The EMBO Journal (2008) 27, 155–167 | © 2008 European Molecular Biology Organization | All Rights Reserved 0261-4189/08
www.embojournal.org

THE EMBO JOURNAL

Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step

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To address the biochemical mechanisms underlying the coordination between the various proteins required for nucleotide excision repair (NER), we employed the immobilized template system. Using either wild-type or mutated recombinant proteins, we identified the factors involved in the NER process and showed the sequential comings and goings of these factors to the immobilized damaged DNA. Firstly, we found that PCNA and RF-C arrival requires XPF 5′ incision. Moreover, the positioning of RF-C is facilitated by RPA and induces XPF release. Concomitantly, XPG leads to PCNA recruitment and stabilization. Our data strongly suggest that this interaction with XPG protects PCNA and Polδ from the effect of inhibitors such as p21. XPG and RPA are released as soon as Polδ is recruited by the RF-C/PCNA complex. Finally, a ligation system composed of FEN1 and Ligase I can be recruited to fully restore the DNA. In addition, using XP or trichothiodystrophy patient-derived cell extracts, we were able to diagnose the biochemical defect that may prove to be important for therapeutic purposes.

The EMBO Journal (2008) **27,** 155–167. doi:10.1038/sj.emboj.7601948; Published online 13 December 2007
Subject Categories: genome stability & dynamics
Keywords: DNA resynthesis; nucleotide excision repair; XPG

Introduction

Cells have developed different repair mechanisms such as nucleotide excision repair (NER) to maintain the integrity of the DNA, which is exposed to endogenous and exogenous genotoxic attacks (Hoeijmakers, 2001). This repair pathway eliminates the damage induced by UV and by antitumoral chemicals among others. The primary importance of NER is underlined by the existence of autosomal recessive syndromes known as xeroderma pigmentosum (XP) and trichothiodystrophy (TTD) (Kraemer *et al*, 2007). These syndromes are genetically complex with at least eight complementation groups for XP (XP-A to XP-G and variant) and three for TTD (XP-B, XP-D and TTD-A). The products of the XP genes are proteins involved in the different steps of NER and comprise three damage-recognition proteins, two helicases and two endonucleases (de Laat *et al*, 1999). Mutations in specific repair factors have been shown to affect genome stability (Lehmann, 2003). The broad range of clinical features as well as the variability in the severity of each phenotype cannot be explained by only a deficiency in DNA repair. Thus, it was demonstrated that some of the NER factors play a role in the DNA replication (Johnson *et al*, 1999) and transcription processes (Compe *et al*, 2005; Ito *et al*, 2007). Furthermore, in the sole context of global genome NER (GG-NER), the relationship between the mutations and the phenotypes displayed by patients is further complicated by the network of interactions between NER factors that can modulate their stability, positioning on the damaged DNA and enzymatic activities. Therefore, it is not surprising that a mutation in a given protein such as in XPB/fs740 will disturb the activity of another one like the XPF endonuclease, which acts in a later step of NER (Araujo *et al*, 2000; Coin *et al*, 2004).

Considerable progress has been made in recent years toward determining the function of each of the NER components starting from the recognition of the DNA damage with XPC, to the excision of the damaged oligonucleotide and DNA resynthesis (Aboussekhra *et al*, 1995; Shivji *et al*, 1995). However, little is known about the intricate network that regulates the comings and goings of the 11 NER factors onto the damaged DNA template and about the protein/protein interactions that lead to the formation of the various intermediate DNA repair complexes (Araujo and Wood, 1999; Riedl *et al*, 2003). While the first steps leading to the removal of the DNA damage have been described (Araujo *et al*, 2000; Volker *et al*, 2001; Riedl *et al*, 2003), little is known on the link between the dual incision and the resynthesis (Gillet and Scharer, 2006). How are PCNA, RF-C and DNA polymerase recruited? Do the dual incision factors play a role in the formation of the resynthesis complex? What is the fate of XPG, XPF and RPA?

To obtain a more detailed picture of the NER mechanism, we used an immobilized template. We thus were able to recruit and identify from HeLa nuclear extracts (NE) all the NER factors that participate in the three steps of the NER: dual incision, resynthesis and ligation. Moreover, by using either wild type and mutated recombinant proteins or XP- and TTD-patient-derived cell extracts, we were able to show how the NER factors were sequentially coming on and going off the immobilized damaged DNA in a 'ballet' similar to what was observed by confocal microscopy in the cell. We also document how a mutation in a single NER factor can disturb the formation of the successive intermediate complexes and their enzymatic activities. Indeed, the understanding of the mechanistic defects leading to the XP or TTD has

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Received: 22 August 2007; accepted: 14 November 2007; published online: 13 December 2007

been beneficial in unravelling the NER reaction and vice versa. From this combined data emerged an unexpected role for XPG, which is as important as RPA in initiating the resynthesis step of the NER.

Results

Sequential arrival of NER factors on local UV-irradiated DNA

We studied the dynamics of the NER factors in living cells, using local UV irradiation technology combined with fluorescent immunostaining (Volker *et al*, 2001). Confocal microscopy showed that 10 min post-UV irradiation, TFIIH, one of the first factors to target the damaged DNA (Figure 1A, panels a–d), colocalized with the cyclopyrimidine dimers (CPD) photoproduct (Riedl *et al*, 2003). At that time, neither PCNA nor the DNA polymerase delta (Polδ), which are involved in DNA resynthesis, was detected (Figure 1B and C, panels a–d). However, 30 min after UV irradiation, both PCNA (Miura, 1999) and Polδ accumulated at the irradiated sites (Figure 1C, panels e–h), supporting the sequential occurrence of the dual incision and the resynthesis. As local UV irradiation generates several hundreds of lesions, as visualized by CPD antibodies, the merging of TFIIH and either PCNA or Polδ signals results from the simultaneous and close presence of damaged DNA complexes at different stages of the repair reaction. At 60 min after UV irradiation, we also observed the arrival of the histone chaperone chromatin assembly factor 1 (CAF-1), which colocalizes with PCNA (Figure 1D). CAF-1 is the key player involved in the dynamics of the nucleosome assembly process at UV-damage sites *in vivo* (Green and Almouzni, 2003; Polo *et al*, 2006).

Sequential arrival of NER factors on immobilized damaged DNA

To better understand the transition steps between dual incision and resynthesis and to pinpoint the role of each factor, we followed their kinetics *in vitro*. The 'comings and goings' of the NER factors on a DNA template were analysed by incubating NE with an immobilized damaged DNA containing a single 1,3 intrastrand d(GpTpG) cisplatin–DNA crosslink, also called the 'immobilized template assay' (Lainé *et al*, 2006). At different time points, the immobilized DNA was washed with 0.05 M KCl and the recruitment of the repair factors was monitored by immunoblotting, while the release of the damaged oligonucleotide and the DNA resynthesis activity were analysed by autoradiography (Figure 2A and B; Supplementary data 1). In the absence of ATP, which does not allow the DNA unwinding by TFIIH (Riedl *et al*, 2003), neither the XPG endonuclease nor the other dual incision factors were recruited to the damaged DNA (Figure 2A, lanes 1–3; data not shown). In the presence of ATP, TFIIH (as revealed by its p62 subunit) and XPG were bound to the damaged DNA at 10 min and were gradually released from the DNA thereafter (Figure 2A, lanes 4–7; Figure 2C, upper panel). At 40 min, when the removal of the damaged oligonucleotide was optimal (Figure 2B, lane 5), the level of RPA increased (Figure 2A, lanes 4 and 5; Figure 2C, upper panel). In parallel, the resynthesis factors RF-C, PCNA and Polδ (Shivji *et al*, 1992, 1995) replaced the dual incision factors on the DNA template, reaching a maximum at 120 min (Figure 2A, lanes 4–6; Figure 2C, middle panel). At this time, the DNA resynthesis was optimal (Figure 2B, lane 6) and RPA was removed from the template (Figure 2A, lanes 6 and 7). Within the next 60 min, RF-C and Polδ were released, leaving PCNA on the DNA (Figure 2A, lanes 6–7; Figure 2C, middle panel). The recruitment of Ligase I and FEN 1 was initiated after 40 min (Figure 2A, lanes 6 and 7; Figure 2C). Once the newly synthesized DNA was ligated to the 3′ end of the excision, we observed the arrival of CAF-1 at 180 min, indicating the end of the repair process (Figure 2A, lane 7). It is also worthwhile to mention that this fishing method allowed us to identify RF-C, Polδ, FEN 1 and Ligase I as part of the NER system, being selectively and functionally recruited by the immobilized cisplatinated DNA from NE (Supplementary data 3A). In the absence of either ATP (lanes 2–4), none of these NER factors were specifically recruited.

