Interplay between human DNA repair proteins at a unique double-strand break *in vivo*

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DNA repair by homologous recombination is essential for preserving genomic integrity. The RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) play important roles in this process. In this study, we show that human RAD51 interacts with RAD51C-XRCC3 or RAD51B-C-D-XRCC2. In addition to being critical for RAD51 focus formation, RAD51C localizes to DNA damage sites. Inhibition of RAD51C results in a decrease in cellular proliferation consistent with a role in repairing double-strand breaks (DSBs) that occur naturally. To monitor a single DNA repair event, we developed immunofluorescence and chromatin immunoprecipitation (ChIP) methods on human cells where a unique DSB can be created *in vivo*. Using this system, we observed a single focus of RAD51C, RAD51 and 53BP1, which colocalized with γ -H2AX. ChIPs revealed that endogenous human RAD51, RAD51C, RAD51D, XRCC2, XRCC3 and MRE11 proteins are recruited in the S–G2 phase of the cell cycle, while Ku80 is recruited during G1. We propose that RAD51C ensures a tight regulation of RAD51 assembly during DSB repair and plays a direct role in repairing DSBs *in vivo*.

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Introduction

Maintenance of genome stability relies on the accurate repair of double-strand breaks (DSBs) that arise during DNA replication or from DNA-damaging agents. Failure to repair such breaks can lead to the introduction of mutations, chromosomal translocations, apoptosis and cancer. Hence, in order to

preserve genome integrity, cells have evolved processes to respond and repair DSBs. In higher eukaryotes, the signalling response to DSBs is centered on mammalian ATM (ataxia-telangiectasia mutated), ATR (ATM and Rad3 related) and DNA-PK (DNA-dependent protein kinase). These PI3KKs (phosphatidylinositol-3 kinase-like kinases) trigger cell cycle arrest following DNA damage, therefore allowing DNA repair to take place (Kurz and Lees-Miller, 2004). Moreover, following the induction of DSBs by ionizing radiation, ATM and DNA-PKs rapidly phosphorylate the carboxy-terminal SQE motif of H2AX (to form γ -H2AX foci) along flanking megabase chromatin regions (Rogakou *et al*, 1999; Stiff *et al*, 2004), a process that might contribute to both detection and repair of DSBs. In addition, ATR and DNA-PKs phosphorylate H2AX downstream of replication-associated DSBs (Ward and Chen, 2001).

In mammalian cells, homologous recombination (HR) has emerged as the major mechanism for the error-free homology-directed repair of DSBs. The central activity of HR is conferred by the RAD51 protein, a eukaryotic homolog of the *Escherichia coli* RecA recombinase, which catalyses the invasion of the broken ends of the DSB into the intact sister chromatid. Recently, extensive studies have been dedicated to the identification of proteins involved in HR. Five genes (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) sharing 20–30% sequence identity with human RAD51 were identified (Thompson and Schild, 2001). Strong evidence implicating the vertebrate paralogs in HR came particularly from studies with mutants in hamster and chicken DT40 cells. In hamster, XRCC3 deficiency was shown to lead to a 25-fold decrease in DSB repair (Pierce *et al*, 1999), whereas chicken DT40 cells with knocked-out RAD51 paralogs, although viable, were found to be sensitive to DNA crosslinking agents and to ionizing radiation besides exhibiting recombination/repair-defective phenotypes such as reduced growth rates, chromosomal instability and spontaneous chromosomal breaks (Takata *et al*, 2001). The sensitivity displayed by the paralog mutants was shown to be partially suppressed by RAD51 overexpression in chicken cells, thereby implicating the RAD51 paralogs as RAD51 cofactors. Moreover, the formation of DNA damaged-induced RAD51 foci was shown to be abolished in the absence of RAD51C, XRCC2 and XRCC3 in hamster cells and in RAD51B-, RAD51C-, RAD51D-, XRCC2- and XRCC3-deficient chicken DT40 cells (Bishop *et al*, 1998; French *et al*, 2002; Godthelp *et al*, 2002). The importance of the RAD51 paralogs in recombination and in genome stability was further emphasized by the embryonic lethality of RAD51B, RAD51D or XRCC2 deficiency observed in mouse (Shu *et al*, 1999; Deans *et al*, 2000; Pittman and Schimenti, 2000).

At present, there is much to learn about the biochemical properties and the biological functions of the paralogs. Previous studies using yeast two- and three-hybrid assays have shown numerous protein–protein interactions between the RAD51 paralogs, including RAD51B with RAD51C, RAD51C with XRCC3, RAD51C with RAD51D, RAD51D with

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XRCC2 and XRCC3 with RAD51 (Schild *et al*, 2000). Immunoprecipitations experiments from human cells revealed the presence of two complexes of the RAD51 paralogs (Masson *et al*, 2001a,b; Liu *et al*, 2002; Miller *et al*, 2002; Wiese *et al*, 2002). One complex, referred to as BCDX2, consists of RAD51B, RAD51C, RAD51D and XRCC2, whereas the CX3 complex contains RAD51C and XRCC3. Evidence for subcomplexes were also found (Miller *et al*, 2002). Residues required for RAD51C binding to XRCC3 (Tyr139 and Phe249) have been mapped (Kurumizaka *et al*, 2003), as well as domains for interactions between the paralogs (Miller *et al*, 2004). Both complexes of the paralogs might have overlapping and distinct functions. In *Arabidopsis*, for example, RAD51C and XRCC3 mutants are meiosis deficient, while XRCC2 and RAD51B mutants are not (Bleuyard *et al*, 2005; Li *et al*, 2005).

