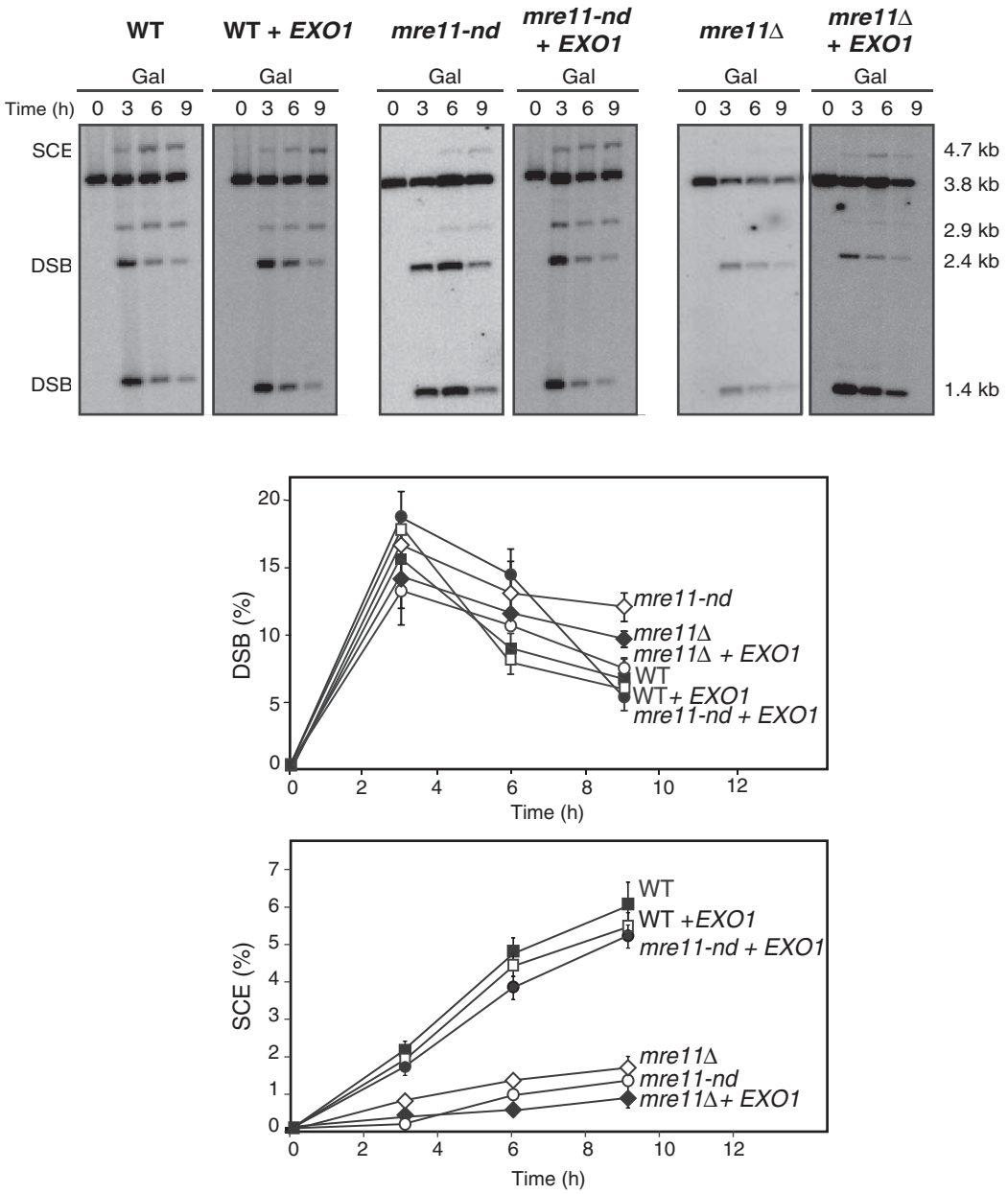

Figure 3. Overexpression of Exo1 exonuclease the in *mre11-nd* mutant suppresses its SCR defect. Kinetic analysis of DSB induction and SCR repair using the TINV system in isogenic wild type, *mre11*Δ and *mre11-nd* strains transformed with pRS314 (empty plasmid) or with pSM502 overexpressing Exo1 exonuclease under control of the *GAL1,10* promoter. Other details as in Figure 1.

deficiency in Mre11 nuclease activity (Figure 4B). Collectively, these results suggest an unanticipated active competition between the functions promoting resection (resulting in HR) and stabilization (resulting in NHEJ) of ends of DSBs generated during replication, and an active role of Yku70 in the choice of the repair mechanism by competition with the resection machinery.