To further assess and underline the role of each of the identified factors in the NER, we used purified DNA repair factors (Figure 2D), components of the reconstituted incision system (RIS: XPC-HR23B, TFIIH, XPA, RPA, XPG and ERCC1-XPF) the reconstituted resynthesis system (RRS: RF-C, PCNA and DNA Polδ) and the reconstituted ligation system (RLS: FEN 1 and Ligase I). Their enzymatic activities were checked with *in vitro* repair assays (Supplementary data 1). Our reconstituted system is close to the efficiency of the NE system, as the dual incision and resynthesis efficiencies are ~87 and ~70%, respectively (compared to 90 and 85%, respectively, with the NE; Supplementary data 2). The comings and goings of the repair factors present in RIS, RRS and RLS on the immobilized damaged DNA were comparable to those obtained with NE (Figure 2E): the recruitment of the dual incision factors occurs early and their release is concomitant with the coming of PCNA, RF-C and Polδ (upper and middle panels). It should be noted that the reaction is slower. For example, at 210 min, significant amounts of RPA and XPG are still present on the DNA template (middle panel). This might explain the late arrival of Ligase I (Figure 2E, lower panel). It is also likely that the variations in the kinetic curves might reflect differences in the stoichiometry, the post-translational modifications and specific activities between the endogenous and the recombinant NER factors. We cannot exclude the possibility that some additional proteins that are not yet identified could participate in the NER reaction.

XPG and RPA recruit PCNA and RFC to allow DNA resynthesis

We next focused our attention on the transition between dual incision and DNA resynthesis. At different time points, we quantified dual incision and resynthesis activities. The removal of the damaged oligonucleotides strongly increased to reach a plateau at 40 min, while the gap filling was slightly delayed by 10 min (Figure 3A, upper panels). Both the kinetic curves exhibited a similar slope, suggesting a close coordination between these two steps (Figure 3A, graph).

We also investigated whether some of the RIS factors take part in DNA resynthesis. The immobilized damaged DNA was first incubated with RIS to allow dual incision (Figure 3B, Incub I) and then washed either at 0.05 M KCl to remove the nonspecifically bound proteins or at 2 M KCl to remove all proteins from the DNA. The gapped DNA was then incubated with RRS alone or in combination with one of the RIS factors (Incub II). In the absence of certain RIS factors, there is no

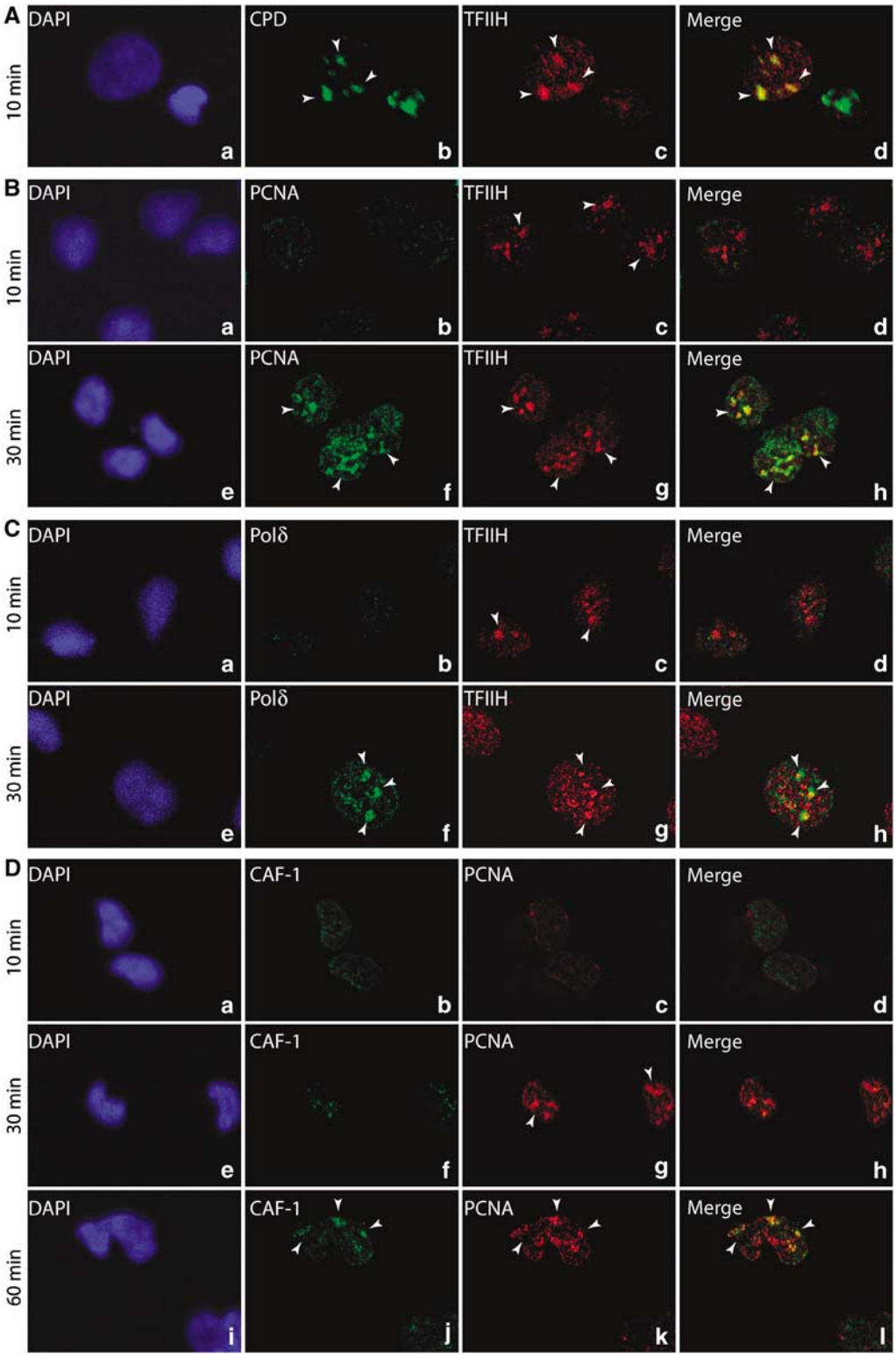

Figure 1 *In vivo* sequential recruitment of NER factors. Rescued XPCS2BA human fibroblasts were locally UV irradiated and labelled at 10, 30 and 60 min after UV irradiation with the indicated MAbs or PAbs. Colocalization of (**A**) CPD and TFIIH (XPB) (panels a–d), (**B**) TFIIH and PCNA (panels a–h), (**C**) TFIIH and Polδ (panels a–h) and (**D**) PCNA and CAF1 (panels a–l). Nuclei were counterstained with DAPI, and pictures were merged.

DNA resynthesis (Figure 3B, lanes 3 and 2). However, the incubation of the 2 M KCl-washed DNA with RRS and either RPA or XPG led to an optimal DNA resynthesis (compare lanes 6 and 7 and lane 2). On the contrary, incubation of RRS with XPA, TFIIH or XPF did not allow resynthesis (lanes 4, 5 and 8), thus establishing their exclusive role in the dual incision step. This result suggests that XPG as well as RPA plays a key role in dual incision and in DNA resynthesis.