RAD51C is the central core of both RAD51C-XRCC3 and RAD51B-C-D-XRCC2 complexes. A number of studies suggest that these complexes are involved in HR. RAD51B-RAD51C alleviates the inhibitory effect of RPA *in vitro* and also promotes ATP-independent strand exchange (Sigurdsson *et al*, 2001; Lio *et al*, 2003). BCDX2 has shown preferential binding to Y-shaped DNA and synthetic Holliday junction (HJ) (Yokoyama *et al*, 2004). Branch migration and resolution activities are dependent on the presence of the RAD51 paralogs (Liu *et al*, 2004).

Although these studies show a role for the RAD51 paralogs in HR *in vitro*, it is not clear whether these proteins interact directly with DSBs *in vivo* and how they mediate repair. For instance, foci of RAD51C have not been observed by immunofluorescence in mitotic cells. Using immunoprecipitation analyses, the recombinase RAD51 was found to be exclusive of the endogenous paralog complexes (Miller *et al*, 2002). Here, we report the *in vitro* and *in vivo* interaction between RAD51 and RAD51C. Furthermore, using a recombination reporter system where a single DSB can be created, we show that RAD51C, together with several repair factors, binds a DSB *in vivo* as determined by immunofluorescence and chromatin immunoprecipitation (ChIP) strategies.

Results

BCDX2 and CX3 complexes interact with RAD51 *in vitro*

Previous studies have shown that the two paralog complexes may assist Rad51 during recombinational repair by acting as cofactors. Two- and three-hybrid studies have shown a network of interactions between the RAD51 paralogs. Hence, we wanted to test whether purified CX3 or BCDX2 complexes could interact with RAD51 *in vitro* (Figure 1A). We observed that anti-RAD51 pulled down a complex between CX3 and RAD51 (lanes 7–10). Similarly, BCDX2 was pulled down together with RAD51 (lanes 11–14). Quantification revealed that almost all RAD51 was bound to the paralog complexes. The interactions were strong and complex formation was resistant to 1 M NaCl washes (lanes 10 and 14). Control experiments showed that preimmune sera failed to pull down RAD51 or any of the RAD51 paralogs (lanes 2 and 3) and anti-RAD51 failed to immunoprecipitate BCDX2 or CX3 (lanes 5 and 6). Additional controls revealed that human RAD52 failed to interact with CX3 or BCDX2 and that RAD51-CX3 or RAD51-BCDX2 complexes were detected when incubated in 2 mg of *E. coli* or HeLa whole-cell extracts (data not

shown). Interestingly, immunoprecipitations of RAD51 and RAD51C coexpressed in insect cells show that this interaction is direct (Figure 1B). These results suggest that both RAD51 paralog complexes can interact specifically with RAD51.

RAD51C interacts with RAD51 *in vivo* and localizes to DNA damage sites

To provide further evidence for the specific nature of the BCDX2 and CX3 interaction, we performed immunoprecipitation analyses on cells transfected with YFP-RAD51C or CFP-RAD51. Given the difficulties to detect interactions between the endogenous RAD51 and the paralog proteins (Masson *et al*, 2001a; Miller *et al*, 2002), the rationale was to verify whether we could detect an interaction with endogenous RAD51C or RAD51 when the corresponding heterologous partner was expressed transiently. To do this, YFP-RAD51C and CFP-RAD51 fusions were used and proper fusions were confirmed by Western blotting (Figure 1C and D). When cells expressing YFP-RAD51C were immunoprecipitated for endogenous RAD51, a complex between RAD51 and YFP-RAD51C was observed (Figure 1E). Conversely, a complex between endogenous RAD51C and CFP-RAD51 was observed (Figure 1F). Quantifications revealed that about 20% of RAD51 is bound to RAD51C. Control experiments revealed that anti-RAD51 immunoprecipitated endogenous RAD51 and anti-RAD51C pulled down endogenous RAD51C (Figure 1E and F, lower panels). Taken together, these results suggest that RAD51 and RAD51C can interact *in vivo*.

The interaction between RAD51C and RAD51 suggests that RAD51C might assist RAD51 in DNA repair during HR in the nucleus. In order to support this, two experiments were performed. First, we looked at the localization of RAD51 and RAD51C within the cell. Cellular fractionation revealed that both RAD51 and RAD51C were located in the nuclear fraction of human fibroblast cell line DR95. DNA damage with etoposide did not alter the localization, mobility or the abundance of RAD51 or RAD51C proteins (Figure 2A). Consistent with this observation, the RAD51C-green fluorescent protein (GFP) fusion was specifically located in the nucleus in all DR95 cells that were transfected (Figure 2B). Since DR95 cells can produce a functional GFP following successful HR (about 5% of the cells by flow cytometry), the nuclear localization of RAD51C-GFP was also monitored in other cell lines. RAD51C-GFP was located in the nucleus in HEK293T and SKN-SH cells (data not shown). It is well known that DNA damage induced by genotoxic agents results in the recruitment of several DNA repair proteins, including RAD51, to DNA damage-induced foci. To our knowledge, DNA damaged-induced foci of RAD51C have not been observed in mitotic cells. Using a polyclonal antibody generated against full-length RAD51C, we observed RAD51C foci formation by immunofluorescence after etoposide treatment (Figure 2C). RAD51C foci colocalized with γ -H2AX, a marker of DNA damage. Identical results were observed with another RAD51C polyclonal antibody (data not shown). These results show that the nuclear protein RAD51C localizes to sites of DNA damage.