Defective MRX- and Sae2-dependent resection increases NHEJ

Given the competition between resection and core NHEJ proteins suggested by the above results, we anticipated that an impairment of resection would decrease HR events in favor of NHEJ. Both a decrease in HR and an increase in NHEJ have been independently reported for *sae2*Δ mutants (22,24,28,30,31), but not in a system in which both types of events can be monitored together. Consequently, it has not been possible to evaluate the relative importance of each pathway for the repair of a particular DSB. To address this issue, we developed a system (Figure 6A) in which a central fragment of the *URA3* gene was removed from plasmid pRS316-LEU2 by restriction digestion. This broken minichromosome can be repaired by NHEJ or by HR (using the endogenous *ura3-1* allele as template) resulting in loss or restoration of the central fragment, respectively, once introduced into the cells. The difference in size derived from each type of

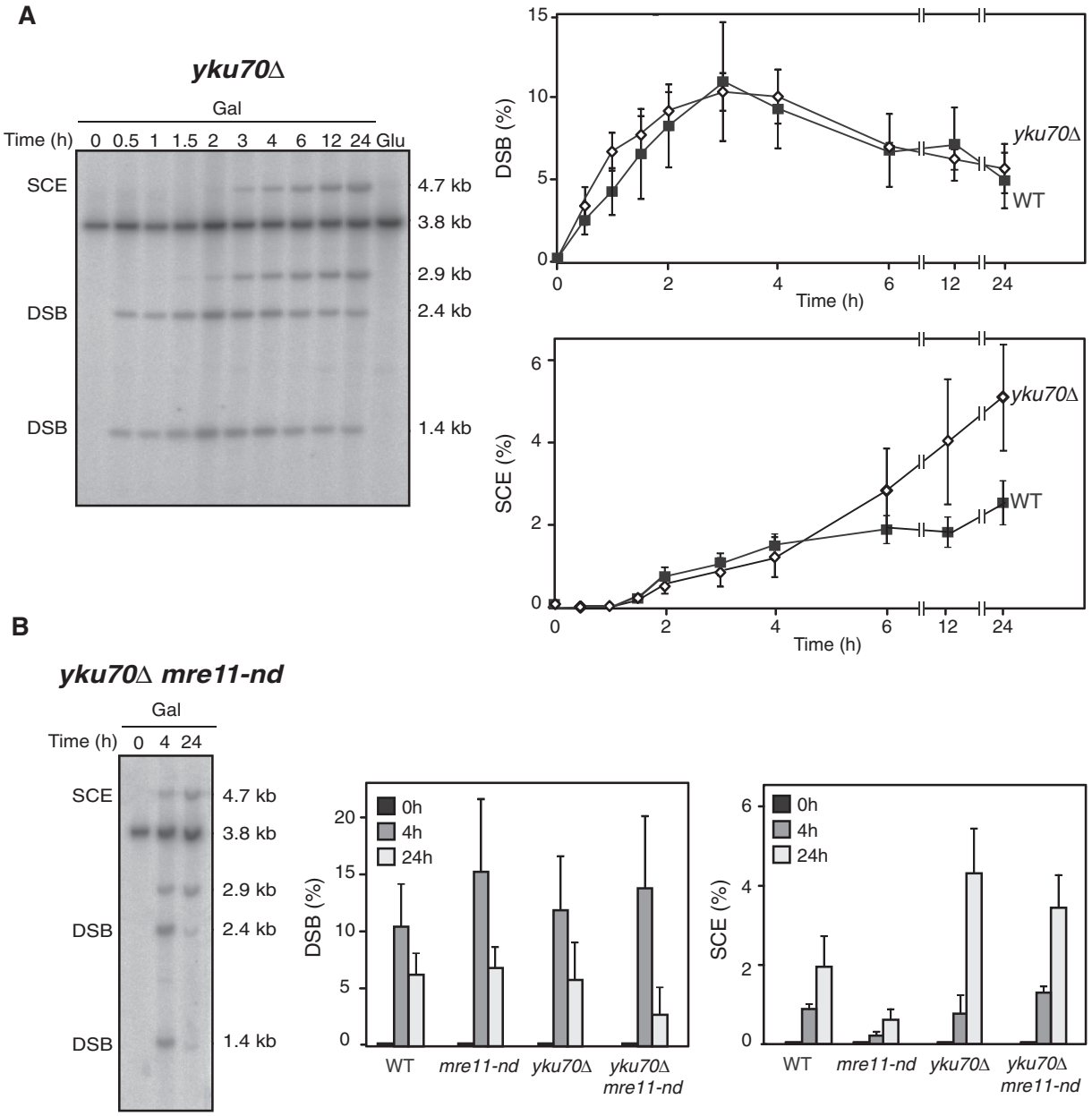

Figure 4. *YKU70* deletion suppresses MRX nuclease deficiency. **(A)** Kinetic analysis of DSB induction and repair by SCR using the TINV system in isogenic *yku70*Δ cells. **(B)** SCR analysis of the *yku70*Δ *mre11-nd* double mutant. The same data of WT, *mre11-nd* and *yku70*Δ of Figures 1 and (A) are plotted to facilitate comparison of results. Other details as in Figure 1.