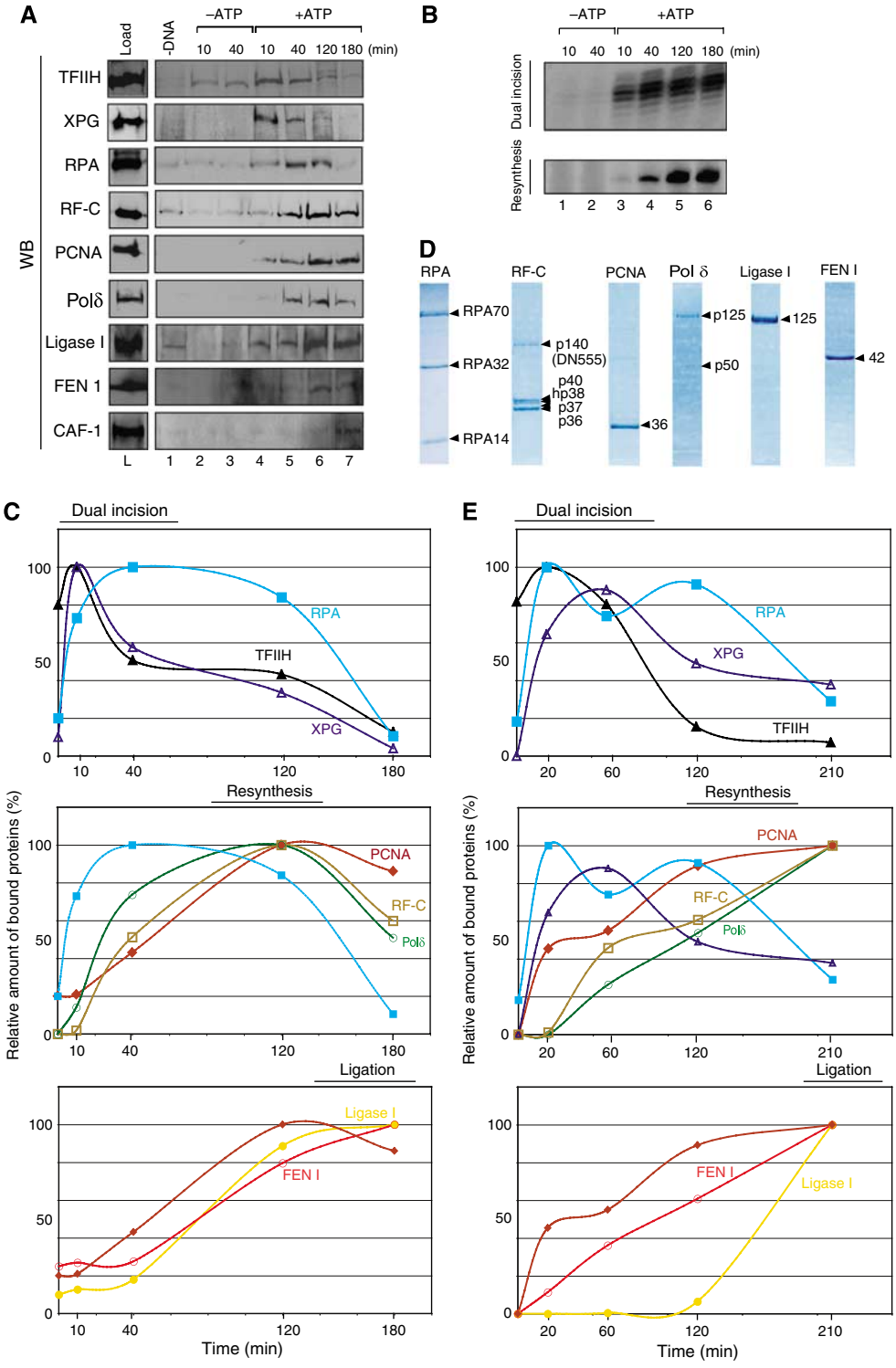

Figure 2 *In vitro* sequential recruitment of the NER factors. (**A**) The immobilized damaged DNA fragment was incubated with NE. At different time points, the immobilized DNA was washed with 0.05 M KCl and the remaining bound factors were further analysed by western blot. (**B**) The damaged fragment removal (Dual Incision) and the gap filling (Resynthesis) activities were also followed through time (Supplementary data 1). (**C**) The WB signals were quantified using Genetool (Syngene) and plotted on the graphs as a percentage of the maximal binding to the DNA. (**D**) Coomassie staining of the highly purified human NER resynthesis factors RPA, RF-C, PCNA, Polδ, Ligase I and FEN 1. (**E**) The same recruitment experiment as in (B) was carried out with our complete reconstituted system (dual incision, resynthesis and ligation factors). All these experiments were carried out at least two times.

To document the role of XPG and RPA in the recruitment of certain RRS factors, we incubated the immobilized cisplatinated DNA with RIS and a combination of RRS factors (Figure 3C, Incub I). The resulting gapped DNA/protein complex was washed at 0.05 M KCl and further incubated with a combination of RRS factors (Incub II). In such a

