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ORIGINAL ARTICLE

XRCC4 in G1 suppresses homologous recombination in S/G2, in G1 checkpoint-defective cells

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Non-homologous end joining (NHEJ) and homologous recombination (HR) are two pathways that can compete or cooperate for DNA double-strand break (DSB) repair. NHEJ was previously shown to act throughout the cell cycle whereas HR is restricted to late S/G2. Paradoxically, we show here that defect in XRCC4 (NHEJ) leads to over-stimulation of HR when cells were irradiated in G1, not in G2. However, XRCC4 defect did not modify the strict cell cycle regulation for HR (i.e. in S/G2) as attested by (i) the formation of Rad51 foci in late S/G2 whatever the XRCC4 status, and (ii) the fact that neither Rad51 foci nor HR (gene conversion plus single-strand annealing) events induced by ionizing radiation were detected when cells were maintained blocked in G1. Finally, both γ-H2AX analysis and pulse field gel electrophoresis showed that following irradiation in G1, some DSBs reached S/G2 in NHEJ-defective cells. Taken together, our results show that when cells are defective in G1/S arrest, DSB produced in G1 and left unrepaired by XRCC4 can be processed by HR but in late S/G2.
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Introduction

Faithful genome transmission requires the coordination of a network of pathways including cell cycle checkpoint, DNA replication, repair and recombination. DNA double-strand breaks (DSBs), which can be produced by ionizing radiation (IR) or replication inhibition (Jeggo *et al*., 1995; Rothstein *et al*., 2000; Saintigny *et al*., 2001), are highly toxic lesions which can lead to profound genome rearrangements. On the other hand, DSBs can also be advantageously used to generate genetic diversity in physiological processes such as meiosis or V(D)J recombination (Kleckner, 1996; Smith and Jackson, 1999; Cohen and Pollard, 2001; Jung and Alt, 2004).

Two general alternative strategies can compete to repair DSBs. The first one is homologous recombination (HR), which takes advantage of a partner sharing homologies with the damaged molecule. The second and alternative strategy is non-homologous end joining (NHEJ), which ligates the DNA ends without requiring sequence homologies between the two interacting molecules. Defects in HR or in NHEJ can lead to genome instability and tumorigenesis (Liu *et al*., 1998; Sonoda *et al*., 1998; Difilippantonio *et al*., 2000; Ferguson *et al*., 2000a; Bertrand *et al*., 2003). However, both functional HR and NHEJ can be responsible for genome rearrangements: (i) functional HR between repeated sequences dispersed through the genome can also lead to genome rearrangements (Purandare and Patel, 1997; Richardson and Jasin, 2000b; Bertrand *et al*., 2004), and (ii) functional Ku autoantigen protein (KU)-dependent as well as KU-independent NHEJ can generate genetic rearrangements (Guirouilh-Barbat *et al*., 2004). This shows the importance of a precise DSB repair regulation in mammalian cells for the equilibrium between genetic stability and diversity. When faced with DSBs, cells must coordinate the various alternative DNA repair systems, in connection with cellular pathways such as the cell cycle checkpoint and apoptosis.

The cell cycle may impact on the choice of DSB repair pathway. HR appears to be active from mid-S to G2 as attested by the fact that: (i) HR-defective vertebrate cells are more sensitive when irradiated in the late S/G2 phase (Cheong *et al*., 1994; Takata *et al*., 1998; Rothkamm *et al*., 2003; Hinz *et al*., 2005); (ii) Rad51 foci do not occur in G1 (Yuan *et al*., 2003; Aten *et al*., 2004); (iii) phosphorylation by cyclin-dependent kinase of BRCA2, an essential factor for Rad51 foci assembly, consistently disrupts BRCA2-Rad51 interaction only in G1 phase (Esashi *et al*., 2005); (iv) conservative HR has been proposed to occur preferentially in S phase (Saleh-Gohari and Helleday, 2004). However, one study challenged such interpretations as it found Rad51 foci in G1 (Kim *et al*., 2005). The cell cycle dependency of NHEJ is even more contested, and in many reviews it is

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proposed to act only in G1 phase. This is due to the fact that a large majority of NHEJ-defective cells are hypersensitive to IR when irradiated in G1 but not in G2 (Stamato *et al*., 1988; Jeggo, 1990; Lee *et al*., 1997; Takata *et al*., 1998; Wang *et al*., 2001). Recently, this interpretation found molecular support by data showing that KU80 preferentially binds to DSBs in the G1 phase (Rodrigue *et al*., 2006). However, this view is challenged by the fact that NHEJ has been proposed to act in S phase (Saintigny *et al*., 2001; Mills *et al*., 2004) and throughout the cell cycle (Rothkamm *et al*., 2003). Supporting the latter view, crosstalk between HR and NHEJ has been described, suggesting overlaps during the cell cycle. The two processes can compete or in contrast be coupled for DSB repair (Brouillette and Chartrand, 1987; Belmaaza *et al*., 1990; Belmaaza and Chartrand, 1994; Richardson and Jasin, 2000a; Pierce *et al*., 2001; Allen *et al*., 2002; Delacote *et al*., 2002). Consequently, the over-stimulation of HR in NHEJ-defective cells (Pierce *et al*., 2001; Allen *et al*., 2002; Delacote *et al*., 2002) could easily be explained by defects in NHEJ in G2. To test this hypothesis, we measured IR-induced HR following irradiation in G1/early S or late S/G2 of *XRCC4*$^-$ or complemented cells. Surprisingly, IR-induced HR over-stimulation occurred only when *XRCC4*$^-$ cells were irradiated in G1/early S. This result raises two hypotheses: (1) NHEJ defects abolish repression of HR in G1; (2) NHEJ does not affect HR cell cycle regulation; therefore, DSBs unrepaired in G1 might progress through S phase and be processed by HR in late S/G2. Here, we show that an XRCC4 defect does not modify cell cycle regulation of HR and the present data support the latter hypothesis. The importance of coupling DNA repair and cell cycle arrest to secure genome stability will be discussed.

Results

HR is stimulated in XRCC4-deficient cells irradiated in G1/early S but not in G2

Cells were synchronized in late G1/early S by double thymidine block, then irradiated at different times after the block release, that is either in G1/early S or in late S/G2 (Figure 1a and b). *XRCC4*$^-$ cells were more sensitive to IR when irradiated in G1/early S, but not significantly more sensitive when irradiated in the late S/G2 phase (Figure 1a), as described previously (Stamato *et al*., 1988). We show here that IR-induced HR was increased 9- to 13-fold in *XRCC4*$^-$ cells irradiated in G1 compared to control cells irradiated in G1 (Figure 1b). Surprisingly, irradiation in late S/G2 similarly stimulated HR in the *XRCC4*$^-$ and in *XRCC4*-complemented cells (Figure 1b). Importantly, both irradiated and unirradiated cells were synchronized, thus we specifically monitored HR induced by IR and not by any putative effect of the synchronization protocol. Nevertheless, we repeated this type of experiment using another synchronization protocol that does not involve replication perturbation. Cells were blocked at mitosis by nocodazole, then were irradiated at different times after block release, that is at different cell cycle phases, in progressing cells (Figure 1c). Using this new protocol, we confirmed that *XRCC4*$^-$ cells were more sensitive to IR than complemented cells, when irradiated in G1 but not in late S/G2 phase (Figure 1d). We also show that the increase in IR-induced HR observed in *XRCC4*$^-$ cells corresponds to cells irradiated in G1, but not in late S/G2 phase (Figure 1e). Noticeably, *XRCC4*$^-$ cells were sensitive to IR in mid-S phase and showed an intermediate level of HR over-stimulation, suggesting an involvement of NHEJ during S phase, as already proposed (Saintigny *et al*., 2001; Mills *et al*., 2004). These results were further confirmed with a third protocol, using mimosine to block cells at late G1/early S (see below, Figure 3).

