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The structure-specific endonuclease Mus81–Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks

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Repair of interstrand crosslinks (ICLs) requires multiple-strand incisions to separate the two covalently attached strands of DNA. It is unclear how these incisions are generated. DNA double-strand breaks (DSBs) have been identified as intermediates in ICL repair, but enzymes responsible for producing these intermediates are unknown. Here we show that Mus81, a component of the Mus81–Eme1 structure-specific endonuclease, is involved in generating the ICL-induced DSBs in mouse embryonic stem (ES) cells in S phase. Given the DNA junction cleavage specificity of Mus81–Eme1 *in vitro*, DNA damage-stalled replication forks are suitable *in vivo* substrates. Interestingly, generation of DSBs from replication forks stalled due to DNA damage that affects only one of the two DNA strands did not require Mus81. Furthermore, in addition to a physical interaction between Mus81 and the homologous recombination protein Rad54, we show that *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ ES cells were as hypersensitive to ICL agents as *Mus81*$^{-/-}$ cells. We propose that Mus81–Eme1- and Rad54-mediated homologous recombination are involved in the same DNA replication-dependent ICL repair pathway.

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Introduction

A DNA interstrand crosslink (ICL) covalently connects the two complementary strands of the DNA double helix, thereby blocking important DNA transactions, such as transcription and replication, that require unwinding of the two DNA strands. Because they cause such a dramatic block to accessing genetic information, ICL-inducing agents are extremely cytotoxic (Dronkert and Kanaar, 2001). ICL-inducing agents, such as mitomycin C, nitrogen mustards, platinum compounds and psoralens, are more cytotoxic to proliferating cells compared to nondividing cells and therefore they are widely used in chemo- and phototherapy of cancers and skin diseases.

Owing to the nature of ICLs, mechanism(s) for their repair are complex. ICLs damage both DNA strands at the same, or very close, nucleotide positions. Therefore, repair mechanisms involving a simple excision followed by templated resynthesis are not sufficient. In *Escherichia coli* and *Saccharomyces cerevisiae*, ICL repair requires nucleotide excision repair (NER), homologous recombination and translesion DNA synthesis (Dronkert and Kanaar, 2001; McHugh *et al*, 2001). In these organisms, NER seems to be involved in generating the incision(s) near the ICL. Homologous recombination on the other hand has several roles in ICL repair (Dronkert and Kanaar, 2001). One important role for homologous recombination is the repair of DNA double-strand breaks (DSBs), which can result from ICL processing in *S. cerevisiae* cells (McHugh *et al*, 2001). Another role, documented for *E. coli* RecA-mediated homologous recombination *in vitro*, is the generation of the substrate for a second round of strand incisions by NER enzymes (Cheng *et al*, 1991). Alternative roles proposed for homologous recombination repair or homology-directed repair in ICL repair include mechanisms involving break-induced replication, single-strand annealing or the generation of substrates for translesion DNA synthesis (Dronkert and Kanaar, 2001; Niedernhofer *et al*, 2005).

In vertebrate cells, homologous recombination and translesion DNA synthesis are involved in ICL repair as well (Dronkert and Kanaar, 2001). However, an interesting difference between *S. cerevisiae* and higher eukaryotes is the role of the NER proteins in ICL repair. Most, if not all, NER proteins in *S. cerevisiae* cells are involved in ICL repair as deduced from the ICL hypersensitivity of the respective mutants. By contrast, in mammalian cells, mutations in *XPF* and *ERCC1* confer extreme ICL sensitivity, but mutations in other genes essential for NER, including *XPA*, *XPG* and *CSB*, are not dramatically ICL hypersensitive (Dronkert and Kanaar, 2001; De Silva *et al*, 2002). This suggests that XPF-ERCC1, a heterodimeric structure-specific endonuclease (de Laat *et al*, 1998), plays a central role in ICL repair that is largely independent of NER. In addition to an NER-independent role in ICL repair, XPF-ERCC1 functions in at least two subpathways of homology-directed DNA repair. Both in *S. cerevisiae* and mammalian cells, XPF-ERCC1 is involved in DSB repair, through single-strand annealing, and in homologous gene targeting (Paques and Haber, 1999; Adair *et al*, 2000; Sargent *et al*, 2000; Niedernhofer *et al*, 2001; Langston and Symington, 2005).

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Unraveling the mechanism(s) of ICL repair requires answers to two central questions; what are the intermediates in ICL repair at the DNA level and how are they generated? Recently, it has become clear that one pivotal intermediate that can arise during the repair of an ICL is a DSB (Akkari *et al*, 2000; De Silva *et al*, 2000; Niedernhofer *et al*, 2004; Rothfuss and Grompe, 2004). The formation of this DSB intermediate requires DNA replication, suggesting that a stalled replication forks at the site of the ICL is recognized and processed by a structure-specific endonuclease into a DSB. Owing to the ICL hypersensitivity of cells mutated in the XPF–ERCC1 complex and the biochemical properties of this complex, it has been suggested that XPF–ERCC1 would convert ICLs to DSBs. However, recent studies have demonstrated that this conversion is XPF–ERCC1 independent (De Silva *et al*, 2000; Niedernhofer *et al*, 2004), thus leaving the question of how DSBs are generated from ICLs partially unanswered.

Recently, a structure-specific endonuclease, Mus81–Eme1, with amino-acid sequence similarity to XPF-ERCC1 has been identified in yeast and mammalian cells (Heyer, 2004). In yeast, *mus81* mutants exhibit sensitivity to hydroxyurea, UV-light and methyl methanesulfonate but not to ionizing radiation (Interthal and Heyer, 2000; Doe and Whitby, 2004; Doe *et al*, 2004). This sensitivity profile is consistent with a role for Mus81–Eme1 in processing stalled DNA replication forks. The biochemical properties of the enzyme complex are also consistent with its involvement in forming DSBs at DNA structures resembling replication forks. Like XPF–ERCC1, Mus81–Eme1 also cleaves branched DNA structures (Boddy *et al*, 2001; Chen *et al*, 2001; Constantinou *et al*, 2002; Kaliraman *et al*, 2001; Ciccia *et al*, 2003; Ogrunc and Sancar, 2003; Whitby *et al*, 2003). However, XPF–ERCC1 prefers three-way branched junctions containing two single-stranded DNA arms, whereas Mus81–Eme1 has a preference for three-way junctions that are more double-stranded in nature, such as 3′-flap and replication fork-like structures (Heyer, 2004). *Mus81* and *Eme1* mutant embryonic stem (ES) cells, which have recently become available, display hypersensitivity to ICL-inducing agents such as mitomycin C and cisplatin (Abraham *et al*, 2003; McPherson *et al*, 2004).

Results

Mus81 is involved in processing ICLs into DSBs

To test whether the Mus81–Eme1 structure-specific endonuclease is involved in processing ICLs into DSBs, we first constructed mouse ES cells lacking Mus81 (Supplementary Figure 1). Next, we analyzed ICL-induced DSB formation in Mus81-proficient and -deficient ES cells. Proliferating ES cells were treated with different doses of mitomycin C for 24 h and ICL-induced DSBs were detected using PFGE (Figure 1A). As we previously demonstrated, mitomycin C treatment resulted in an increase in broken DNA in wild-type ES cells (Niedernhofer *et al*, 2004). The increase was dose dependent (Figure 1A) and was not owing to DNA fragmentation during apoptosis (Supplementary Figure 2). In ES cells lacking ERCC1, mitomycin C-induced DSBs were observed (Figure 1A). However, cells lacking Mus81 mitomycin C failed to induce DSBs, even at the highest dose of mitomycin C used (Figure 1A). Similarly, in response to another ICL-inducing agent, cisplatin, wild-type ES cells showed a dose-dependent increase in broken DNA that was not observed in *Mus81*$^{-/-}$ ES cells (Figure 2A). We conclude that the structure-specific endonuclease Mus81–Eme1 is involved in processing ICLs into DSBs.

