# Replication protein A safeguards genome integrity by controlling NER incision events

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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 evolutionarily 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 accumulation of unprocessed repair intermediates. We monitored NER regulatory events in vivo using sequential UV irradiations. Under conditions that allow incision yet prevent completion of repair synthesis or ligation, preincision factors can reassociate with new damage sites. In contrast, replication protein A remains at the incomplete NER sites and regulates a feedback loop from completion of DNA repair synthesis to subsequent damage recognition, independently of ATR signaling. Our data reveal an important function for replication protein A 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 aging (CS, XP/CS, TTD) (Tanaka and Wood, 1994).

In vitro–reconstituted NER systems (Aboussekhra et al., 1995; Mu et al., 1995; Bessho et al., 1997; Araújo et al., 2000) originally identified ∼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 preincision step of NER), which includes the basal transcription factor TFIIH, replication protein A (RPA), XPA, and the structure-specific endonucleases XPG and XPF/ERCC1

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Abbreviations used in this paper: 6-4 PP, 6-4 photoproducts; AraC, cytosine-β-arabinofuranoside; ChIP, chromatin immunoprecipitation; CPD, cyclobutane pyrimidine dimer; HU, hydroxyurea; NER, nucleotide excision repair; NHF, normal human fibroblast; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; XP, xeroderma pigmentosum.



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(Gillet and Schärer, 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 (Polδ), ε (Polε; Moser et al., 2007) and κ (Polκ; Ogi and Lehmann, 2006; Ogi et al., 2010). The remaining nicks are sealed by either XRCC1-DNA Ligase IIIα (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; Riedl et al., 2003), it has been suggested that release of preincision factors (with the exception of RPA) occurs before or after dual incision and/or recruitment of post-incision factors to NER sites. XPC is the first to depart from the complex with the arrival of XPG within the preincision complex, i.e., even before incision (Riedl et al., 2003). The recruitment of XPF/ERCC1 resulting in 5′ incision leads to release of XPA and TFIIH that may rejoin new incision complexes, while XPG and XPF/ERCC1 remain bound to the incised DNA. RPA is the only preincision factor found together with post-incision NER factors and might protect the undamaged strand from nuclease attack, promote arrival and positioning of RFC (Riedl et al., 2003; Mocquet et al., 2008), and enhance NER-mediated DNA synthesis (Shivji et al., 1995).

More than 30 years ago, it was observed that addition of DNA Polδ and ε inhibitors cytosine-β-arabinofuranoside (AraC) and hydroxyurea (HU) to UV-exposed cells led to an accumulation of nonrepairable 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/m$^2$ 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 (Smith and Okumoto, 1984; Mullenders et al., 1985; Moser et al., 2007). Together, these data suggested that inhibition of the post-incision step of NER by HU and AraC leads to inhibition of further repair incision events. Slow or incomplete sealing of repair gaps is of physiological relevance. Differentiated cells such as lymphocytes display increased frequency of gaps after UV related to deficient DNA repair synthesis, likely due to low intracellular deoxyribonucleotide pools (Green et al., 1994). Notably, the retarded post-incision step is detrimental for UV-irradiated lymphocytes as shown by the lethality rescue after addition of deoxyribonucleosides (Green et al., 1996). Moreover, noncycling human fibroblasts exposed to high doses of UV show ATR-dependent signaling, suggesting that under these conditions the post-incision step is retarded (Vrouwe et al., 2011). In these cells, ubiquitin-modified proliferating cell nuclear antigen (PCNA) is formed and translesion synthesis polymerases are involved in gap filling, indicating replication stress (Ogi et al., 2010).

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 Schärer, 2006). Yet it still remains an enigma; how does inhibition of the DNA synthesis step prevent subsequent incisions? One possible mechanism could be the activation of the phosphoinositol-3-kinase–like kinase ATR by perturbed gap filling. ATR phosphorylates downstream targets like p53 and histone H2AX and activates RNF-8–dependent ubiquitylation of histone H2A as part of UV-induced DNA damage response (Bergink et al., 2006; Marteijn et al., 2009). Initiation of signaling might impair further incision events, but other mechanisms can contribute as well.

In this study, we provide mechanistic insight into the regulation of NER stages in vivo and the central role of RPA therein. We show that dissociation of NER preincision factors (including XPC) from the damage sites requires incision. Inhibition of repair synthesis or ligation impairs removal of photolesions independent of ATR and leads to a persistent engagement of RPA and NER post-incision factors at sites of UV damage that undergo repair. In contrast, preincision proteins other than RPA can dissociate and freely relocate to other damage sites. Failure of RPA to relocate to other damage sites 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.

## 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 Polδ and ε inhibitors (HU and AraC) leads to linear increase of NER-mediated incisions (measured as accumulated breaks) that comes to saturation at UV doses of 2–5 J/m$^2$. Moreover, treatment with these inhibitors appeared to be inhibitory to the bulk repair of photolesions (Fig. 1 A; Snyder et al., 1981; Smith and Okumoto, 1984; Mullenders et al., 1985; Moser et al., 2007). 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 inhibitory role of ATR-dependent signaling on repair of UV photolesions in the presence of inhibitors. ATR signaling is initiated by perturbed gap filling (O'Driscoll et al., 2003; Matsumoto et al., 2007) and mediated by ATR binding to the single-stranded DNA-binding protein complex RPA. To measure repair of 6-4PP, cells were stained with a 6-4PP–specific antibody and lesion removal was measured by quantitative immunofluorescence (Moser et al., 2005). We compared 6-4PP repair kinetics in the presence and absence of HU and AraC in UV-irradiated noncycling normal human fibroblasts (NHFs) and ATR-deficient Seckle syndrome cells (O'Driscoll et al., 2003). Fig. 1 A shows similar levels of inhibition in NHF and Seckle cells, demonstrating that ATR deficiency does not rescue the HU and AraC–mediated inhibition of 6-4PP repair. The severe reduction of H2AX phosphorylation in UV-irradiated Seckle cells compared with NHF cells (Fig. 1 B), but the normal level recruitment of XPB (a subunit of TFIIH) confirmed that these cells have an impaired