RAD51C inhibition results in a decrease of cellular proliferation and RAD51 foci formation

We reasoned that if RAD51 and RAD51C act in concert during HR, inhibition of the expression of RAD51C should decrease

Figure 1 (A) Interactions between purified human RAD51 and RAD51C-XRCC3 or RAD51B-C-D-XRCC2 and between RAD51 and RAD51C *in vivo*. Human RAD51, or a mixture with CX3 or BCX2, were immunoprecipitated with preimmune serum or the anti-hRAD51 as indicated, and visualized by Western blotting using anti-hRAD51 mAb 14B4 and anti-RAD51C mAb 2H11. Lane 1, purified RAD51C_{his10} and RAD51. CX3 and RAD51 (lane 2) or BCDX2 and RAD51 (lane 3) were immunoprecipitated with RAD51 preimmune antibody. As controls, RAD51 (lane 4), CX3 (lane 5) or BCDX2 (lane 6) were immunoprecipitated with anti-hRAD51 pAb. CX3 and RAD51 (lanes 7–10) or BCDX2 and RAD51 (lanes 11–14) were immunoprecipitated with anti-hRAD51 pAb and complexes were washed with buffer containing NaCl, as indicated. (B) Direct interaction between RAD51 and RAD51C. SF9 cells were infected with RAD51 baculovirus (lane 2) or a mixture of RAD51 and RAD51C (lane 3) and immunoprecipitated with the anti-hRAD51C. Complexes were washed in lysis buffer containing 1 M NaCl and visualized by Western blotting using anti-hRAD51 mAb 14B4. Lane 1, purified human RAD51. (C) Expression of YFP-RAD51C in HEK293 cells. Lane 1, purified RAD51C_{his10}; lane 2, cells transfected with the empty vector pEYFP-N1; lane 3, cells transfected with pEYFP-N1-51C encoding a fusion between YFP and RAD51C. RAD51C was visualized using the anti-RAD51C mAb 2H11. (D) Expression of CFP-RAD51 in HEK293 cells. Lane 1, purified RAD51; lane 2, cells transfected with the empty vector pECFP-N1; lane 3, cells transfected with pECFP-N1-51 encoding a fusion between CFP and RAD51. RAD51 was visualized using the anti-RAD51 mAb 14B4. (E) Co-immunoprecipitation of endogenous RAD51 with YFP-RAD51C. Extracts from HEK293 cells transfected with pEYFP-N1-51C were prepared and protein complexes were precipitated using control IgG (lane 2) or anti-RAD51 pAb (lane 3), and visualized by Western blotting with anti-RAD51C and RAD51, as indicated. Lane 1: marker protein (RAD51C_{his10}). (F) Co-immunoprecipitation of endogenous RAD51C with CFP-RAD51. Extracts from HEK293 cells transfected with pECFP-N1-51 were prepared and protein complexes were precipitated using control IgG (lane 2) or anti-RAD51 pAb (lane 3) and visualized by Western blotting with anti-RAD51 and RAD51C, as indicated. Lane 1: marker protein (RAD51).

cellular proliferation because natural or induced DSBs would not be repaired. siRNA inhibition of RAD51C during 24 h led to a decrease of 80% in the soluble pool of RAD51C as observed by Western blotting (Figure 3A). Western blotting of other RAD51 paralogs was conducted to verify the specificity of the siRNA (Figure 3B). While RAD51B, RAD51D and XRCC2 levels were unaffected, siRNA against RAD51C results in a concomitant repression of XRCC3 protein levels as described previously (Lio *et al*, 2004). To further evaluate the specificity of RAD51C siRNA, we transfected the cells with an siRNA-resistant construct (Figure 3C). In addition, siRNA against XRCC3 was used as a control (Figure 3D). Colony formation assays revealed a 92 and 90% reduction of colonies when cells were inhibited for the expression of RAD51C by double transfection of siRNA against RAD51C or XRCC3, respectively (Figure 3E, middle panel and Figure 3F). Next, cells were transfected once with RAD51C siRNA to preserve viability before treatment (a 46% decrease in viability was observed without damage; Figure 3E, top panel and Figure 3F) and challenged with etoposide. In these condi-

tions, knockdown of RAD51C led to a decrease of 75% in the number of colonies (Figure 3E, bottom panel and Figure 3F). Expression of an siRNA RAD51C-resistant construct restored the number of colonies with and without DNA damage to approximately wild-type levels (Figure 3F). These results show that siRNA against RAD51C and XRCC3 result in a similar decrease of cellular proliferation, highlighting a close functional relationship.

Immunofluorescence studies in cells treated with mitomycin C revealed a significant decrease in RAD51 foci formation following RAD51C inhibition (compare Figure 4A and B). RAD51 foci in RAD51C knockdown cells were reduced by 38%. Interestingly, expression of the siRNA-resistant RAD51C construct restored RAD51 foci formation to 91% of the wild-type cells. Consistent with this observation, immunofluorescence studies in hamster and chicken DT40 cells suggest that the RAD51 paralogs act before RAD51 (Bishop *et al*, 1998; Takata *et al*, 2001; French *et al*, 2002; Godthelp *et al*, 2002). These results establish a pre-RAD51 role for RAD51C during the repair of DSBs in human cells.

Figure 2 RAD51C is a nuclear protein and forms foci following DNA damage. (A) Cell-free extracts were prepared from mock-treated DR95 cells or DR95 cells treated with etoposide and fractionated into cytosolic (Cyt) and nuclear (Nuc) fractions. These fractions (25 µg) were analyzed for the presence of RAD51C, RAD51, γ -H2AX and GAPDH were used as controls for the cytoplasmic and nuclear fraction by Western blotting. (B) Localization of GFP-RAD51C in DR95 cells. (C) RAD51C foci formation following etoposide treatment (50 µM, 1 h). Immunofluorescence of RAD51C, γ -H2AX and merge pictures are depicted.