event can be analyzed by PCR with plasmid-specific primers. As seen in Figure 6B, total repair, represented as the percentage of DSBs repaired, can be estimated from the TE (Leu+) of the fragment related to that of the intact pRS316-LEU2 plasmid, as previously described. Quantification analyses revealed that both the *mre11-nd* and *sae2*Δ mutants displayed a non-significant reduction in repair events (13.4 and 11.3% repair, respectively; $P > 0.3$) when compared with wild-type cells (16.6%). PCR analyses of independent wild-type transformants revealed that 87% of the breaks were repaired via HR and 13% via NHEJ (Figure 6C), consistent with HR being the preferred DSB repair pathway in *S. cerevisiae*. Notably, this ratio was shifted in favor of NHEJ events in *mre11-nd* (57% HR versus 43% NHEJ) and *sae2*Δ (64% HR versus 36% NHEJ) mutants, with a high-statistical significance compared with wild type ($P < 0.001$). Our results are, therefore, consistent with a model in which resection mainly influences DSB repair pathway choice but not overall repair efficiency.

Sgs1 and Exo1 define two pathways for SCR repair of replication born DSBs

As mentioned earlier, MRX and Sae2 are involved in the initial processing of DSB ends. However, extensive resection, which is critical for the recombination process, is carried out by other components, such as the Sgs1

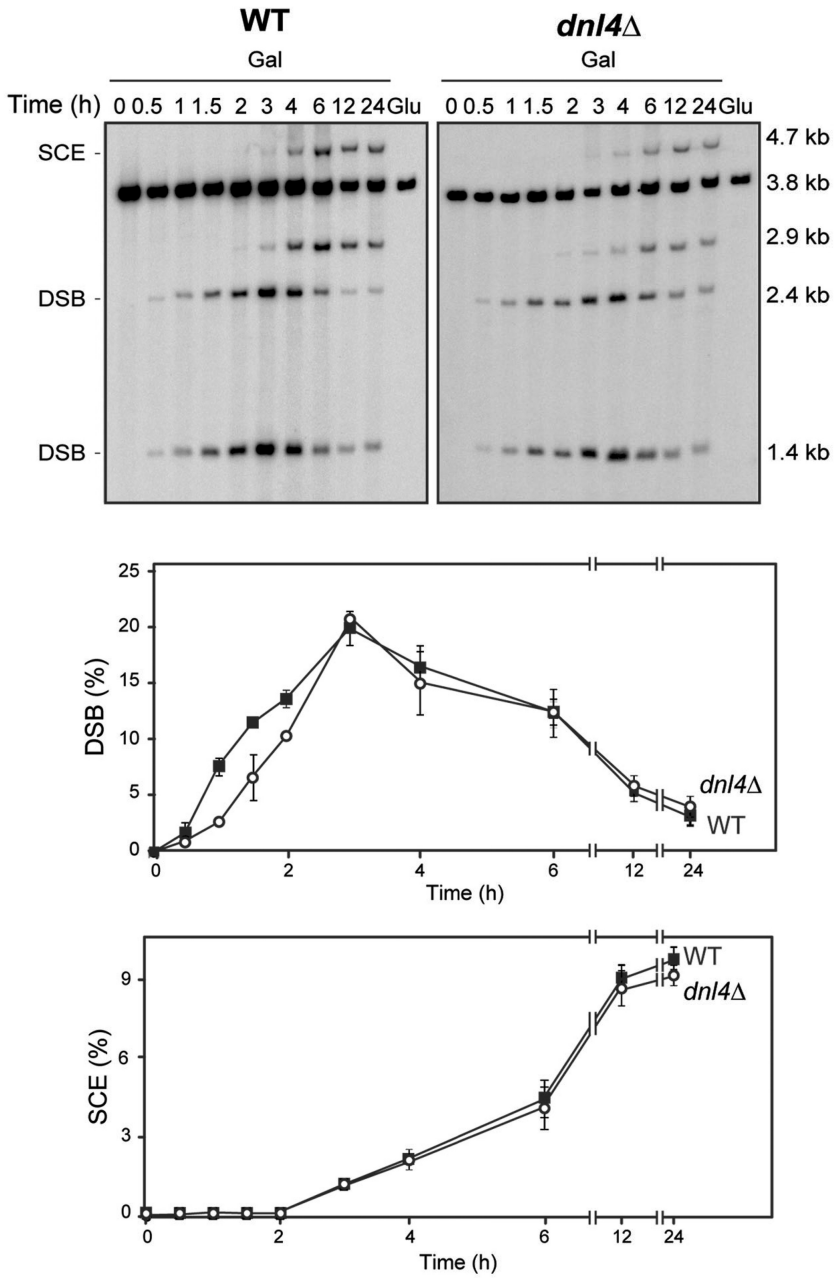

Figure 5. *DNL4* deletion does not affect DSB repair by SCR. Kinetic analysis of DSB induction and repair by SCR using the TINV system in WT and isogenic *dnl4*Δ cells.

