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Nucleotide excision repair : a multi-step mechanism required to maintain genome integrity

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REPLICATION PROTEIN A SAFEGUARDS GENOME INTEGRITY BY CONTROLLING NER INCISION EVENTS

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Submitted

SUMMARY

Single stranded DNA gaps that might arise by futile repair processes can lead to mutagenic events and challenge genome integrity. Nucleotide excision repair (NER) is an evolutionary conserved repair mechanism, essential for removal of helix-distorting DNA lesions. In the currently prevailing model, NER operates through coordinated assembly of repair factors into pre- and post-incision complexes; however, its regulation in vivo is poorly understood. Notably, the transition from dual incision to repair-synthesis should be rigidly synchronized as it might lead to unprocessed repair intermediates. Employing a novel approach of sequential UV-irradiations we monitored NER regulatory events in vivo. Under conditions that allow incision yet prevent completion of repair-synthesis or ligation, pre-incision factors can disassemble and associate with new damage sites. In contrast, RPA remains associated at the incomplete NER sites regulating a feedback loop from complete repair-synthesis to subsequent damage recognition independently of ATR signaling. Our data reveal a pivotal role of RPA in averting further generation of DNA strand breaks that could lead to mutagenic and recombinogenic events.

INTRODUCTION

To counteract genotoxic challenges and maintain genomic integrity, cells have evolved an interrelated network of biological responses including DNA damage detection, signaling and DNA repair systems such as nucleotide excision repair (NER). NER removes DNA helix distorting lesions including DNA photolesions induced by ultraviolet light (UV) i.e. cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). DNA damage processed by NER is differentially recognized depending on whether the damage is located throughout the genome (global genome repair, GG-NER) or specifically blocks transcription (transcription-coupled repair, TC-NER). The consequences of defective NER are apparent from the clinical symptoms of individuals affected by the rare recessive inherited disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) that characteristically display severe photosensitivity, as well as high incidence of cancer (XP), multi-system clinical malfunctions, neurological abnormalities and features of premature ageing (CS, XP/CS, TTD) (Tanaka and Wood, 1994).

In vitro reconstituted NER systems (Aboussekhra et al., 1995; Araujo et al., 2000; Bessho et al., 1997; Mu et al., 1995) originally identified approximately 30 polypeptides required for GG-NER and assigned specific roles to the various factors that were later confirmed by in vivo studies (Sugasawa et al., 1998; Volker et al., 2001; Tapias et al., 2004; Moser et al., 2005). The UV-DDB and the XPC-hHR23B heterodimers are responsible for DNA lesion recognition and efficient assembly of the core NER complex (the pre-incision step of NER), which includes the basal transcription factor TFIIH, replication protein A (RPA), XPA, and the structure-specific endonucleases XPG and ERCC1-XPF (Gillet and Scharer, 2006). After excision of the damaged DNA, the gap is filled by DNA repair synthesis (the post-incision step of NER) involving DNA polymerases δ (Pol δ), ϵ (Pol ϵ) (Moser et al., 2007) and κ (Pol κ) (Ogi and Lehmann, 2006; Ogi et al., 2010). The remaining nicks are sealed by either XRCC1-DNA Ligase III α (XRCC1-Lig3) or DNA Ligase I (Lig1) (Moser et al., 2007). Even though the key NER factors involved in the repair of NER substrates have been identified, the coordination between the two stages of NER (pre- and post-incision steps) is still poorly understood. Based on data from reconstituted NER reactions (Wakasugi and Sancar, 1998), it has been suggested that dual incision and/or recruitment of post-incision factors to NER sites lead to the differential release of pre-incision factors with the exception of XPC and RPA. XPC was shown to depart from the complex when positioning of XPG within the pre-incision complex occurs i.e. before incision is complete (Riedl et al., 2003). On the other hand, XPG might reside longer at the repair sites than XPF/ERCC1, since 5' incision generated by ERCC1/XPF does not lead to dissociation of XPG and does not require the catalytic activity of XPG (Staeresincic et al., 2009). Among the other factors, RPA was the only pre-incision factor that was found together with post-incision NER factors (Riedl et al., 2003) and it was shown to enhance NER mediated DNA synthesis (Shivji et al., 1995).

More than 30 years ago, it was observed that addition of DNA Pol δ and ϵ inhibitors, cytosine- β -arabino-furanoside (AraC) and hydroxyurea (HU) to UV-exposed cells, led to an accumulation of non-repairable DNA single strand breaks in the genome (Dunn and

Regan, 1979). The number of accumulated breaks was saturated at a dose of 2-5 J/m² and coincided with complete inhibition of photolesion removal (Snyder et al., 1981). Later it was shown that the saturation of breaks was due to the inhibition of NER-associated DNA synthesis (Moser et al., 2007; Mullenders et al., 1985; Smith and Okumoto, 1984). Together these data suggested that inhibition of the post-incision step of NER by HU & AraC leads to inhibition of further repair incision events. Despite numerous breakthroughs in understanding NER the actual mechanism that controls incision is still unknown. Tight regulation of new repair/incision events when gap-filling/sealing is incomplete is essential to prevent generation of DNA strand breaks that could lead to mutagenic and recombinogenic events (Gillet and Scharer, 2006). Yet it still remains an enigma; how does inhibition of the DNA synthesis step prevent subsequent incisions?

In this study we provide mechanistic insights into the regulation of NER stages *in vivo* and the central role of RPA therein. We show that dissociation of NER pre-incision factors (including XPC) from the damage sites requires incision. Inhibition of repair synthesis or ligation impairs removal of photolesions and leads to persistent accumulation of all NER factors at sites of UV damage that undergo repair. Under these conditions, we find that pre-incision proteins can dissociate and freely relocate to other damage sites, whereas RPA and post-incision proteins remain engaged at the initial sites of repair. Failure of RPA to relocate to other damages leads to incomplete NER complexes that are unable to perform dual incision. Our data reveal a central role for RPA in coupling NER mediated incision to DNA repair synthesis thereby precluding the initiation of further incision events that could lead to genomic instability.

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RESULTS

Impaired DNA repair synthesis/ligation leads to prolonged binding of NER factors and inhibition of repair independent of ATR.

Treatment of UV-irradiated human cells with DNA Pol δ and ϵ inhibitors (HU & AraC) leads to linear increase of NER mediated incisions (measured as accumulated breaks) that comes to saturation at UV-doses of 2-5 J/m². Moreover, treatment with these inhibitors appeared to be inhibitory to the bulk repair of photolesions (Moser et al., 2007; Snyder et al., 1981; Mullenders et al., 1985; Smith and Okumoto, 1984). To unravel the mechanism that underlies the saturation of incision events at low UV doses and the inhibition of damage removal in the presence of DNA polymerase inhibitors, we first addressed a possible role of ATR-dependent signalling events initiated by the perturbed gap filling (Matsumoto et al., 2007; O'Driscoll et al., 2003). For this purpose, we compared 6-4PP repair kinetics in the presence and absence of HU & AraC in UV-irradiated non-cycling normal human fibroblasts (NHF) and ATR deficient Seckle syndrome cells (O'Driscoll et al., 2003). Figure 1A shows similar levels of inhibition in NHF and Seckle cells, demonstrating that ATR deficiency does not rescue the HU & AraC mediated inhibition of 6-4PP repair. The severe reduction of H2AX phosphorylation in UV-irradiated Seckle cells compared to NHF cells (Figure 1B) but the normal level recruitment of XPB (a subunit of TFIIH) confirmed that these cells have an impaired ATR-dependent signalling, yet efficient NER complex formation. To