Mus81 operates in S phase

Given the biochemical activity of Mus81–Eme1 on splayed arm DNA substrates and the lack of mitomycin C-induced DSBs in *Mus81*$^{-/-}$, the role of Mus81–Eme1 in ICL repair is likely the conversion of ICL-stalled replication forks into DSBs. Thus, Mus81–Eme1 would function during S phase. Consistent with this notion, culturing the cells in the continuous presence of mitomycin C or cisplatin resulted in their accumulation in S phase (Figures 1B and 2C, respectively). If Mus81–Eme1 acts in S phase on stalled replication forks, then the induction of DSBs by mitomycin C should be slow, in contrast to DSB induction by agents that directly act on DNA such as ionizing radiation. Indeed, in wild-type ES cells cultured in the presence of 1.0 μg/ml mitomycin C, DSB induction became apparent after around 12–18 h and subsequently increased over time (Figure 1C). Again, no DSBs were induced in *Mus81*$^{-/-}$ cells, even after 30 h of incubation with mitomycin C.

If Mus81–Eme1 functions on ICL-stalled replication forks, then active replication would be required to detect Mus81-dependent, ICL-dependent DSBs. To test this premise, we blocked replication by the addition of thymidine, which resulted in accumulation of the cells in S phase (Figure 3A and B). When mitomycin C was added, no increase in DSBs was detected, either in wild-type or *Mus81*$^{-/-}$ ES cells (Figure 3C). In normal growth conditions, without added thymidine to block replication, Mus81-dependent DSBs were detected upon the addition of mitomycin C. Furthermore, when cells treated with mitomycin C, under conditions of thymidine-blocked replication (Figure 4A), were allowed to resume replication by incubating them in media lacking thymidine and mitomycin C, Mus81-dependent DSBs were detected (Figure 4C, compare lanes 7 and 14). Based on the results of the PFGE analysis, the cell cycle analysis and the hypersensitivity of *Mus81*$^{-/-}$ cells to both mitomycin C and cisplatin (Figures 2B and 7C, respectively), we conclude that Mus81–Eme1 is involved in DSB formation when DNA replication forks are blocked by an ICL.

In the experiments described above, the cells were continuously incubated in the presence of mitomycin C. This resulted in accumulation of the cells in S phase (Figure 1B) and allowed us to observe the involvement of Mus81 in converting ICLs to DSBs. In contrast, when cells were incubated for 1 h in the presence of mitomycin C and subsequently placed in fresh media without mitomycin C, the cells did not accumulate in S phase, irrespective of their genotype (Figure 5A). Instead, 12 h after the mitomycin C pulse most cells were in late S phase, whereas G2, G1 and early S phase cells were detected by 36 h. Under these conditions, DNA breaks were observed in both wild-type and *Mus81*$^{-/-}$ ES cells (Figure 5B).

Mus81-mediated DSB formation is DNA lesion selective

Next, we asked whether Mus81–Eme1 is required, in general, to produce DSBs under conditions where DNA damage leads to replication fork stalling. Therefore, instead of using mitomycin C, UV light was used to stall replication through DNA

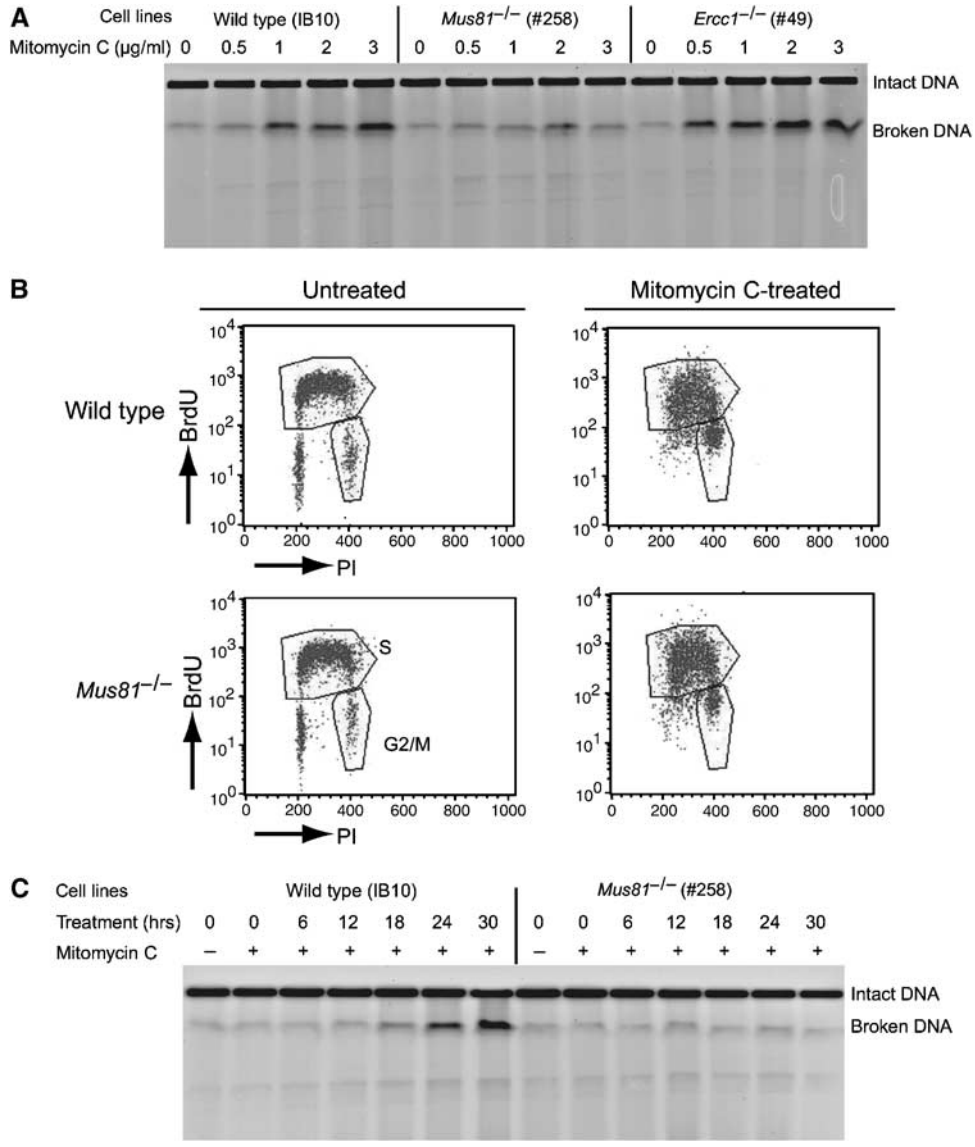

Figure 1 Analysis of mitomycin C-induced DSB formation in wild-type, *Ercc1*$^{-/-}$ and *Mus81*$^{-/-}$ ES cells. (**A**) Using PFGE, DSB formation was analyzed. Cells of the indicated genotype were treated with increasing concentrations of mitomycin C for 24 h, collected into agarose plugs and their DNA was separated by size on an agarose gel. Under the electrophoresis conditions used, high molecular weight genomic DNA remains in the well, whereas lower molecular weight DNA fragments (several Mbp to 500 kbp) migrate into the gel and are compacted into a single band. (**B**) Cell cycle profiles of wild-type and *Mus81*$^{-/-}$ ES cells after continuous treatment with mitomycin C for 24 h. Using a FACscan, the cell cycle profile of cells pulse labeled with BrdU was analyzed by total DNA content as determined by propidium iodide (PI) staining (*x*-axis) and replication status as determined by BrdU incorporation (*y*-axis). Cells were either untreated or incubated with 2.0 μg/ml mitomycin C. (**C**) Time course of mitomycin C-induced DSB formation in wild-type and *Mus81*$^{-/-}$ ES cells. Cells were treated with 1.0 μg/ml mitomycin C, indicated by (+). The untreated control sample is indicated by (−).