helicase and the Exo1 nuclease. To gain new insights into the mechanism of SCR generated by replication-born DSBs, we used the TINV system to characterize the contribution of these latter two proteins. This revealed that *sgs1*Δ or *exo1*Δ as single mutations, or in a double mutant state, had no impact on the kinetics of DSB induction (Figure 7). On the other hand, SCR was unaffected in *exo1*Δ cells but greatly diminished in *sgs1*Δ cells, reaching only 50% of wild-type levels. This reduction, although strong, was not as dramatic as that observed in the absence of MRX or Sae2 functions (compare with Figures 1 and 2). Interestingly, the double *sgs1*Δ *exo1*Δ mutant further reduced SCR to levels equivalent to those of *mre11*Δ and *sae2*Δ cells. These results suggest that Sgs1 and Exo1 define two pathways for the repair by SCR of DSBs generated during replication, the Sgs1-mediated repair being the prevalent mechanism, whereas the role of Exo1 is only clearly observed in a *sgs1*Δ background.

DNA-PKcs inhibition suppresses the resection defect caused by CtIP depletion

To extend our yeast results to the mammalian system, we used U2OS human osteosarcoma cells that express an shRNA targeted toward the human counterpart of Sae2, CtIP (14,22,31). As a control, we used U2OS cells

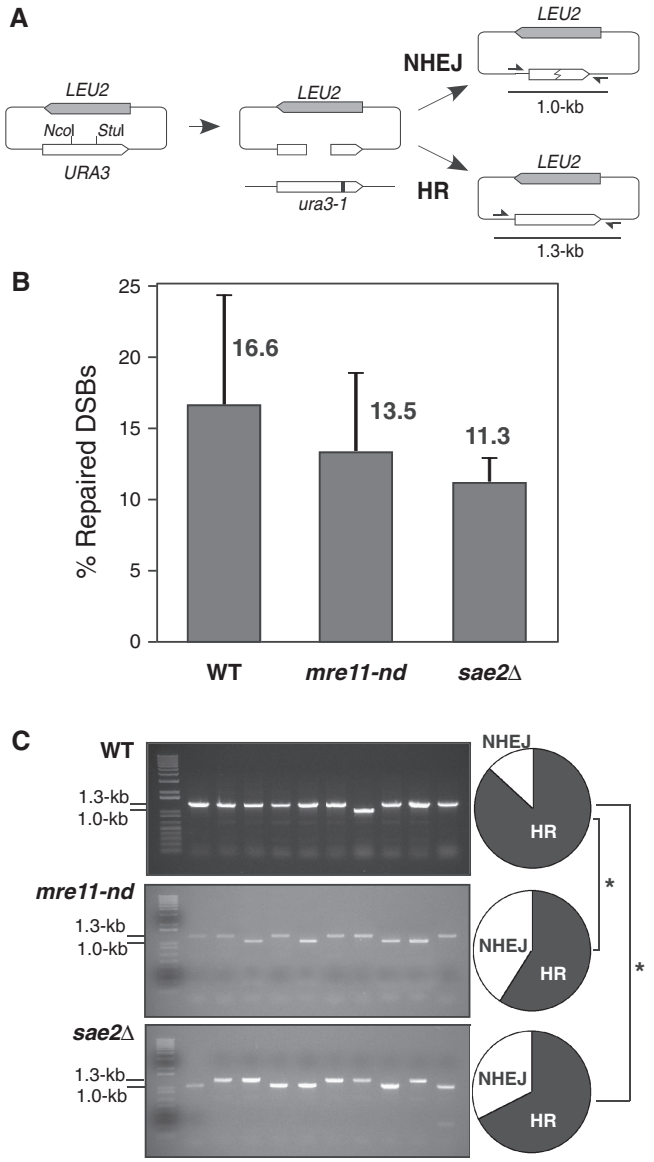

Figure 6. Analysis of HR and NHEJ. **(A)** Scheme of the system used to analyze repair by HR and NHEJ. The NcoI–StuI fragment of the *URA3* gene is removed from plasmid pRS316-LEU2. Once transformed into cells, the break can be repaired by NHEJ or by HR, in the latter case using the endogenous *ura3* allele as template. PCR with plasmid-specific primers generates products of different sizes for each kind of event (1.3 kb for HR and 1.3 kb for NHEJ). **(B)** Quantification of the percentage of repaired DSBs in wild-type, *mre11-nd* and *sae2*Δ mutants, estimated from the TE (Leu+) of the fragment related to that of pRS316-LEU2 closed plasmid. Statistical analysis of data with respect to the wild type was performed according to a Student's *t*-test, resulting in non-statistically significant differences. **(C)** Analysis by PCR of independent transformants and quantification of the percentage of repair by HR *vs.* NHEJ. Statistical analysis of data with respect to the wild-type values was performed according to a 'Chi-square' Pearson test.