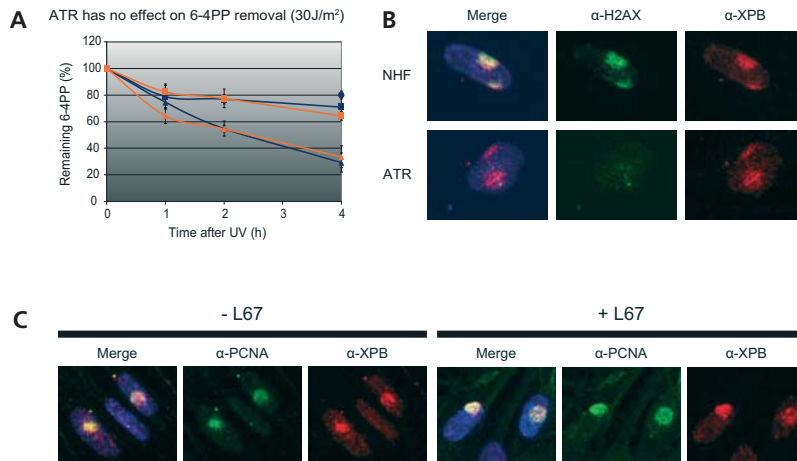


Figure 1. Impairment of DNA repair synthesis and ligation inhibits repair independently of ATR. (A) Removal of 6-4PP in time is measured in the presence (■) and absence (▲) of inhibitors following global UV-irradiation (30 J/m^2) in NHF (blue lines) and ATR deficient cells (orange lines); NHF cells are treated with L67 for 4 hours (h) prior to irradiation (●). (B) Seckle syndrome cells (ATR) show impaired γH2AX staining at sites of UV damage compared to NHF. Cells are irradiated with 30 J/m^2 1 h prior to fixation and processed for immunofluorescent staining with antibodies against XPB and γH2AX . (C) NHF, treated and non-treated with L67 for 4 h prior to local UV irradiation (30 J/m^2), are stained for XPB and PCNA localisation.

test whether inhibition of 6-4PP repair is a consequence of incomplete gap filling step or merely the result of HU & AraC treatment, we treated confluent NHF with L67, a potent inhibitor of both DNA ligases Lig1 and Lig3 (Chen et al., 2008; Moser et al., 2007) prior to UV irradiation. As shown in Figure 1C, inhibition of DNA ligation by L67 has no effect on the recruitment of either pre or post-incision NER factors to the damage site, however it severely impairs removal of 6-4PP in NHF similar to the HU & AraC treatment (Figure 1A).

To assess the effect of inhibition of UV-induced repair synthesis on assembly and disassembly of NER subcomplexes from sites that undergo repair, we measured the kinetics of (dis)assembly of NER factors in UV-irradiated NHF treated and non-treated with DNA synthesis inhibitors. We then made a comparison with the (dis)assembly of these factors in NER deficient cells that are unable to perform dual incision such as the XP-A and XP-F fibroblasts.

In agreement with earlier reports, pre- and post-incision factors (such as XPB, RPA and PCNA, DNA Pol δ , respectively) accumulate at local UV-spots (i.e. regions of locally induced UV damage) shortly after irradiation (30 min, Figure 2A, 2B). In the absence of inhibitors, XPB is barely visible 4 and 8 hrs following UV-irradiation (Figure 2A and S1A) closely mimicking the repair kinetics of 6-4PP in NHF (Figure 1A) (Volker et al., 2001; Wang et al., 2003). On the other hand, Pol δ and PCNA can be found at UV-spots up to 8 hrs after irradiation (Figure 2B). Moreover, RPA (a pre-incision factor) is still present at UV-spots 8 hrs following UV irradiation (Figure 2B) similar to post-incision factors and in line with observations from in vitro reconstituted repair systems indicating involvement of RPA in

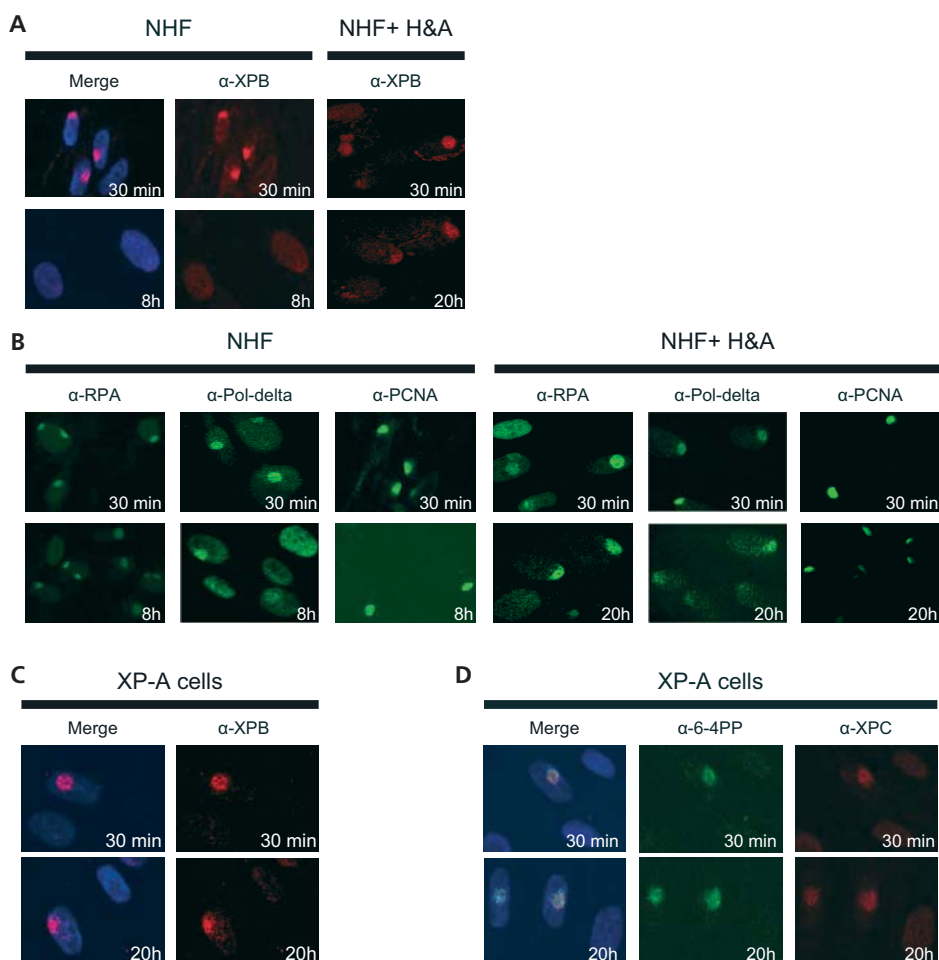


Figure 2. Prolonged accumulation of pre- and post-incision factors at NER sites in the presence of replication inhibitors. (A) Fluorescent immunostaining of XPB localization at damage sites in confluent NHF, and NHF treated with DNA synthesis inhibitors (HU & AraC) after local UV irradiation (30 J/m^2) at time points as indicated. 'Merge' refers to the combined image of DAPI and the antibody used for the panel. (B) Immunolocalization of RPA, Pol δ and PCNA in NHF in the presence or absence of HU & AraC at different repair times after 30 J/m^2 local UV-irradiation. (C) Immunolocalization of XPB in confluent XP-A cells at different repair times after 30 J/m^2 local UV-irradiation. (D) XPC and 6-4PP are visualised by immunostaining following local UV-irradiation; cells are irradiated with 30 J/m^2 UV and incubated for either 30 min or 20 h.

the repair synthesis step (Shivji et al., 1995). Interestingly, this kinetic analysis revealed that the prolonged binding of post-incision factors (PCNA) at damage sites is independent of TC-NER or CPD repair, as demonstrated by similar kinetics in α -amanatin treated cells and XP-E cells (lacking CPD repair), respectively (Figure S1A). Despite defective repair of 6-4PP (Figure 1A) and similar to the treatment with L67 inhibitor (Figure 1C), treatment of non-cycling NHF with HU & AraC does not prevent accumulation of NER subcomplexes at sites

of UV-damage when monitored 30 minutes after irradiation (Figure 2A, 2B). Nevertheless, impairment of the DNA repair synthesis step prevents dissociation of NER subcomplexes, since all factors tested (including pre-incision factor XPB) remained visible at the damage spots for up to 20 hrs (Figure 2A, 2B). Closely resembling the effect of DNA repair synthesis inhibition, defective incision leads to persistent binding of pre-incision factors (XPB, XPC) to UV-spots 20 hrs after UV irradiation in XP-A cells (Figure 2C, 2D) whereas accumulation of post-incision factors is absent in these cells (Green and Almouzni, 2003; Moser et al., 2007).