damage induction (Courcelle and Hanawalt, 2001; Branzei and Foiani, 2005). Wild-type, *Mus81*$^{+/-}$ and *Mus81*$^{-/-}$ ES cells were treated with increasing doses of UV light and the cells were analyzed for DSB formation after 4 h. After the treatment, the cells accumulated in S phase, just as was observed after treatment with the ICL-inducing agents (Figure 6C). A dose-dependent increase in broken DNA was observed, irrespective of whether Mus81 was functional (Figure 6A). Consistent with this observation, Mus81 was not required for cell survival in response to UV-light treatment (Figure 6B). By contrast, *Mus81*$^{-/-}$ cells were hypersensitive to ICL-inducing agents (Figures 2B and 7C, and McPherson *et al*, 2004; Dendouga *et al*, 2005). We conclude that not all stalled replication forks are equivalent and that the replication fork cleavage activity of Mus81–Eme1 depends on the lesion that causes the stalling.

Physical and genetic interactions between Mus81 and Rad54

A one ended-DSB such as generated by Mus81–Eme1 from stalled replication forks is an ideal substrate for the initiation of homologous recombination as a next step in ICL repair. Consistent with this notion we observed a reduction of mitomcyin C-induced sister chromatid exchanges (SCEs) in *Mus81*$^{-/-}$ cells compared to wild-type ES cells. Wild-type cells treated with 0.2 μg/ml mitomycin C displayed 40.8 ± 4.0

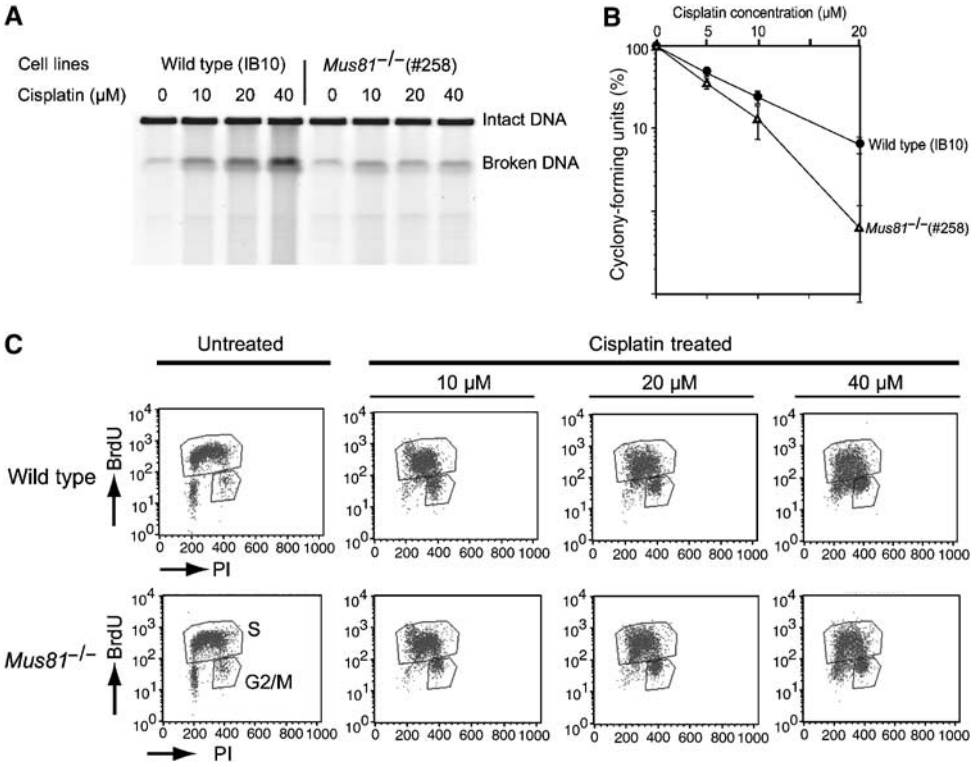

Figure 2 Analysis of cisplatin-induced DSB formation in wild-type and *Mus81*$^{-/-}$ ES cells. (**A**) Cells of the indicated genotype were treated with increasing concentrations of cisplatin for 24 h and their DNA was analyzed by PFGE. (**B**) Clonogenic survival curve of wild-type and *Mus81*$^{-/-}$ ES cells in response to increasing doses of cisplatin. (**C**) Cell cycle profiles of wild-type and *Mus81*$^{-/-}$ ES cells after continuous treatment with increasing doses of cisplatin for 24 h. Bi-parameter (BrdU and PI) FACscan plots are shown.

SCE per metaphase, whereas *Mus81*$^{-/-}$ cells showed 31.5 ± 1.9 SCE per metaphase. Interestingly, the spontaneous level of SCEs was already slightly, but significantly ($P<0.01$) reduced in *Mus81*$^{-/-}$ cells compared to wild type from 9.9 ± 0.8 to 7.2 ± 0.5 SCEs per metaphase. Possibly, repair of replication forks stalled due to endogenous DNA damage is less likely to proceed through a DSB intermediate in the absence of Mus81.

In addition to reduced mitomycin C-induced SCEs level in *Mus81*$^{-/-}$ ES cells, evidence for a link between Mus81 and homologous recombination is also provided by the interaction between *S. cerevisiae* Mus81 and the homologous recombination protein Rad54 in a two-hybrid assay and in co-immunoprecipitation experiments (Interthal and Heyer, 2000). We asked whether a physical and genetic interaction exists between mouse Mus81–Eme1 and Rad54. Whole-cell extracts were prepared from an ES cell line that carries a Rad54 knockout allele and an HA-tagged Rad54 knock-in allele, which expresses HA-tagged and fully functional Rad54 protein from the endogenous promoter (Tan *et al*, 1999). HA-tagged Rad54 protein was precipitated with immobilized anti-HA antibodies. Immunoblotting of the precipitated samples was used to detect the presence of Mus81, the HA epitope and Rad54 (Figure 7A and B). Co-immunoprecipitation of Mus81 with Rad54 was detected. In contrast, Mus81 was not detected when the precipitation was performed using extracts prepared from an isogenic ES cell line in which HA-tagged Rad54 was absent. The interaction is likely protein-mediated, because DNA in the extracts was digested with DNase I before the immunoprecipitation.

The physical interaction between Mus81–Eme1 and Rad54 is consistent with a function of these proteins in the same ICL repair pathway. The DNA intermediate from which homologous recombination during ICL repair would be initiated is the DSB generated by Mus81–Eme1. Therefore, inactivating mutations in *Mus81* should be epistatic to mutations in *Rad54* in the context of ICL repair. To test this premise, we generated *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ double knockout ES cells and compared their degree of mitomycin C sensitivity to that of either of the single mutants. *Rad54*$^{-/-}$ ES cells were about three-fold more sensitive to mitomycin C than wild-type ES cells (Figure 7C and Essers *et al*, 1997). The *Mus81*$^{-/-}$ ES cells displayed a seven-fold increase in mitomycin C sensitivity. The *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ double knockout ES cells were as sensitive to mitomycin C as the *Mus81*$^{-/-}$ cells (Figure 7C). We conclude that *Mus81* is epistatic to Rad54 with respect to repair of ICLs. Consistent with this notion, mitomycin C-induced DSBs were observed in *Rad54*$^{-/-}$ ES cells, but not in *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ ES cells (Figure 7D).