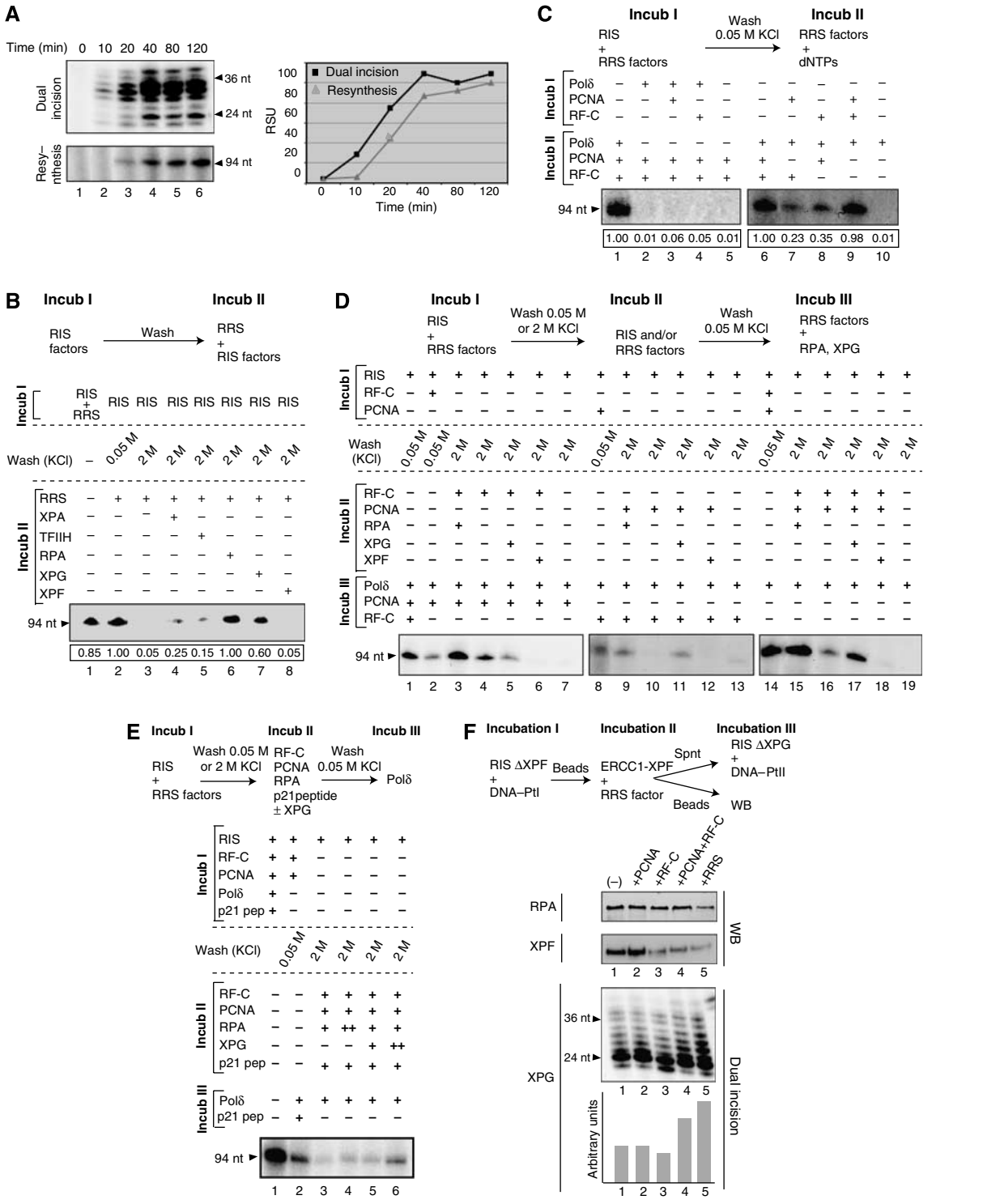

Figure 3 Molecular events during the resynthesis. (**A**) Time course of the dual incision (upper panel) and the resynthesis (middle panel). Signals were quantified (Genetool, Syngene) and plotted in a graph (square for dual incision; triangle for DNA resynthesis) (lower panel). (**B**) The immobilized DNA-Pt was incubated with RIS + /−RRS (Incub I). After washes at either 0.05 M KCl or 2 M KCl, DNA resynthesis were performed using RRS supplemented with some of the RIS factors as indicated (Incub II). The relative intensity of each signal is indicated at the bottom of the gel. (**C**) DNA-Pt was incubated with RIS either alone or in combination with the indicated RRS factors (Incub I). After 0.05 M KCl wash, the complementary RRS factors were added for DNA resynthesis (Incub II). The relative intensity of each signal is indicated at the bottom of the gel. (**D**) DNA-Pt was incubated with RIS either alone or with the indicated RRS factors (Incub I). After washes with either 0.05 M KCl or 2 M KCl, Pt-DNA was further incubated with the indicated RIS and/or the RRS factors (Incub II). Following a 0.05 M KCl wash, and the addition of the complementary RRS factors (Incub III), DNA resynthesis was checked. (**E**) The experiment was carried out similarly as described in (D), except that we added 2 nmol of a peptide corresponding to the domain of interaction between the p21 CDK inhibitor and PCNA as indicated. (**F**) RIS-ΔXPF and DNA-Pt were incubated with the RRS factors as indicated at the top of the panels. Following Incub I + Incub II, and a 0.05 M KCl wash, amounts of RPA (upper panel) and XPF (middle panel) remaining on the DNA fragment were checked by western blot. The released XPG (lower panel) was tested in dual incision with RIS-ΔXPG, containing DNA-PtII as a challenge template (Riedl *et al*, 2003). A graph depicts the relative intensity of each signal.

complementation assay, the level of the resynthesis activity demonstrates whether the various factors are correctly integrated in an active intermediary complex that depends on accurate protein/protein interactions. Incubation of the RIS with Polδ alone or in combination with PCNA or RF-C (Incub I) did not allow DNA resynthesis (lanes 2–4). The incubation of the RIS with either PCNA or RF-C resulted in a weak DNA resynthesis (lanes 7–8), unless they were added together, in which case, optimal recruitment of the Polδ and DNA resynthesis is observed (lane 9). It thus seems that XPG and RPA, together with RF-C and PCNA constitute the loading platform for Polδ.

The recruitment of RF-C/PCNA on the gapped DNA was examined by carrying out a three-step resynthesis complementation experiment (Figure 3D). The gapped DNA obtained after incubation with RIS (Incub I) was washed at 2 M KCl and incubated (Incub II) with a combination of XPG, RPA, XPF/ERCC1, RF-C and PCNA. After being washed at 0.05 M KCl, the immobilized DNA was incubated with RRS factors as indicated (Incub III) to complement the resynthesis reaction. RPA (Incub II) stimulated the recruitment and/or the stabilization of RF-C, whereas XPG and XPF weakened or even prevented its binding (Figure 3D, lanes 3–6). Both XPG and RPA were able to place and stabilize the PCNA molecules present around the gapped DNA (lanes 9–12). Accordingly, RF-C + PCNA were recruited and stabilized by XPG with the assistance of RPA (lanes 15–17). We noticed that the binding of RF-C + PCNA to the gapped DNA was prevented by the presence of XPF (lane 18). These data further illustrate that the recruitment and stabilization of RFC and PCNA, which are likely to be mediated by RPA and XPG, respectively, first require the release of XPF from the DNA substrate.

To further illustrate the new role of XPG in initiating DNA resynthesis, we carried out a similar assay as in Figure 3D in which we used a peptide corresponding to the p21 domain (p21-pep) that interacts with PCNA (Cooper *et al*, 1999). Indeed, the p21 cyclin-dependent kinase inhibitor is known to induce cell-cycle arrest by preventing the PCNA/Polδ interaction (Oku *et al*, 1998). Preincubation of p21-pep with the PCNA/RF-C/RPA complex bound to the gapped DNA (Incub II) does not allow Polδ (Incub III) to synthesize DNA (Figure 3E, compare lanes 3 and 4 to lane 1). DNA resynthesis was restored upon the addition of increasing amounts of XPG (lanes 5 and 6) but not RPA (lanes 3 and 4), demonstrating a competition between XPG and p21-pep for targeting PCNA. This underlines the role of XPG in stabilizing the replication complex.

To investigate the fate of XPF, RPA and XPG, we incubated the immobilized damaged DNA with XPC, TFIIH, XPA, RPA and XPG before addition of XPF/ERCC1 together with a combination of the RRS (Figure 3F). After a wash at 0.05 M KCl, RPA and XPF remaining on the DNA were analysed by western blot, and the supernatant was tested for dual incision activity to check the release of XPG. The release of RPA only occurred upon simultaneous addition of PCNA, RF-C and Polδ (Figure 3F, upper panel). This result showed that RPA is released as soon as the Polδ is recruited. Similarly, the recruitment of RF-C alone or combined with PCNA, promotes the release of XPF (middle panel). Dual incision assays carried out on the supernatant of the various fractions showed that the release of XPG was stimulated upon the addition of RF-C + PCNA together and evenmoreso in the presence of the complete RRS (lower panel and quantifications).

Altogether, our data strongly show that in the context of NER, XPG and RPA are the key players in initiating DNA resynthesis, a replication-like process. We have demonstrated that XPG recruits and stabilizes PCNA to the gapped DNA with its replicative partners RF-C and Polδ. Our results even suggest that this interaction could protect PCNA from inhibitors like p21.

The ligation of the newly synthesized DNA fragment

Finally, we checked whether the newly synthesized DNA strand was ligated to the DNA template. After being incubated with NE and radiolabelled nucleotides (Supplementary data 3A), the immobilized DNA was digested by a combination of restriction enzymes (*Ban*I and *Apa*LI or *Cla*I or *Eco*RV, see Figure 4A). Whether the digested fragments are radiolabelled or not is dependent on the number of radiolabelled dCTPs incorporated per DNA molecule and also on the position of the ligation site. For instance, (i) digestion by *Apa*LI and *Ban*I, resulted in 156 and 201 bp labelled fragments demonstrating that DNA resynthesis encompassed the restored *Apa*LI site (Figure 4A and B, lane 1), (ii) digestion by *Ban*I and *Cla*I resulted in the 140 and 217 bp labelled fragments (lane 2), and (iii) digestion by *Eco*RV and *Ban*I resulted in a single labelled 261 bp DNA fragment, demonstrating that the ligation occurs between the *Eco*RV and *Cla*I sites (beyond the 3′ XPG incision site, which is located on the *Cla*I site) (lane 3). Therefore, the NER ligation occurred after a 'nick translation' process originated by the ongoing Polδ (Ayyagari *et al*, 2003). Knowing the number of incorporated radiolabelled dCTPs in the 201 and 217 bp fragments and their intensity on the radiograph, it is possible to estimate the number of incorporated radiolabelled dCTPs in the ligated fragments and thus determine the position of the ligation site. This one is located around 10 nucleotides after the XPG incision site when using Ligase I and FEN1.

We identified LigaseI/FEN1 (Figure 2A) as candidates to carry out the ligation once DNA resynthesis was performed on the damaged DNA (Shivji *et al*, 1995; Araujo *et al*, 2000). The immobilized DNA was then incubated with RIS + RRS in addition to Ligase I and FEN1 as indicated in Figure 4D, before digestion by *Eco*RV and *Ban*I. We observed that (i) the DNA fragments of a size smaller than 160 nt result from the nick translation process, bypassing the *Eco*RV restriction site (lane 1) and escaping ligation; (ii) the products between 180 and 250 nt are characteristic of unligated DNA between the damage site and the *Eco*RV site; (iii) the ligated DNA corresponds to the 261 nt signal (lanes 1–5). Addition of Ligase I did not allow a proper ligation of the newly synthesized DNA (lane 2) unless associated with FEN1 (lanes 3 and 4). Indeed, FEN1 works in association with the Polδ and Ligase I to limit the nick translation process and, therefore, stimulates the ligation. However, FEN1 does not block the ongoing Polδ or promote the ligation by itself (lane 5) (Ayyagari *et al*, 2003).