Using three different synchronization protocols, which act on different metabolism pathways and synchronize in different cell cycle phases, our results consistently show that defects in NHEJ led to over-stimulation of IR-induced HR, but only when cells were irradiated in G1/early S phase. Two hypotheses can account for these results: (1) a defect in NHEJ in G1 leaves DSBs accessible for HR factors and consequently HR would become possible in G1 phase; (2) DSBs produced in G1 and left unrepaired by NHEJ can progress through S phase and be processed by HR in late S/G2 phase. With the substrate used here (see Figure 1b), both single-strand annealing (SSA) and gene conversion (GC), associated or not with crossing over, can lead to G418-resistant cells. We focused on GC because it is possible to follow Rad51 protein, the pivotal component of GC, after genotoxic stresses. Thus, we first measured the kinetics of Rad51 foci assembly, after IR in G1 vs in G2.

IR-induced Rad51 foci assembly occurs in late S/G2 independently of NHEJ status

After genotoxic stress, Rad51 assembles into nuclear foci at DNA damage sites, associated with γ-H2AX-irradiated chromosome domains, and these are thus thought to represent recombination/repair centers (Haaf *et al*., 1995; Raderschall *et al*., 1999; Tashiro *et al*., 2000; Aten *et al*., 2004). In addition, Rad51 foci assembly seems to represent a pre-requisite as until now no HR events have been recorded when Rad51 assembly is impaired. Finally, in CHO cells and using a similar substrate and strategy, it has been shown that most of IR-induced HR are RAD51-dependent and thus correspond to GC and not to SSA (Lambert and Lopez, 2000).

In wild-type cells, Rad51 foci do not assemble in G1 (Yuan *et al*., 2003; Aten *et al*., 2004). However, as IR-induced HR is stimulated in *XRCC4*$^-$ cells irradiated in G1 (not in G2), this raises the question whether the NHEJ defect modifies the cell cycle dependency program of HR, allowing Rad51 foci assembly in G1 phase. Here, we irradiated (6 Gy) cells synchronized by double thymidine block either in G1/ early S or in late S/G2 and analysed Rad51 foci at different times after irradiation (Figure 2a).

As with asynchronous cells (Delacote *et al*., 2002), the frequency of synchronized cells with Rad51 foci was

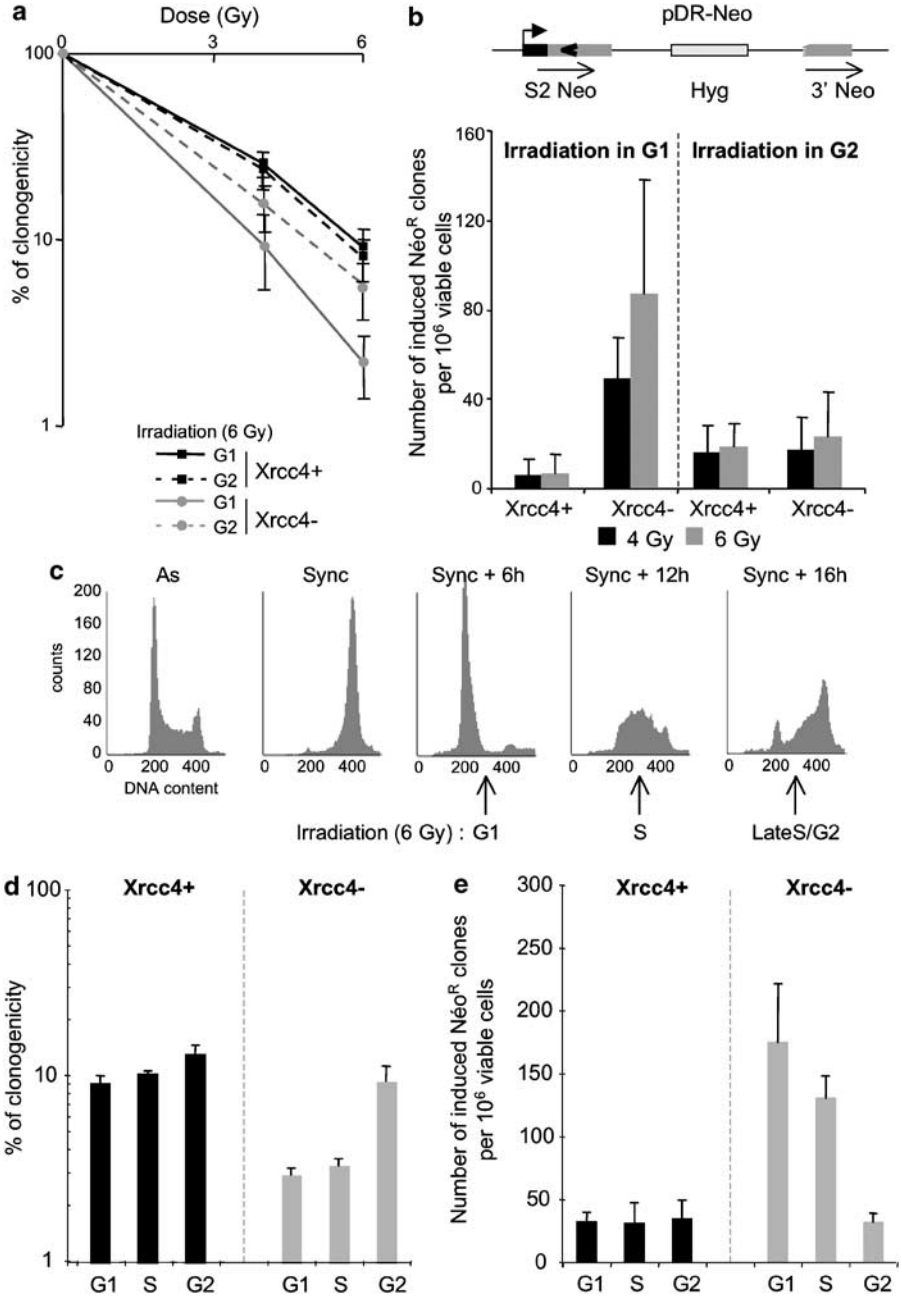
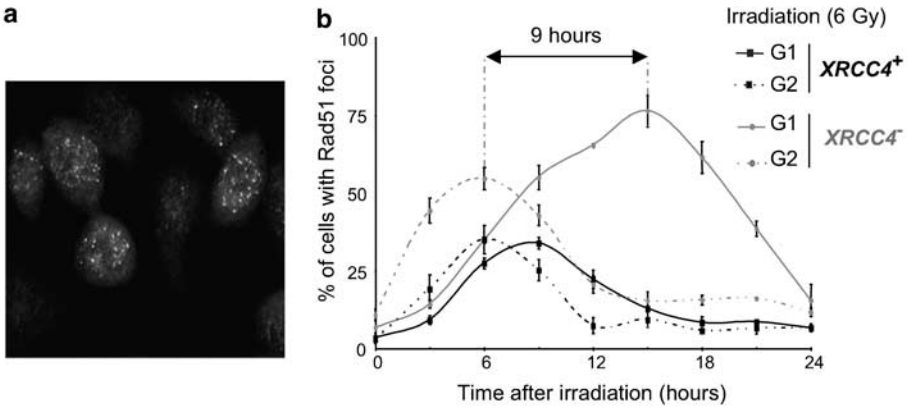