Discussion

A DNA ICL covalently links both strands of the DNA double helix and thus its repair requires incisions not only on both sides of the crosslink but also in both DNA strands. Previously, DSBs have been identified as intermediates in ICL repair. Here, we identify Mus81–Eme1 as a structure-specific endonuclease involved in converting ICLs to DSBs in a DNA replication-dependent manner.

ICL-inducing agents cause very heterogeneous types of DNA distortions (Dronkert and Kanaar, 2001). Therefore,

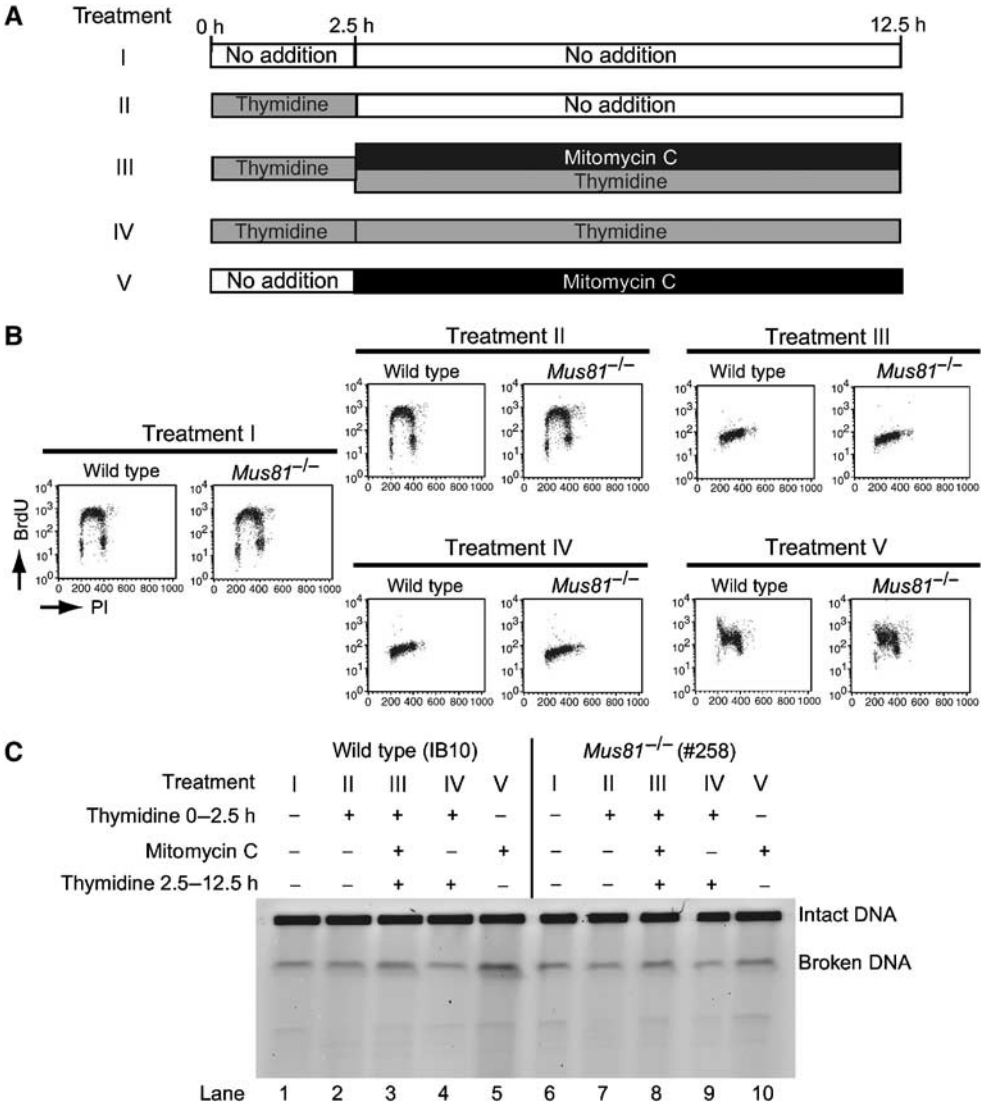

Figure 3 Inhibition of replication suppresses Mus81-dependent DSB formation in response to ICLs. (**A**) Schematic representation of the experimental protocol. Replication in wild-type and *Mus81*$^{-/-}$ ES cells was inhibited by incubating them in media containing 20 mM thymidine for 2.5 h. Next, the cells were treated with 2 μg/ml mitomycin C and 20 mM thymidine for 10 h (III). Control cells were either untreated (I), treated with 20 mM thymidine for 2.5 h (II) or 12.5 h (IV), or treated with 2 μg/ml mitomycin C for 10 h (V). (**B**) Cell cycle profiles of cells treated as described in panel (A) were determined by bi-parameter (BrdU and PI) FACS analysis. (**C**) Wild-type and *Mus81*$^{-/-}$ ES cells were treated as described above and the DSB formation was analyzed by PFGE.

recognition of ICLs posses a problem because recognition based on chemical and three-dimensional structure would require multiple recognition proteins. Instead, cells probably rely on detection methods that do not require direct recognition of the ICL, such as ICL-induced transcription or replication stalling. For cells in S phase, a replication fork stalled by an ICL can provide a branched DNA structure that triggers the required strand cleavages. However, classical excision repair pathways such as NER or base excision repair alone are not sufficient for ICL repair because these pathways have evolved to cleave only one of the two DNA strands. While it has been established that ICLs are converted into DSBs in a replication-dependent manner (Akkari *et al*, 2000; De Silva *et al*, 2000; Niedernhofer *et al*, 2004; Rothfuss and Grompe, 2004), the identity of nucleases responsible for this conversion had not been determined.

Mammalian cells contain at least two structure-specific endonucleases that cleave branched DNA structures: XPF-ERCC1 and Mus81–Eme1 (Heyer *et al*, 2003; Heyer, 2004). XPF-ERCC1, first identified for its function in NER, prefers three-way branched junctions containing two single-stranded DNA arms, whereas Mus81–Eme1 cleaves three-way junctions with at least two double-stranded arms, such as 3′ flaps and structures resembling replication forks. *Ercc1*$^{-/-}$ cells are extremely sensitive to ICL-inducing agents, but the XPF–ERCC1 complex is not involved in the generation of ICL-induced DSBs and probably plays a role in another step of ICL repair (Niedernhofer *et al*, 2004). We have generated *Mus81*$^{-/-}$ ES cells to address whether Mus81 was part of the structure-specific endonuclease complex responsible for DSB formation after treatment with crosslinking agents. *Mus81*$^{-/-}$ cells, as well as *Eme1*$^{-/-}$ cells, are hypersensitive to mitomycin C and cisplatin (Figures 2B and 7C, and Abraham *et al*, 2003; McPherson *et al*, 2004; Dendouga *et al*, 2005). Culturing wild-type ES cells in the continuous presence of an ICL-inducing agent results in the accumulation of the cells in