Localization of endogenous repair proteins to a single-double strand break *in vivo*

Most studies use DNA-damaging agents to study foci formation. However, this causes a difficulty when it is time to look at a single repair event within the nucleus since DNA damage is caused randomly. In order to study DNA repair at the resolution of a single lesion, we used the DR95 cell line, which bears a modified GFP gene in which an *I-SceI* restriction site has been engineered. As far as is known, the *I-SceI* restriction enzyme does not cut elsewhere in the genome and therefore is specific for the modified GFP. In this way, a unique DSB can be created in a known nucleotide sequence. Following transfection of DR95 cells with pCBASce (a plasmid encoding *I-SceI*), DSB induction was efficient as most of the cells were cleaved in DR-GFP 4 h after transfection as judged by LM-PCR (data not shown). Following transfection with pCBASce, immunofluorescence studies allowed the visualization of a single focus formation of RAD51C, RAD51, and 53BP1, which all colocalized with γ -H2AX (Figure 5). The single focus was not apparent in the absence of *I-SceI* expression. A direct role for RAD51C in repairing DNA is therefore established. Since cells undergo cycles of *I-SceI* cleavage and repair, unique DSBs were also present 24 h after transfection. We also synchronized cells in G2 and performed the same analysis. Double RAD51 foci representing newly replicated sister chromatids that have been cut by *I-SceI* were observed (Figure 6).

It is not clear whether RAD51C binds very close to DSBs *in vivo*. RAD51C is part of the HJ resolvosome that could migrate junctions far from the break site (Liu *et al*, 2004). One attractive strategy is to examine the assembly of RAD51C on a

DSB using ChIPs. First, we looked whether the levels of RAD51C changed during the cell cycle after cell synchronization. RAD51C levels did not change during the cell cycle (Figure 7A). Early after transfection with pCBASce, cells were fixed with formaldehyde and the chromatin was solubilized by sonication and purified. Immunoprecipitations were conducted with antibodies raised against RAD51, RAD51C, Ku80 or MRE11. Control experiments revealed that RAD51 antibodies immunoprecipitated endogenous RAD51 (Figure 1E) and RAD51C antibodies pulled down CX3 and BCDX2 paralog complexes (Masson *et al*, 2001a, b). Similarly, Ku80 and MRE11-RAD50-NBS1 were pulled down with the corresponding antibodies (data not shown). After reversal of the formaldehyde crosslinks, DNA samples were deproteinized. DNA was isolated and amplified by real-time PCR with primer pairs specific to regions of interest near the DSB created by *I-SceI* (Figure 7B). All reactions were normalized against a control primer pairs for sequences near the RAD51 gene or the AFP locus, which allowed us to control for DSB-independent effects on protein occupancy. This highly informative approach allowed the detection of repair proteins on the DSB (Figure 7C–E). We monitored events in cells arrested at the G1 and S–G2 phases of the cell cycle. Control experiments revealed that *I-SceI* cleavage was equivalent in the G1 and S–G2 phase based on γ -H2AX foci formation. When cells were synchronized so *I-SceI* would cut in the G1 phase of the cell cycle (73.2% of the cells, as determined by flow cytometry), an enrichment in Ku80 (10-fold) was observed at 94–378 bp from the break and decreased further from the break (3.3-fold at 675–1044 bp and 1.4-fold at 901–1210 bp) (Figure 7C). MRE11 and RAD51C were not detected, but RAD51 was found to be present at the DSB, but this may account for the S–G2 cells present in this sample (24.6%). When cells were synchronized for DSB formation in S–G2 (70.2% of the cells, as determined by flow cytometry), Ku80 was not detected, whereas the levels of RAD51 (16-fold), RAD51C (8.5-fold) and MRE11 (13-fold) were vastly increased very close to the break (94–378 bp) and decreased away from the break (RAD51 (10.2-fold), RAD51C (7.5-fold), MRE11 (6.1-fold), all at 675–1044 bp from the break) (Figure 7D). Given that RAD51C is part of BCDX2 and CX3 complexes, we performed ChIP analysis of RAD51D, XRCC2 and XRCC3 in S–G2 cells to distinguish whether BCDX2 or CX3 were bound to DSBs *in vivo*. Control experiments revealed that all antibodies were able to pull-down paralog complexes. As expected, the levels of enrichment were lower than those obtained for RAD51C, since the enrichment of RAD51C on the unique DSB may represent the sum of each paralog complexes. RAD51D, XRCC2 and XRCC3 were present at 94–378 bp (1.62-, 1.56- and 1.65-fold, respectively). The levels of RAD51D and XRCC2 increased at 675–1044 bp (2.58- and 2.2-fold), whereas XRCC3 was stable (1.65-fold). Surprisingly, both RAD51D and XRCC2 were present at 901–1210 bp (2.2- and 2.17-fold).