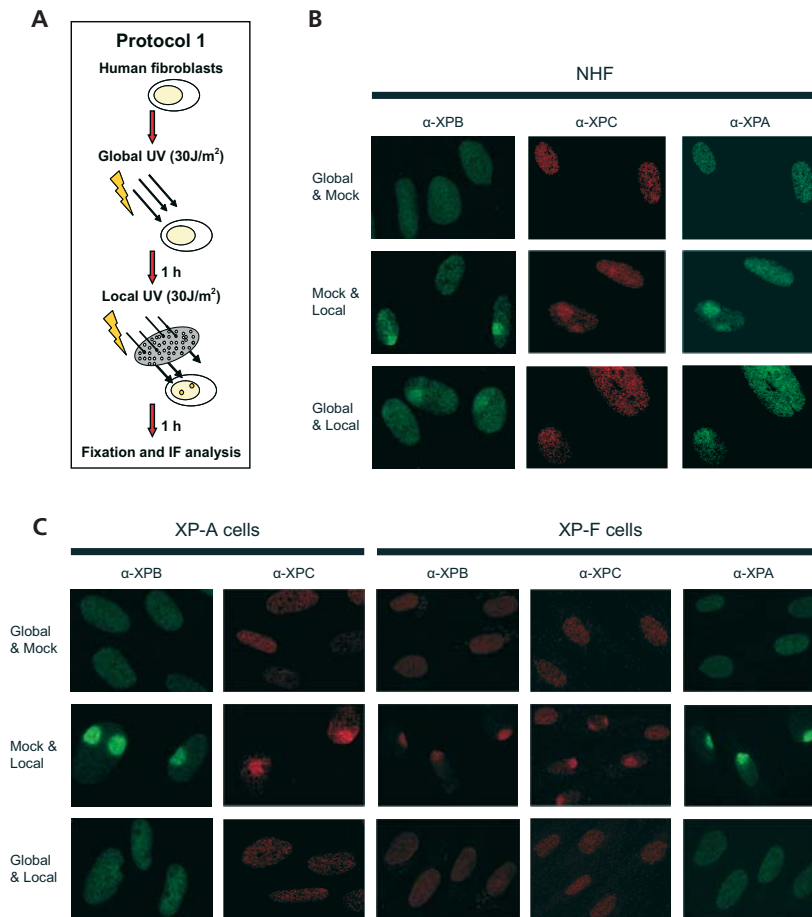


Figure 3. Pre-incision factors remain associated to initial repair sites in the absence of incision. (A) Schematic representation of Protocol 1. Cells are globally UV-irradiated (30 J/m^2) in the absence of HU & AraC, recover for 1 h and subsequently irradiated with the same dose of local UV. (B) Release of XPB, XPC and XPA from repair complexes is dependent upon functional incision. Confluent NHF are treated according to Protocol 1 (A) and immunostained with the indicated antibodies. Exposure times within experiments are equal. (C) Immunolocalisation of XPB, XPC and XPA antibodies in XP-A, XP-F cells treated according to Protocol 1 (A).

NER-mediated incision is required for the release and re-localisation of pre-incision factors to new repair sites.

The persistent localisation of pre-incision factors at local UV spots that we observed in the incision deficient XP-A cells (Figure 2C, 2D) is suggestive of the stable formation of pre-incision NER complex at sites of incomplete repair. However, these data are also consistent with a dynamic equilibrium of processes that allow assembly and disassembly of NER factors at photolesions within the UV-spot (which contains a number of damage sites). To distinguish between the different possibilities we designed an experimental approach i.e. *in vivo* competition by using two sequential doses of UV irradiation with sufficient time in-between the UV-exposures to allow assembly of repair complexes. The rationale behind this experimental set-up is that NER factors released from DNA damages induced by the first UV irradiation would be targeted to damages induced by the second UV-irradiation, allowing differentiation between stable and dynamic association of endogenous NER factors at sites that undergo repair. In these experiments, cells are exposed to global UV-irradiation of 30 J/m², a dose that saturates GG-NER (Smith and Okumoto, 1984; Erixon and Ahnstrom, 1979), cultured for 1 hr and subsequently exposed to local UV (30 J/m²) or mock-irradiation (Figure 3A i.e. Protocol 1). Following the second UV-irradiation, cells are incubated for 1hr prior to analysis. For Protocol 1, cells were UV-irradiated in the absence with DNA synthesis inhibitors (Figure 3A). In some experiments the order of UV-irradiations was reversed (Figure 4A i.e. Protocol 2).

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To validate the experimental setup (Protocol 1) we analysed the distribution of pre-incision NER factors in NHF. Accumulation of XPB, XPC and XPA is observed at damage sites induced by the second (local) UV irradiation (Figure 3B), demonstrating that the experimental protocol allowed detection of the released repair factors from the initially formed (globally irradiated) NER sites after completion of repair. On the other hand, experiments in XP-A and XP-F cells revealed that the same global UV-irradiation of 30 J/m² engaged all available XPB, XPC and XPA to UV lesions induced by the initial global irradiation as these cells lack accumulation of the repair factors at damages induced by the subsequent local UV-irradiation (Figure 3C). Similarly, local accumulation of pre-incision factors in XP-F cells (reverse order of UV irradiation, Protocol 2, Figure 4A) could not be competed out by the second globally induced UV damage (Figure S2A, in the absence of inhibitors). Taken together these data demonstrate that release of pre-incision factors from NER sites *in vivo* is dependent upon incision and/or formation of a functional pre-incision complex. Furthermore, our data suggest that assembly of NER pre-incision complexes at UV damages in the absence of XPA or XPF/ERCC1 leads to incomplete fixed complexes that are unable to dissociate from the specific repair sites.

Inhibition of DNA repair synthesis differentially affects dissociation of NER subcomplexes from damage sites.

We next examined the dynamic nature of the prolonged accumulation of NER factors that was induced by the DNA repair synthesis inhibition (Figure 2A, B). For this purpose, we carried out competition experiments with NHF in the presence of HU & AraC according to Protocol 2 (Figure 4A). Notably, XPA and XPB are able to dissociate from NER sites with

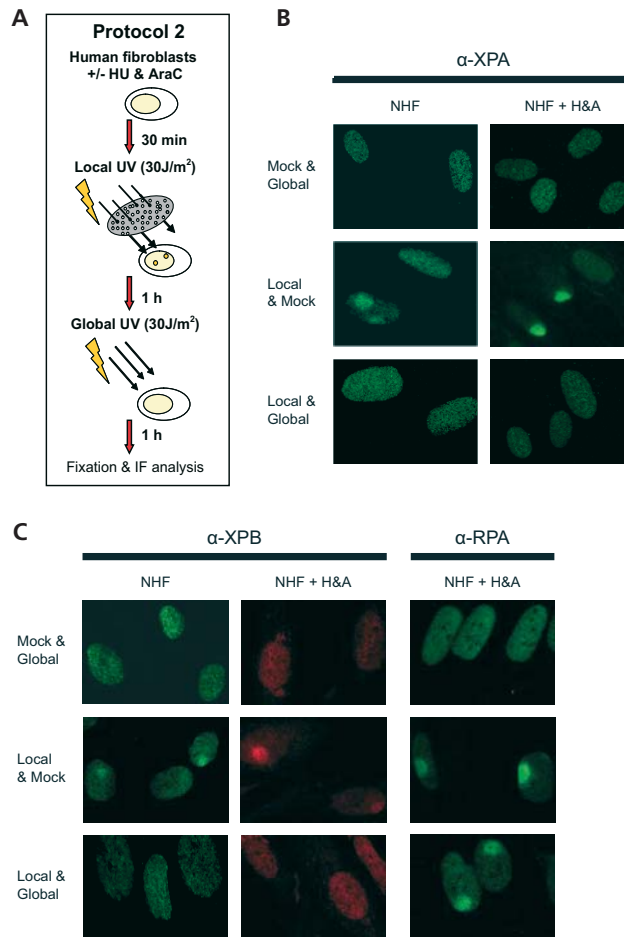


Figure 4. Dissociation of pre-incision factors from repair sites requires incision whereas dissociation of RPA requires DNA synthesis. (A) Schematic representation of the competition experiments according to Protocol 2. Cells are incubated in the presence or absence of inhibitors 30 min prior to the first (local) UV irradiation, which is subsequently followed by the second (global) irradiation. (B) Confluent NHF are irradiated according to Protocol 2 in the absence (NHF) or presence of inhibitors (NHF + H&A) and stained with XPA antibody. (C) NHF treated as in (B) stained with XPB and RPA antibodies.

incomplete NER synthesis engaging in new repair events (Figure 4B, 4C). These data clearly demonstrate that damage incision and not completion of the gap filling/sealing stage of NER is the prerequisite for the release of pre-incision factors. In contrast, RPA remained firmly bound at the initially induced damaged sites and could not be challenged away by the second global UV-irradiation before DNA repair synthesis was complete (Figure 4C) underlining its essential role in the post-incision step of NER.