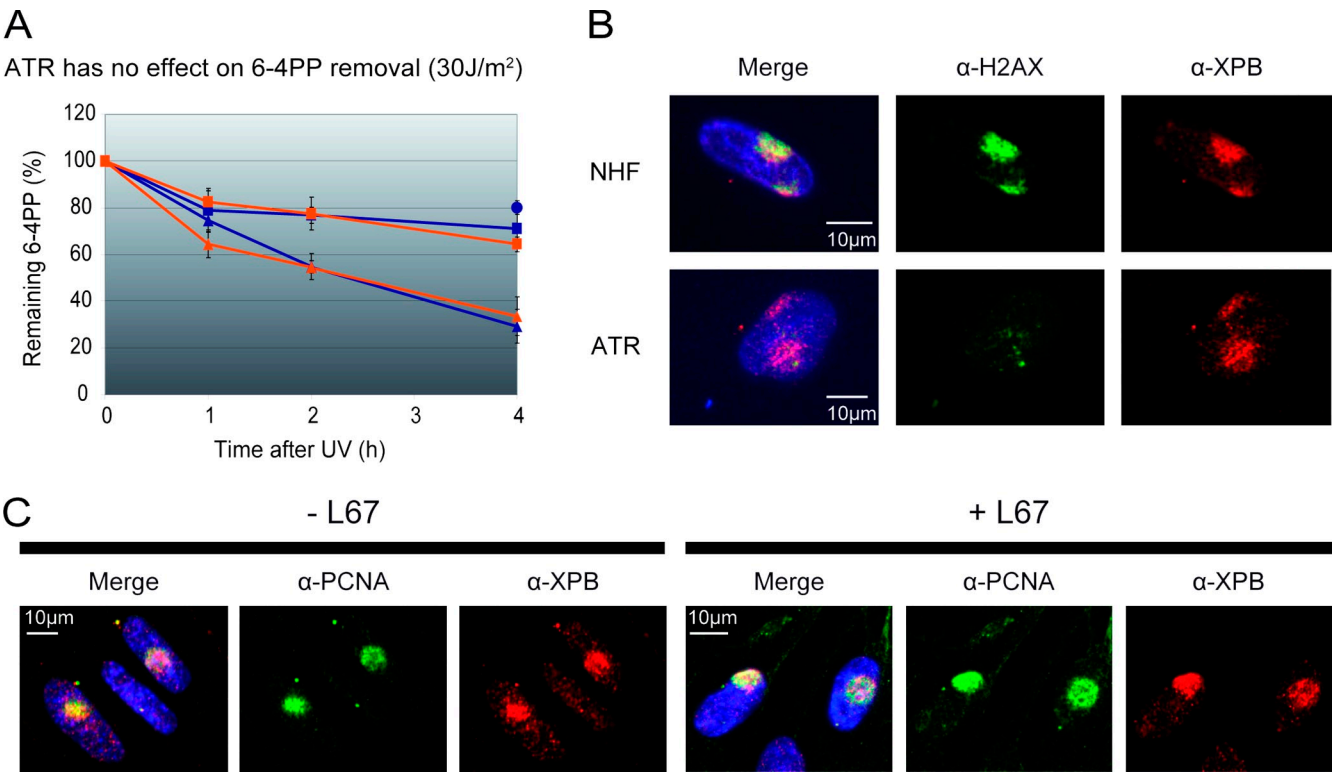


Figure 1. **Impairment of DNA repair synthesis and ligation inhibits repair independently of ATR.** (A) Removal of 6-4PP in time was measured in the presence (■) and absence (▲) of inhibitors after global UV irradiation (30 J/m$^2$) in NHF (blue lines) and ATR-deficient cells (orange lines); NHF cells were treated with L67 for 4 h before irradiation (●). Cells were stained with a 6-4PP–specific antibody and lesion removal was measured by immunofluorescent staining and quantification of at least three independent experiments with over 40 cells per point. (B) Seckle syndrome cells (ATR) show impaired γ-H2AX staining at sites of UV damage compared with NHF. Cells were irradiated with 30 J/m$^2$ 1 h before fixation and were processed for immunofluorescent staining with antibodies against XPB and γ-H2AX. (C) NHFs, treated and nontreated with L67 for 4 h before local UV irradiation (30 J/m$^2$), were stained for XPB and PCNA localization.

ATR-dependent signaling, yet efficient NER complex formation. To test whether inhibition of 6-4PP repair is a consequence of incomplete gap-filling step or merely the result of HU and AraC treatment, we treated confluent NHF with L67, a potent inhibitor of both DNA ligases Lig1 and Lig3 (Moser et al., 2007; Chen et al., 2008) before UV irradiation. As shown in Fig. 1 C, 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 further removal of 6-4PP in NHF similar to the HU and AraC treatment (Fig. 1 A).

To assess the impact of inhibited 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 NHFs, treated and nontreated 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 Polδ, respectively) accumulate at local UV spots (i.e., regions of locally induced UV damage) shortly after irradiation (Fig. 2, A and B, 30 min). In the absence of inhibitors, XPB is barely visible 4 and 8 h after UV irradiation (Fig. 2 A and Fig. S1 A), closely mimicking the repair kinetics of 6-4PP in NHF (Fig. 1 A; Volker et al., 2001; Wang et al., 2003). On the other hand, PCNA can be found at UV spots 8 h after irradiation; however, after 20 h the signal of PCNA is greatly reduced (Fig. 2 A). Notably, RPA (a preincision factor) is still present at UV spots 8 h after UV irradiation (Fig. 2 A), similar to PCNA and Polδ and in line with observations from in vitro–reconstituted repair systems, indicating involvement of RPA in the repair synthesis step (Shivji et al., 1995). Moreover, we recently showed a decline of RPA in UV spots at 20 h in NHFs, similar to PCNA (Vrouwe et al., 2011). Interestingly, this kinetic analysis revealed that the prolonged association of post-incision factors (PCNA) at damage sites is independent of TC-NER or CPD repair, as demonstrated by similar kinetics in α-amanitin–treated cells and XP-E cells (lacking CPD repair), respectively (Fig. S1 B). Despite defective repair of 6-4PP (Fig. 1 A), treatment of non-cycling NHF with HU and AraC does not prevent accumulation of NER subcomplexes at sites of UV damage when monitored 30 min after irradiation (Fig. 2 B). Nevertheless, impairment of the DNA repair synthesis step prevents release of NER subcomplexes because all factors tested (including preincision factor XPB) remained visible at the damage spots for up to 20 h (Fig. 2 B).

Closely resembling the effect of DNA repair synthesis inhibition, defective incision leads to prolonged accumulation of pre-incision factors (XPB, XPC) in UV spots 20 h after UV irradiation of XP-A cells (Fig. 2, C and D). Accumulation of post-incision factors is absent in these cells (Green and Almouzni, 2003; Moser et al., 2007).