Ligase III and XRCC1 are known to be involved in base-excision repair and single-strand break repair pathways (Mortusewicz *et al*, 2006; Almeida and Sobol, 2007). Recent data suggest that both factors are also involved in the NER ligation (Moser *et al*, 2007). Therefore, we checked over the ligation activity of Ligase III and XRCC1 after a Polδ-dependent resynthesis. We have found that these two factors,

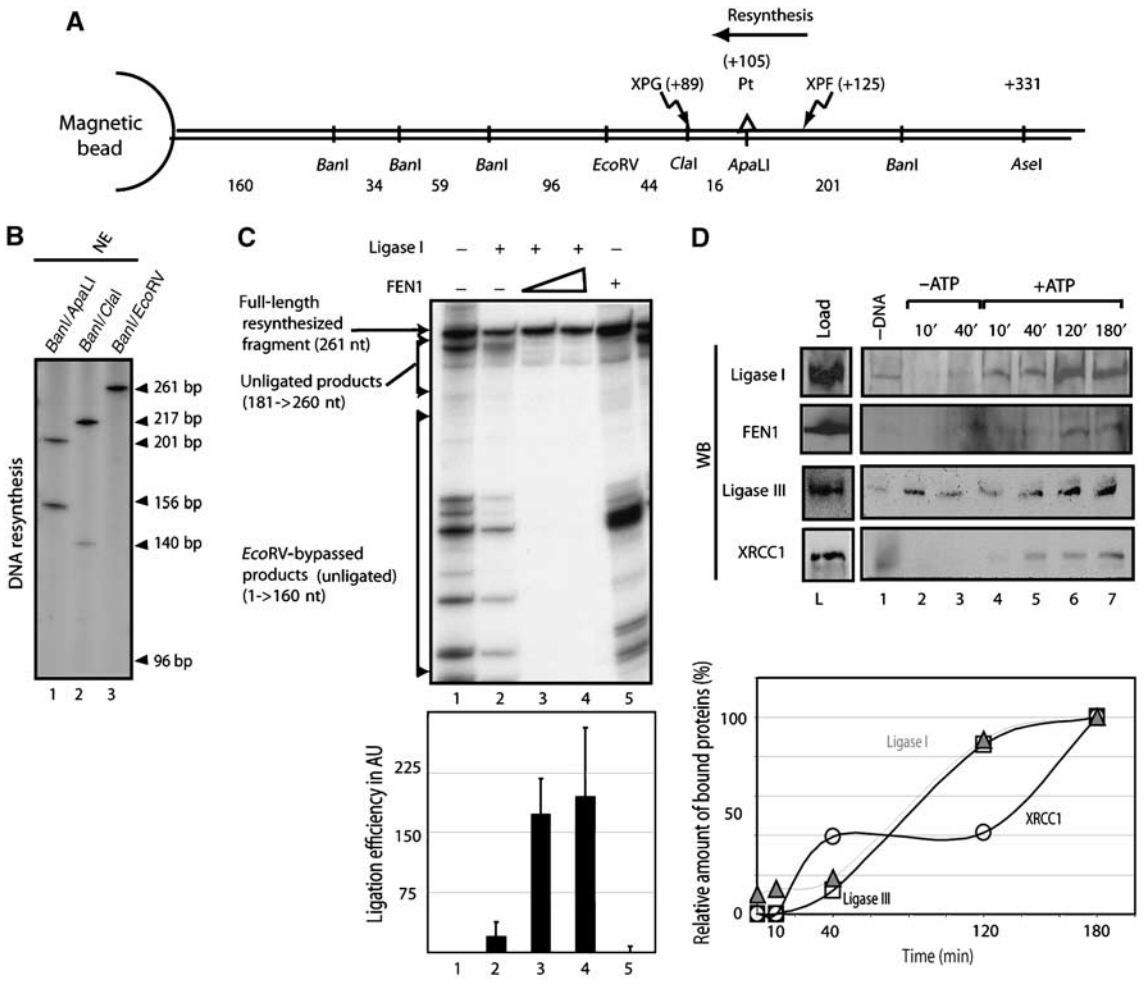

Figure 4 The ligation in NER. (**A**) Scheme of the substrate used with the position of the lesion, the restriction sites and the endonucleases cut sites. The sense of resynthesis is indicated. (**B**) DNA-Pt was incubated with a NE. The repaired DNA was then digested with indicated restriction enzymes. The absence of the 96 nt signal proved that the ligation occurred between *Cla*I and *Eco*RV (lane3). (**C**) The ligation activity was investigated by incubating the DNA with the RIS + RRS and combinations of the ligation system (RLS) before digestion by *Eco*RV and *Ban*I. The unligated DNA due to the absence of ligation could be easily discriminated from the unligated DNA due to nick translation process, bypassing the *Eco*RV site. To evaluate the ligation efficiency, the ratio between the full-length resynthesized DNA and all the forms of unligated DNA was calculated. (**D**) The recruitment of Ligase III and XRCC1 on the damaged DNA was investigated with a method described in Figure 2. Western blot (WB) signals (upper panel) were quantified and plotted (lower panel) (open circle: XRCC1; open triangle: Ligase III).

coming from a NE, were recruited to the damaged DNA following a kinetic close to that of LigaseI and FEN1 (Figure 4D); however, their ligation activities were much lower (Supplementary data 3).

Biochemical defects originating from mutations in NER factors

To unravel the sequential interactions between the NER factors and to determine the exact role of XPG in recruiting the replicative complex, we investigated how mutations found within XP and TTD patients (Figure 5A) disturb NER at the molecular level. The following experiments were performed (as described in Figure 2) using NE from the XP, XP/CS or TTD patients or using recombinant NER proteins. Each of these mutated proteins prevents the elimination of the DNA damage, as we could not generate any dual incision or resynthesis activity (Figure 5B).

By using a mutated XPC in which the C-terminus was deleted (XPC/C814st), we observed a delay and a decrease in the binding of this mutant, TFIIH and RPA to the damaged DNA. We detected minimal opening of the DNA when XPC + TFIIH were bound in the presence of ATP (Figures 2C and 5C; unpublished results). Under those conditions, XPG cannot be recruited (Figure 5C), which explains the absence of dual incision and why PCNA and the other factors cannot target the DNA despite the presence of RPA. This demonstrates how crucial the simultaneous presence of RPA and XPG are for recruiting PCNA, which is essential for DNA resynthesis (as also observed Figure 3D).

TFIIH containing the XPB/fs740, which was shown to prevent the removal of the damaged oligonucleotide (Araujo *et al*, 2000), was incubated with the complete reconstituted system (Figure 5E). Although the recruitment of TFIIH/XPB/fs740, RPA, XPG and XPF dual incision factors is the same as with TFIIHwt (Figures 2E and 5E), their release from the immobilized DNA does not occur (Oh *et al*, 2007). Although this XPB mutation allows DNA opening as well as the recruitment of XPF and XPG, we only observed the 3′ cut performed by XPG (Coin *et al*, 2004) with XPF being present but inactive (Figure 5E). As a consequence, the recruitment of

A

Factor	Mutation	Phenotype	Repair deficiency	References
XPC	C814 Stop	XP-C	Damage recognition	Unpublished data
XPD	R683 W	XP-D	p44/XPD interaction	Dubaele *et al*, 2002
p8	R56 Stop	TTD-A	DNA opening	Coin *et al*, 2006
XPB	fs741	XP-F like	5′ XPF cut	Coin *et al*, 2004
XPA	R228 Stop	XP-A	Damage recognition	Unpublished data
XPG	K917fs962fs1186	XP-G	3′ XPG cut	Lalle *et al*, 2002