expressing an shRNA corresponding to a scrambled version of the CtIP sequence with no target in the human genome. As seen in Figure 8A and B, IR induced the appearance of RPA foci in a substantial proportion of cells, which in part reflects activation of DNA end resection. In agreement with earlier published results (14,26), CtIP depletion reduced the number of cells that were positive for RPA foci. Importantly, however, no clear difference was observed in the number of cells accumulating DSBs as determined by the appearance of γH2AX foci (data not shown). To test whether, as in yeast cells, the resection defect of CtIP-depleted cells depended on the actions of Ku and associated proteins, we used a small-molecule inhibitor of DNA-PKcs (32), which in higher eukaryotes associates with the Ku heterodimer to form the DNA-PK complex that functions in NHEJ (33,34). DNA-PKcs inhibition, measured as an impairment of its auto-phosphorylation, was essentially complete (Supplemental Figure S2). Under these conditions, the number of cells containing RPA foci was increased, and the difference between CtIP depleted and non-depleted cells was lost (Figure 8A, white bars; see Figure 8C for CtIP depletions), suggesting that competition between resection and NHEJ factors is conserved from yeast to mammalian cells.

DISCUSSION

In this study, we determined how the different functions reported to influence DNA end resection of enzymatically mediated DSBs affect the repair of a replication-born DSB by SCR. A strong dependency of SCR repair is shown on all members of the MRX complex, with a particular role of the Mre11 nuclease activity and Sae2. This requirement is alleviated by either overexpression of Exo1 or deletion of *YKU70*, consistent with the role of the MRX nuclease activity in SCR. Furthermore, we show that long-range resection activities such as Exo1 and especially Sgs1 are also required for replication-induced SCR in a partially redundant fashion. Collectively, our results demonstrate that during replication DNA-end resection plays an active role in the repair of replication-born DSBs mediated by MRX, Sae2, Sgs1 and Exo1 functions and in the choice between SCR and NHEJ as two active DSB repair mechanisms.

Traditionally, the MRX complex has been considered the main function promoting DSB resection. The fact that nuclease-deficient *mre11* mutants show proficient resection of HO-induced DSBs and unaffected levels of mitotic HR (21,35–37) suggested instead a regulatory rather than enzymatic role of the complex, possibly by promoting access of other nucleases to DNA ends. However, this possibility is not consistent with the fact that nuclease-dead mutations in Mre11 do cause hypersensitivity to IR and methyl methanesulfonate (MMS), although less severe than that of null MRX mutants. This has been explained by the 'dirty' nature of most DSB ends generated by these DNA-damaging agents, and a specific role of Mre11 nuclease activity in processing blocked termini (27,38–41). Consistent with this idea, the activity of MRX is fully required for the resection of meiotic DSBs, which are blocked by covalently bound Spo11 protein (42–44).

Our study, however, shows identical SCR defects in *mre11-nd* and null *mre11*Δ mutants. This, to our knowledge, is the first time that a major mitotic HR defect is linked to nuclease deficient activity in Mre11, and suggests