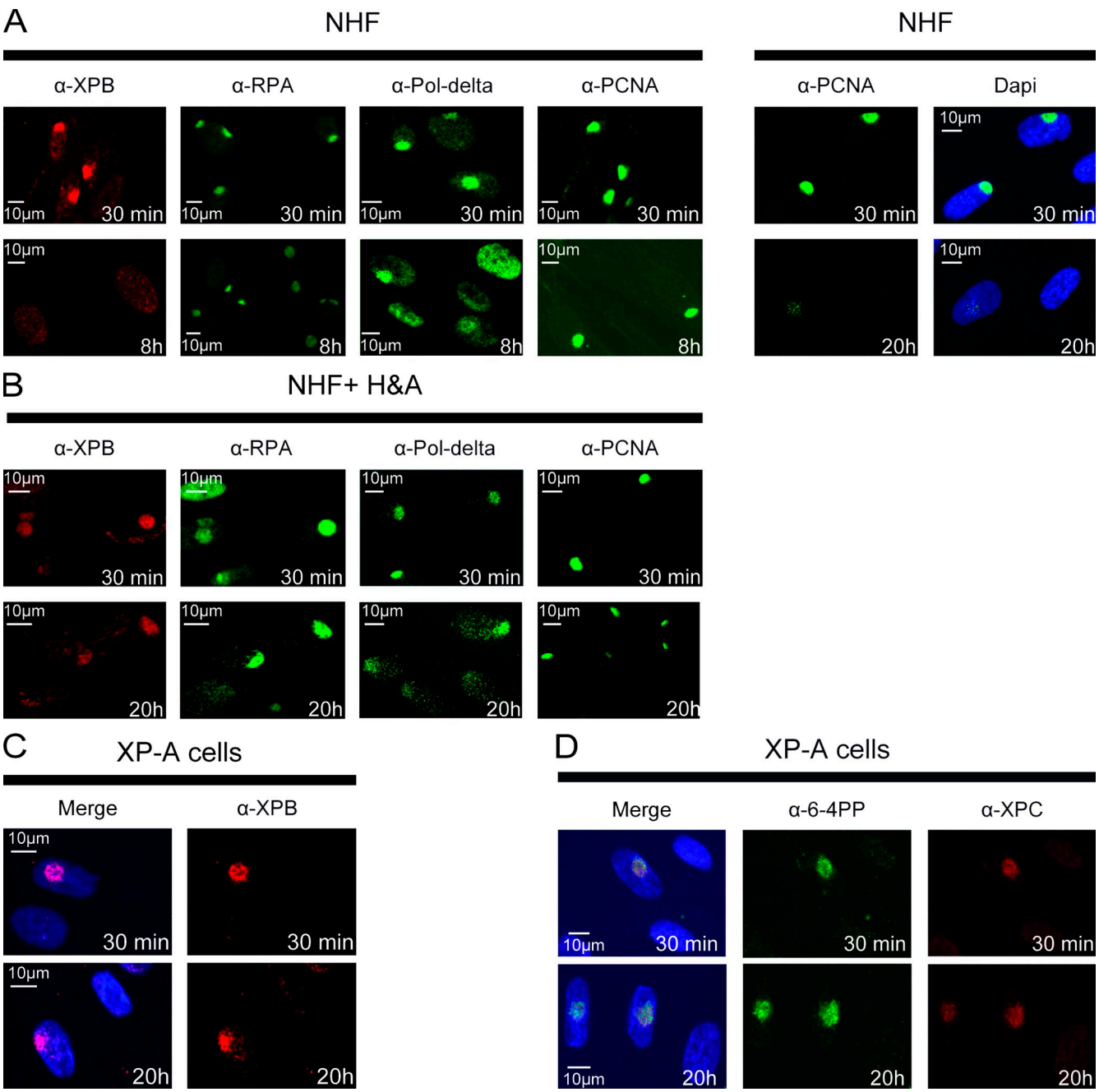


Figure 2. **Prolonged accumulation of pre- and post-incision factors at NER sites in the presence of replication inhibitors.** (A) Fluorescent immunostaining of XPB, RPA, Polδ, and PCNA localization at damage sites in confluent NHF after local UV irradiation (30 J/m$^2$) at time points as indicated. "Merge" refers to the combined image of DAPI and PCNA staining. (B) Immunolocalization of XPB, RPA, Polδ, and PCNA in NHF in the presence of HU and 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 were visualized by immunostaining after local UV irradiation; cells were irradiated with 30 J/m$^2$ UV and incubated for either 30 min or 20 h.

### NER-mediated incision is required for the release and relocalization of preincision factors to new repair sites

Although persistent accumulation of XPB and XPC at local UV spots in XP-A cells (Fig. 2, C and D; Volker et al., 2001) is suggestive of a stable preincision NER complex, live-cell imaging studies favor a model in which preincision NER factors dynamically interact with high affinity to their substrate, i.e., damaged DNA (Overmeer et al., 2010). Hence, the data are also consistent with a dynamic equilibrium of processes that may allow assembly and disassembly of NER factors at photolesions within the UV spot (which contains multiple 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 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

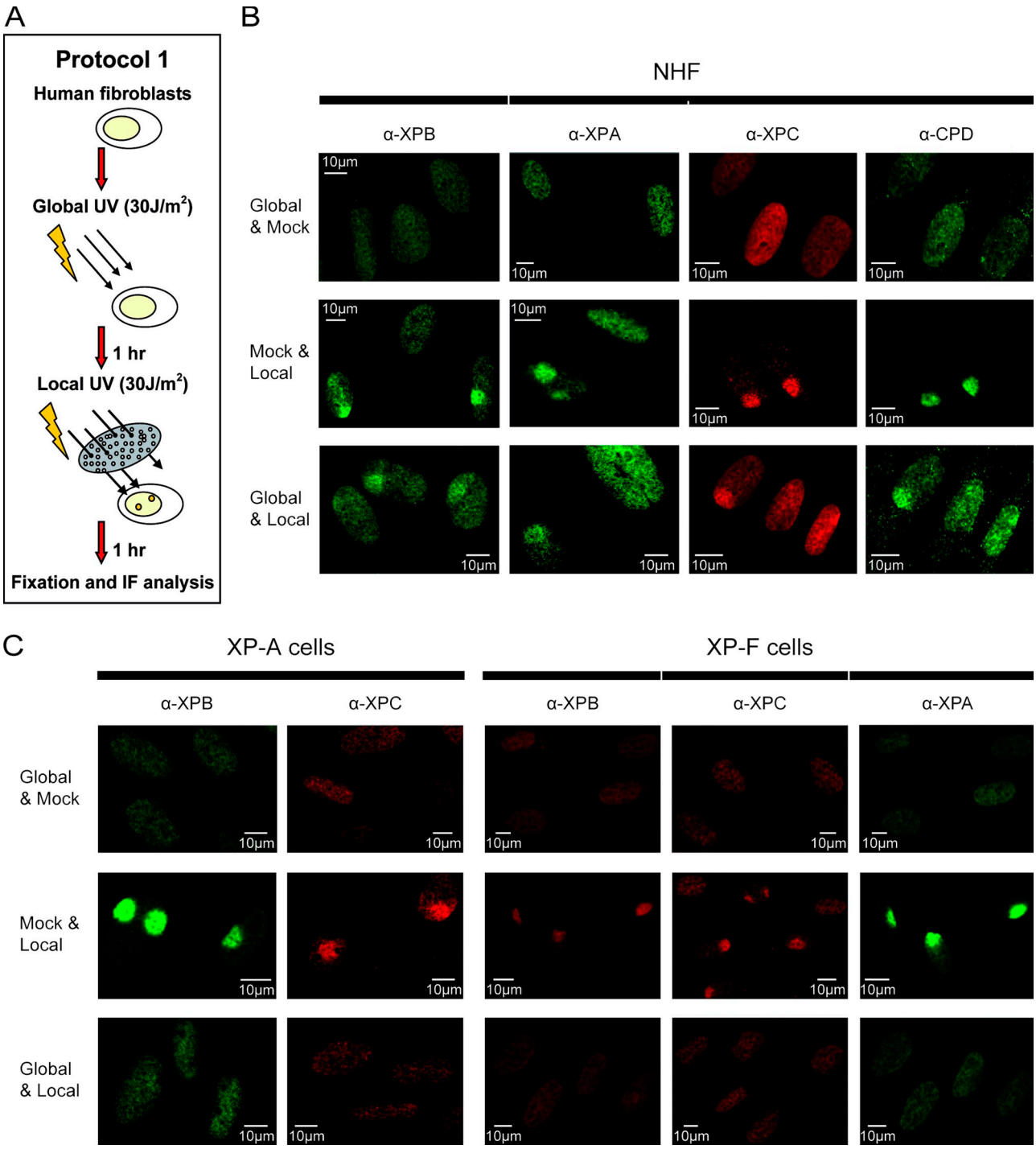


Figure 3. **Preincision factors remain associated to initial repair sites in the absence of incision.** (A) Schematic representation of Protocol 1. Cells were globally UV irradiated (30 J/m$^2$) in the absence of HU and AraC, recovered for 1 h, and were subsequently irradiated with the same dose of local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

damages induced by the second UV irradiation. This allows 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/m$^2$, a dose that saturates GG-NER (Erixon and Ahnström, 1979; Smith and Okumoto, 1984), cultured for 1 h, and subsequently exposed to local UV (30 J/m$^2$) or mock irradiation (Fig. 3 A; Protocol 1, global-local UV irradiation). After the second UV irradiation, cells are incubated for 1 h before analysis.