Figure 1 Toxicity and HR induced by irradiation in G1, S or G2. (**a**, **b**) Double thymidine block synchronization before 4 or 6 Gy irradiation. (**a**) Survival. (**b**) HR induced by irradiation in G1/early S or in late S/G2. On the top of the figure is presented the intrachromosomal HR substrate used: two inactive copy of the neomycin-resistant gene (S2neo and 3′neo) are separated by an hygromycin-resistant gene. Parental cells are sensitive to G418, HR restores one functional neomycin-resistant gene and the recombinant colonies are thus G418-resistant. (**c–e**) Nocodazole synchronization before 6 Gy irradiation. (**c**) Cell cycle distribution after nocodazole release. As: asynchronous cell population. (**d**) Survival. (**e**) HR induced by irradiation in G1/early S or in late S/G2. The phases of irradiation correspond to the cell cycle distribution indicated in (**c**). For each experiment, values and bars correspond to the mean of three independent experiments and s.d.

higher in *XRCC4*$^-$ cells than in the complemented cell population. Interestingly, the differences between *XRCC4*$^-$ and *XRCC4*$^+$ cells were much more pronounced when cells were irradiated in G1/early S (Figure 2b). As SSA is independent of RAD51 and that IR-induced HR is mainly RAD51-dependent (Lambert and Lopez, 2000), the present data also suggest that all DSBs not repaired by NHEJ (*XRCC4*-defective cells) are not repaired by SSA and that some of them should be processed by GC.

We then compared the occurrence of Rad51 foci and the cell cycle distribution, after IR in G1/early S or in late S/G2. A direct correlation, with a good coefficient, was found between the number of cells with foci and the



c

Time after irradiation		0h		6h		12h		18h		24h		Correlation coefficient Rad51 foci and G2
Line	Irradiation phase	% foci	% G2	% foci	% G2	% foci	% G2	% foci	% G2	% foci	% G2	
XRCC4+	G1	3,3	3,1	27,3	45,3	22,2	62,3	8,3	27,8	6,6	20,1	0,73
XRCC4+	G2	2,8	87,2	35,1	73,2	7,4	35,1	5,6	19,5	6,6	17,6	0,74
XRCC4-	G1	6,6	2,4	34,6	22,4	65,3	72,8	61,5	66,3	15,5	48,7	0,78
XRCC4+	G2	10,8	83,2	54,7	74,6	20,6	61,5	15,5	46,5	11,6	39,5	0,76

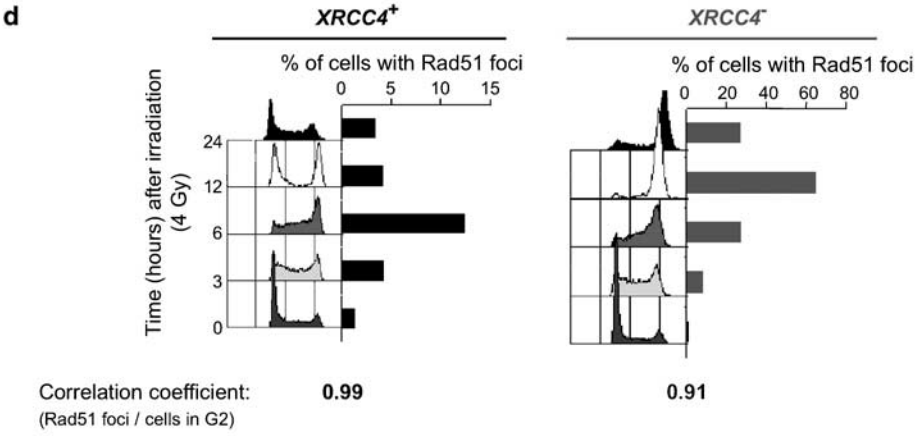


Figure 2 Rad51 foci assembly induced by irradiation of a synchronized or asynchronous cell population. (**a**) Example of radiation-induced Rad51 foci. (**b**, **c**) Synchronization by double thymidine block before 6 Gy irradiation. (**b**) Rad51 foci kinetics of *XRCC4*$^-$ (gray circle) or *XRCC4*$^+$ (black square) cells irradiated in G1/early S or in late S/G2. (**c**) Correlation between the frequency of cells with Rad51 foci and cells in late S/G2/M after irradiation in G1/early S (G1) or in late S/G2 (G2). (**d**) Cell cycle distribution and corresponding percentages of Rad51 foci-positive cells at different times after 4 Gy irradiation of asynchronous *XRCC4*$^+$ (left panel) and *XRCC4*$^-$ (right panel) cells. Correlation coefficient between the frequency of cells with Rad51 foci and cells in late S/G2/M, after irradiation (4 Gy) of asynchronous cells.

number of late S/G2/M cells, whatever the *XRCC4* status and the cell cycle phase of irradiation (Figure 2c). Importantly, we also showed that in the absence of any synchronization treatment, Rad51 foci were correlated with late S/G2 phase in both *XRCC4*$^-$ and *XRCC4*$^+$ cell lines (Figure 2d).

Altogether, our results demonstrate that the cell cycle regulation of Rad51 foci assembly (i.e. in S/G2) is not affected by an NHEJ defect, suggesting that DSBs left unrepaired by NHEJ in G1 progress through S phase, that at least some of them are not processed by SSA and can be processed by GC in late S/G2. Consistently, the peak of cells with Rad51 foci was delayed by 9 h in *XRCC4*$^-$ cells irradiated in G1 compared to irradiation in G2, a timing compatible with the S phase duration in this cell line (Figure 2b). This hypothesis implies that irradiated cells are not arrested at the G1/S transition, which is the case in our *p53*$^-$ hamster cell lines. To verify the latter hypothesis, we measured the impact of a prolonged G1 arrest on IR-induced HR, after irradiation in G1.