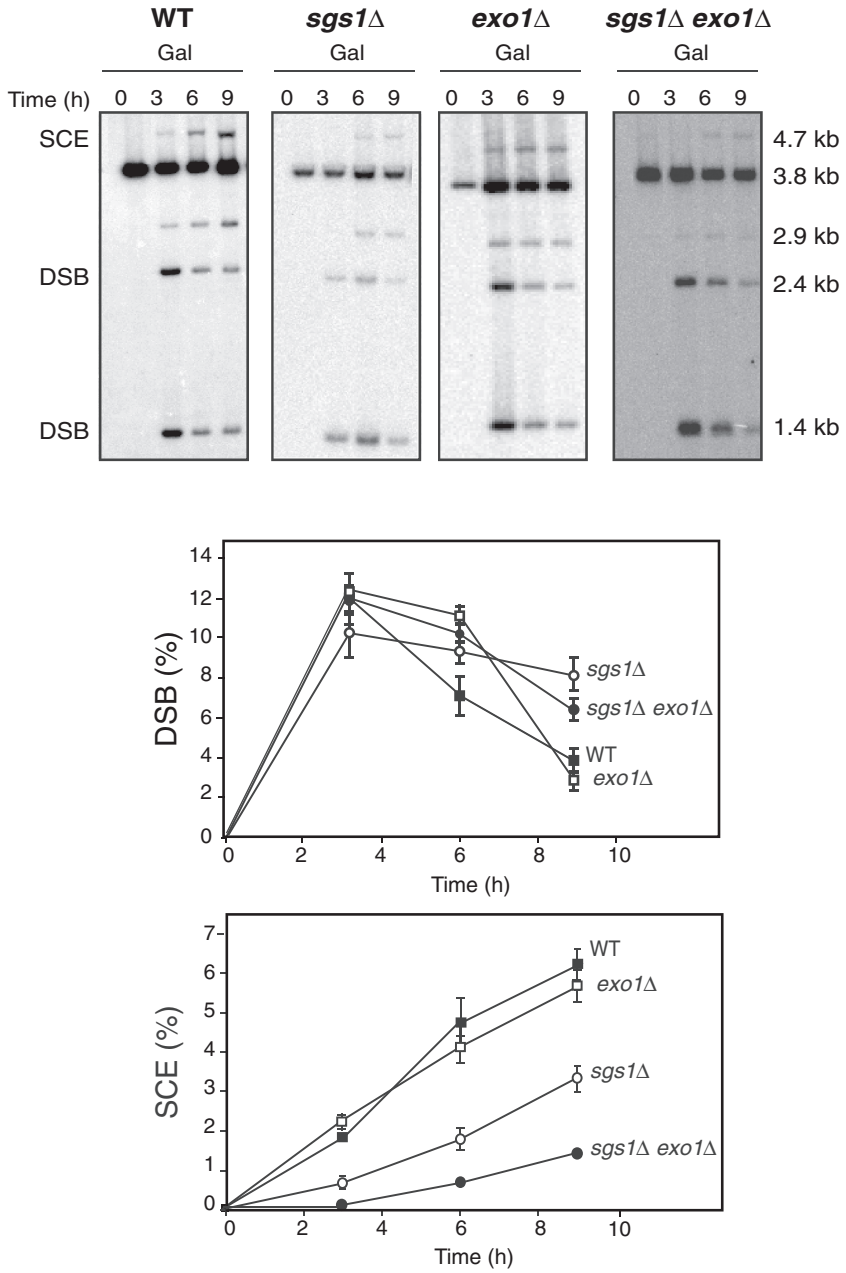

Figure 7. Role of Sgs1 helicase and Exo1 exonuclease in SCR. Kinetic analysis of DSB induction and repair by SCR using the TINV system in isogenic wild-type, *sgs1*Δ, *exo1*Δ, and *sgs1*Δ *exo1*Δ cells. Other details as in Figure 1.

that the main function of MRX in the repair of DSBs generated by replication failure, the most common form of spontaneously formed DSBs, is directly related to DNA end processing. Importantly, in the TINV system used in this study, DSBs are 'clean' and the defect caused by the *mre11-nd* mutation cannot be explained by a specific function putatively required for processing blocked termini. It is worth noting that this replication-associated role of MRX could also explain the hypersensitivity of nuclease-deficient *mre11* mutants to DNA damage, as at least an important fraction of DSBs induced by most DNA damaging agents arise during replication. Consistent with this idea, MRX null mutants are impaired in genetically scored SCR events that are induced by DNA-damage agents, such as MMS or HU, but not in those induced by enzymatically induced DSBs at a full 117 bp HO site (36,45,46). In addition, despite the similarity of the SCR defects in MRX mutants, one important difference is observed between nuclease-deficient and null *mre11* mutants. While DSBs accumulate in *mre11*Δ, they disappear in *mre11-nd* cells with the same kinetics as in the wild type. This suggests that the physical presence of MRX further influences DSB-end processing and/or repair by other mechanisms non-dependent on its enzymatic activity. For example, disappearance of DSBs could be due to the possibility of the repair of unresected breaks by NHEJ, which would be also affected in null mutants. This is consistent with the