To validate the experimental setup of Protocol 1 we analyzed the distribution of preincision NER factors in NHFs in the absence of DNA synthesis inhibitors. Accumulation of XPB, XPC, and XPA is observed at damage sites induced by the second (local) UV irradiation (Fig. 3 B), demonstrating that the

experimental protocol allowed detection of 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 a similar initial global UV irradiation of 30 J/m$^2$ engages all available XPB, XPC, and XPA at UV lesions, as these cells lack accumulation of the repair factors at damages induced by the subsequent local UV irradiation (Fig. 3 C). To verify the latter we reversed the order of UV irradiations (Fig. S2 A; i.e., Protocol 2, local-global UV irradiation). We found that, in the absence of inhibitors, local accumulation of preincision factors in XP-F cells could not be competed out by the second globally induced UV damages (Fig. S2 B). Taken together, these data demonstrate that release of preincision factors from NER sites in vivo is dependent on incision and/or formation of a functional preincision complex. Furthermore, our data suggest that assembly of NER preincision complexes at UV damages in the absence of XPA or XPF/ERCC1 leads to incomplete repair complexes that are unable to associate with new damage 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 after inhibition of DNA repair synthesis (Fig. 2, A and B). For this purpose, we performed competition experiments with NHF in the presence of HU and AraC according to Protocol 2 (Fig. S2 A, local-global UV irradiation). Notably, XPA and XPB are able to dissociate from NER sites with impaired DNA synthesis and to engage in new repair events (Fig. S2, C and D). These data clearly demonstrate that damage incision and not the completion of the gap-filling/sealing stage of NER is the prerequisite for the release of preincision factors. In contrast, RPA remains firmly bound at the initially induced damaged sites and cannot be challenged away by the second global UV irradiation before DNA repair synthesis is complete (Fig. S2 D), underlining its essential role in the post-incision step of NER.

To exclude any interference between local (through 8-µm pores) and global UV irradiation, we replaced the global by an additional local UV irradiation (same dose of 30 J/m$^2$) through 3-µm pores (Fig. 4 A, Protocol 3). In NHF, XPA accumulates at the initial (larger) sites of damage as well as the subsequently induced (smaller) sites, both in the absence and presence of DNA synthesis inhibitors (Fig. 4 B), confirming the results of Fig. S2 C. We then repeated the same experimental approach for XPB, RPA, and PCNA as well as XPG and ERCC1 (Fig. 4 C and Fig. S3 A). In the absence of inhibitors, all factors behave similar to XPA and accumulate at both large and small UV spots within the same cell and with similar intensities. Also, in the presence of the inhibitors, XPB, XPG, and ERCC1 behave similar to XPA (Fig. 4 C and Fig. S3 A). In contrast, PCNA and RPA cannot be competed out by the second irradiation (small damage spots) because these proteins are absent from the small spots and remain confined at the initial repair sites (large damage spots; Fig. 4 C). We note that in few double UV-irradiated cells minor quantities of RPA are visible in the small damage spots (among 55 cells examined, 85% had no detectable or very weak RPA staining of small spots, whereas 15% had reduced RPA staining). This is most likely due to incomplete inhibition (~10% residual repair synthesis) of NER gap filling by the replication inhibitors (Mullenders et al., 1985). Although we cannot exclude that XPG might reside longer than XPF/ERCC1 at NER sites (Staresincic et al., 2009), our data clearly show that XPG (Fig. S3 A) behaves similar to the other NER preincision factors, i.e., it is able to dissociate from the incomplete NER sites in the presence of HU and AraC. We performed competition experiments (Protocol 3) with Seckle syndrome cells (with impaired ATR-dependent signaling) in the presence of HU and AraC to assess the dynamic nature of the accumulated NER factors. These experiments reveal that pre- and post-incision factors such as XPB, PCNA, and RPA behave in Seckle syndrome cells similar to NHF (Fig. 4 D, unpublished data).

We showed (Fig. 3 C) that, in vivo, XPC is unable to leave the NER sites before incision and/or a functional preincision complex has been formed. This observation contrasts in vitro observations suggesting that XPC leaves the complex before incision takes place (Wakasugi and Sancar, 1998). To further verify our data, we analyzed the localization of XPC in incision-deficient cells lacking either XPA or XPF/ERCC1 proteins using 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 after a dose that saturates NER (100 J/m$^2$ local UV irradiation; Smith and Okumoto, 1984), XPC remains engaged at the initial nonincised damage sites in XP-A (Fig. S3 B) and two XP-F cell lines (either expressing a severely truncated XPF protein or a catalytically dead XPF protein; Staresincic et al., 2009; Fig. S3 C), confirming that, similar to the other preincision factors, dissociation of XPC from NER sites requires incision and/or all preincision factors to be present.

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

Single-strand DNA gap intermediates generated by NER-mediated incisions in nondividing human cells trigger the phosphorylation of histone H2AX (Ser 139) in an ATR-dependent manner (Matsumoto et al., 2007). To examine whether accumulation of preincision factors to subsequently induced DNA damages in the presence of DNA synthesis inhibitors leads to new incisions, we performed competition experiments (Protocol 3) and γ-H2AX/XPB costaining in quiescent NHFs. Under normal conditions, γ-H2AX is observed at both the initial and subsequently induced UV spots, coinciding with XPB (Fig. 5 A). However, in the presence of HU and AraC, phosphorylation of H2AX occurs at the initial large (8 µm) UV spot but is absent from the subsequent small (3 µm) spots, in contrast to XPB. As expected, ATR-deficient Seckle cells lack phosphorylation of H2AX (Fig. 1 B).