Prolonged G1 arrest abrogates IR-induced HR

After synchronization in late G1/early S phase by mimosine, cells were irradiated and thereafter either the block was immediately released to allow cells to proliferate, or the block was maintained for 24 additional hours before release (Figure 3a). This additional

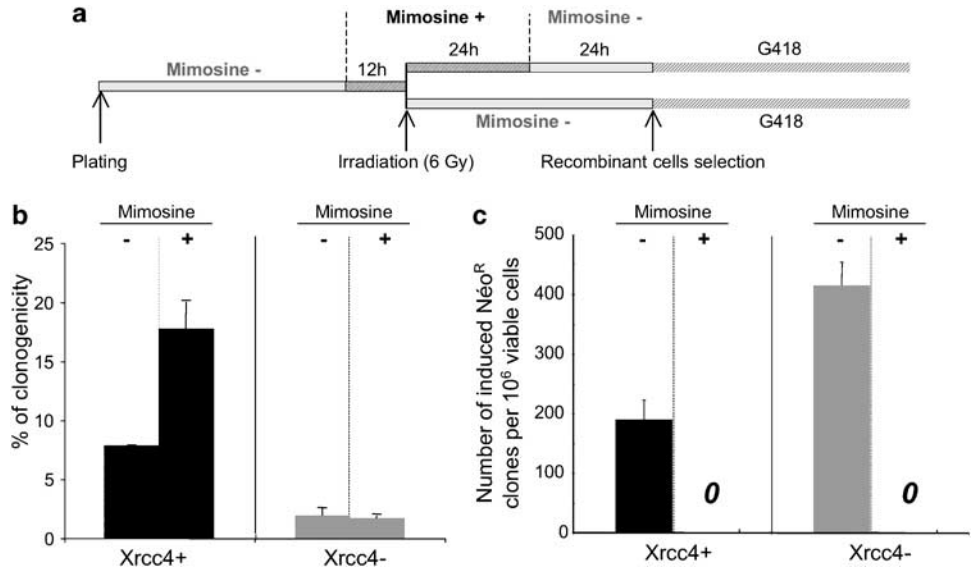


Figure 3 Impact of prolonged arrest in G1 on HR induced by IR. (**a**) Experimental design. (**b**) Survival of cells irradiated (6 Gy) in G1 and maintained arrested in G1 (mimosine+) or not (mimosine−). (**c**) Recombination induced by G1 irradiation (6 Gy) of cells maintained blocked in G1/early S (mimosine+) or not (mimosine−). '0' means that no recombinant was scored in this condition. For each experiment, values and bars correspond to the mean of three independent experiments and s.d., respectively.

arrest time was supposed to mimic G1/S arrest allowing repair of IR-induced damage before resuming proliferation. An additional block of 24 h had no effect on the survival of *XRCC4*$^-$ cells, whereas it increased twofold the resistance of complemented cells (Figure 3b). This shows that the block is not toxic and behaves as a sort of 'pharmacological' G1 arrest, actually providing extra time for repair and viability rescue. IR-induced HR events were recorded when cells were not maintained blocked in the G1 phase. *XRCC4*$^-$ cells showed higher levels of IR-induced HR compared to complemented cells (Figure 3c), as already shown using different synchronization methods (see Figure 1). When cells were blocked for 24 additional hours, no IR-induced HR event was detectable, even with *XRCC4*$^-$ cells (Figure 3c).

These results show that IR-induced HR is highly inefficient when cells are maintained blocked (even transiently) in late G1/early S phase. Importantly, as no G418 colonies were found, this shows that both SSA and GC were inhibited by the treatment. More particularly, these data show that SSA is inefficient in cells maintained in G1/early S phase. Thus, the absence of delay at the G1/S transition is essential to stimulate IR-induced HR in the *XRCC4*$^-$ cells (irradiated in G1). As GC corresponds to the main IR-induced HR pathway (Lambert and Lopez, 2000), we measured the effects on Rad51 foci formation of blocking cells in G1/early S phase.

Prolonged G1 arrest abrogates IR-induced Rad51 foci assembly

Cells were treated by mimosine for 12 h to synchronize them in G1/early S, and then irradiated (6 Gy), and mimosine arrest was maintained or not (Figure 4a). Proliferating (-mimosine) or G1/early S-blocked (+mimosine) cells were harvested at different times after irradiation to analyse (i) cell cycle distribution (Figure 4b), (ii) Rad51 foci formation (Figure 4c) and (iii) Rad51 protein level (Figure 4d).

XRCC4$^-$ (Figure 4b) as well as complemented cells (data not shown) were permanently blocked by mimosine treatment in G1/early S. In proliferating cells (-mimosine), Rad51 foci occurred several hours after IR and block release from mid-S to G2 phase (compare Figure 4b and c). *XRCC4*$^-$ cells showed a higher frequency of cells with Rad51 foci compared to complemented cells (Figure 4c), as already shown with double thymidine block (see Figure 2a). When cells were maintained blocked at the late G1/early S phase, no Rad51 foci were recorded whether or not cells were defective for *XRCC4* (Figure 4c). This defect in Rad51 foci was not due to absence of Rad51 protein at G1 phase as Western blot analysis showed that the steady-state level of Rad51 protein did not vary significantly at different times after G1 irradiation in *XRCC4*$^-$ or in *XRCC4*$^+$ cells (Figure 4d).

The present data show that a defect in NHEJ does not allow Rad51 to assemble into foci in G1 phase even though Rad51 protein is present in substantial amounts. Moreover, our data suggest that the absence of G1/S arrest is essential for IR-induced HR and Rad51 foci stimulation, in *XRCC4*-defective cells.

In conclusion, the stimulation of IR-induced HR in *XRCC4*$^-$ cells corresponds to cells irradiated in G1/early S but occurs in late S/G2 phase. This conclusion is consistent with the delay of Rad51 foci formation in *XRCC4*$^-$ cells irradiated in G1 compared to a G2 irradiation, by a time compatible with S phase duration (see Figure 2b). Thus, we assume that unrepaired DSBs produced in the G1 phase could progress through