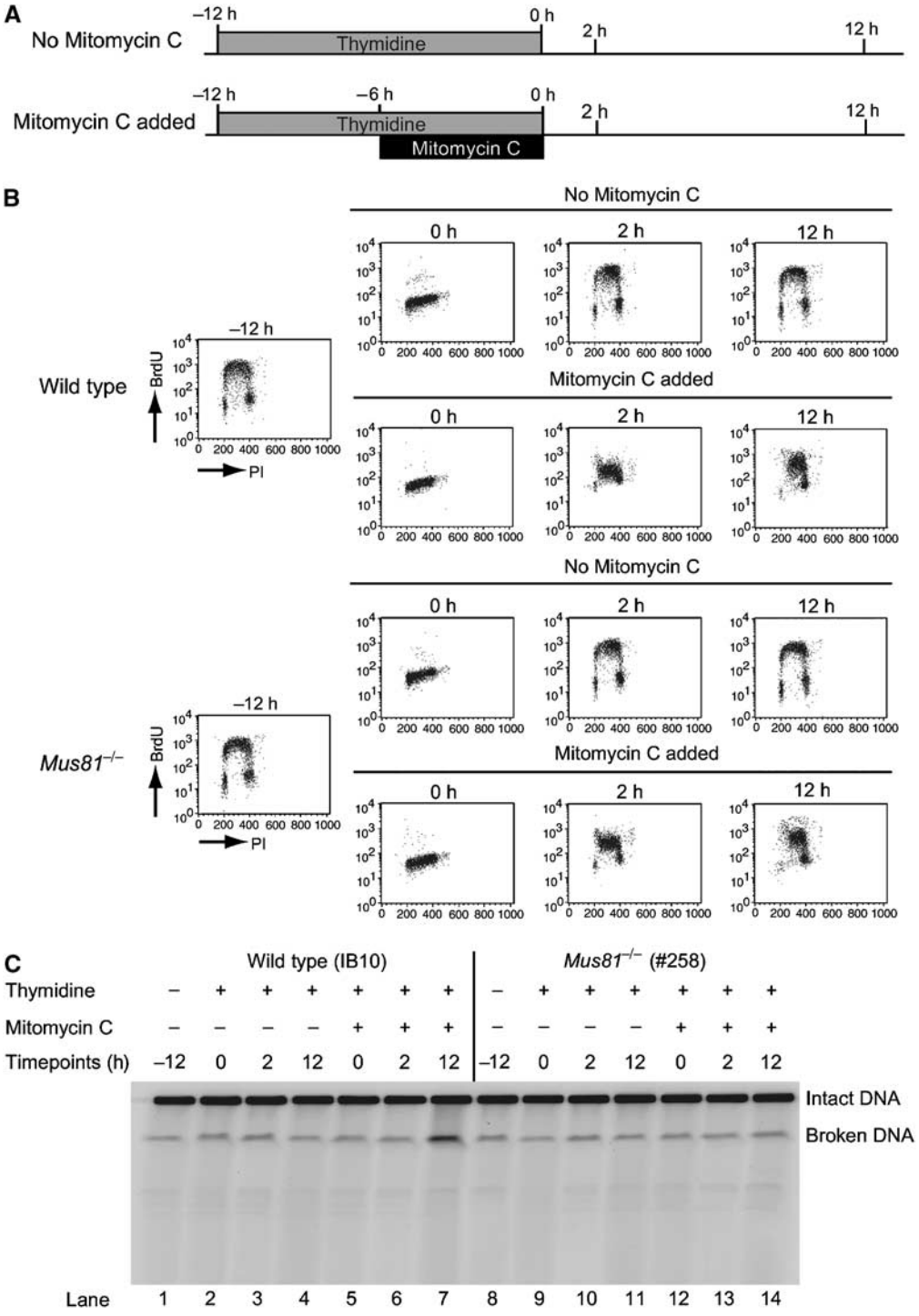

Figure 4 Mus81-dependent generation of mitomycin C-induced DSBs occurs during S phase. (**A**) Schematic representation of the experimental protocol. Wild-type and *Mus81*$^{-/-}$ ES cells were incubated in 5 mM thymidine for 12 h. For the last 6 h of the incubation, mitomycin C was added to a final concentration of 2 μg/ml. Control cells were incubated with thymidine-containing media only. Next, the cells were washed twice with PBS, incubated in fresh medium to allow resumption of replication, and collected at the indicated times. (**B**) Cell cycle profiles of cells treated as described in panel (A), shown as BrdU incorporation versus PI plots. (**C**) The relative amount of broken DNA in wild-type and *Mus81*$^{-/-}$ ES cells treated as described in panel (A) was assessed by PFGE.

S phase (Figures 1B and 2C) and in DSB formation that increases in a time- and dose-dependent manner (Figures 1C, A and 2A). By contrast, no such increase in DSB formation occurs in the absence of Mus81, even through the cells do accumulate in S phase. Inhibition of DNA replication suppresses Mus81-dependent DSBs in response to mitomycin C (Figure 3), whereas resuming replication resulted in their formation (Figure 4). Taken together, the biochemical activity of the Mus81–Eme1 complex, the mitomycin C hypersensitivity of *Mus81*$^{-/-}$ and *Eme1*$^{-/-}$ cells, and the lack of ICL-induced DSB formation in *Mus81*$^{-/-}$ S phase cells suggest that Mus81–Eme1 is a structure-specific endonuclease