To examine whether impairment of NER-mediated ligation would also generate a response similar to HU and AraC, we performed competition experiments (Protocol 3, local-local UV irradiation) in the presence of L67 inhibitor (Chen et al., 2008). Treatment of NHF with L67 for 4 h before UV irradiation leads to recruitment of preincision proteins to newly induced UV spots; in contrast, post-incision factors are confined to the initial UV

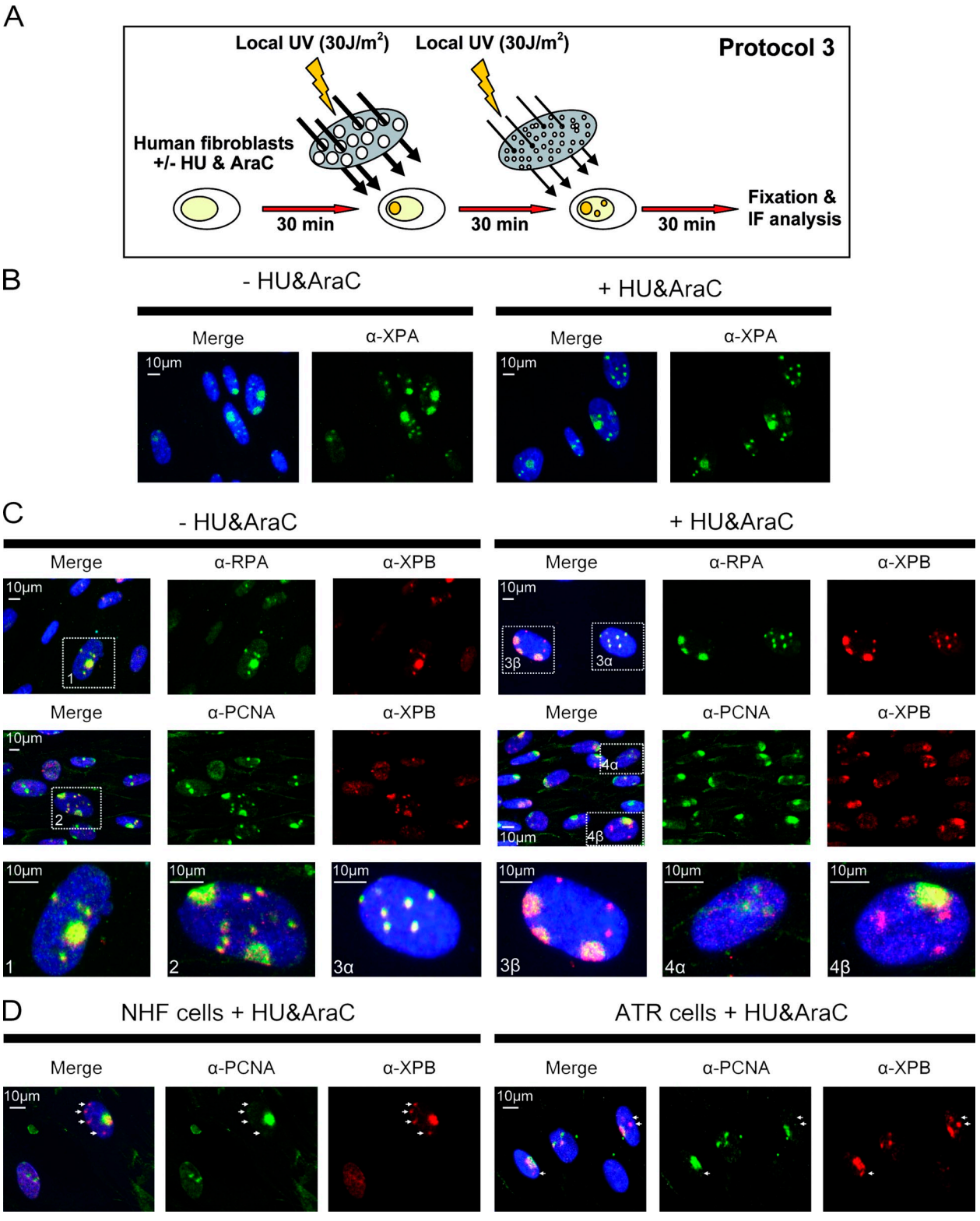


Figure 4. **Impairment of DNA synthesis prevents recruitment of RPA and post-incision factors to de novo UV damage.** For clarity, representative cells (numbered boxes) are enlarged and depicted below. (A) Schematic representation of competition experiments according to Protocol 3. Cells were treated or mock-treated with DNA synthesis inhibitors 30 min before the first local (8 μm) UV irradiation, which was followed (after 30 min) by the second local (3 μm) irradiation. Cells that have been hit twice by UV irradiation contain both large and small spots. (B) Competition experiments (Protocol 3) confirm dynamic association of XPA with damage sites in the presence and absence of DNA synthesis inhibitors in confluent NHFs. (C) Confluent NHFs treated as in B and co-stained with RPA, PCNA, and XPB antibodies. (D) Confluent NHFs and Seckle syndrome cells (ATR) were treated according to Protocol 3 in the presence of HU and AraC, followed by immunofluorescent staining of PCNA and XPB. For clarity, small spots are indicated with arrows.