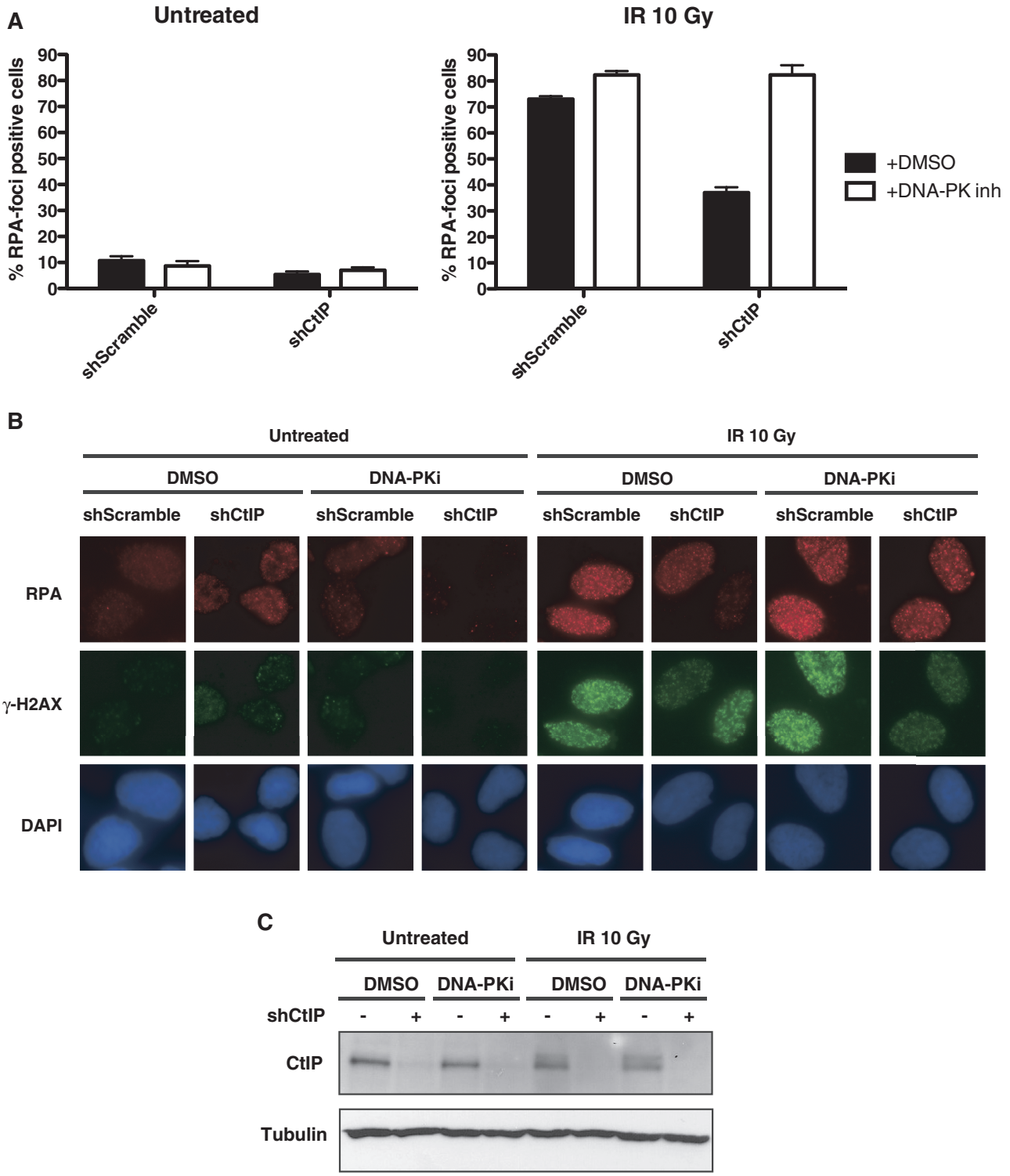

Figure 8. DNA-PKcs inhibition suppresses the resection defect caused by CtIP depletion in response to IR. **(A)** RPA and γH2AX focus accumulation in response to treatment with (right panel) or without (left panel) 10 Gy of IR in human U2OS cells depleted of CtIP (shCtIP) or not (shScramble) with or without chemical inhibition of DNA-PKcs (NU7441, 10 μM). **(B)** Representative images of cells upon different treatment combinations are shown. The panel showing shRNA CtIP treated with DMSO and after irradiation shows a positive (top left) and negative (bottom right) cell for RPA focus accumulation. **(C)** shRNA-mediated depletion of CtIP.

increased frequency of NHEJ observed in *mre11-nd* and *sae2*Δ (Figure 6C).

The lack of mitotic phenotype of nuclease-deficient *mre11-nd* mutants, together with the observation that null mutants display increased levels of interhomolog recombination, has also led to the proposal that MRX could function specifically in SCR and not in other types of HR. This hypothesis is supported by different genetic data (36,40,47) but, most importantly, by the biochemical capacity of MRX to tether DNA molecules, which suggests that MRX could hold both ends of a DSB together and/or each end to the sister chromatid, facilitating thus repair by SCR (44,48–52). It has indeed been shown that, after induction of a break, DNA ends flanking the DSB in wild-type cells remain adjacent while loss of MRX results in DNA ends being dispersed (51). Our findings, however, indicate that, at least for replication-born DSBs, a relevant function of the MRX complex in SCR not previously anticipated is related to the nuclease activity of Mre11, although we cannot rule out this possible structural component being affected by the *mre11-nd* mutation. Indeed, the *sae2*Δ mutant, which displays identical phenotype to *mre11-nd* in our assay, shows defects in DSB-end bridging (25).

We show that overexpression of Exo1 or the absence of Yku70 fully suppresses the SCR defect of the *mre11-nd* mutant in the TINV system. This suggests a competition between resection and NHEJ factors for DNA ends that arise during replication, the outcome of which finally dictates processing of the ends and subsequent repair. Such a competition has been proposed previously based on genetic data and on the study of directly induced DSBs, but never studied for breaks linked to replication (28,53–57). In these type of breaks, Ku binding could even be deleterious, particularly in the case of one-ended breaks generated by replication-fork breakage. In such cases repair by NHEJ is not possible due to the lack of a second DNA end with which to ligate, whereas HR would be prevented by Ku-binding, therefore, compromising cell viability. One could consider the possibility of Ku binding being constitutively inhibited during replication by resection-independent processes, which would render repair of replication-born breaks insensitive to deficiencies in NHEJ. We find, however, that this is not the case as *yku70*Δ mutation increases SCR and suppresses the defect of *mre11-nd* and *sae2*Δ mutants. Ku, therefore, is also active on breaks generated during replication, where it exerts an inhibitory role on repair by SCR. It is resection mediated by MRX and Sae2 that counteracts this negative impact of Ku.