To exclude possible interference between local (through 8 μ m pores) and global UV-irradiation we replaced the global by an additional local irradiation (30 J/m²) through

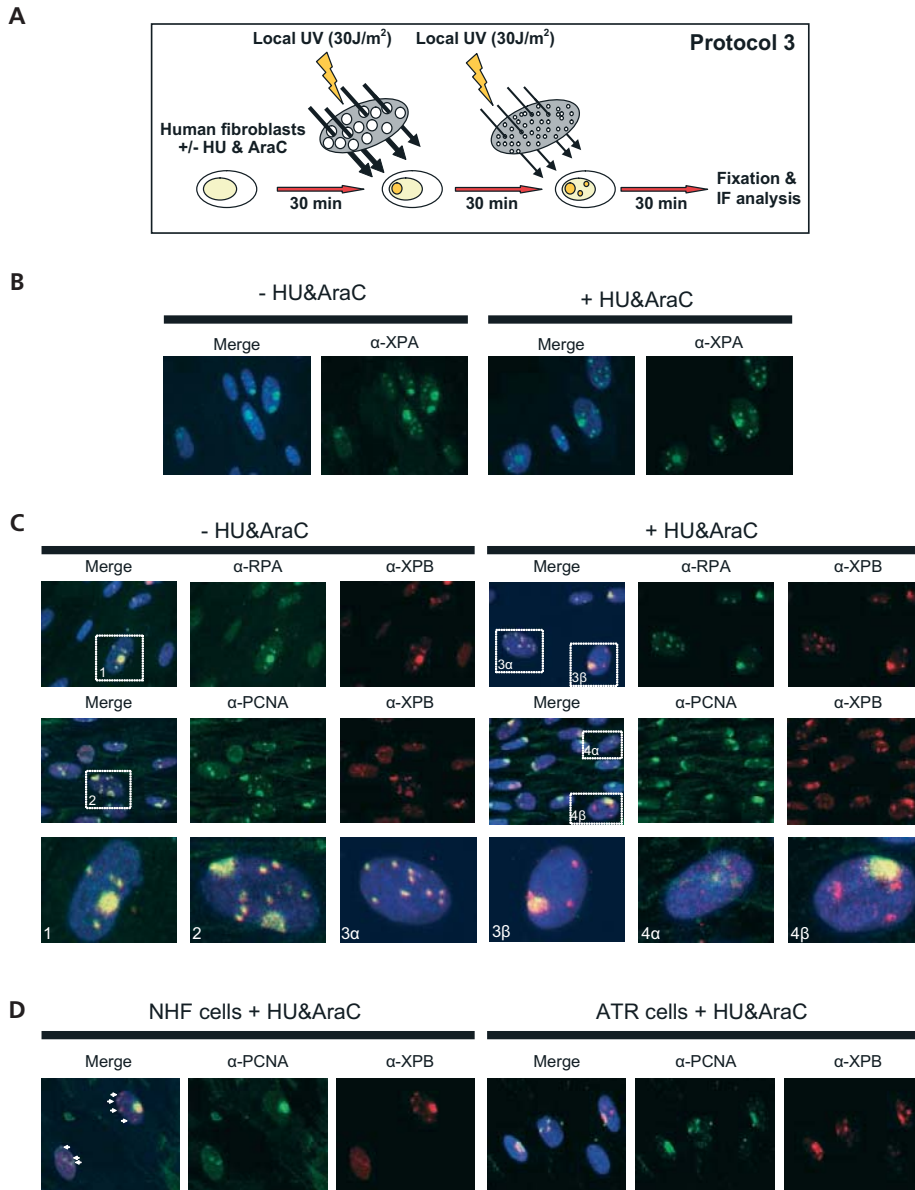


Figure 5. Impairment of DNA synthesis prevents recruitment of RPA and post-incision factors to *de novo* UV damages. For clarity, representative cells (numbered boxes) are enlarged and depicted below. (A) Schematic representation of competition experiments according to Protocol 3. Cells are treated or mock-treated with DNA synthesis inhibitors 30 min prior to the first local (8 μm) UV-irradiation, which is followed (after 30 min) by the second local (3 μm) irradiation. (B) Competition experiments (Protocol 3) confirm dynamic association of XPA with damage sites in the presence and absence of DNA synthesis inhibitors in confluent NHF. (C) Confluent NHF treated as in (B) and co-stained with RPA, PCNA and XPB antibodies. (D) Confluent NHF and Seckle syndrome cells (ATR) are treated according to Protocol 3 in the presence of HU & AraC, followed by immunofluorescent staining of PCNA and XPB. For clarity small spots are indicated with arrows.

3 μm pores (Figure 5A, Protocol 3). Under these conditions, XPA dissociates from the initial (larger) and is recruited to the subsequently induced (smaller) damage sites, both in the absence and presence of DNA synthesis inhibitors (Figure 5B, cells that have been irradiated twice contain both large and smaller spots), confirming the results of Figure 4B. We then repeated the same experimental approach for XPB, RPA and PCNA as well as XPG and ERCC1 (Figure 5C and S3A). In the absence of inhibitors all factors behave similar to XPA and are recruited to both large and small UV spots within the same cell and with similar intensities. In the presence of the inhibitors however, XPB, XPG and ERCC1 behave similar to XPA (Figure 5C and S3A) whereas PCNA and RPA cannot be competed out by the second irradiation since they are only visible and remain confined at the initial repair sites where synthesis is impaired (Figure 5C). Although we cannot exclude that XPG might reside longer than ERCC1/XPF at NER sites (Staresincic et al., 2009), our data clearly show that XPG (Figure S3A) behaves similar to the other NER pre-incision factors i.e. it is able to dissociate from the incomplete NER sites in the presence of HU & AraC.

Competition experiments (Protocol 3) in the presence of HU & AraC to assess the dynamic nature of the prolonged accumulation of NER factors (pre- and post-incision) in cells with impaired ATR-dependent signalling (Seckle syndrome cells) show similar results to NHF (Figure 5D).

We showed (Figure 3C) that, in vivo, XPC is able to leave the NER sites only after incision has taken place and a functional pre-incision complex has been formed. This result contrasts in vitro observations suggesting that XPC leaves the complex before incision takes place (Wakasugi and Sancar, 1998). To further verify our result, we analysed the localization of XPC in incision deficient XP-A cells employing Protocol 3 (in the absence of inhibitors) and either 30 or 100 J/m^2 of local UV-irradiation (equivalent to a global dose of 4.5 J/m^2 or 15 J/m^2 , respectively). The results clearly show that following 100 J/m^2 local UV-irradiation (a dose that saturates NER) (Smith and Okumoto, 1984) XPC is permanently immobilised at the initial non-incised damage sites (Figure S3B) confirming that, similar to the other pre-incision factors, XPC is not able to dissociate from UV-damaged sites in vivo when NER mediated incisions are impaired.

Impediment of NER gap filling and ligation inhibits further incision events.

Single strand DNA gap intermediates generated by NER mediated incisions in non dividing human cells trigger the phosphorylation of histone H2AX (Ser 139) in an ATR-dependent manner (Matsumoto et al., 2007). To examine whether accumulation of the released preincision factors to subsequently induced DNA damages that we observed in the presence of DNA synthesis inhibitors leads to new incisions, we performed competition experiments (Protocol 3) and $\gamma\text{H2AX}/\text{XPB}$ co-staining in quiescent NHF. Under normal conditions, γH2AX is observed at both the initial and subsequently induced UV-spots, coinciding with XPB (Figure 6A). However, in the presence of HU & AraC, phosphorylation of H2AX occurs only at the initial 8 μm UV spot but is absent from the subsequent 3 μm spots in contrast to XPB.