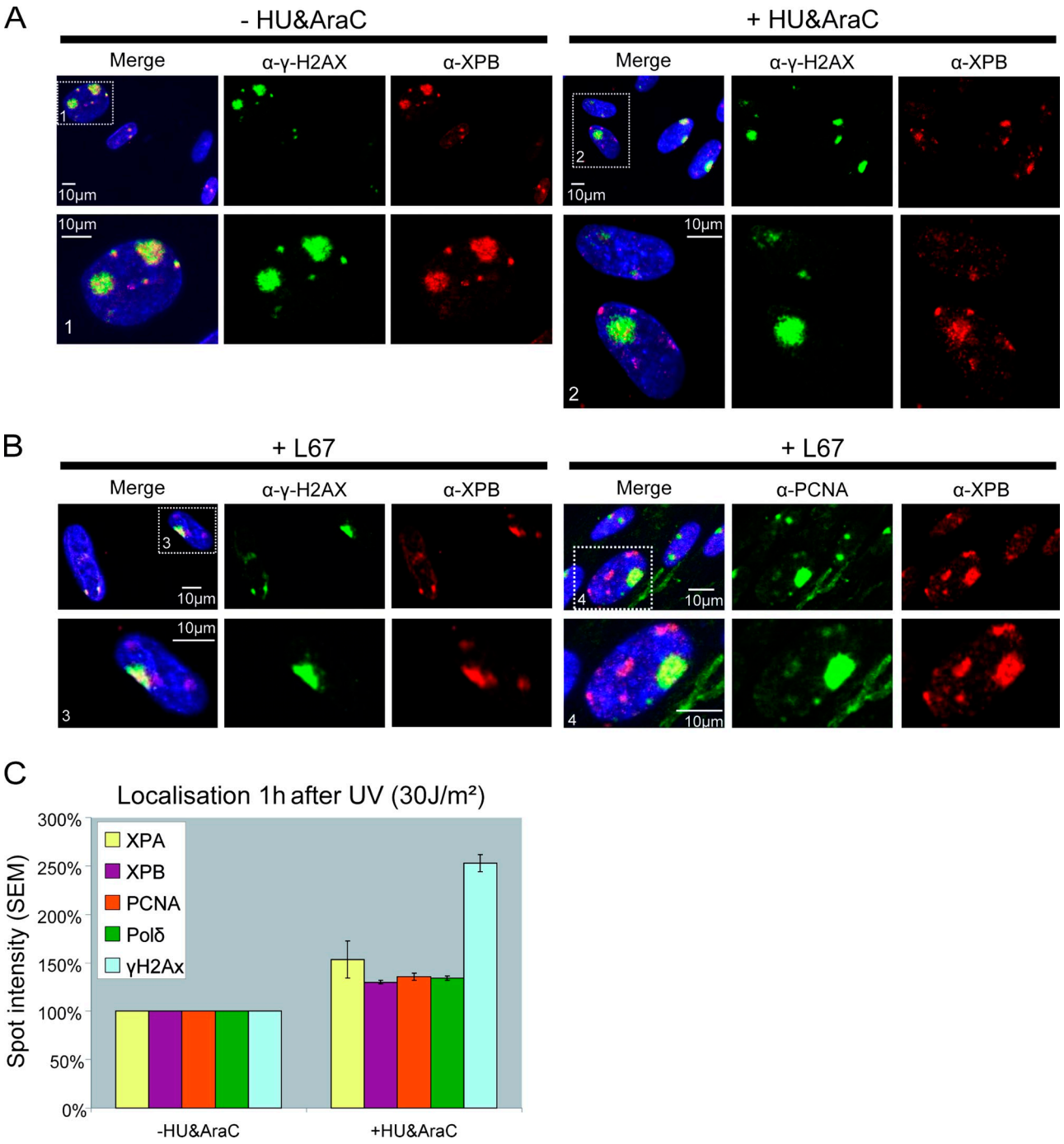


Figure 5. **Inhibition of DNA repair synthesis or ligation prevents novel incision events and leads to prolonged accumulation of post-incision factors.** (A) Quiescent NHFs were irradiated according to Protocol 3. Incision events are visualized by γ-H2AX staining; counterstaining for XPB revealed areas of damage induction. Γ-H2AX accumulation increases in time, thus the intensity is lower at the second UV spots when compared with the initially induced damage. Also, incubation with inhibitors increases γ-H2AX accumulation, therefore microscopic exposure time for γH2AX is threefold shorter when cells are irradiated in the presence of inhibitors. (B) NHFs were irradiated according to Protocol 3 in the presence of L67 inhibitor and stained for PCNA, XPB, and γ-H2AX. For clarity, small spots are indicated with arrows. (C) NHFs were locally irradiated in the presence or absence of inhibitors, fixed 1 h later, and stained for XPA or XPB in combination with PCNA, Polδ, or γH2AX. Average intensity inside local spots was 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.

spots (Fig. 5 B). Similarly, phosphorylation of H2AX occurs only at the initial UV spots, suggesting that under conditions of inhibited ligation, relocalization 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 intensity of γ-H2AX at the initial UV damages is enhanced ∼2.5-fold in the presence of inhibitors (Fig. 5 C), suggesting that the level of H2AX phosphorylation greatly increases when gap filling is perturbed. Approximately 1.2-fold increased intensity is observed for all NER factors at UV-damaged spots in the presence of HU and AraC (single UV dose, Fig. 5 C).

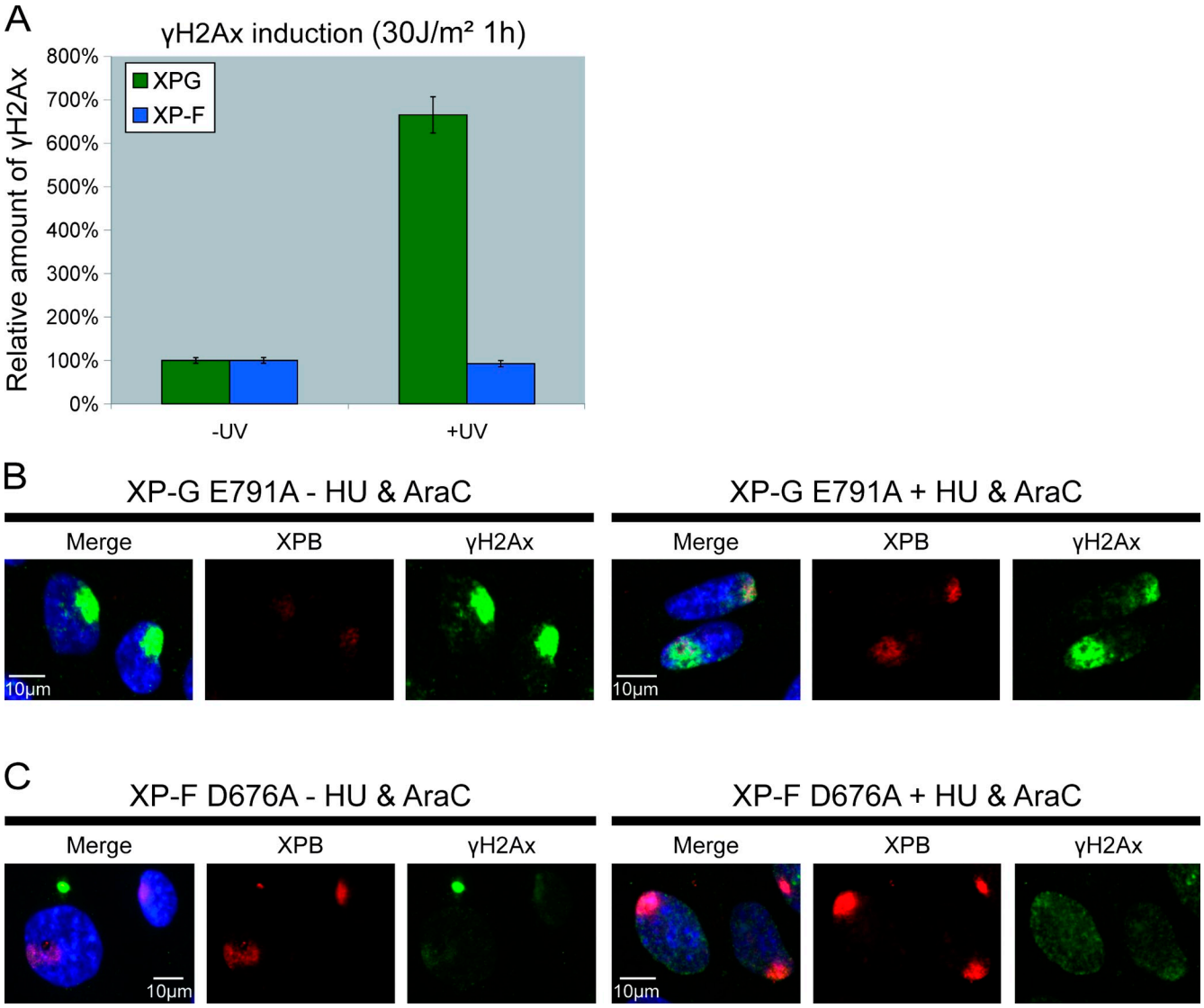


Figure 6. **Single 5′ incision leads to γ-H2AX phosphorylation independent of DNA synthesis.** (A) Catalytically dead XP-G and XP-F cells were stained for γ-H2AX 1 h after UV or mock irradiation with 30 J/m$^2$. Average intensity per nucleus was quantified and normalized to mock treatment. (B) Catalytically dead XP-G and XP-F were locally irradiated with 30 J/m$^2$ in the presence or absence of inhibitors. 1 h later, cells were fixed and stained for XPB and γ-H2AX.