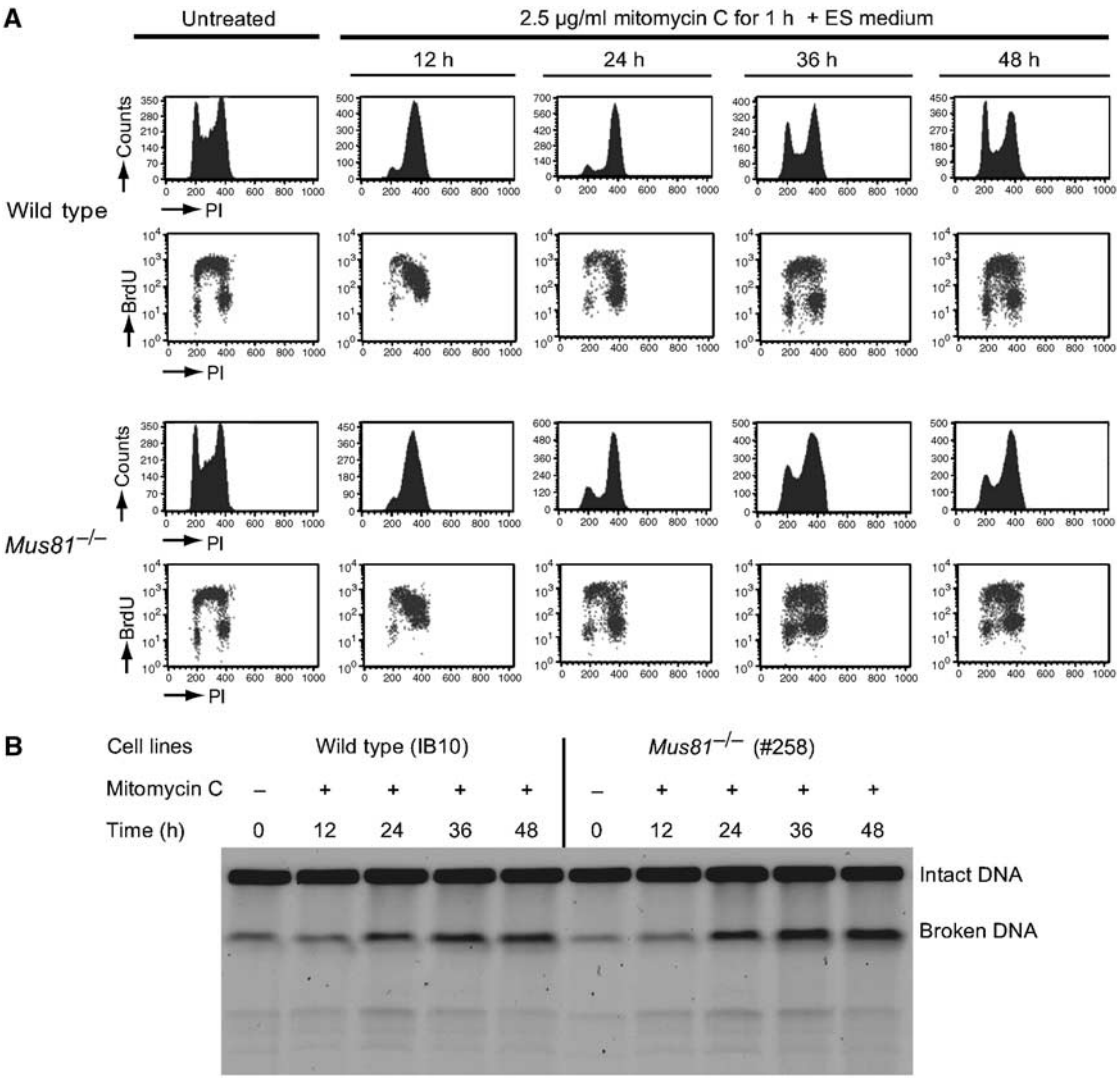

Figure 5 Analysis of DSB formation after pulse treatment of cells with mitomycin C. (**A**) ES cells of the indicated genotype were treated for 1 h with mitomycin C and continued to be incubated in media without mitomycin C for the indicated amount of time before their cell cycle profile was determined by FACS analysis. The cell count versus PI staining and BrdU incorporation versus PI profile are shown. (**B**) Wild-type and *Mus81*$^{-/-}$ ES cells were treated with mitomycin C for 1 h. Cells were incubated in media without mitomycin C for the indicated amount of time and the amount of broken DNA was determined by PFGE.

involved in cleaving one of the branched arms of replication forks stalled by ICL lesions.

While Mus81 is involved in cleaving replication forks stalled at ICLs, it does not seem to be responsible for dealing with DNA damage-associated replication stalling in general. After treatment with UV light, *Mus81*$^{-/-}$ cells accumulate a similar amount of DSBs as wild-type cells (Figure 6). Encountering DNA damage that affects only one strand, such as induced by UV light, will cause problems for a replicative polymerase but may not stop a replicative helicase. The incomplete replicated regions resulting in this case are apparently not substrates for Mus81. However, the DNA structures created, and the challenges to restarting replication, when a fork encounters an ICL are likely to be much different. Because the integrity and movement of a replication fork is determined by the presence and movement of a replicative helicase, halting this enzyme, as an ICL will, will likely cause complete disruption of fork movement. DNA synthesis will stop on both strands and the complex assemblies of replication proteins may disassociate from each other and their DNA templates.

While the results of our experiments reveal the involvement of Mus81–Eme1 in converting ICLs into DSBs, they also demonstrate the importance of the assay to detect these DSBs. When cells are continuously exposed to mitomycin C, they accumulate in S phase and Mus81-dependent DSBs can be detected (Figure 1). On the other hand, when they are treated with a short pulse of mitomycin C, cells do not accumulate in S phase and while DSBs are detected they are not Mus81-dependent (Figure 5), consistent with the results of a previous study (Dendouga *et al*, 2005). It is possible that unrepaired or partially repaired ICL damage, for example gaps or crosslinks between sister chromatids, are still present in G2 cells. Once such cells go through mitosis, this damage can impair proper chromosome segregation, for example as evidenced by an increase in anaphase bridging especially under conditions of reduced ICL repair (Niedernhofer *et al*, 2004), and lead to mechanical breakage

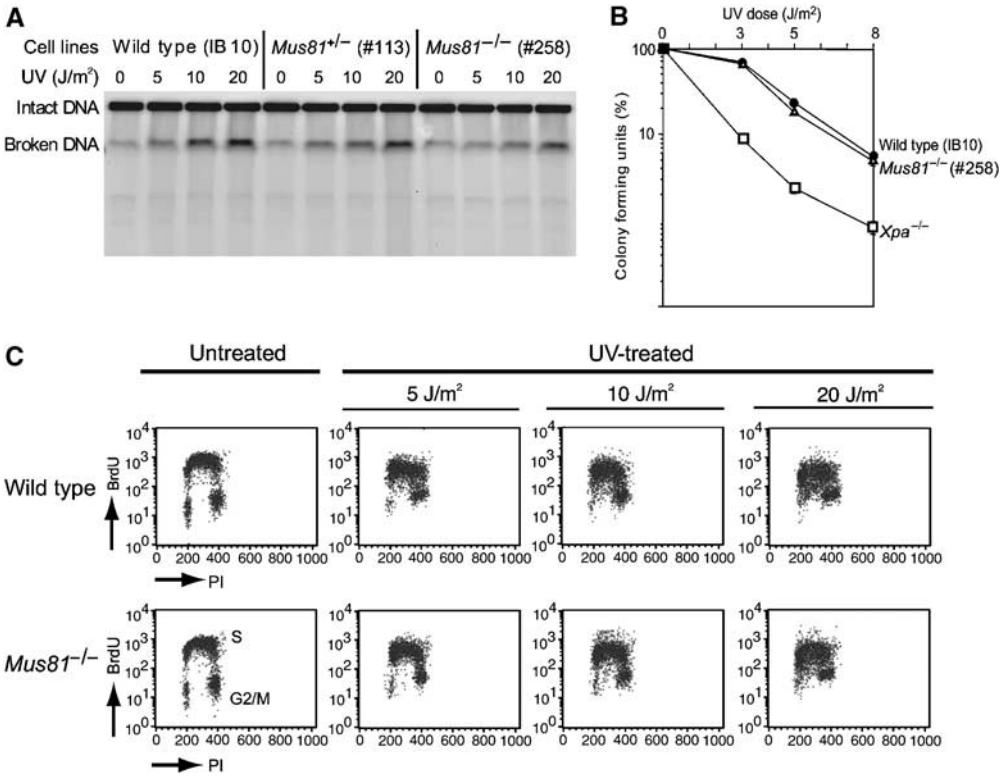

Figure 6 Analysis of UV-light-induced DSB formation in wild-type and *Mus81*$^{-/-}$ ES cells. (**A**) UV-light-induced DSB formation in wild-type, *Mus81*$^{+/-}$ and *Mus81*$^{-/-}$ ES cells as analyzed by PFGE. (**B**) Survival curve in response to UV light for wild-type, and *Mus81*$^{-/-}$ ES cells. *Xpa*$^{-/-}$ ES cells served as a control. (**C**) Cell cycle profiles of wild-type and *Mus81*$^{-/-}$ ES cells 4 h after treatment increasing doses of UV light. The BrdU incorporation versus PI profiles are shown.

of DNA molecules, both in wild-type and Mus81-deficient cells. Alternatively, the Mus81-independent breaks might occur in the subsequent S phase. The conversion of mitomycin C mono-adducts to ICLs is slow (Warren *et al*, 1998). Therefore, the ratio of ICLs to mono-adducts might be low in the pulse treatment assay because cells do not accumulate in S phase. When DNA-containing mono-adducts will be replicated in the subsequent S phase, the mono-adducts could trigger Mus81-independent DNA cleavage in a manor analogous to UV-light-induced DNA damage (Figure 6).