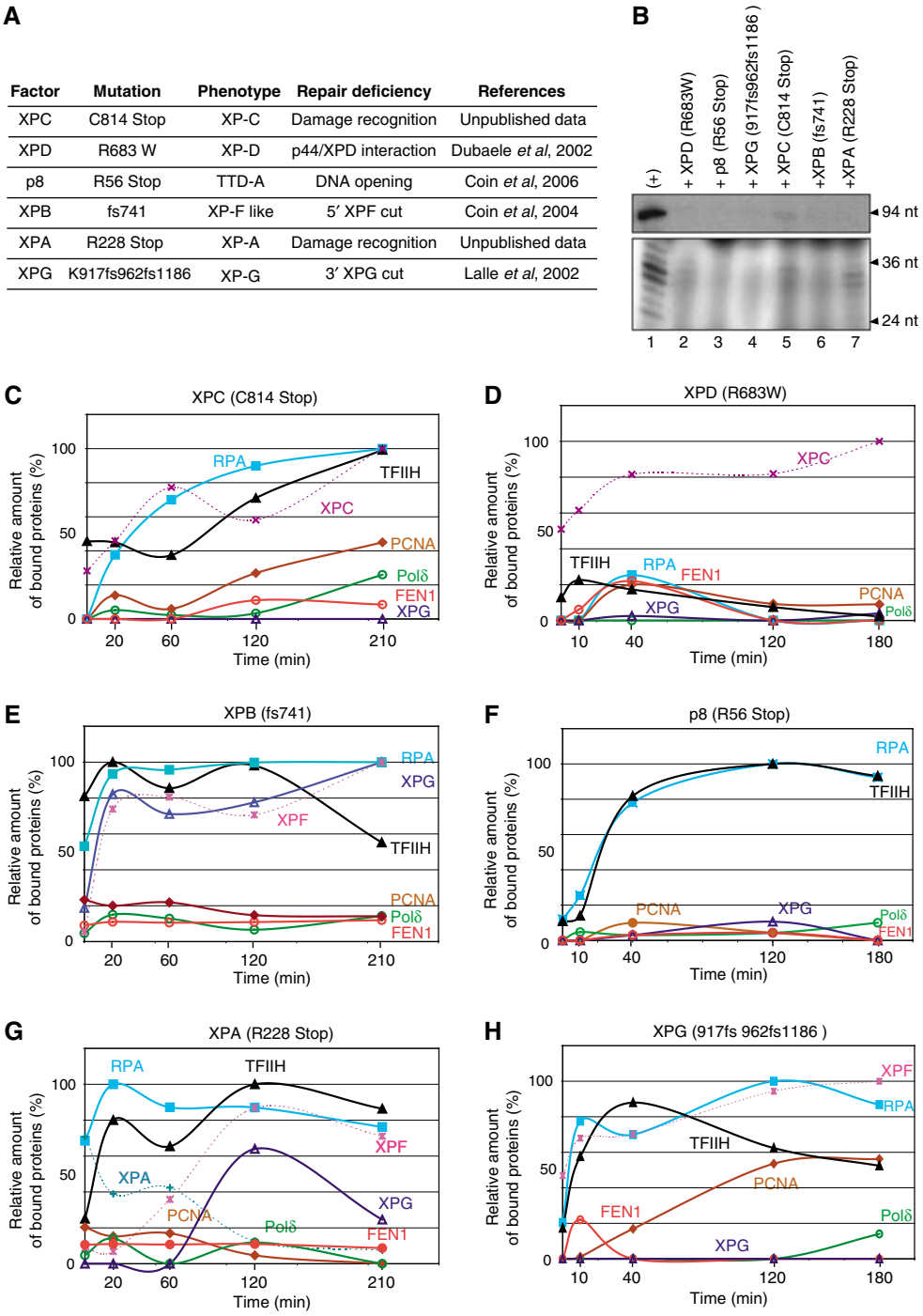

Figure 5 Recruitment of the NER machinery involving mutated factors. (**A**) Data concerning the mutated factors used and references. (**B**) Dual incision and resynthesis assays were carried out with each mutant (lanes 2–8). WT factors were used for the positive control (lane 1). The recruitment analysis of the NER factors was carried out as described previously with mutated forms of (**C**) XPC, (**E**) XPB and (**G**) XPA used in a reconstituted system and (**D**) XPD, (**F**) p8 and (**H**) XPG coming from patient cell extracts.

PCNA (and partners such as Polδ and RF-C) that requires the XPF release from the damaged DNA, does not occur. These data (Figures 2E and 5E) demonstrate that the recruitment of PCNA is dependent not only on the presence of both XPG and RPA on the damaged DNA but also on the 5′ incision by XPF.

We next focused our attention on the XPD/R683W mutation that weakens the XPD/p44 interaction within TFIIH and inhibits the XPD helicase activity (Dubaele *et al*, 2003). XPCwt that was bound to the immobilized damaged DNA is unable to recruit and/or stabilize TFIIH/XPD/R683W, explaining the absence of the other NER factors (Figure 5D). It thus can be concluded that preserving the protein/protein interactions as well as the optimal XPD helicase activity of TFIIH is a prerequisite for the formation of the ternary XPC/TFIIH/damaged DNA complex and the recruitment of the other NER factors.

TFIIH containing the TTD-A/p8/R56st mutated subunit present in cell extracts from patients can still bind to the

XPC/damaged DNA complex (Figure 5F). Neither XPG nor the resynthesis factors can be recruited. Interestingly, we noticed that RPA binds to the damaged DNA already targeted by TFIIH (Figure 5E–H), suggesting the presence of a single-stranded DNA (ssDNA) region (Bochkareva *et al*, 2001). It thus seems that the p8 defect in stimulating the XPB ATPase activity (Coin *et al*, 2006) impairs not only the proper damaged DNA opening but also the further recruitment of NER factors.

XPA/R228st does not efficiently bind to the damaged DNA already targeted by XPC, TFIIH and RPA (Figure 5G). As XPA/R228stop is unable to maintain the opened damaged DNA (Tapias *et al*, 2004; unpublished results), the recruitment of XPF is delayed and not optimal (Figure 5G) in agreement with published data (Volker *et al*, 2001; Oh *et al*, 2007). XPG has been recently shown to be tightly associated to TFIIH (Ito *et al*, 2007) explaining its early recruitment *in vivo* (Volker *et al*, 2001). By using highly purified TFIIH, it could be explained why we observed an XPG recruitment in an XPA-dependent manner (Riedl *et al*, 2003; Figure 5G). The delayed recruitment of the endonucleases might explain the deficiency in the NER activity (Figure 5B, lane 7). In such conditions, although XPG and RPA are present on the damaged DNA, neither PCNA nor Polδ can be recruited.

XPG/917fs962fs1186 present in the XP3BR patient cell extract (Lalle *et al*, 2002), did not bind to the damaged DNA already opened by TFIIH and XPA (Figure 5H; Evans *et al*, 1997). We also noticed the presence of RPA and XPF. Despite the absence of XPG and the lack of the 3′ incision, PCNA is partially recruited. At this point, the absence of Polδ and the partial recruitment of PCNA corroborate the role of XPG in stabilizing PCNA to the gapped DNA.

Discussion

To address the biochemical mechanisms underlying the coordination between the various proteins required for NER, we employed the immobilized template assay. This approach combines the recruitment of the factors with functional enzymatic assays allowing NER to occur. Having identified the main components of the NER, we thus were able to follow the NER reaction in which 11 highly purified factors (representing 33 polypeptides) repair the immobilized damaged DNA, thereby mimicking the *in vivo* situation. In addition, using NER factors that contain mutations found in XP and TTD patients, we were able to pinpoint the biochemical defects. Finally, the present study provides new insights into the mechanisms by which dual incision and repair synthesis factors work together to fill the gap formed after dual incision, which contributes to a more detailed understanding of the first steps of NER (Figure 6).

From damage recognition to DNA incision

The distortion induced by the lesion is first recognized by the XPC/HR23B complex (Sugasawa *et al*, 1998; Mocquet *et al*, 2007), which then recruits TFIIH (Volker *et al*, 2001; Riedl *et al*, 2003). Here, we observed the essential role of XPC in positioning TFIIH (Tapias *et al*, 2004), allowing it to unwind the damaged DNA.

In the presence of ATP, p44 stimulates the XPD helicase activity (Dubaele *et al*, 2003), whereas p52, upon p8 mediation, stimulates the XPB ATPase activity (Coin *et al*, 2006,