Discussion

Despite the fact that the current model of HR was proposed about 20 years ago, its biochemical complexity is not fully understood in mitotic cells. Since RAD51C is at the crossroads of at least two stable complexes of the RAD51 paralogs (a dimeric complex of RAD51C-XRCC3 and a larger complex composed of RAD51B-C-D-XRCC2), its characterization is

Figure 3 Knockdown of RAD51C and XRCC3 affects cellular proliferation. (A) Small interfering RNA inhibition of RAD51C. Lane 1, purified RAD51C_{his10}. Whole-cell extracts from mock-transfected DR95 cells (lane 2) or DR95 cells transfected with RAD51C siRNA (lane 3) were subjected to Western blotting with RAD51C or GAPDH antibodies (as a loading control). (B) Inhibition of RAD51C by siRNA destabilizes XRCC3. Whole-cell extracts from mock-transfected DR95 cells (lane 2) or DR95 cells transfected with RAD51C siRNA (lane 3) were subjected to Western blotting using anti-RAD51B, anti-RAD51D, anti-XRCC2 and anti-XRCC3. Lane 1, purified RAD51B_{his10}, RAD51D, XRCC2, XRCC3_{his6}. (C) RAD51C siRNA can knockdown wild-type RAD51C but not an siRNA-resistant form. Lane 1, purified RAD51C_{his10}. Whole-cell extracts from cells transfected with pcDNA3 (lane 2), pcDNA-51C (lane 3) or the siRNA-resistant RAD51C construct pcDNA-51C-Res (lane 4), RAD51C siRNA-treated cells transfected with pcDNA3 (lane 5), pcDNA-51C (lane 6) or pcDNA-51C-Res (lane 7) were subjected to Western blotting with RAD51C or GAPDH antibodies. (D) RNAi inhibition of XRCC3. Lane 1, purified XRCC3_{his6}. Whole-cell extracts from mock-transfected DR95 cells (lane 2) or DR95 cells transfected twice with XRCC3 siRNA (lane 3) were subjected to Western blotting with XRCC3 or GAPDH antibodies. (E) Cellular proliferation of mock-treated or cells transfected once (top panel) or twice with an siRNA specific for RAD51C (middle panel). Bottom panel: wild-type or RAD51C siRNA-transfected cells challenged with 50 μ M etoposide for 1 h followed by growth and recovery in fresh media. (F) Quantification of colony-forming assays. Cells were either untreated or treated with 50 μ M etoposide for 1 h followed by growth and recovery in fresh media, as indicated. White bars indicate cells transfected once with RAD51C siRNA and gray bars designate cells transfected twice with RAD51C or XRCC3 siRNA. Black bars indicate wild-type cells. These experiments were repeated three times.

Figure 4 Knockdown of RAD51C affects RAD51 foci formation in human cells. Immunofluorescence was performed on DR95 cells treated with 600 nM mitomycin C (A) or cells pretreated with RAD51C siRNA followed by DNA damage with mitomycin C (B). DNA staining by DAPI (blue) and immunofluorescence of RAD51 (green), γ -H2AX (red) and merge pictures are depicted.

Figure 5 Foci formation of RAD51, RAD51C and 53BP1 on a unique DSB *in vivo*. DR95 cells were transfected with pCBASce and immunofluorescence was conducted with the indicated antibodies. Confocal micrographs depict DNA stained with DAPI (blue); RAD51 (top panel), anti-RAD51C (middle panel) and 53BP1 (bottom panel) (green); γ -H2AX (red). The merge picture is an overlay of the three channels.

Figure 6 RAD51 foci formation in DR95 cells after DNA replication. Confocal micrographs depict DNA stained with DAPI (blue), RAD51 (green) and γ -H2AX (red). The merge picture is an overlay of the three channels.

essential to gain insights into the roles of both paralogs complexes in HR. In this study, we characterized further the roles of RAD51C in DNA repair, but also developed sophisticated techniques allowing the detection of repair proteins at a single repair event at the cellular and chromosomal level.

RAD51C interacts with RAD51

Since their discovery, the functions of the RAD51 paralogs have been attributed to HR. On the basis of sequence homology, it seemed very likely that these proteins would interact with RAD51. In contrast, several studies have shown that both BCDX2 and CX3 complexes were distinct from RAD51 (Masson *et al*, 2001a; Miller *et al*, 2002). Here, we observed an interaction between purified BCDX2 or CX3 complexes and the RAD51 recombinase. Secondly, we observed an interaction between endogenous RAD51 or RAD51C with transiently expressed YFP-RAD51C and CFP-RAD51, respectively. Studies indicate that the composition of the damage-induced foci is dynamic. Photobleaching studies indicate that several RAD proteins have very different diffusion coefficients, suggesting that they may not exist together as a preassembled protein complex (Essers *et al*, 2002). The dynamic nature between different DNA repair complexes or

Figure 7 Detection of repair proteins on a unique DSB *in vivo* at a high resolution by ChIPs. (A) RAD51C protein levels do not fluctuate during the cell cycle. Cells were synchronized, whole-cell extracts were prepared and subjected to Western blot analysis using RAD51C 2H11 mAb. The percentage of cells at each phase is represented. (B) Schematic representation of the position of primers used for real-time PCR quantification of ChIPs respective to the unique DSB created by I-SceI *in vivo*. (C–E) ChIPs of endogenous RAD51, RAD51C, Ku80 and MRE11 on a unique DSB. The presence of the proteins was verified during the G1 (C) or S–G2 phase (D) of the cell cycle. Real-time PCR on ChIP samples were carried out at 94–378, 675–1044 and 901–1210 nucleotides from the break (red, yellow and blue bars, respectively). (E) ChIP analysis of RAD51D, XRCC2 and XRCC3. Fold-enrichment represents the enrichment of the proteins compared to an IgG control (normalized with a PCR internal control to a locus other than the DSB). These experiments were repeated three times and the PCR reactions were performed in triplicate.

the fact that they should interact on DNA may explain why it is difficult to see an interaction between RAD51 and RAD51C.