### Single 5′ incision can initiate ATR signaling

We considered the possibility that absence of γ-H2AX in the small damage spots might be due to the fact that inhibition of repair synthesis only allows 5′ incision to occur, as the second 3′ incision may depend on initiation of repair synthesis (Staresincic et al., 2009). Hence, a single 5′ incision may not be sufficient to induce ATR-dependent H2AX phosphorylation. To approach this question we first examined whether a single incision is capable to induce γ-H2AX signaling at NER sites. We used XP-G and XP-F cells complemented with the corresponding catalytically dead XPG and XPF mutants (provided by Dr. Orlando Scharer, Stony Brook University, NY) that execute 5′ incision by XPF/ERCC1 only or no incision at all, respectively (Staresincic et al., 2009). Fig. 6 A shows that, in XP-G–complemented cells, global UV irradiation induces a significant increase of H2AX phosphorylation, which is absent in XP-F–complemented cells. We then examined H2AX phosphorylation in locally UV-irradiated XP-G cells complemented with the catalytically dead XPG mutant. A profound H2AX phosphorylation, which was present also in the presence of HU and AraC, is observed (Fig. 6 B); in contrast, XP-F cells, expressing a catalytically dead XPF mutant, are not capable to carry out incision and thus lack H2AX phosphorylation upon UV exposure (Fig. 6 C). These experiments reveal that a single 5′ incision by XPF/ERCC1 at NER sites can provoke ATR-dependent signaling and H2AX phosphorylation even when repair replication is inhibited.

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

We find that RPA is confined to sites of incomplete DNA repair synthesis, whereas the rest of the preincision factors are able to reassociate with newly induced UV damages. To exclude any limitation of our approach to detect relocalization of RPA to small spots (Protocol 3), we assessed the effect of RPA knockdown (KD) on the recruitment of NER subcomplexes to locally

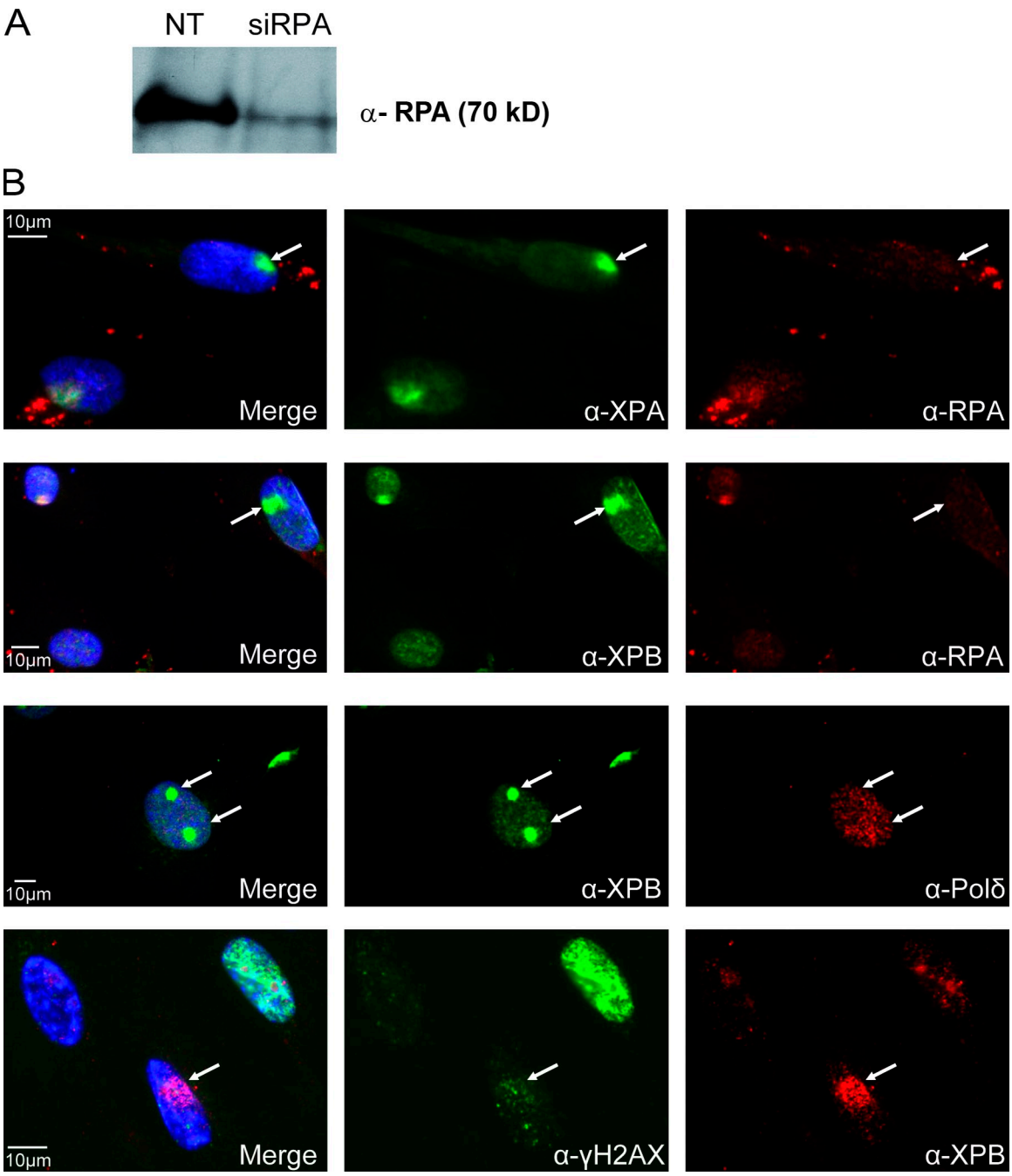


Figure 7. **RPA is prerequisite for the functional assembly of NER subcomplexes.** (A) Western blot analysis of equal amounts of whole cell lysates prepared from cells treated or nontreated (NT) with siRNA against RPA p70. (B) Cells treated with siRNA against RPA p70 were locally UV irradiated (30 J/m$^2$), fixed 1 h later, and stained with XPA, XPB, Polδ, and γ-H2AX antibodies. Absence of RPA was verified with RPA costaining (arrows).

induced UV damage. KD of RPA p70 (Fig. 7 A) has no effect on the accumulation of preincision NER factors such as XPB and XPA at UV spots, but prevents recruitment of Polδ and incision, as indicated by the absence of H2AX phosphorylation (Fig. 7 B). Hence, RPA KD does not impinge on the recruitment of preincision factors to UV damage, yet it is absolutely required for NER-mediated incision and the assembly of post-incision factors.

We hypothesized that, in the presence of inhibitors, depletion of RPA by virtue of its participation in post-incision complexes prevents its subsequent assembly into preincision complexes and hence impedes further incisions (Fig. 5 B, γ-H2AX staining). To support this hypothesis, we assessed the amount of RPA in nuclear extracts isolated from nondividing NHFs 1 h after exposure to UV in the presence or absence of HU and AraC. In the absence of inhibitors, we monitored a dose-dependent decrease of RPA in the nuclear fraction with the largest depletion in cells exposed to 30 J/m$^2$ (Fig. S3 C). Interestingly, in the presence of DNA synthesis inhibitors, this maximum depletion is observed with a dose as low as 5 J/m$^2$ and no further depletion is found at increasing doses (Fig. S3 C). Due to the high amounts of RPA present in chromatin before 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 classical chromatin immunoprecipitation (ChIP) of in vivo cross-linked confluent NHFs and analyzed the coprecipitated proteins by Western blotting (Fousteri et al., 2006; Moser et al., 2007; Coin et al., 2008). In line with our previously published data (Moser et al., 2007), XPB-specific ChIP yields an increased interaction of XPB with preincision factors such as XPA and RPA in UV-irradiated cells, whereas post-incision factors, i.e., Polδ, Lig3, and XRCC1, are virtually absent (Fig. 8 A), 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 and AraC before UV irradiation, the XPB interaction with XPA is further enhanced (Fig. 8 A). This is consistent with the 1.2-fold increased accumulation of NER factors that we observed at UV spots in the presence of inhibitors (Fig. 5 C). However, treatment of cells with HU and 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 perform ChIP-on-Western with an XRCC1 antibody; a clear UV-increased interaction can be found between XRCC1, RPA, and Polδ, which is further enhanced in the presence of HU and AraC (Fig. 8 B). This is in agreement with the increased association of DNA synthesis factors (including RPA) at sites of incomplete DNA repair synthesis. As reported previously (Moser et al. 2007), the ratio between XRCC1 and Lig3 is not significantly altered by the UV irradiation.