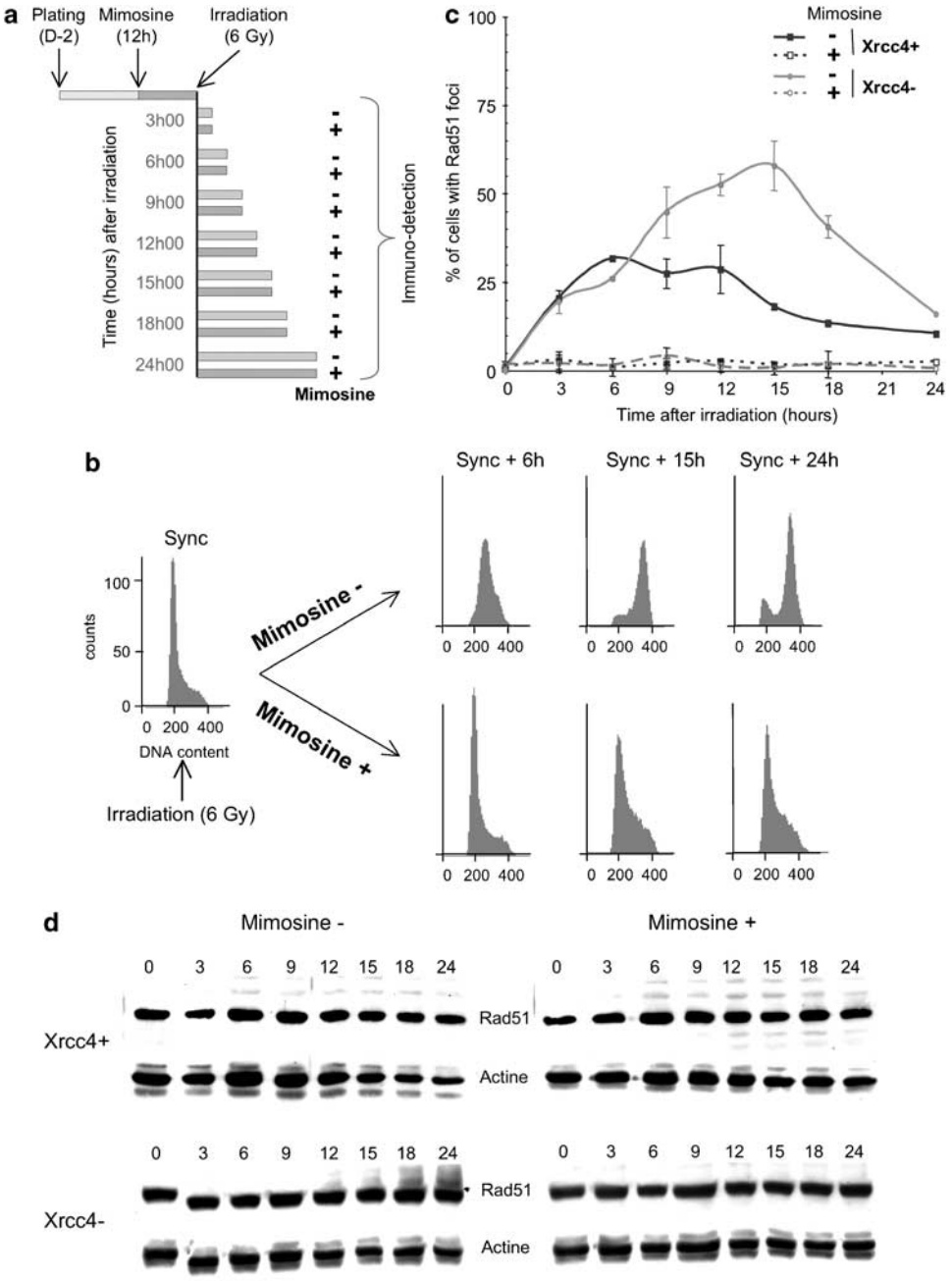


Figure 4 Kinetics of Rad51 foci assembly after irradiation (6 Gy) of cells maintained blocked or not in G1. (**a**) Experimental design. (**b**) Cell cycle distribution of *XRCC4*$^-$ cells irradiated in G1 and maintained arrested in G1 (mimosine +) or not (mimosine −). (**c**) Rad51 foci assembly at different times after irradiation in *XRCC4*$^+$ and in *XRCC4*$^-$ cells maintained or not blocked in G1. Values and bars correspond to the mean of three independent experiments and to s.d., respectively. (**d**) Western blot quantification of Rad51 protein at different times after irradiation in cells maintained blocked in G1 or not.

S phase and be processed by HR in the late S/G2. To confirm this hypothesis, we determined whether DSBs could be detected during S phase in *XRCC4*$^-$ cells irradiated in G1/early S.

DSBs not repaired in the G1 phase persist in late S phase
To measure the persistence of DSBs in S phase, (1) cells were synchronized at the late G1/early S phase by mimosine; (2) irradiated and the block was immediately released; (3) at different times after IR and block release, cells were analysed by fluorescence-activated cell sorter (FACS) to measure cell cycle distribution (see Figure 4b) and DSBs by the analysis of γ-H2AX foci formation (Figure 5a).

After irradiation, histone H2AX is phosphorylated and assembles into nuclear foci at the DSB sites (Redon *et al*., 2002; Aten *et al*., 2004). Unirradiated cells in S phase exhibited fluorescence. Brighter foci were generated by irradiation (Figure 5a, left panel); we monitored such structures as an estimation of DSBs.