Direct *in vivo* evidence for the roles of RAD51C in DNA repair and cell survival

Consistent with a nuclear function, cellular fractionation with and without DNA damage revealed that both RAD51 and RAD51C were found in the nucleus. Hamster RAD51C has been localized to the nucleus and the nuclear localization was dependent on the C-terminus of the protein (French *et al*, 2003). In eukaryotes, DNA repair is choreographed by multi-protein complexes that are organized in focal assemblies. Although studies have attributed a role for RAD51C in repairing DNA by HR *in vitro*, no foci of RAD51C have been observed *in vivo* following DNA damage. Very interestingly, we generated an antibody that detected RAD51C foci by immunofluorescence following DNA damage. Consistent with this, siRNA inhibition of RAD51C resulted in a decrease in cellular proliferation. Our results suggest a role for RAD51C in repairing natural endogenous DSBs that occur during each cell cycle as well as DNA damage-induced DSBs in human cells.

Human RAD51C: early and late roles in HR

During genetic recombination and the recombinational repair of chromosome breaks, DNA molecules become linked at points of strand exchange. Branch migration and resolution of these crossovers, or HJs, complete the recombination process. It has been shown that extracts from cells carrying mutations in the recombination/repair genes RAD51C or XRCC3 have reduced levels of HJ resolvase activity. Moreover, depletion of RAD51C from fractionated human extracts caused a loss of branch migration and resolution activity, but these functions were restored by complementation with a variety of RAD51C complexes containing RAD51C (Liu *et al*, 2004). These results established that RAD51C is part of a complex for HJ migration and cleavage. However, the catalytic enzyme promoting branch migration and resolution in human cells remains to be identified.

We observed that RNAi of RAD51C resulted in a decrease of 38% in RAD51 foci formation following MMC treatment. These results suggest that RAD51C plays a role upstream of RAD51 during HR. This is supported by the following observations: (i) *in vitro* studies have shown that BCDX2, when immobilized on DNA, could recruit limiting amounts of RAD51 as judged by electron microscopy (Masson *et al*, 2001b); (ii) chicken and hamster cell lines deficient in

RAD51C also showed impaired Rad51 foci formation in response to DNA damage (Takata *et al*, 2001; French *et al*, 2002; Godthelp *et al*, 2002). Hence, our current study proposes that RAD51C is a mediator at early stages of HR, most likely during the invasion step of HR.

Although BCDX2 and CX3 complexes have been associated to different stages of HR, it is not totally clear which paralog complex contribute to early and late steps of the HR pathway. XRCC3 is recruited early to DSBs (Forget *et al*, 2004), but defective processing of recombination intermediates is observed in both hamster and *Arabidopsis* XRCC3^{-/-} cells during the later stages of HR (Brenneman *et al*, 2002; Bleuyard and White, 2004). Certainly, CX3 and BCDX2 complexes may have overlapping and different functions since deletion of RAD51D and XRCC3 in chicken DT40 cells lead to an additive phenotype (Yonetani *et al*, 2005). During our ChIP experiment in S–G2 phase of the cell cycle, we observed that the levels of RAD51C, unlike those of RAD51 and MRE11, did not decrease from 94 to 1044 bp. Since the polyclonal antibody used is able to pull down both CX3 and BCDX2 complexes, this observation raises the possibility that we are looking at different paralog complexes depending on the distance from the I-SceI cut: a RAD51-recruiting complex at 94–378 bp and a HJ migration/resolution complex at 675–1210 bp. Our data provide new insights into this, as RAD51D, XRCC2 and XRCC3 levels were similar at 94–378 bp, while RAD51D and XRCC2 levels increased at 675–1210 bp. This might suggest that BCDX2 and CX3 are both involved in RAD51 loading close to the break and RAD51D–XRCC2 might contribute to branch migration/resolution. Therefore, this complex is found further away from the break. However, it should be noticed that ChIPs rely on antibodies. The absence of RAD51C at 901–1210 bp could mean that the epitopes recognized by the antibody are masked by another protein. This could also explain why MRE11 is not found in G1, although a role in NHEJ was attributed for this protein (Paull and Gellert, 2000).

Recombination protein foci mark the site of DSB repair

We report here the first molecular-level characterization of regional DNA repair factors binding a unique DSB in human cells. To date, these events have been defined primarily at the resolution of light microscopes. Following I-SceI cleavage, a single focus of RAD51, RAD51C and 53BP1 was observed in the nucleus of DR95 cells. All signalling and repair protein foci colocalized with γ -H2AX. 53BP1 and NBS1 appear to directly bind the phosphorylated SQE motif of H2AX, suggesting that it may act as a scaffold to anchor or facilitate the assembly of signalling and repair proteins (Kobayashi *et al*, 2002; Ward *et al*, 2003). One concern about this system is that we can also detect endogenous DNA damage rather than the I-SceI cut. We observed G2 cells with two closely positioned foci of RAD51 representing the cleavage of both sister chromatids. The frequency of cells having these spots argues that it is very unlikely that this represents random DNA damage in the nucleus. In agreement with this, two foci of Rad51 has also been observed at HO endonuclease-induced DSB in the MAT locus of *Saccharomyces cerevisiae* (Miyazaki *et al*, 2004). Our results also suggest that resection of the DSB goes as far as 1 kb in human cells since RAD51 was not present at 901–1210 bp from the break.

We took advantage of the DR95 cell line where a unique DSB can be produced and performed ChIPs on endogenous DNA repair proteins. In yeast, temporal analysis of the recruitment of budding yeast proteins on an HO-induced DSB revealed that Rad51 binds first, followed by Rad52, Rad55 and finally Rad54. Consistent with biochemical studies indicating a requirement for Rad55 in mediating Rad51 filament formation, inactivation of *RAD55* lead to a decrease in the recruitment of Rad51 (Wolner *et al*, 2003). Certainly, our system now allows the possibility to study whether this order of recruitment is conserved in human cells.