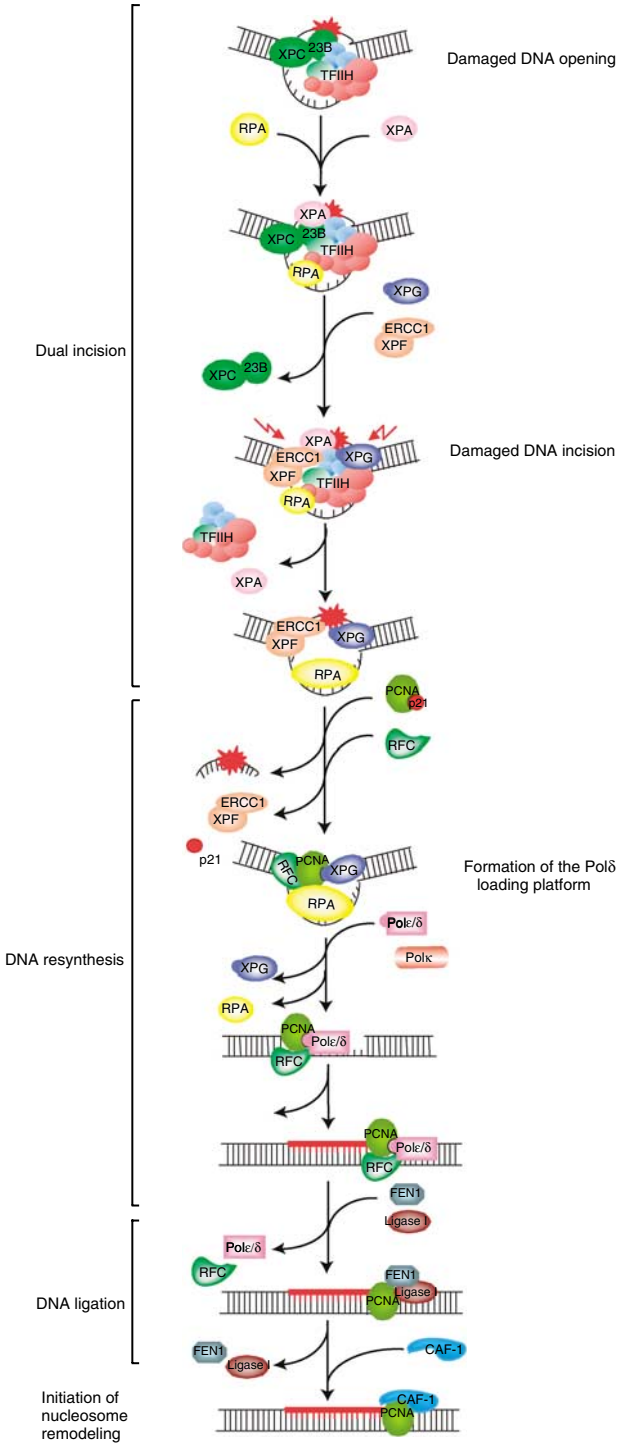

Figure 6 Sequential complexes in NER from the damage recognition to the ligation of newly synthesized DNA fragment. The function of the damaged DNA/XPC–HR23B/TFIIH complex was previously described by Riedl *et al* (2003) and Coin *et al* (2004, 2006, 2007). After the recruitment of XPA, RPA, XPG and finally XPF, the incision by the endonucleases allows the removal of the patch and the recruitment of the resynthesis factors. RF-C is stabilized by RPA and induces the release of XPF, whereas PCNA is stabilized by XPG and RPA. The presence of XPG could protect PCNA from the inhibitory effect of p21. The further recruitment of the Polδ is then possible and provokes the release of XPG (which protective effect is not any more needed) and RPA. After gap filling, we suggest that FEN1 and Ligase I stop the ongoing Polδ due to interactions with PCNA and allow ligation. Finally, the nucleosome assembly is carried out with by CAF-1.

2007). This allows the opening of the damaged DNA, a prerequisite for the recruitment of the other factors. Mutations in either XPD or in p8/TTD-A, disturb the opening, thus underlining their specific roles—XPD/R683W mutation prevents TFIIH recruitment, p8/TTD-A allows the recruitment of TFIIH and RPA—which suggest that the presence of TFIIH *per se* is sufficient to promote the recruitment of RPA. Corroborating these observations, we also found that the use of XPA/R228st does not impair the arrival of RPA (Figure 5G; Rademakers *et al*, 2003), that is likely to be devoted to single-stranded-DNA protection (Bochkareva *et al*, 2001). We do not exclude the possibility that XPA and RPA might bind to the DNA synergistically; the stabilization of the opened DNA by XPA may favour the positioning of the RPA molecules.

XPA then stabilizes the ternary complex and increases the local DNA unwinding around the lesion (Evans *et al*, 1997; Tapias *et al*, 2004), allowing the recruitment of both endonucleases XPG and ERCC1-XPF, which incise the damaged oligonucleotide at the 3′ and 5′ sides, respectively (O'Donovan *et al*, 1994; Sijbers *et al*, 1996). During the course of the dual incision, XPC is recycled concomitantly with the positioning of XPG, respectively (Riedl *et al*, 2003). TFIIH and XPA are most likely released after the incision/excision of the damaged oligonucleotide, as a 40 aa residue of XPB is necessary for the ERCC1-XPF cut (Figure 5B), and XPA stimulates the activities of endonucleases (Bartels and Lambert, 2007).

RPA and XPG mediate the transition between dual incision and resynthesis

At this point, it is worthwhile to note that not only RPA but also the 5′ endonuclease XPG plays a central role in initiating the DNA resynthesis step, a 'replication-like process'. Our data demonstrate that dual incision and DNA resynthesis are closely coordinated and that such a transition requires (i) the DNA opening by TFIIH (Figures 2C and 5E), (ii) the XPF incision (Figure 5E) and (iii) the simultaneous presence of RPA and XPG on the damaged DNA (Figure 5). Furthermore, XPG then would be responsible for recruiting PCNA either alone or together with RF-C (Gary *et al*, 1999; Miura, 1999) to the vicinity of the gap (Figure 3). Simultaneously with the recruitment of PCNA by XPG, RF-C interacts with (Yuzhakov *et al*, 1999) and is recruited by RPA onto the 30 nt gap (Figure 3). This would consequently lead to position RF-C together with PCNA at the 3′ primer template junction, as the loading of RF-C + PCNA is associated with the release of the XPF endonuclease as well as to the damaged oligonucleotide (Figures 3D, F; data not shown).

A phospho/dephosphorylation process occurring after UV stress, may direct RPA towards either the repair or replication pathways (Henricksen *et al*, 1996). Similarly, the phosphorylation of XPG (Winkler *et al*, 2001) could modulate its own activity and the recruitment of the resynthesis factors. This kind of partnership promoted by potential post-translational modifications (Araujo and Wood, 1999) would speed up the transition between dual incision and DNA resynthesis and, thus, the efficiency of the global repair. Additionally, this would avoid the persistence of multiple small ssDNA gaps, which could lead to double-strand breaks, as already observed in XP/CS cells (Berneburg *et al*, 2000; Theron *et al*, 2005).

The recruitment of Polδ is associated with the release of XPG and RPA

During NER, RFC and PCNA form a complex similar to what occurs during replication with the sliding clamp (Tsurimoto and Stillman, 1991) and dependent on the RF-C ATPase activity (Gomes *et al*, 2001). Together with RPA and XPG, these proteins constitute a stable platform ready to be targeted by Polδ (Nishida *et al*, 1988; Zeng *et al*, 1994). It is worthwhile to point out the role of XPG in both stabilizing and recruiting PCNA on the gapped DNA, which might prevent inhibition of DNA resynthesis by p21 (Shivji *et al*, 1998). Then the release of XPG (Figure 3) parallels the initiation of DNA resynthesis. As the DNA synthesis proceeds, RPA, which protects ssDNA (Bochkareva *et al*, 2001), is also released. We do not exclude the possibility that other DNA polymerases could substitute for Polδ in the NER process. Despite the fact that Polε and Polκ are suggested to be candidates for NER *in vivo* (Shivji *et al*, 1995; Ogi and Lehmann, 2006), our confocal microscopy experiments (Figure 1) as well as the immobilized template assay with NE (Figure 2) clearly identified Polδ at the sites of damage. Recent findings corroborate this point (Moser *et al*, 2007).

PCNA mediates the transition between resynthesis and ligation

The last phase of the NER pathway starts with the arrival of FEN 1 and Ligase I (Prigent *et al*, 1994; Araujo *et al*, 2000), which occurs concomitantly with the release of RF-C and Polδ (Figure 4B). PCNA, still present on the DNA, would bridge the resynthesis and the ligation steps, in a pathway similar to what occurs during the maturation of the Okazaki fragments (Montecucco *et al*, 1998). As Polδ, FEN1 and Ligase I target the same PCNA domain (Warbrick, 2000), it is likely that they are sequentially recruited onto PCNA and that their presence is mutually exclusive. In a manner similar to RPA, PCNA is subjected to post-translational modifications such as ubiquitination after UV irradiation (Essers *et al*, 2005).

It has recently been shown that Ligase III associated with XRCC1 could be involved in the ligation step of NER in a cell-cycle-dependant manner and following DNA resynthesis by Polδ (Moser *et al*, 2007). With our non-synchronized cell extracts, we indeed observed their recruitment in the later times of the reaction. Nevertheless in our hands, using Polδ, their ligation efficiency is lower than the one performed with Ligase I and FEN1. It is possible that the Ligase III/XRCC1 efficiency could be improved with other DNA polymerases such as Polε (Shivji *et al*, 1995) or Polκ (Ogi and Lehmann, 2006). It thus can be hypothesized that different resynthesis and ligation systems can coexist in the cell and their involvement in DNA repair would depend on their availability at the time of repair.