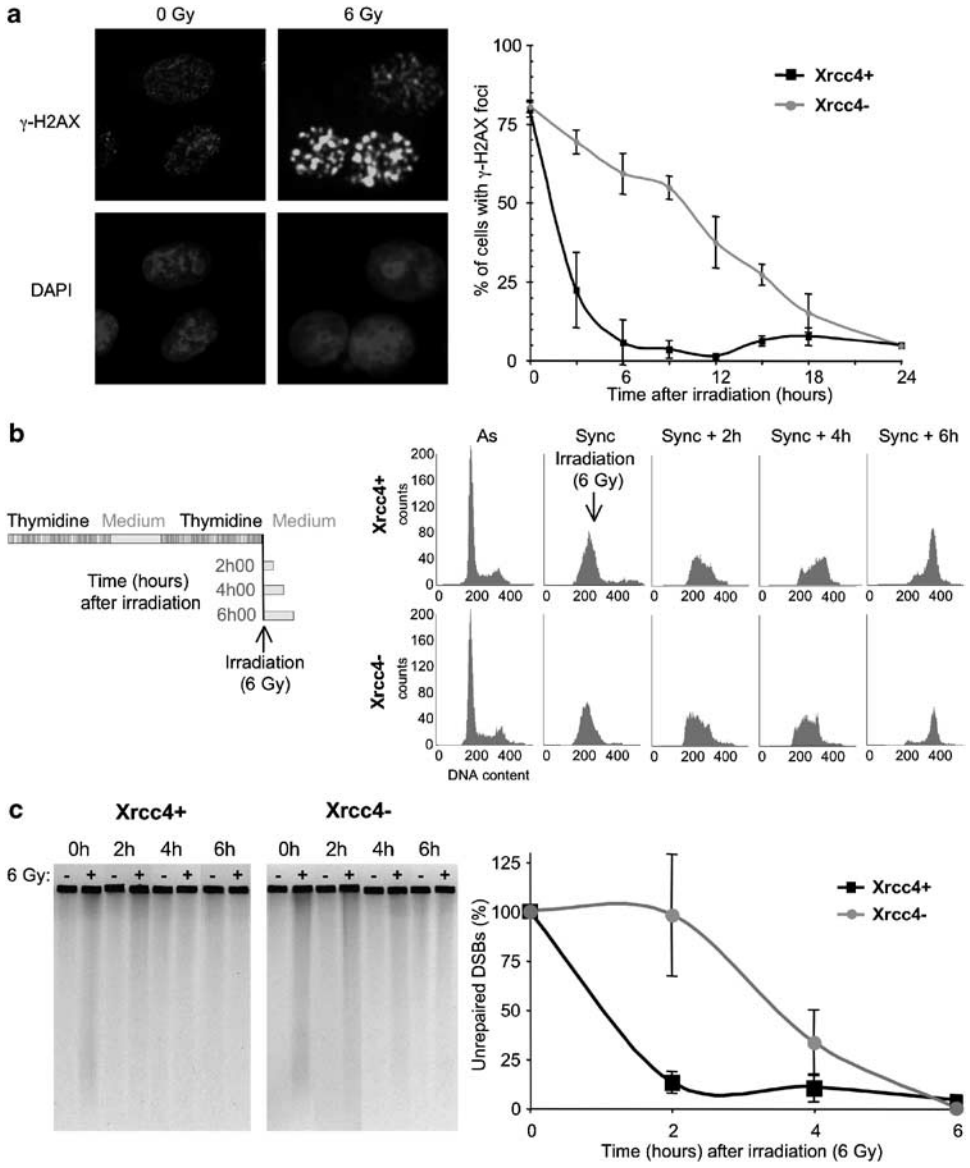


Figure 5 Impact of XRCC4 status on disappearance of G1-induced DSBs, (**a**) Left panels: example of IR-induced γ-H2AX foci in S phase; upper panels: labeling with anti-γ-H2AX antibody; lower panels: DNA labeling with 4,6-diamidino-2-phenylindole. The doses are indicated in the figure. Right panel: Kinetics of IR-induced DSB disappearance after irradiation in G1 of XRCC4-proficient (black line) or deficient (gray line) cells. Synchronization by mimosine (the corresponding cell cycle distribution are shown in Figure 4b). (**b**) Experimental design after double thymidine block (left panel). Right panel: Cell cycle distribution after 6 Gy irradiation and block release. (**c**) Kinetics of DSB disappearance measured by PFGE after irradiation (6 Gy) in G1/early S of *XRCC4*$^-$ or *XRCC4*$^+$ cells. Right panel: example of PFGE. Left panel: Quantification performed using five independent experiments.

In complemented cells, the frequency of cells with foci dropped from 80 to 20% and almost to the background 3 and 6 h after block release and irradiation, respectively (Figure 5a, right panel). In *XRCC4*-defective cells, 62% of cells presented foci 6 h after block release, and 37% of cells still exhibit foci 12 h after block release and irradiation. As 3, 6, and 12 h after block release and irradiation, cells are in early S, late S and G2 phases, respectively (compare with Figure 4b), these data show the persistence of IR-induced DSBs in S phase in *XRCC4*-defective cells irradiated in G1.

We then confirmed these conclusions by using another synchronization method and a more direct method for DSB measurement: pulse field gel electrophoresis (PFGE): (1) cells were synchronized by double thymidine block at the late G1/early S phase; (2) irradiated and the block was immediately released; (3) at different times after IR and block release, cells were analysed by FACS and PFGE to measure cell cycle distribution and DSBs, respectively (Figure 5b and c). We used a PFGE protocol allowing to monitor DSBs generated with as little as 6 Gy irradiation (Boucher *et al*., 2004), corresponding to the physiological doses used in the above experiments.

Two hours after irradiation and block release, most cells were in early S phase, and although almost all

DSBs had disappeared in complemented cells, 95% of IR-induced DSBs were still present in the *XRCC4*$^-$ cells (Figure 5c). Four hours after irradiation and block release, most cells were in late S phase and 35–40% of IR-induced DSBs were still present in *XRCC4*$^-$ cells (Figure 5c). These data are highly consistent with all the above results and conclusions.

Discussion

NHEJ and HR indirectly compete through S phase for DSB repair, in the absence of G1 arrest

Defects in NHEJ lead to a over-stimulation of HR specifically induced by DSBs (Pierce *et al*., 2001; Allen *et al*., 2002; Delacote *et al*., 2002). As HR does not act in G1, and NHEJ has been proposed to act throughout the cell cycle (Saintigny *et al*., 2001; Rothkamm *et al*., 2003; Saleh-Gohari and Helleday, 2004; Esashi *et al*., 2005), the simplest explanation was that the HR over-stimulation reflected the defect in NHEJ in S and G2 phases. Surprisingly, we show here that, (1) an NHEJ defect in G1, but not in G2, stimulated IR-induced HR; (2) the cell cycle restriction for HR, that is in S/G2 phase, was not affected by the NHEJ defect; (3) the absence of delay at the G1/S transition was essential for the over-stimulation of IR-induced HR in *XRCC4*$^-$ cells; this allowed the presence in S/G2 of unrepaired DSB generated in G1.

Prolonged arrest at the G1/S transition abolished the over-stimulation of IR-induced HR in *XRCC4*$^-$ cells (both GC and SSA). This underlines the importance of coupling the regulation of the alternative DNA repair pathways and the control of the G1/S transition. Consistently, all the cell lines used in the different laboratories, showing over-stimulation of HR in NHEJ-defective backgrounds (Pierce *et al*., 2001; Allen *et al*., 2002; Delacote *et al*., 2002), were defective in G1 arrest. They were either *p53*$^-$ hamster cells or mouse embryonic stem cells, in which p53 is cytoplasmic and unable to control the G1 checkpoint (Aladjem *et al*., 1998). The absence of G1 arrest thus permits the progression through S phase of damage generated in G1, and its processing in S/G2. This raises the question of the fate of DNA ends generated in G1, during S phase (Figure 6a). As KU86/KU70 can efficiently bind to DSBs in G1 (Rodrigue *et al*., 2006) it should be able to protect the ends. In addition, various other proteins such as γ-H2AX, 53BP1 and RAD50/MRE11/NBS1 are good candidates for protection of DNA ends, allowing DSBs to progress through S phase. Single-strand tails (the first step of SSA or GC) can be generated in early S, and replication protein A, could coat the single-stranded DNA, protecting it and preparing it for SSA or HR at the late S phase (Figure 6a). Such a sequence has been demonstrated in budding yeast, under the control of the cyclin-dependent kinase CDK1 (Ira *et al*., 2004). In mammalian cells, resection by MRE11 in S and G2 phase under the control of the kinase ATM (ataxia telangiectasia mutated) and requiring CDK kinase activity has recently been described (Jazayeri *et al*., 2006). However, in mammalian cells, progression of unrepaired damage in S phase should be deleterious. Indeed, the fact that *XRCC4*$^-$ cells are not sensitive in G2 could mean either that NHEJ is marginal or that HR can fully compensate for NHEJ defects during this phase. The latter hypothesis is supported by the fact that, when irradiated in G2, cells with Rad51-foci were twofold more frequent with *XRCC4*$^-$ cells than with complemented cells (see Figure 2). In contrast to G2, when irradiated in G1, even though HR could process in S/G2 some damages left unrepaired in G1, *XRCC4*$^-$ cells are highly sensitive, (i) confirming the paramount importance of NHEJ in G1 for cell survival, (ii) showing that HR (GC and SSA) in S/G2 is unable to fully compensate for NHEJ defects in G1, therefore suggesting that the progress through S phase of damaged DNA should be toxic.

Impact on genome stability maintenance

The 'trans-S DSB repair' process described here should be blocked by either an efficient NHEJ, as shown here, and/or by an efficient G1 arrest. Alteration of both p53 and the KU/DNA-PKcs/XRCC4/Lig4 NHEJ pathway strongly increased genome rearrangements in very complex products (complicons) and tumorigenicity (Ferguson *et al*., 2000b; Zhu *et al*., 2002). KU alternative NHEJ pathway(s), which are error-prone and can participate in genome rearrangements (Guirouilh-Barbat *et al*., 2004), should presumably participate. However, HR should also be involved. First, SSA can generate translocation when two DSBs are generated (Richardson and Jasin, 2000b). Second, GC is generally an error-free repair pathway, but excess or uncontrolled GC can lead to genome instability: GC with one pseudogene can inactivate the functional gene (Amor *et al*., 1988); GC between two heteroalleles can result in loss of heterozygosity and crossing-over between repeat sequences dispersed through the genome can lead to complex rearrangements such as deletions, amplification, translocation (Bertrand *et al*., 2004). Third, mice deficient in both NHEJ and p53, develop lymphomas, resulting from rearrangements initiated by V(D)J recombination-activating protein (RAG)-induced DNA cleavage and involving a break-induced replication (BIR) pathway (Vanasse *et al*., 1999; Difilippantonio *et al*., 2002; Zhu *et al*., 2002). BIR is a DSB repair process requiring sequence homology and in part Rad51-dependent in yeast (Kraus *et al*., 2001; Malkova *et al*., 2005).