To examine whether impairment of NER mediated ligation would also generate a response similar to HU & AraC, we performed competition experiments (Protocol 3) in

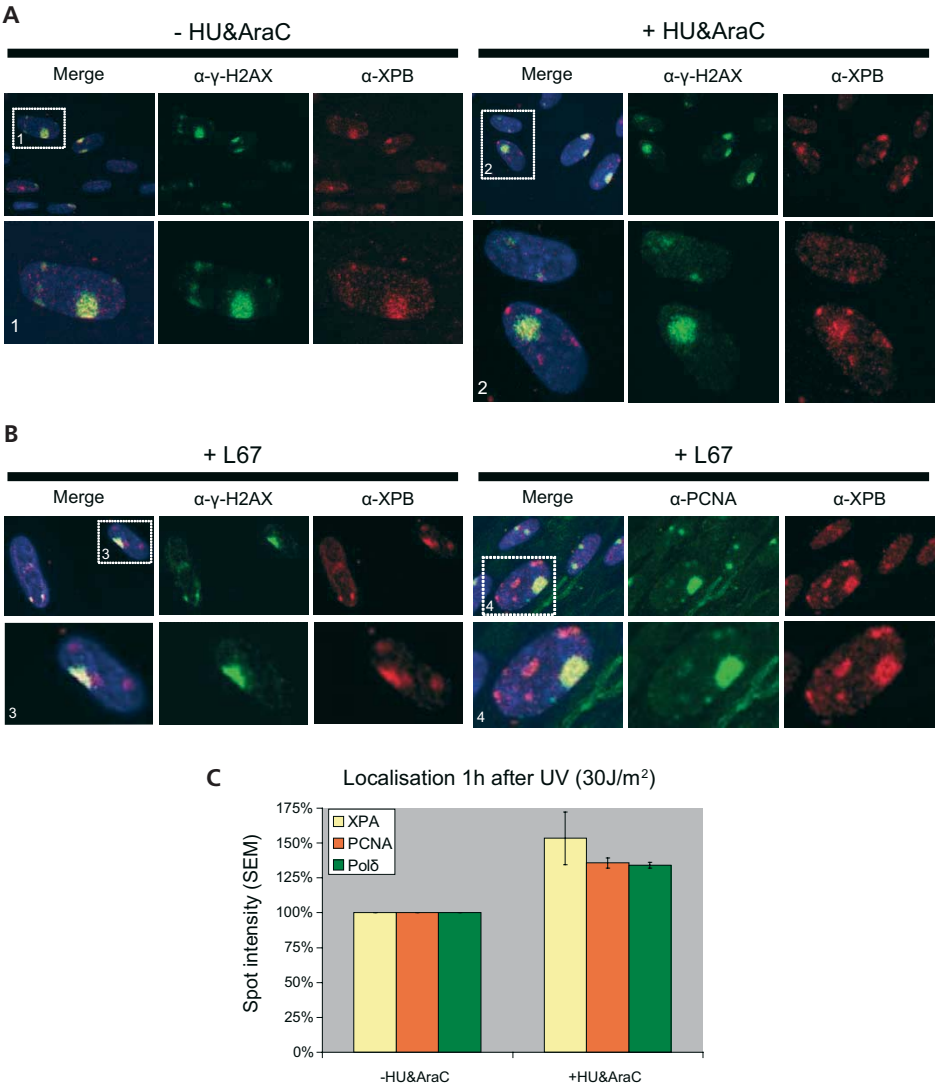


Figure 6. Inhibition of DNA repair synthesis or ligation prevents novel incision events and leads to prolonged accumulation of post-incision factors. (A) Quiescent NHF were irradiated according to Protocol 3. Incision events are visualized by γ H2AX staining; counterstaining for XPB revealed areas of damage induction. γ H2AX accumulation increases in time, thus the intensity is lower at the second UV spots when compared to the initially induced damage. Also, incubation with inhibitors increases γ H2AX accumulation, therefore microscopic exposure time for γ H2AX is 3 fold shorter when cells are irradiated in the presence of inhibitors. (B) NHF are irradiated according to Protocol 3 in the presence of L67 and stained for PCNA, XPB and γ H2AX. For clarity small spots are indicated with arrows. (C) NHF are locally irradiated in the presence or absence of inhibitors, fixed 1 hr later and stained for XPA in combination with PCNA or Pol δ . Average intensity inside local spots is measured and normalized to normal conditions (no inhibitors). Error bars represent the SEM values of 40 nuclei per experiment; out of at least three independent experiments.

the presence of L67 inhibitor (Chen et al., 2008). Treatment of NHF with L67 for four hours before UV irradiation leads to recruitment of pre-incision proteins to newly induced UVspots; in contrast, post-incision factors and RPA are immobilised at the initial UVspots (Figure 6B). Similarly, phosphorylation of H2AX occurs only at the initial UVspots, suggesting that under conditions of incomplete DNA repair synthesis, re-localisation of preincision factors (with the exception of RPA) to new NER sites does not lead to further incisions.

In line with published data (Matsumoto et al., 2007), we find that the observed intensity of γ H2AX at the initial UV damages is enhanced 3 fold in the presence of inhibitors suggesting that the level of H2AX phosphorylation greatly increases when gap-filling is perturbed. The same increased intensity is observed for all NER factors at UV-damaged spots in the presence of HU& AraC (single UV dose, Figure 6C).

Inhibition of DNA synthesis modulates the ability of RPA to associate with newly formed NER complexes.

We find that RPA is immobilised at incomplete DNA repair synthesis sites whereas the rest of pre-incision factors are able to re-associate to newly induced UV damages. To exclude any limitation of our approach to detect re-localisation of RPA to small spots (Protocol 3), we assessed the effect of RPA knockdown (KD) on the recruitment of NER subcomplexes to

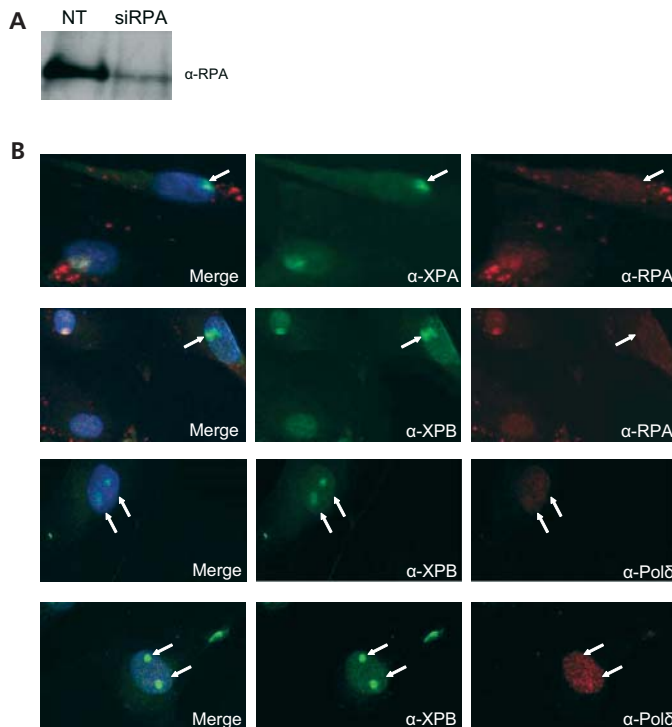


Figure 7. RPA is prerequisite for the functional assembly of NER subcomplexes. (A) Western blot analysis of equal amounts of whole cell lysates prepared from cells treated or non-treated (NT) with siRNA against RPA p70. (B) Cells treated with siRNA against RPA p70 are locally UV-irradiated (30 J/m²) fixed 1 h later and stained with XPA, XPB or Polδ antibodies. Absence of RPA is verified with RPA co-staining (arrows).

locally induced UV damage. KD of RPA p70 (Figure 7A) had no effect on the accumulation of pre-incision NER factors such as XPB and XPA at UV spots, but prevented recruitment of Pol δ (Figure 7B). Hence, RPA KD does not impinge on the recruitment of pre-incision factors to UV damage, yet it is absolutely required for the assembly of the post-incision components.

We hypothesised that depletion of RPA by virtue of its binding in post-incision complexes in the presence of inhibitors prevents its subsequent assembly into pre-incision complexes and hence impedes further incisions (γ H2AX staining, Figure 6A, B). To support this hypothesis, we assessed the amount of RPA and XPB in the soluble nuclear fraction isolated from non-dividing NHF one hour after exposure to UV in the presence or absence of HU & AraC. In the absence of inhibitors, we monitored a dose dependent decrease of RPA and XPB in the nuclear fraction with the largest depletion in cells exposed to 30 J/m² (Figure 8A). Interestingly, in the presence of the DNA synthesis inhibitors, this maximum depletion is observed with a dose as low as 5 J/m² and no further depletion is found at increasing doses (Figure 8A). Due to the high amounts of RPA present in chromatin prior to UV exposure we were unable to measure significant changes in this fraction after UV.