Our results indicate that Mus81–Eme1 is involved in cleaving a replication fork stalled by an ICL to produce a DSB, which itself is a genotoxic intermediate that has to be repaired before it causes further damage to the genome (Dronkert and Kanaar, 2001; McHugh *et al*, 2001). In mammalian cells, DSBs can be repaired through two mechanistically distinct pathways; homologous recombination and non-homologous end joining (van Gent *et al*, 2001). Homologous recombination mutants are hypersensitive to ICL-inducing agents, suggesting that recombination is the main pathway involved in DSB-associated ICL repair (McHugh *et al*, 1999; De Silva *et al*, 2000). The one-ended DSBs created by Mus81–Eme1 cleavage at a stalled replication fork (Supplementary Figure 3) are substrates for homologous recombination rather than nonhomologous end joining (Cromie *et al*, 2001). Thus, it would be advantageous if Mus81–Eme1 endonuclease would be directly linked to the recombination machinery for efficient processing of the DSBs it generates. Indeed, Mus81 has first been identified in *S. cerevisiae* through a two-hydrid interaction with the homologous recombination protein Rad54 (Interthal and Heyer, 2000). Here we show, in mammalian cells, that the Mus81 and Rad54 proteins interact (Figure 7A) and that they are genetically involved in the same ICL survival pathway (Figure 7C). Furthermore, we show that mitomycin C-induced SCEs are reduced in the absence of Mus81. These results are consistent with the notion that the Mus81-generated DSBs are further processed by Rad54-mediated homologous recombination. The additional sensitivity of the *Mus81*$^{-/-}$ ES cells compared to *Rad54*$^{-/-}$ ES cells indicates that alternative pathways exists for repair of the ICL-induced DSBs that do not involve Rad54, consistent with the mild homologous recombination defect of *Rad54*$^{-/-}$ ES cells (Essers *et al*, 1997; Dronkert *et al*, 2000). One such alternative pathway might involve the Rad54 paralog Rad54B (Wesoly *et al*, 2006). Both Rad54 paralogs interact with Rad51, which provides the catalytic core of homologous recombination. Rad51 assembles into nucleoprotein filaments on single-stranded DNA and promotes homology recognition and DNA strand exchange, which can result in repair of DSBs (Wyman *et al*, 2004). In a possible scenario to limit the genotoxicity of the DSB intermediate in ICL repair, Rad51 could assemble on single-stranded DNA arising at the ICL-stalled DNA replication fork, before the DSB has occurred. As Rad54 in mammalian cells interacts with Rad51 upon the induction of DNA damage (Tan *et al*, 1999), it is possible that it is the Rad51 nucleoprotein filament that attracts Rad54. This notion has previously been proposed as a mechanism to target Rad54, which acts on the duplex DNA, to the intact homologous template DNA (Mazin *et al*, 2000; Solinger *et al*, 2001). As Rad54 interacts with Mus81, the structure-specific

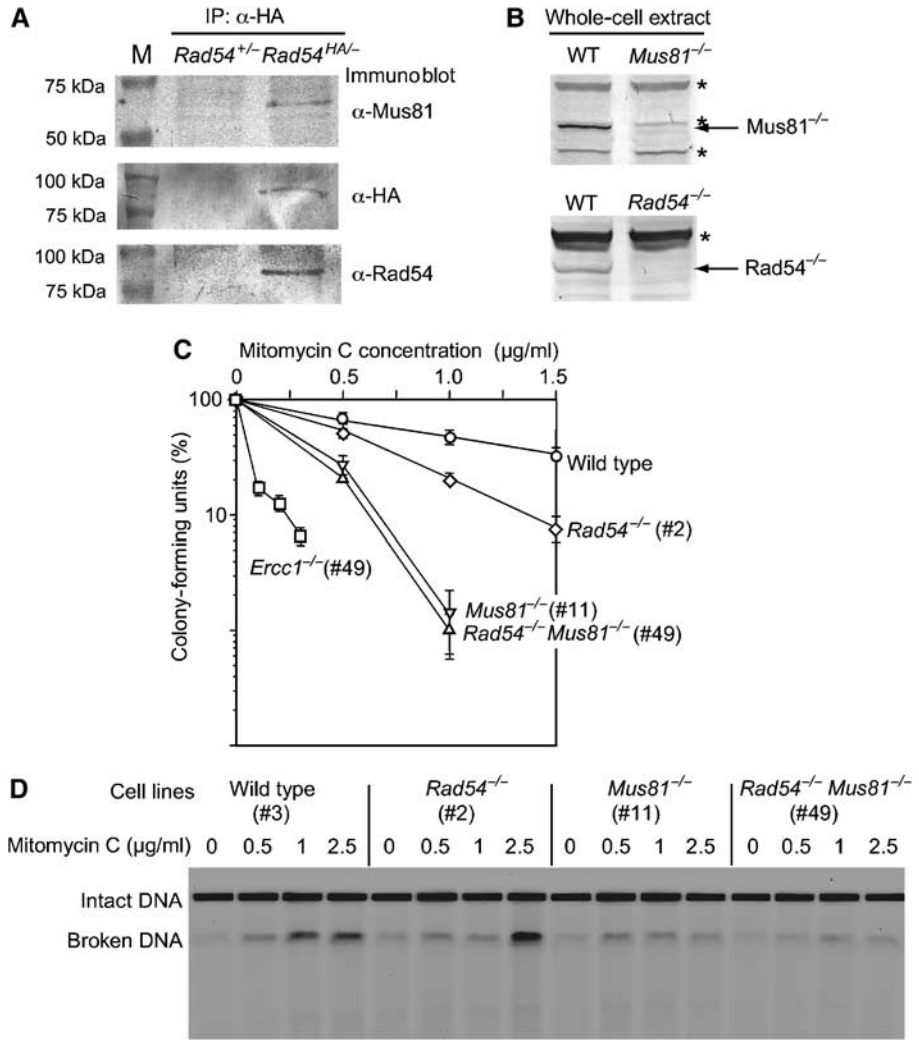

Figure 7 Analysis of relationship between Mus81 and Rad54 with respect to ICL repair. (**A**) Immunoprecipitation (IP) of Rad54 and Mus81. Using an anti-HA-antibody, HA-tagged Rad54 protein was precipitated from HA-tagged Rad54 knock-in ES cells. The precipitated material was analyzed by immunoblotting using antibodies against Mus81, HA and Rad54. As a negative control, *Rad54*$^{+/-}$ cells (cell line #18) were used, because the cell line is isogenic to the *Rad54*$^{HA/-}$ cell line, except for the HA-tag on Rad54. (**B**) Identification of the Mus81 and Rad54 proteins in the input material for the immunoprecipitation. Immunoblots using whole-cell extracts representing 1% of the material used for the immunoprecipitation. As controls for the identification of Mus81 and Rad54, extracts from *Mus81*$^{-/-}$ and *Rad54*$^{-/-}$ ES cells were used. Signals from nonspecific proteins are indicated by an asterix. (**C**) Comparison of mitomycin C sensitivity of wild-type, *Mus81*$^{-/-}$, *Rad54*$^{-/-}$ and *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ ES cells. ES cells of the indicated genotype were treated with increasing doses of mitomycin C for 1 h after which the medium was refreshed. Colonies were fixed, stained and counted after 5–8 days. Error bars indicate the standard error of the mean. (**D**) Analysis of mitomycin C-induced DSB formation in wild-type, *Rad54*$^{-/-}$, *Mus81*$^{-/-}$ and *Mus81*$^{-/-}$ *Rad54*$^{-/-}$ ES cells using PFGE.

endonuclease would only get in the proximity of the stalled replication fork when the critical DSB repair proteins are already in place.