Very elegantly, using chicken DT40 cell lines deficient in Ku70^{-/-} and Rad54^{-/-} cells and synchronised cells, the Takeda group has found that NHEJ pathway plays a dominant role in repairing γ -radiation-induced DSBs during G1–early S phase, while recombinational repair is preferentially used in late S–G2 phase of the cell cycle (Takata *et al*, 2000). We extend this study and show directly that Ku80 is bound to a unique DSB specifically in the G1 phase of the cell cycle, while proteins representative of the HR pathway (such as RAD51, RAD51C, RAD51D, XRCC2, XRCC3 and MRE11) are bound during the S–G2 phase of the cell cycle. Interestingly, by correlating our immunofluorescence data with our high-resolution ChIP analysis, these results clearly define that a focus represents a mark of repair of a DSB in human cells.

Materials and methods

Cell culture and plasmids

The DR95hyg-xt cell line was donated by Dr Maria Jasin (Pierce *et al*, 2001). HEK293T cells are derived from human embryonic kidney cells and express Ad5 E1A, E1B proteins and large T antigen (a gift from Dr Josée Lavoie) and SKN-SH are neuroblastoma cells. DR95hyg-xt, HEK293T and SKN-SH cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The human RAD51C and RAD51 genes were inserted in pEYFP-N1 and pECFP-N1 (BD Biosciences) to generate pEYFP-RAD51C and pECFP-RAD51, respectively. pCBASce is an I-SceI expression vector (Pierce *et al*, 2001). The siRNA-resistant RAD51C plasmid was constructed using site-directed mutagenesis of pcDNA3-RAD51C using the oligonucleotide 5'-ATAATCACCTTCTGCTCGGCGCTAGATGATATTCTT.

Cell fractionation, synchronization and colony-forming assay

DR95 cells (1×10^6) were mock treated or treated 2 h with 50 μ M etoposide. Cells were harvested, washed once with PBS1X, centrifuged and resuspended in 400 μ l of Schaffner lysis buffer (10 mM HEPES-NaOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, aprotinin (0.019 TIU/ml) and leupeptin (3 μ g/ml)) and allowed to swell on ice for 15 min. NP-40 was added to a final concentration of 0.55%, cells were vortexed vigorously for 10 s and centrifuged for 30 s at 13 000 r.p.m. The supernatant was kept as the cytoplasmic fraction while the nuclear proteins were extracted from the pellet by incubation in nuclear extraction buffer (20 mM HEPES-NaOH, pH 7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, aprotinin (0.019 TIU/ml) and leupeptin (3 μ g/ml)) on a rotary shaker for 15 min at 4°C. The nuclei were centrifuged for 5 min and the supernatant was collected as the nuclear extract.

Cells were synchronized according to Zhu *et al* (2000) with minor modifications. DR95 cells were treated with 2 mM thymidine for 14 h. The cells were washed with PBS1X and released in fresh media for 11 h. Subsequently, cells were treated with aphidicolin at 1 μ g/ml for 14 h. Cells were then released in fresh media.

Cellular viability of DR95 cells or DR95 cells transfected with RAD51C or XRCC3 siRNA was determined by colony formation. When required, pcDNA3, pcDNA-51C or pcDNA-51C-Res were

electroporated 24 h prior to siRNA transfection. A total of $1-5 \times 10^5$ mock- and siRNA-transfected cells released by trypsinization were plated onto 100 mm dishes. After 12–16 days, colonies were fixed with methanol and stained using methylene blue (4 g/l in methanol) and counted. Cells were treated with 50 μ M of etoposide for 1 h, allowed to recover for 1 h in fresh media and their colony-forming ability was monitored as above.

RNA interference

RNAi-mediated knockdown of RAD51C was conducted using an siRNA (Qiagen) with CACCTTCTGTTCAGCACTAGA as a target sequence. Knockdown of XRCC3 was performed using two siRNA against CAGAATTATTGCTGCAATTAA and CAGCCAGATCTTCATCG AGCA. siRNA transfection was performed using RNAiFect (Qiagen). In brief, cells were seeded in six-well plates at 2×10^5 cells/cm² 24 h prior to transfection. For each transfection, 5 μ g of siRNA was diluted in culture medium (containing serum and antibiotics) to a final volume of 100 μ l and incubated 15 min at room temperature with 15 μ l of RNAiFect. The RNAiFect-siRNA complex was then added dropwise to the 75% confluent cells and incubated at 37°C for 24 h. When required, cells were retransfected the next day for another 24 h. As a control, cells were mock transfected with RNAiFect alone.

Western blot analysis

RAD51C expression was assayed by Western blotting 24 h following siRNA transfection. A total of 5×10^5 cells were harvested, washed with PBS1X and lysed in 100 μ l IP buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.5% NP-40) containing 1 mM PMSF, aprotinin (0.019 TIU/ml) and leupeptin (3 μ g/ml). After 30 min of incubation on ice, cells were sonicated two times for 5 s with a Branson sonifier at 30% burst and centrifuged 10 min at 132 000 r.p.m. at 4°C. Cell lysate (25 μ g) was boiled and subjected to SDS-PAGE electrophoresis. After transfer to nylon membrane, the membranes were blocked overnight in PBS1X–0.05% Tween containing 5% skim milk (for RAD51, RAD51C or GAPDH antibodies) or PBS1X–0.05% Tween containing 5% BSA (for γ -H2AX mAb). Primary antibodies RAD51 (Genetex), RAD51C (mAb 2H11), GAPDH (Research diagnostics) or γ -H2AX (Upstate cell signaling solutions) were added followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin and revealed using enhanced chemoluminescence (Perkin-Elmer).