To further examine the presence of RPA in the two putative NER subcomplexes at sites of UV damage, we performed a modified protocol of the classic chromatin immunoprecipitation (ChIP) of *in vivo* crosslinked confluent NHF and analysed the coprecipitated proteins by western blotting (ChIP-western) (Fousteri et al., 2006; Moser et al., 2007; Coin et al., 2008). In line with our previously published data (Moser et al., 2007),

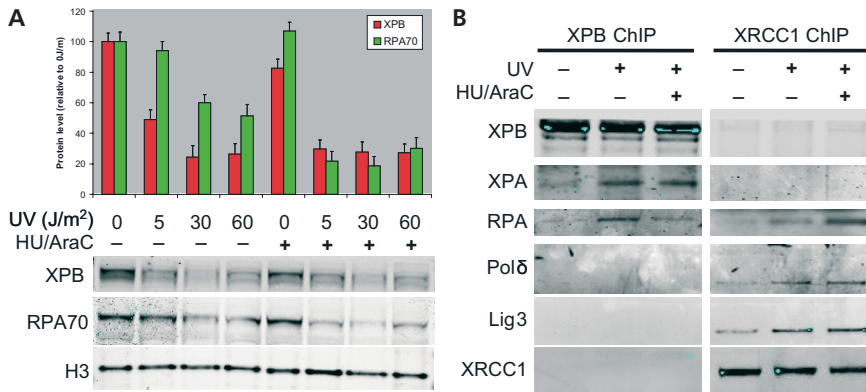


Figure 8. DNA synthesis inhibitors sequester RPA to sites of incomplete NER repair synthesis. (A) NHF irradiated with increasing UV doses (as indicated) in the presence and absence of inhibitors. Nuclear fractions are then isolated 1 h after UV and analysed by western blot analysis using XPB and RPA primary antibodies and fluorescently labelled secondary antibodies, signals are quantified using Odyssey 2.1 and normalised to the loading control. The amount of free nuclear RPA in non-irradiated cells without inhibitors is set at 100%. (B) Confluent NHF are (mock) irradiated with 20 J/m² in the presence or absence of inhibitors, let to recover and crosslinked 40 min later. ChIP is performed with XPB and XRCC1 specific antibodies and western blot analysis of the co-precipitating proteins is performed with antibodies as indicated.

XPB specific ChIP yields an increased interaction of XPB with pre-incision factors such as XPA and RPA in UV-irradiated cells whereas post-incision factors i.e. Pol δ , Lig3 and XRCC1 are virtually absent (Figure 8B), confirming that, also in vivo, NER subcomplexes involved in pre- or post-incision stages reside at different repair sites. When cells are treated with HU & AraC prior to UV irradiation, the XPB interaction with XPA is further enhanced (Figure 8B). This is consistent with the increased accumulation of NER factors and γ H2AX staining that we observed at UV spots in the presence of the inhibitors (Figure 6C). However, treatment of cells with HU & AraC has the opposite effect on the interaction between XPB and RPA. These results suggest that the number of chromatin-bound NER subcomplexes that contain both XPB and RPA are reduced when NER synthesis is impaired. The opposite is evident when we performed ChIP-on-western with an XRCC1 antibody; a clear UV increased interaction can be found between XRCC1 and RPA as well as Pol δ and Lig3, which is further enhanced in the presence of HU & AraC (Figure 8B). This is in agreement with the increased accumulation and immobilisation of DNA synthesis factors (including RPA) that we observed at sites of incomplete DNA repair synthesis. Similar to XPB specific ChIP, no interaction is observed between XRCC1 and pre-incision factors (Figure 8B) as reported previously (Moser et al., 2007).

DISCUSSION

The results reported here provide novel mechanistic insights in the regulation of NER in human cells. We find that in non-dividing NHF impairment of late NER events (i.e. gap filling and/or ligation) results in a persistent accumulation of NER factors at sites of UV damage and overall repair inhibition in an ATR independent manner. Under these conditions, pre-incision factors may dynamically assemble and disassemble freely associating with other damage sites whereas RPA and post-incision factors remain associated with the perturbed repair intermediates. RPA, an essential component of both NER subcomplexes, plays a unique role in controlling the transition from pre- to post-incision stages by linking the initiation of new repair events to completion of on-going DNA gap filling/sealing events. Incomplete pre-incision complexes, containing RPA, that are unable to perform incision (i.e. XP-A, XP-F cells) are stably bound at damage sites, demonstrating that their disassembly is dependent upon NER-mediated incision. These results reveal an unprecedented role for RPA in regulating NER by coupling novel incisions to completion of already initiated repair events.

Differential requirements for (dis)assembly of NER subcomplexes.

Dissociation of XPC from NER core complexes prior to incision has been observed in in vitro experiments (Riedl et al., 2003; Wakasugi and Sancar, 1998). On the other hand competition experiments in incision deficient XP cells demonstrated that, in vivo the situation might be different given that XPC is stably assembled in NER pre-incision complexes and cannot be challenged away (Figure S3B). Hence, NER-mediated incision is the key determinant for the release of pre-incision NER proteins except RPA. NER incision is also required for efficient recruitment of the post-incision factors (Aboussekhra and

Wood, 1995) and, compared to the pre-incision factors, post-incision factors including RPA remain visible at repair sites for extended periods of time. The average time to repair a UV-lesion i.e. from detection to final DNA ligation is estimated to be four minutes (Erixon and Ahnstrom, 1979), closely mimicking the residence time of most NER pre-incision factors at UV-damage (Mone et al., 2004; Politi et al., 2005). We find that the prolonged association of post-incision factors with repair sites is not due to ongoing NER (i.e. TC-NER or the slower repair of CPD by GG-NER) (Figure S1) suggesting that completion of NER events (i.e. gap-filling and ligation) does not lead to disassembly of proteins involved in the post-incision step in spite of their dynamic nature (Essers et al., 2005; Mone et al., 2004; Zotter et al., 2006). We speculate that this extended association might have a functional role in the restoration of chromatin structure at the repaired sites. In line with this, PCNA has been shown to be the preferred target for chromatin assembly factor CAF1 (Moggs et al., 2000) and NER mediated DNA synthesis occurs in concert with chromatin assembly (Green and Almouzni, 2003) suggesting that these two processes are mechanistically linked.

RPA is indispensable for NER complex stability and incision.

When NER mediated incision is followed by inhibition of repair patch synthesis/ligation, the pre-incision factors but none of the post-incision or RPA can dissociate from sites of ongoing repair and re-associate with unprocessed UV photolesions. Notably, XPF/ERCC1 can also be recruited independently of RPA, raising questions on the exact mechanism of its recruitment. While RPA greatly enhances the binding of XPF to artificial structures *in vitro* (de Laat et al., 1998; Matsunaga et al., 1996), XPF/ERCC1 was not recruited to sites of damage in XP-A cells despite the presence of RPA (Volker et al., 2001). Taken together, these results suggest that one of the key roles of RPA in NER (in concert with XPA) is its involvement in the correct orientation and activation of the endonucleases XPG and XPF/ERCC1 rather than their recruitment. A number of studies suggest that XPA and TFIIH are sufficient to recruit XPF and XPG, respectively (Li et al., 1994; Park and Sancar, 1994; Volker et al., 2001; Iyer et al., 1996), however in the absence of RPA this will lead to a non-functional pre-incision complex that lacks incision activity (this work).

The number of incisions made by NER in the presence of HU & AraC reaches a maximum one hour after irradiation and at doses as low as 2-5 J/m² (Smith and Okumoto, 1984; Mullenders et al., 1985; Berneburg et al., 2000; Snyder et al., 1981) indicating that under these conditions NER is non-catalytic, i.e. NER can only create a limited number of incisions at one time. This limited number of incisions goes along with the impaired removal of 6-4PP (Moser et al., 2007) (Figure 1A), the depletion of the pool of soluble nuclear RPA and XPB (Figure 8A), the enhanced H2AX phosphorylation (Matsumoto et al., 2007) and the increased accumulation of NER factors at UV damage spots (Figure 6C). In addition, no H2AX phosphorylation is observed when pre-incision factors are re-localised to sites of the second UV-irradiation in the presence of NER synthesis or ligation inhibitors (Figure 6A, 6B) suggestive of no incision events at these sites.