Based on the results described above, we propose a model for a Mus81-dependent ICL repair pathway (Supplementary Figure 3). ICL lesions prevent DNA unwinding required for processive replication and thereby induce stalling of replication forks. The exact structure of DNA strands at an ICL stalled fork is not known. A replicative helicase may stop some base pairs ahead of the crosslinked nucleotides. The presence of single-stranded gaps in the nascent strands will depend on the resulting mis-coordination between leading and lagging strands. Regression of the newly synthesized strands or other recombination-mediated DNA strand exchanges may be needed to assure that the yet to be replicated parental and new daughter DNA strands remain associated for fork recovery after ICL removal. Many scenarios can be envisioned that would result in branched DNA structures that match the *in vitro* nuclease activity of Mus81–Eme1 for cleavage to a DSB. Although potentially dangerous, DSBs are needed to remove ICLs and may facilitate subsequent repair processes. A DSB may serve as an exit point for stalled replication proteins as well as a release for accumulated positive DNA supercoiling, which inhibits most DNA-binding proteins. In both cases, DSB formation would promote recruitment of repair enzymes, including incision nuclease(s). The ICL is still in place after Mus81 cleavage (Supplementary Figure 3) and therefore additional strand incisions are required for its removal. The hypersensitivity of *Ercc1*$^{-/-}$ and *XPF* mutant cells to ICL-inducing agents highlights the importance of this complex for ICL repair. The XPF–ERCC1 endonuclease can incise ICL-containing DNA *in vitro*

(Bessho *et al*, 1997; Kuraoka *et al*, 2000). This activity is consistent with a role in incising DNA at one side of an ICL, in the unreplicated DNA region of the cleaved fork shown in Supplementary Figure 3. Subsequently, rotating the crosslinked base out of the helix creates in effect, a single-stranded gap (Kaye *et al*, 1980; Matsumoto *et al*, 1989; De Silva *et al*, 2000). This idea is supported by the observation that ICL-induced single-stranded breaks and single-stranded gaps are decreased in XPF- and ERCC1-deficient cell lines (De Silva *et al*, 2000; De Silva *et al*, 2002; Rothfuss and Grompe, 2004). Homologous recombination between the daughter DNA molecules could re-establish a replication fork. The Mus81-generated DSB would be processed such that its 3′ end invades and pairs with the other daughter molecule to create a primer for the polymerase. DNA synthesis over the site of the crosslink would likely require a translesion polymerase to restore the duplex and eventually result in re-establishing a complete replication fork.

The coordinated cooperation of several DNA repair pathways is clearly required during Mus81-dependent repair of ICLs during S phase. Additional complexities to understanding ICL repair are provided by the possibility that other Mus81-independent ICL repair pathways must exist. For example, the ICL sensitivity of *Ercc1*$^{-/-}$ ES cells is much greater than that of *Mus81*$^{-/-}$ cells (Figure 7C). Outside of S phase, ICLs cannot be detected by stalled DNA replication, yet they will still be highly toxic owing to their interference with transcription. Recently, compelling evidence has been provided for the existence of a G1 phase ICL repair in *S. cerevisiae*, which is dependent on NER and a translesion DNA polymerase (Sarkar *et al*, 2006).

Materials and methods

Cell lines

The cell lines used in this study are described in Supplementary Table 1. The method of *de novo* isolation of ES cells is described previously (Essers *et al*, 2000). The generation of *Mus81*$^{-/-}$ ES cells is described in the Supplementary data. Subconfluent cultures of ES cells were treated with DNA-damaging agents or thymidine as indicated.

Detection of DSBs by pulse-field gel electrophoresis

Subconfluent cultures of wild-type, *Mus81*$^{+/-}$, *Mus81*$^{-/-}$, *Rad54*$^{-/-}$, *Rad54*$^{-/-}$ *Mus81*$^{-/-}$ and *Ercc1*$^{-/-}$ ES cells were treated with mitomycin C or cisplatin for 24 h, unless otherwise indicated. In case of UV-light treatment, cells were washed with 10 ml phosphate-buffered saline (PBS), exposed to UV light and incubated in fresh medium to allow repair and cell cycle progression for 4 h. Cells were harvested after trypsinization and agarose plugs containing 10^6 cells were prepared with a CHEF-disposable plug mold (Bio-Rad). The cells were lysed by incubation of the plugs in 1 mg/ml proteinase K in 100 mM EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine for 48 h at 37°C and then washed repetitively with 10 mM Tris–HCl, pH 8.0, 100 mM ETDA. Electrophoresis was performed for 23 h at 13°C through 0.9% agarose in Tris–borate–EDTA buffer using a Biometra Rotaphor apparatus with the following parameters: interval, 30–5 s log; angle, 120°–110° linear; 180–120 V log). The DNA was stained with ethidium bromide and visualized using a Typhoon 9200 scanner (Amersham Pharmacia Biotech). The electrophoresis conditions were specifically designed to compact lower molecular weight DNA fragments (several Mbp to 500 kbp) into a single band, while keeping high molecular weight genomic DNA in the well. The lower molecular weight DNA fragments are the results of DSBs in the chromosomal DNA. Thus, the assay allows broken DNA to be readily detected. However, in the context of DSBs arising during DNA replication stalling, the assay has limited sensitivity and is not quantitative. When a DSB occurs at a DNA replication fork, the broken DNA is still attached to the template chromosome. To detect DSBs in the assay, two relatively closely (several Mbp) spaced independent DSBs have to occur.

Flow cytometric analysis

Before collection after the various indicated treatments, ES cells were incubated with 10 μM BrdU for 5 min at 37°C, harvested by trypsynization and fixed overnight with 70% ethanol at 4°C (Smits *et al*, 2000). After the ethanol was washed away, the cells were treated with 0.1 N HCl containing 0.5 mg/ml pepsin (Merck) for 20 min at room temperature. Next, the cells were treated with 2 N HCl for 12 min at 37°C, followed by the addition of borate buffer (pH 8.5). The cells were washed with PBS containing 0.5% Tween-20 and 0.1% BSA and incubated with FITC-conjugated anti-BrdU antibodies (Becton Dickinson) for 1 h at 4°C. After washing, the cells were counterstained with a solution containing PI (10 μg/ml) and RNase (10 μg/ml) for 30 min at 37°C. The cells were analyzed on a fluorescence-activated cell sorter (Becton Dickinson) using CellQuest software.

Colony survival assays and SCE analysis

Sensitivity of ES cells to increasing doses of mitomycin C was determined as described previously (Budzowska *et al*, 2004). Briefly, ES cells of the indicated genotypes were plated in 60 mm dishes, at various dilutions. After overnight incubation, cells were treated with mitomycin C for 1 h. Subsequently, the cells were washed twice with PBS and incubated with fresh medium for 6–8 days. Cells were fixed, stained and colonies were counted. All experiments were performed in triplicate. Similar protocols were followed to determine sensitivities to cisplatin and UV light. Analysis of SCEs was carried out as described (Dronkert *et al*, 2000).

Immunoprecipitation

Subconfluent cultures of ES cells were harvested after trypsinization and lysed in IP lysis buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5% Triton X-100, 150 mM NaCl, 10% glycerol, protease inhibitors Complete (Roche)) for 1 h on ice. The lysate was centrifugated for 1 h at 4°C at 46 000 r.p.m. using SW60 rotor (Beckman) and the supernatant was collected into a new tube. To get rid of DNA, 2 mM $MgCl_2$ and DNase I were added and the mixture was incubated at room temperature for 15 min. An anti-HA antibody attached to an agarose matrix (Roche) was added to the lysate followed by an overnight incubation at 4°C. The precipitate was washed four times with Wash buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 200 mM NaCl, 0.5 % Triton X-100).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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