Using similar cells and substrates than here, it has been shown that IR-induced HR is mainly a Rad51-dependent event (Lambert and Lopez, 2000). In addition, DSB repair via HR has been shown to occur mainly via GC between sister chromatids (Johnson and Jasin, 2000). These data strongly suggest an important role of GC in IR-induced HR after replication of the sister chromatids, in agreement with the Rad51 foci data presented here. In G1, HR would be forced to use the homologous chromosome or either intrachromatid or ectopic repeated sequences dispersed through the genome, with the risk of generating genetic

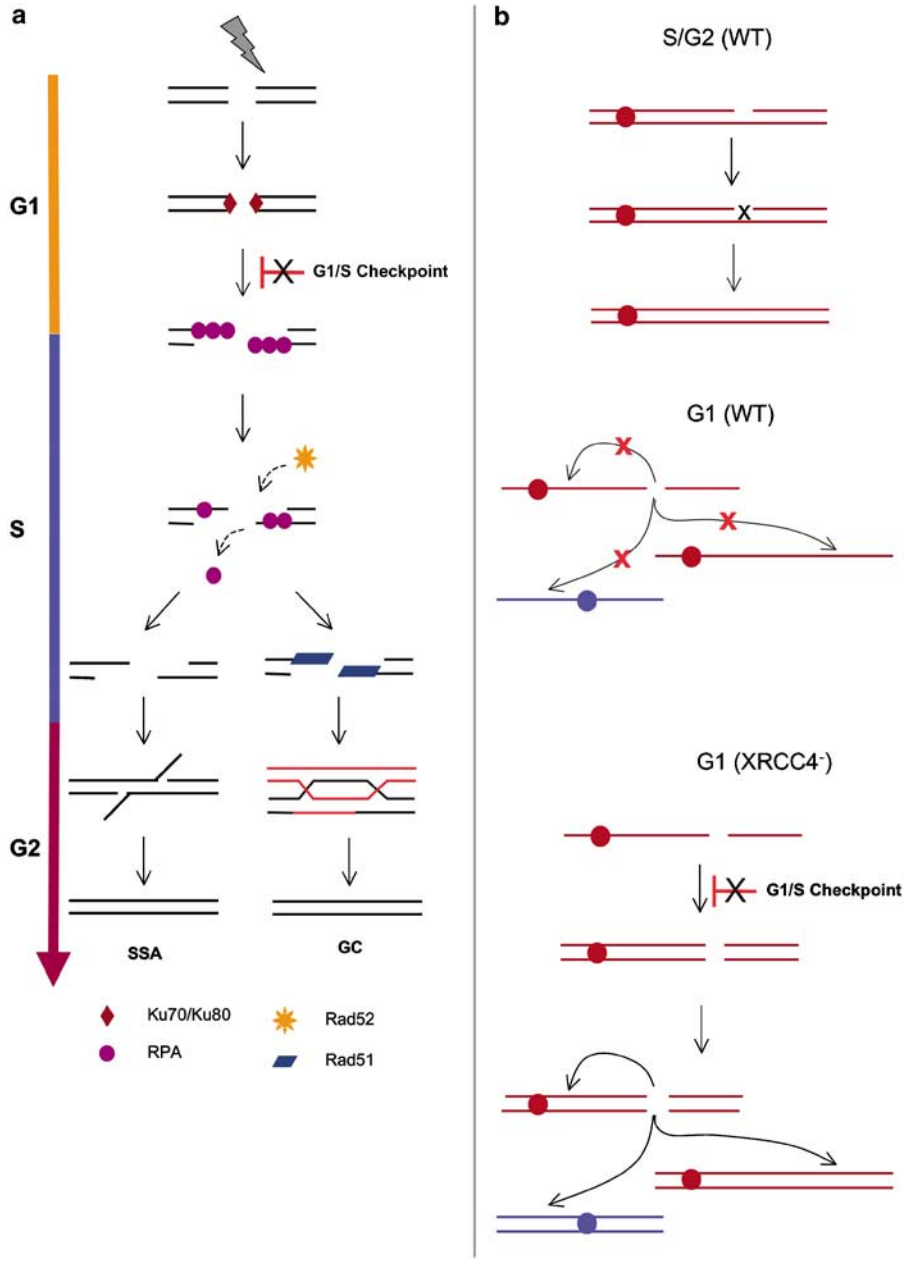


Figure 6 Unrepaired DSBs produced in the G1 phase. (**a**) Potential successive processing steps of unrepaired DSBs in G1: KU80/KU70 can efficiently bind to the DNA in the G1 phase (Rodrigue *et al*., 2006) and can protect the DNA ends against extensive degradation. If the G1 checkpoint is inefficient, cells with unrepaired DSBs can progress through cell cycle. Then, the sequence of events could be similar to that described in budding yeast (Ira *et al*., 2004): (1) in early S phase, resection of the DNA ends generates single-strand tails which are early intermediates of both SSA and GC; (2) replication protein A then coats and protects the ssDNA. (3) In late S/G2, Rad52 might promote SSA or load Rad51 for GC. (**b**) Consequences on genetic stability. At a post-replication stage (S/G2), DSB repair can efficiently use sister chromatid GC (Johnson and Jasin, 2000). In the G1 phase, the sister chromatids are not synthesized and HR would be force to use the homologous chromosome or repeated intrachromatid or ectopic sequences dispersed through the genome, with the risk of genetic rearrangements. However, HR is blocked at such cell cycle phase. If the G1 checkpoint is inefficient or if the DSBs are produced after the checkpoint, cells with unrepaired DSB can progress through S phase resulting in a DSB on each chromatid at the same locus. Cytogenetic analysis frequently described such situation, at a chromosome level. Consequently, the DSBs cannot be repaired by sister chromatid GC but would be forced to use homologous chromosome or intrachromatid or ectopic repeated sequences dispersed through the genome and can thus generate genetic instability.

rearrangements (Figure 6b). Therefore, restricting HR at a post-replication phase more efficiently secures genome stability. Indeed, at a post-replication stage, HR can efficiently use the sister chromatid for DSB repair (Fabre *et al*., 1984; Kadyk and Hartwell, 1992; Johnson and Jasin, 2000). HR between the two sister chromatids, which are identical, should not promote genetic rearrangements. In the present data, if unrepaired DSBs can progress through S phase, DSBs should be present on both chromatids at the same locus

after replication (Figure 6b). Cytogenetic analysis frequently described such situation, at a chromosome level. In such a situation, sister chromatid GC becomes unable to repair the DSBs and the process would be channeled to ectopic or intrachromatid (SSA or GC) or unequal sister chromatid events (Figure 6b), all processes that increase the risk of genetic rearrangements. Fortunately, the substrate used here monitored the two latter classes of events and therefore allowed us to detect the over-stimulation of HR in *XRCC4*-defective cells irradiated in G1.