We considered two possible mechanisms underlying the limited occurrence of incision events and the impaired repair of photolesions observed when the late steps of NER are inhibited. First, it is conceivable that DNA damage induced signalling (manifested by H2AX

phosphorylation) prevents further incisions when gap filling is not completed. UV-induced signalling has been shown to depend on ATR. Most likely, this signalling is activated by the formation of RPA-bound single stranded DNA patches formed by NER mediated incisions (Matsumoto et al., 2007; Marini et al., 2006; Marti et al., 2006; O'Driscoll et al., 2003) or alternatively by displacement of single strand DNA generated temporally by the initial 5' to the lesion XPF/ERCC1 incision followed by the 3' to the lesion XPG incision (Staresincic et al., 2009). Nevertheless, ATR deficiency has no effect on the persistent accumulation of post-incision proteins and does not lead to further incisions or increased removal of 6-4PP. We therefore conclude that regulation of NER incision does not depend on ATR signalling in confluent human cells.

A second mechanism could be that one or more NER factors becomes 'trapped' inside existing yet inhibited NER complexes and hence unable to associate with other photolesions.

It has been shown that incision by ERCC1-XPF occurs first, allowing the free 3-OH that is generated to initiate repair synthesis before XPG performs the incision 3'to the lesion (Staresincic et al., 2009). This could potentially lead to XPG 'trapping' when repair synthesis step is inhibited. Nevertheless, our data show that in normal cells, under conditions that impair repair synthesis (yet allow the recruitment of post-incision factors), XPG is able to leave the complex suggesting that in the presence of inhibitors the repair site might be a gap generated by dual incision. Based on the current findings, RPA is the only NER protein that is recruited prior to incision and remains associated at the site of repair after incision and recruitment of post-incision factors consistent with *in vitro* studies (Riedl et al., 2003). The persistent binding of RPA at the initial sites of damage induction and repair in the competition experiments in the presence of DNA synthesis/ligation inhibitors (Figure 4C, 5C, 6B) implies that RPA remains bound to DNA until completion of gap-filling and ligation. Thus, in the presence of inhibitors, all available (free-nuclear) RPA becomes sequestered and trapped in post-incision complexes, thereby preventing it from associating in new repair initiation events. As a consequence, abortive pre-incision repair complexes are formed, which are incapable of incision. In support of this hypothesis, ChIP-on-western experiments (Figure 8B) show an increased interaction of RPA with post-incision factors and a decreased interaction with pre-incision factors under conditions that impair NER mediated DNA synthesis and ligation.

The current observation that NER is independent of ATR in confluent or non-cycling cells (G1, G0) was confirmed by a recent study (Auclair et al., 2008) showing that efficient NER depends on ATR in S but not G1 cells. It should be noted that RPA is released from PML bodies upon UV-irradiation and recruited to UV-induced damages (Park et al., 2005) and this release occurs and requires the kinase activity of ATR in S-and G2-phase but not G1 cells (Barr et al., 2003). Together these observations suggest that during S-phase, due to its large engagement in replication, RPA is unable to associate with repair complexes, and that additional RPA is released from PML bodies in the cell in an ATR dependent manner in order to carry out NER. This is obviously not the case in G1 cells where ATR deficiency has no effect and nuclear RPA is recruited to the damage sites. We speculate that, similar to stalled replication complexes, blocked repair synthesis complexes in G1/

G0 cells also sequesters the majority of free nuclear RPA (indicated by the recruitment of RPA to chromatin after low UV doses in the presence of HU & AraC, Figure 8A), thereby preventing additional NER-mediated incision events to take place.

We propose a model wherein RPA regulates NER by safeguarding the transition from pre- to post-incision stages and coordinating the initiation of new repair events only after completion of on-going repair synthesis (Figure 9). According to this model, pre-incision factors are recruited to sites of DNA damage. In the presence of RPA, NER mediated incision occurs, allowing recruitment of post-incision and release of pre-incision factors. Under normal conditions, the released RPA is able to associate with, and subsequently stabilize and activate newly formed pre-incision complexes enabling further incision events and continuation of repair. In contrast, the persistent association of RPA in post-incision complexes at sites of incomplete repair synthesis prevents release of RPA and thus shields the genome from uncontrolled incisions by coupling repair initiation to completion of DNA repair synthesis and ligation events.

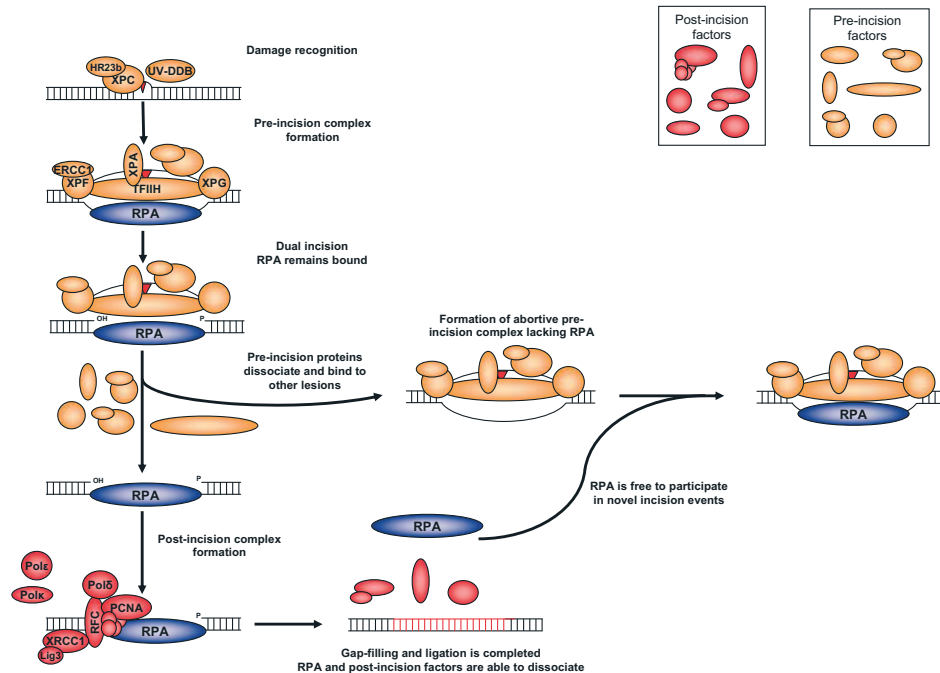


Figure 9. Schematic depiction of regulation of NER mediated incision events in vivo. Upon lesion recognition by UV-DDB and XPC-hHR23B, local opening of DNA by TFIIH provides access to the core GG-NER machinery i.e. XPA, RPA, XPG and ERCC1-XPF. RPA binds to the undamaged single-stranded DNA stabilizing the complex. Subsequently incision is followed by the release of core NER factors, which are then free to associate with other damages with the exception of RPA that, remains bound to the repair site, probably on the undamaged single-stranded DNA. The later stages of repair are carried out by RFC loading PCNA onto the incised DNA, the recruitment of DNA polymerases polδ/ polε/polκ and XRCC1-Lig3/Lig1 to fill in and ligate the gap, respectively. After ligation, post-incision factors and RPA are able to dissociate. RPA can then stabilize the otherwise abortive preincision complexes, enabling the initiation of new NER events.

MATERIALS AND METHODS

Cell culture. Cells used in this study have been grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO₂ atmosphere and include i) primary and telomerase hTert immortalized human fibroblasts each of normal NHF (VH10), as well as NER deficient XP-A (XP25RO), XP-F (XP24KY and XP51RO) and XP-E (XP23PV) fibroblasts and ii) ATR deficient Seckle cells (GM18366).

UV-irradiation and immunofluorescence. Global and local UV-irradiation using a 3 or 8µm filter is performed essentially as described (Moné et al., 2001; Volker et al., 2001). After irradiation, the cells are returned to culture conditions for time periods indicated. Cytosine-β-arabino-furanoside (Fluka) and hydroxyurea (Fluka) at final concentrations of 10 µM and 100 mM respectively, are added to the medium 30 minutes prior to irradiation and remained present throughout the time course of the experiment. Ligase inhibitor L67 (Chen et al., 2008) is added 4 hours prior to irradiation at a final concentration of 25 µM. Where required α-amanitin has been added 5 hours prior to irradiation at a concentration of 1 µg/ml. Blockage of transcription by α-amanitin has been verified by measuring 3H incorporation after pulse labelling with 3H-Urd for one hour (van Oosterwijk et al., 1996), which predominantly represents RNA polymerase II transcription.