Finally, we observed the late arrival of CAF-1, likely through PCNA, once the ligation had occurred (Figures 1 and 2A). Indeed, the naked repaired DNA has to be re-chromatinized and the chaperone CAF-1 can recruit histones H3 and H4 (Gaillard *et al*, 1996; Green and Almouzni, 2003; Polo *et al*, 2006). Its presence on the DNA would suggest that nucleosome reassembly or repositioning on the naked DNA starts as soon as the DNA repair process has finished.

While some of the steps of NER-like damage recognition, DNA opening or incision by the endonucleases have

been described many times, the transition between the dual incision and the resynthesis, which is as important as the previous steps for an accurate, quick and errorless DNA restoration, had never been described before (Gillet and Scharer, 2006). Thus, our work underlines the complexity of the network of interactions between NER factors that can modulate their stability, positioning on the damaged DNA and their respective enzymatic activities. Moreover, it allows us to establish and locate the biochemical defects associated with mutations found in patients suffering from repair syndromes.

Materials and methods

Local irradiation and immunofluorescence

XPCS2BA fibroblasts (T293C (F99S) transition), derived from XP\CS patients and corrected (Riou *et al*, 1999), were grown in F10 (Ham) media (Gibco-BRL), 12% FCS and 10 mg/ml gentamicyn in 5% CO_2 for 2 days in two chambers slides (Labtek® II chamber slide w/Cover 2 wells). Cells were rinsed in PBS, individually covered with an Isopore 3.0 μm filter (Millipore) and irradiated locally at 254 nm with 70 J m^{-2} (Volker *et al*, 2001). Following incubation at 37°C, cells were treated as described by Green and Almouzni (2003) to perform immunofluorescence analysis.

Proteins

XPC/HR23B, TFIIH, XPA, RPA, XPG and ERCC1-XPF recombinant proteins involved in dual incision (Aboussekhra *et al*, 1995) have been produced and highly purified as described previously (Araujo *et al*, 2000; Tapias *et al*, 2004). PCNA (Biggerstaff and Wood, 2006), DNA Ligase I (Mackenney *et al*, 1997) and FEN1 (Robins *et al*, 1994) have been purified from *Escherichia coli*. Polδ (Xie *et al*, 2002) as well as RFC (Podust and Fanning, 1997) were produced in insect cells and purified accordingly. Mutated forms of recombinant XPC (C814st), XPA (R228st) and XPB (fs741) as well as extracts from TTDA (p8/R56st), XPD (R683W) and XPG (917fs962fs1186) patients were also used in Figure 5.

DNA substrates

The damaged DNA substrates contain a single 1,3-intrastrand d(GpTpG) cisplatin–DNA crosslink (Shivji *et al*, 1999) and are based on the 105.TS plasmid (Frit *et al*, 2002) known as DNA-Pt. The immobilized damaged substrate was prepared as explained by Lainé *et al* (2006): the DNA was first digested by *Fok*I and then biotin was incorporated to the DNA by incubation of 1 mM of each biotin-dUTP (USB), dCTP and dGTP with Kleenow enzyme (New England Biolabs). Finally, the DNA was digested by *Ase*I and the 722-bp fragment was further purified. A 3 μl volume of Dynabeads M-280 streptavidin were mixed with each 100 ng of DNA in buffer A (10 mM Tris–HCl pH7.2, 1 mM EDTA, 1 M NaCl, 0.02% and NP-40) for 20 min at room temperature. The immobilized damaged DNA was washed two times in buffer B (50 mM HEPES-KOH pH 7.6, 20 mM Tris–HCl pH 7.6, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 10% glycerol and 0.02% NP-40) before being used.

Protein-binding studies on immobilized DNA

A 100 ng portion of immobilized DNA was incubated with cells extracts [Hela(wt), TTD1BR(TTD-A), XP3BR(XP-G), HD2(XP-D)] or with the purified DNA repair factors mutated or not, as indicated. Magnetic beads were collected and supernatants removed. Beads were then washed three times in 200 μl of cold buffer B containing either 50 mM or 2 M KCl and either resuspended in SDS–PAGE loading buffer for western blotting or in a reaction mixture for functional analysis of bound proteins. Studies were carried out with the equivalent of four dual incision reactions for western blotting analysis and with the equivalent of one dual incision reaction for the functional protein-binding assay. Each experiment as been carried out at least two times.

Dual incision and DNA resynthesis assays

Reconstituted dual incision reactions (25 μl) were carried out in a buffer B (Araujo *et al*, 2000; Lainé *et al*, 2006). The dual incision factors XPC-HR23B (50 ng), TFIIH (100 ng), XPA (30 ng), RPA (200 ng), XPG (150 ng) and XPF-ERCC1 (50 ng) were incubated with 100 ng of the immobilized damaged DNA, for indicated times at 30°C in the presence of 2 mM ATP.

Reconstituted resynthesis reactions were carried out in identical buffers as dual incision assay with RIS in addition to PCNA (70 ng), RF-C (150 ng) and Polδ (300 ng). Ligase I (200 ng) and FEN 1 (50 ng) were added when indicated. Proteins were incubated with a mixture of 20 μM of each dATP, dGTP, dTTP, 5 μM of cold dCTP and 2.5 μCi α32P dCTP (3000 Ci/mmol) at 30°C for 2 h. The DNA was washed with buffer A and digested with *Eco*RI and *Nde*I, resulting in a 94-nt fragment containing the resynthesized DNA patch. Restriction reactions were loaded on a denaturating 8% polyacrylamide gel and visualized by autoradiography. Resynthesis with HeLa NE (50 μg) (Dignam *et al*, 1983) was performed in the same conditions as dual incision (Shivji *et al*, 1995) with 20 μM of each dATP, dGTP, dTTP, 5 μM of cold dCTP and 2.5 μCi α ^{32}P dCTP (3000 Ci/mmol). The mixture was incubated at 30°C for the indicated times. The DNA fragment was then purified and digested by *Eco*RI and *Nde*I as described above.

Antibodies

Mouse monoclonal (MAb) and rabbit polyclonal (PAb) antibodies were used as primary antibodies by Riedl *et al* (2003). Following antibodies were used in this study: TFIIH (p62): MAb 3C9 and (p89) S-19: sc-293 (Santa Cruz Biotechnology); RPA32/70: PAb N2.2 (Henricksen *et al*, 1994); XPG: MAb 1B5 raised against peptide amino acids 1167–1186; XPF: MAb Ab-5 (NeoMarkers); PCNA: MAb (PC10): sc-56 (Santa Cruz Biotechnology) and PAb ab2426 (AbCam) (Green and Almouzni, 2003); RF-C: PAb (H-183): sc-20996 (Santa Cruz Biotechnology) (Beckwith *et al*, 1998); DNA Polymerase δ: MAb (A-9): sc-17776 (Santa Cruz Biotechnology) (Zeng *et al*, 1994); FEN 1: PAb (H-300): sc-13051 (Santa Cruz Biotechnology) (Waga *et al*, 1994); Ligase I PAb raised against amino acids 901–919; CAF-1: MAb (SS1 1–13): NB 500–207 (NeoMarkers) (Green and Almouzni, 2003); CPD: MAb TDM2 D194-1 (MBL) (Coin *et al*, 2006).

Supplementary data

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

Acknowledgements

We thank F Coin, R Velez Cruz and B Bernardes de Jesus for fruitful discussions and critical reading of the paper. We thank C Braun and I Kolb for invaluable assistance. We thank also B Stillman for the RF-C p140 antibody, RD Wood for the PCNA expression vector, W Bohr for the FEN1 expression vector, J Hurwitz for the RF-C baculoviruses expression vectors, T Lindahl for the ligase I and III expression vector and P Radicella for XRCC1 expression plasmid. This study has been supported by funds from 'La Ligue contre le Cancer' (équipe labellisée, contract no EL2004), the 'Ministère de l'Education National et de la Recherche', the ACI grants (BCMS no 03 2 535) and the Agence Nationale de la Recherche (ANR-05-MRAR-005-01). M Lee's lab was supported by Phillip Morris USA Inc., Phillip Morris Inernational. VM has been supported by the Institut National de la Santé et de la Recherche Médicale (INSERM/region Alsace fellowship), la Ligue contre le Cancer et l'Association de la Recherche contre le Cancer. JPL was granted by the Association de la Recherche contre le Cancer and EEC grant (QLRT-1999-2002).

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