Immunoprecipitation

Purified human RAD51 (1 μ g) or CX3 or BCDX2 (1 μ g) were incubated in binding buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5% BSA) for 30 min at 37°C. 20 μ l of preimmune serum or RAD51 pAb coupled to Aminolink beads (Pierce) in 500 μ l of buffer were added to the reaction. Protein complexes were pulled down for 1 h at 4°C. Immunoprecipitates were washed with binding buffer containing NaCl, as indicated.

Immunoprecipitations from transfected HEK293T cells or baculovirus-infected cells were performed as follows. HEK293T cells were collected 48 h after transfection with either pEYFP-RAD51C or pECFP-RAD51 and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% NP-40) containing the protease inhibitors PMSF (1 mM), aprotinin (0.019 TIU/ml) and leupeptin (1 μ g/ml), incubated for 30 min on ice and then lysed by sonication. Insoluble material was removed by high-speed centrifugation. Protein complexes in the supernatant (equivalent to approximately 1.5×10^7 cells) were pulled down for 1.5 h at 4°C using preimmune serum or pAbs raised against RAD51 crosslinked to Aminolink beads (Pierce). Complexes were washed four times in lysis buffer containing salt as indicated, and visualized by Western blotting using RAD51 14B4 or RAD51C 2H11 mAb.

Immunofluorescence

Transiently transfected cells grown on coverslips were fixed 18–20 h post-transfection with 4.0% paraformaldehyde in PBS1X for 5 min at room temperature. Next, cells were permeabilized with PBS1X containing 0.5% Triton X-100 for 5 min and washed twice with PBS1X. Cells were then incubated in primary antibody at the appropriate dilution and incubated at room temperature for 30 min. Coverslips were rinsed with PBS1X containing 0.1% Triton X-100 and washed twice with PBS1X prior to a 30-min incubation with an appropriate secondary antibody conjugated to a fluorophore.

Cells were rinsed again with PBS1X containing 0.1% Triton X-100 and washed twice with PBS. Coverslips were mounted onto slides with approximately 10 μ l of a 90% glycerol-PBS-based medium containing 1 mg of paraphenylenediamine/ml and 0.5 μ g of 4',6'-diamidino-2-phenylindole (DAPI)/ml. RAD51C was visualized with a rabbit polyclonal antibody raised against the full-length protein (JYM pAb20), RAD51 (JYM pAb1) was visualized with a rabbit polyclonal antibody and 53BP1 was obtained from Novus Biologicals. Secondary antibodies included both anti-mouse and anti-rabbit antibodies conjugated with either Alexa-Fluor 488, Alexa-Fluor 555 (Molecular Probes) or cyanin 3 (Jackson ImmunoResearch Laboratories Inc.). Images were collected with a Zeiss Laser Scanning Confocal Microscope or on a Leica DMIRE2 inverted microscope.

ChIPs assays

Induction of a single DSB in DR95 cells was performed through electroporation of the I-SceI expression vector pCBASce using the Gene Pulser Xcell apparatus (BioRad). For each electroporation, a total of 2×10^6 cells suspended in 650 μ l PBS were mixed with 50 μ g of circular plasmid and pulsed at 0.25 kV and 1000 μ F in 4 mm cuvettes. Following electroporation, cells were plated onto 100 mm dishes containing fresh medium and returned to incubation at 37°C for 4 h. Cells were treated with 1% formaldehyde for 8 min to crosslink proteins to DNA. Glycine (0.125 M) was added to quench the reaction. Cells were collected using a cell scraper, washed twice in cold PBS1X, washed for 10 min in solution I (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100) and 10 min in solution II (10 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Cells were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) and sonicated for 6 $\times$ 10 s (30% output) to shear chromatin to an average size of 0.5 kb using a Fisher Sonic Dismembrator sonicator. Once centrifuged until clear, the lysate was precleared overnight with Sepharose CL-6B. Immunoprecipitations were performed for 2 h in lysis buffer with polyclonal antibodies against RAD51C, RAD51D, XRCC2, XRCC3, RAD51, MRE11 and Ku80 proteins. Preimmune serum or rabbit anti-human IgG (H + L) antibody (Jackson ImmunoResearch Laboratories) was used as a negative control. Complexes were washed twice with RIPA buffer, once in high salt buffer (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA), once in LiCl buffer (50 mM Tris-Cl, pH 8.0, 250 mM LiCl, 1% NP-40, 0.5% deoxycholate, 1 mM EDTA) and twice in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0). Beads were resuspended in TE containing 50 μ g/ml of RNase and incubated for 30 min. Beads were washed with water and elution buffer (1% SDS, 0.1 M NaHCO₃) was added for 15 min. Crosslinks were reversed by adding 200 mM NaCl followed by an incubation for 6 h at 65°C. Samples were deproteinized overnight with proteinase K and DNA was extracted with phenol-chloroform followed by ethanol precipitation.

Real-time PCR

Quantification of the amount of immunoprecipitated DNA was carried out by real-time PCR using the LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Sciences), which contained Fast Start Taq DNA polymerase and SYBR Green Dye. For each PCR assay, 0.5 μ l aliquots of the DNA sample were amplified in triplicate. The sequence of the primers can be obtained on request. Primers used in the PCR reactions were analyzed for linearity range and efficiency using a LightCycler (Roche). Values were calculated as fold-enrichment compared to the IgG control versus a control locus.

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