Immunofluorescence. Immunofluorescent labelling is performed essentially as described (Moser et al., 2007; Volker et al., 2001). Briefly, cells are washed with cold PBS, fixed and lysed on ice by either, 100% methanol for 10 min, or by 2% paraformaldehyde for 20 min at room temperature (RT), followed by 0.2% Triton X-100 incubation for 5 min at RT. Following fixation, cells are washed with cold PBS, and incubated with 5% bovine albumin in PBS for 30' at RT. The cells are subsequently incubated with primary antibodies, diluted in washing buffer (WB) (PBS containing 0.5% bovine serum albumin and 0.05% Tween-20) for two hours at RT. The cells are washed 3x with WB and thereafter incubated with secondary antibody for 1 hour at RT. Cells are mounted in Aqua/polymount (Polysciences Inc., Warrington, PA) containing DAPI (1.5 µg/ml). Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2007).

Antibodies. The following primary antibodies are used in this study: rabbit polyclonal α-XPA, α-p89 (XPB), α-ERCC1 and mouse monoclonal α-DNA Polδ (Santa-Cruz), mouse monoclonal α-PCNA (PC10), α-XRCC1, α-XPA (Abcam), mouse monoclonal α-DNA ligase 3α (Genetex), mouse monoclonal α-DNA ligase 1 (MBL), mouse monoclonal α-XPG (8H7, Molecular Probes), mouse monoclonal α-RPAp70 and α-RPAp34 (respectively Ab-1 and Ab-3, Oncogene), mouse monoclonal α-6-4PP (Cosmo Bio). Mouse monoclonal α-p89 (XPB), a gift from Dr. J-M. Egly (IGMC, Illkirch, France), affinity-purified rabbit polyclonal α-XPC, a gift from Dr. W. Vermeulen (Erasmus MC, Rotterdam, The Netherlands). Secondary antibodies include Cy3-conjugated goat α-rabbit IgG and FITC-conjugated donkey α-mouse (Jackson Laboratories) and Alexa Fluor 488 goat α-mouse IgG and AlexaFluor 555 goat α-rabbit IgG (Molecular Probes). All secondary antibodies have been used according to the manufacturer's instructions.

In Vivo Cross-linking and ChIP-on -western. Confluent hTert-immortalized NHF treated or mock-treated with HU and AraC are UV-irradiated (20 J/ m²) and incubated at 37°C for 40 min prior to in vivo cross-linking. Lysis of the crosslinked cells, chromatin purification and fractionation as well as chromatin immunoprecipitation and reversal of the crosslinks prior to DNA analysis and protein analysis by western blotting are performed as described previously (Fousteri et al., 2006; Moser et al., 2007).

RNA interference. SmartPool™ siRNA oligos (Dharmacon) are used for all experiments unless otherwise noted. siRNA transfection is performed using HiPerfect™ (QIAGEN) transfection reagent according to the manufacturer's instruction. In typical experiments, 5 nM of siRNA oligos are transfected in suspension, termed 'reverse transfection', and followed by one additional transfection cycle 24 h after the first transfection (double transfection). Experiments are performed 48 h after the first siRNA transfection. Knockdown efficiencies are confirmed by western blotting and immunofluorescence.

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SUPPLEMENTARY FIGURES

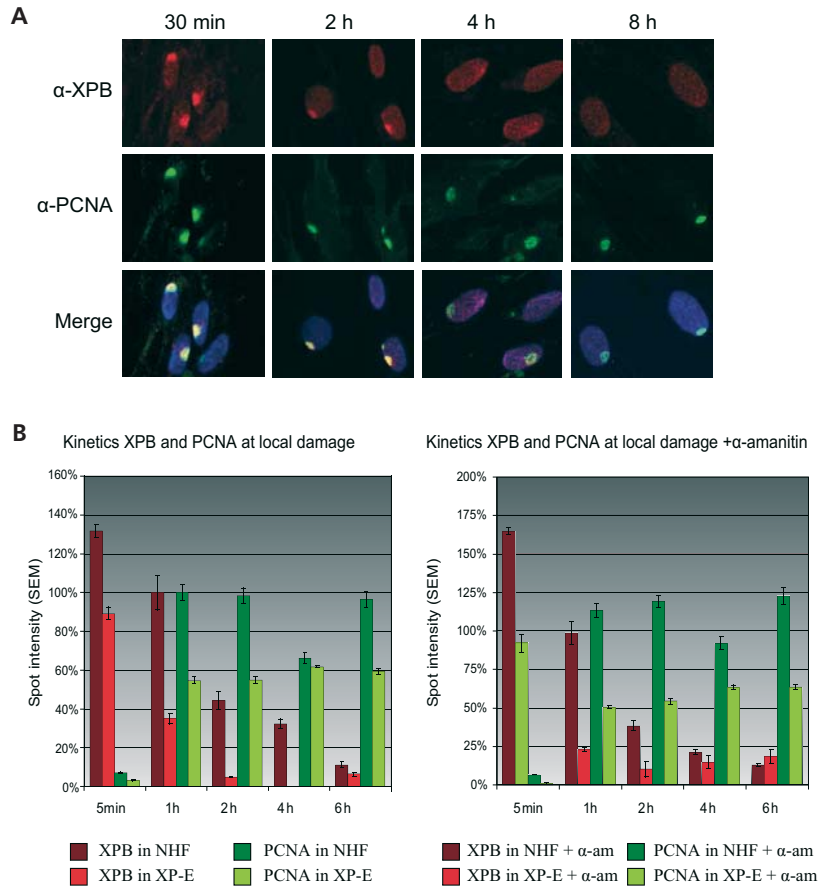


Figure S1. Disassembly kinetics of NER sub-complexes from sites of UV-induced DNA damages and repair. Disassembly is monitored by fluorescent immunostaining of XPB and PCNA in confluent NHF after local UV irradiation (30J/m^2) at different time points prior to fixation. (A) Examples of images used for analysis. (B) Quantitative analysis of the average spot intensity at damage sites as function of time.

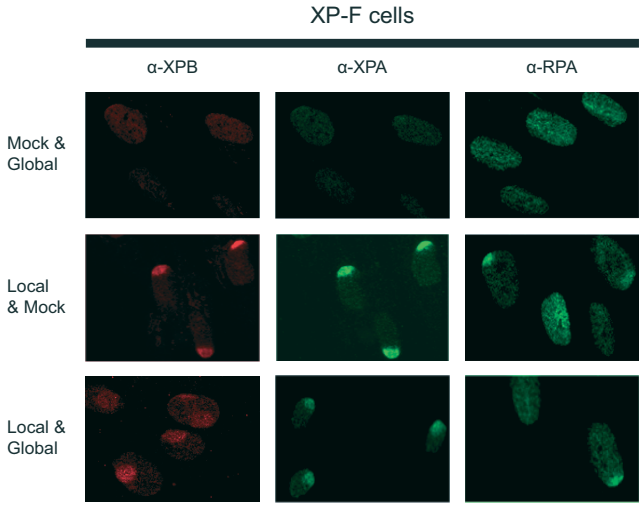


Figure S2. Release of pre-incision factors from NER sites in vivo requires incision. Competition experiments performed in XP-F cells as described in Protocol 2 (first local and then global UV irradiation). The sequential irradiations have been done in the absence of DNA synthesis inhibitors and are followed by immunofluorescent staining of XPB, XPA and RPA antibodies.

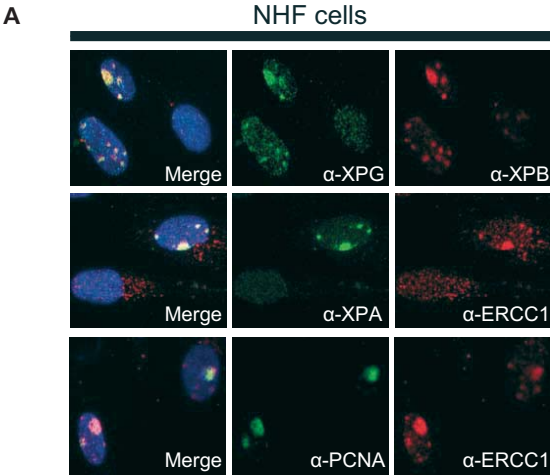


Figure S3. Differential requirements for the (dis)assembly of NER subcomplexes. (A) Confluent NHF are irradiated according to Protocol 3 (first 8 μ m local followed by 3 μ m local) in the presence of DNA synthesis inhibitors and stained with antibodies against XPG, XPB, XPA, ERCC1 and PCNA. (B) XPC remains bound at NER sites in the absence of incision XP-A cells irradiated according to Protocol 3 (in the absence of inhibitors) with two different doses, as indicated, and stained with antibodies against XPC or CPD.