If unrepaired DSBs can progress through S phase, one would expect an increase in the frequency of DSBs in G2, in *XRCC4*$^-$ cells irradiated in G1. The fact that both γ-H2AX and PFGE analysis showed a decrease in DSB frequency in such situation (see Figure 5) means that *XRCC4* alternative pathways can process DSBs in S phase. Such pathways could be NHEJ pathways alternative to *XRCC4* (Wang *et al*., 2003, 2005; Audebert *et al*., 2004) and/or HR, which is highly efficient in S phase. The latter hypothesis is supported by the observation that the frequency of Rad51 foci-positive cells was higher with *XRCC4*$^-$ cells irradiated in G1 compared to G2, whereas this was not the case with complemented cells (see Figure 2b). Finally, p53 protein controls the G1 checkpoint but also directly prevents excess of GC, thus protecting against genome rearrangements, in two complementary ways (for review, Bertrand *et al*., 2004). Consequently, in double NHEJ- and p53-defective cells, HR might participate in the generation of complicons.

DSBs are very harmful lesions, which lead to cell death and/or can generate genome rearrangements. The present data underline the fine and acute regulation between the various alternative repair pathways in association with cell cycle control, in maintaining genome integrity and so, in protecting against neoplastic development.

Materials and methods

DNA manipulations
All DNA manipulations were performed as described (Sambrook *et al*., 1989; Ausubel *et al*., 1999).

Cell culture and synchronization protocols
Cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium without sodium pyruvate, supplemented with 10% fetal calf serum, 2 mM glutamine, 200 IU/ml penicillin and 100 μg/ml streptomycin. The *XRCC4*$^-$ cell lines correspond to the XR-1 bearing a direct repeat recombination substrate and *XRCC4*$^+$ correspond to the *XRCC4*$^-$ complemented with V5-tagged Hs*XRCC4* cDNA cloned in pcDNA6 (Invitrogen, Cergy Pontoise, IDF, France) (Liang *et al*., 1998; Delacote *et al*., 2002).

Cells were grown for 48 h in culture medium before each synchronization. Double thymidine block synchronization: cells were treated with 2 mM thymidine for 20 h, washed three times in phosphate-buffered saline (PBS) then cultured in medium for 10 h, and treated with 2 mM thymidine for 20 additional hours. Nocodazole synchronization: cells were treated with 40 nM nocodazole for 12 h. Mimosine synchronization: cells were treated with 200 μM mimosine for 12 h. In all synchronization experiments, the cell cycle was checked by flow cytometry.

Cell cycle analysis
Trypsinized cells were collected by centrifugation (5 min at 2000 g), resuspended in 500 μl PBS and fixed by adding 1.5 ml of cold ethanol. Cells were then treated with propidium iodide (25 μg/ml) and RNAse (50 μg/ml) and analysed by flow cytometry (Becton Dickinson France SAS, Le Pont-De-Claix, RA, France).

Measurement of recombination
Cells were irradiated in PBS, using a ^{137}Cs source (1.8 Gy/min). After irradiation and indicated treatment, cells were incubated in their medium at 37°C for 24 h. Cells were then trypsinized, counted and divided into two fractions. The first fraction was used to calculate the viability by cloning efficiency. The second fraction was plated under 1 mg/ml G418 selection to measure Neo^R recombinant clones. IR-induced HR corresponds to the number of Neo^R clones in 10^6 surviving cells after synchronization and irradiation subtracted from the number of Neo^R clones of 10^6 synchronized but not irradiated cells.

Western blot analysis
All extract preparation steps were performed at 4°C. After PBS wash, cells were suspended in lysis buffer (25 mM Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 600 mM NaCl, 1 mM dithiothreitol, 0.1% NP40, 5 μg/ml leupeptin, 2 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol) and incubated for 40 min on ice. Extracts were centrifuged for 30 min at 15 000 *g*, supernatant was retrieved and protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad, Marnes-la-Coquette, IDF, France). Boiled protein extract (40 μg) was loaded on a 10% polyacrylamide-sodium dodecyl sulfate gel for electrophoresis. After migration, proteins were electrotransferred onto a nitrocellulose membrane and probed with specific antibodies: anti-Rad51 (Oncogene Research, Fontenay sous Bois, IDF, France) and anti-actin (Sigma, Saint Quentin Fallavier, RA, France) antibodies. Antibodies were visualized using the enhanced chemiluminescence detection kit (Amersham, Orsay, IDF, France).

γ-H2AX and Rad51 foci analysis
Cells cultured on glass coverslips for 48 h were treated as described (synchronization, irradiation), then fixed in methanol (−20°C) for 20 min and acetone for 10 s. The rabbit polyclonal antibody against phosphorylated H2AX was obtained from Upstate Cell Signaling Solutions (Montigny Le Bretonneux, IDF, France). Following staining with the secondary antibody (Cy™2, Jackson ImmunoResearch Laboratories, Newmarket, Suffolk, England), the cells were analysed by epifluorescence microscopy. The Rad51 foci were analysed as described (Haaf *et al*., 1995) using anti-Rad51 antibody (Pharmingen, Le Pont-De-Claix, RA, France).

Pulsed-field gel electrophoresis
G1-synchronized (double thymidine block) cells were washed once with ice-cold PBS before 6 Gy irradiation (^{137}Cs source, 3.5 Gy/min, 4°C). Immediately after irradiation, cells were incubated at 37°C, 5% CO_2 with pre-warmed culture medium for the indicated additional time. Collected cells were kept at 4°C, washed, trypsinized and resuspended at 2×10^6 cells/ml. PFGE plugs of 8.5×10^4 cells were prepared by adding equal volumes of cell suspension to low melting point agarose (1.6% of PBS, Invitrogen) and incubated for 1 h at 4°C. Remaining suspension was used for flow cytometry analysis.

Plugs were placed in 1.5 ml of lysis solution (0.5 M EDTA at pH 7.6, 2% sarkosyl, 1% proteinase K and 2 mM deferoxamine) and incubated at 50°C for 20 h. After two washes (TE + 0.1 mM deferoxamine) and incubation for 1 h at room temperature (TE + 0.12 g/l Pefabloc), plugs were stored at 4°C in 0.05 M EDTA + 0.1 mM deferoxamine. PFGE was performed with a neutral 0.8% agarose gel in tris-acetic acid-EDTA (TAE) buffer using clamped homogenous electric fields (CHEF)-Mapper (Bio-Rad), for 74 h running time at 2 V/cm with a reorientation angle ± 53° (106°) and a 35-min pulse switch time. The molecular weight used was chromosomal DNA of *Schizosaccharomyces pombe* and *Hansenula wingei* (Bio-Rad). This protocol allows monitoring of DSBs generated with as little as 6 Gy irradiation (Boucher *et al*., 2004).

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