Previous studies have explored the interplay between DNA resection and Ku at an HO enzymatically induced DSB (28,53–57). In the model derived from such studies, the MRX complex antagonizes excessive Ku binding to DNA ends and promotes recruitment and activity of the long-range resection nucleases Exo1 and Dna2. Importantly, in the absence of a functional MRX complex or Sae2, Exo1 and Sgs1-Dna2 can perform the initial resection step, but this process is normally inhibited by the presence of Ku. This is in agreement with our observed suppression of *mre11-nd* SCR defect by either overexpression of Exo1 or deletion of *YKU70*, suggesting that this model is extensible to replication-born DSBs. There are, however, important differences that suggest replication-specific roles of the MRX complex. Previous studies show that overexpression of Exo1 partially suppresses IR sensitivity and HO-induced resection defects of both *mre11*Δ and *mre11-nd* mutants (27). In contrast, we observed full suppression of the SCR defect by Exo1 overexpression, but only in *mre11-nd* and not in *mre11*Δ cells, indicating that the action of Exo1 in SCR induced by replication-born DSBs requires the physical presence of MRX. It is tempting to speculate that this difference could be related to a more specific and distinct role of MRX and Exo1 at replication forks (58).

Genetic evidence for a specific role of Mre11 nuclease activity and Sae2 in counteracting Yku action and promoting Exo1 during replication has been recently obtained (28,59). On one hand, *YKU70* deletion or Exo1 overexpression suppress the sensitivity of *mre11-nd* nuclease-dead and *sae2*Δ mutants to the S-phase specific clastogen camptothecin. However, DNA breaks caused by this agent are covalently linked to protein, the removal of which has been shown to require the MRN complex in *Schizosaccharomyces pombe* (60). Therefore, these studies do not exclude the possibility that these phenotypes were related to the removal of a covalently linked protein rather than to a specific role in DSBs generated during replication. In addition, the absence of Yku70 suppresses synthetic lethality of *mre11-nd rad27* and *sae2*Δ *rad27* mutants, which is thought to occur by an increase in S-phase specific DSBs. Our study, in which 'clean' DSBs are generated by replication, supports a replication-specific more that break-type specific role of MRX activity. Importantly, we provide molecular evidence that validates these genetic observations and presents SCR as the main mechanism for MRX-dependent repair of DSBs that arise during replication.

As mentioned earlier, once initial DSB processing occurs, resection is extended by the actions of Sgs1-Dna2 or Exo1. Most studies performed with enzymatically induced breaks suggest that both pathways are redundant and equally important, only ablation of both having a major impact on repair (61–63). Here, we show that this is also essentially the case for replication-generated DSBs. However, in this case, the Sgs1-Dna2 pathway seems to be more prominent, as *sgs1*Δ single mutants already display an important defect in SCR while no effect is observed in *exo1*Δ cells. We speculate that this difference may be related to the fact that both Sgs1 and Dna2 are tightly linked to the replication fork (64,65). Furthermore, Dna2 is phosphorylated by CDK, providing a possible mechanism for cell-cycle regulation of its activity (66). Sgs1 is also involved in the dissolution of recombination intermediates to non-crossover products, so we cannot completely rule out the possibility of the phenotype observed being unrelated to the function of Sgs1 in resection. We consider this unlikely, however, since crossover formation is required for the SCR event detected in TINV, and therefore a defect in the HJ dissolution activity of Sgs1 should not cause a decrease in the SCR events detected in our assays.

Finally, we provided evidence for a role of human DNA-PK in suppressing resection in the absence of CtIP, which suggests that the competition between resection and NHEJ activities is conserved from yeast to higher eukaryotes. Further supporting this idea, the MRN complex and CtIP have been shown to be required for EXO1 recruitment to DNA damage sites in human cells (67). In addition, not only CtIP mutants that reduce DNA resection and recombination (CtIP-T847A) but also mutants that increase DNA resection and hamper NHEJ (CtIP-T847E), lead to an increase in IR-induced genomic instability (26), strengthening the deleterious effect that unscheduled NHEJ or HR can have during replication and the possible consequences that this may have for human health.

Therefore, our study sheds some new light on the resection functions that participate in the repair of DSBs linked to the replication fork, exposing important differences with enzymatically induced DSBs. Specifically, although the players involved are the same for both type of breaks, their relative contributions to repair are different. In summary, our results suggest a specific regulation of resection activities during replication, which is required to counteract the action of Ku and avoid the possible deleterious consequences of NHEJ at this particular stage of the cell cycle.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1 and 2.

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