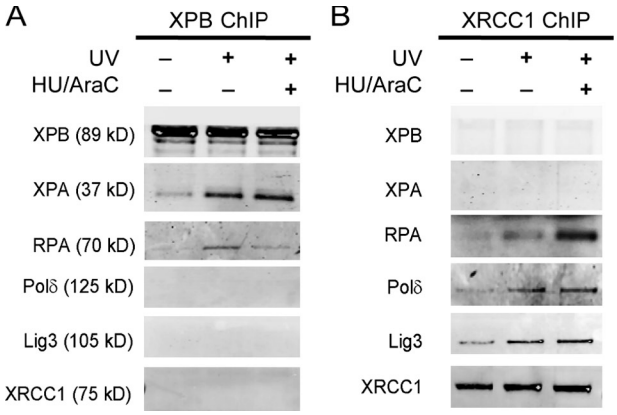


Figure 8. **DNA synthesis inhibitors sequester RPA to sites of incomplete NER repair synthesis.** Confluent NHFs were (mock) irradiated with 20 J/m$^2$ in the presence or absence of inhibitors, let to recover, and cross-linked 40 min later. ChIP was performed with XPB (A) and XRCC1 (B) specific antibodies and Western blot analysis of the coprecipitating proteins was performed with antibodies as indicated.

## Discussion

The results reported here provide novel mechanistic insights in the regulation of NER in human cells. We find that in nondividing NHFs, 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 repair inhibition of UV photolesions in an ATR-independent manner. Under these conditions, preincision 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 pre- and post-incision NER complexes, plays a unique role in controlling the transition from pre- to post-incision stages by linking initiation of new repair events to completion of ongoing DNA gap-filling/sealing events. Incomplete preincision complexes, containing RPA, which are unable to perform incision (i.e., XP-A, XP-F cells), accumulate at damage sites, demonstrating that their disassembly is dependent on 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 before incision has been observed in in vitro experiments (Wakasugi and Sancar, 1998; Riedl et al., 2003). On the other hand, competition experiments with incision-deficient XP cells demonstrate that this situation might be different in vivo, given that XPC stably assembles in NER preincision complexes and cannot be competed away (Fig. S3, B and C). Hence, NER-mediated incision is the key determinant for the release of preincision NER proteins except RPA. NER incision is also required for efficient recruitment of the post-incision factors (Aboussekhra and Wood, 1995) and, compared with the preincision 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 4 min (Erixon and Ahnström, 1979), closely mimicking the residence time of most NER preincision factors at UV damage (Moné 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 repair of CPD by GG-NER; Fig. 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 despite their dynamic nature (Essers et al., 2005; Luijsterburg et al., 2010). 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 dual incision is followed by inhibition of repair patch synthesis/ligation, preincision factors but none of the post-incision factors or RPA dissociates from sites of ongoing repair and reassociate with unprocessed UV photolesions. Notably, XPF/ERCC1 accumulates independently of RPA, raising questions on the exact mechanism of its recruitment. Although RPA greatly enhances the binding of XPF to artificial structures in vitro (Matsunaga et al., 1996; de Laat et al., 1998), 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. Several studies suggest that XPA and TFIIH are sufficient to recruit XPF and XPG, respectively (Li et al., 1994; Park and Sancar, 1994; Iyer et al., 1996; Volker et al., 2001). However, in the absence of RPA this will lead to a nonfunctional preincision complex that lacks incision activity (this paper).

The number of incisions made by NER in the presence of HU and AraC reaches a maximum 1 h after irradiation and at doses as low as 2–5 J/m$^2$ (Snyder et al., 1981; Smith and Okumoto, 1984; Mullenders et al., 1985; Berneburg et al., 2000), indicating that under these conditions, NER is noncatalytic, 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 (Fig. 1 A; Moser et al., 2007), the depletion of soluble nuclear RPA (Fig. S3 C), the enhanced H2AX phosphorylation (Matsumoto et al., 2007), and the increased accumulation of NER factors at UV damage spots (Fig. 5 C). In addition, no H2AX phosphorylation is observed when preincision factors are relocalized to sites of the second UV irradiation in the presence of NER synthesis or ligation inhibitors (Fig. 5, A and B), suggestive of no incision events at these sites. The latter is further highlighted by the challenging observation that a single 5′ incision in a NER complex provokes ATR-dependent signaling and H2AX phosphorylation even when DNA repair synthesis is inhibited.

We considered two possible mechanisms underlying restriction of incision events and impaired repair when completion of NER is inhibited. First, it is conceivable that DNA damage induced signaling (manifested by H2AX phosphorylation) prevents further incisions when gap filling is not completed. UV-induced signaling has been shown to depend on ATR. Most likely, this signaling is activated by the formation of RPA-bound single-stranded DNA patches formed by NER-mediated incisions (O'Driscoll et al., 2003; Marini et al., 2006; Marti et al., 2006; Matsumoto et al., 2007) 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 signaling in confluent human cells, consistent with a previous report (Auclair et al., 2008).

A second mechanism could be that one or more NER factors become "trapped" inside existing yet inhibited NER complexes and hence are unable to associate with other photolesions. It has been shown that incision by XPF/ERCC1 occurs first, allowing free 3-OH 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 the repair synthesis step is inhibited. Nevertheless, under conditions that impair repair synthesis in normal cells (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 is a gap generated by dual incision. Based on the current findings and in vitro studies (Riedl et al., 2003), RPA is the only NER protein that is recruited before incision and remains associated at the site of repair after dual incision and recruitment of post-incision factors. The association of RPA that could not be challenged away from initial sites of damage induction in the presence of inhibitors (Fig. 4 C and Fig. S2 D) implies that RPA remains engaged 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 its engagement in new repair initiation events. As a consequence, abortive preincision repair complexes are formed, which are incapable of incision. In support of this hypothesis, ChIP-on-Western experiments (Fig. 8, A and B) show an increased interaction of RPA with post-incision factors and a decreased coprecipitation with preincision factors under conditions that impair NER-mediated DNA synthesis and ligation.

The current and published (Auclair et al., 2008) observations show that efficient NER depends on ATR in S but not G1 cells. 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 an ATR-dependent manner 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, thereby preventing additional NER-mediated incision events to take place.

In summary, our results substantially extend the findings from in vitro studies (Wakasugi and Sancar, 1998; Riedl et al., 2003; Mocquet et al., 2008). Although in vitro experiments show persistent binding of RPA, the current study adds mechanistic insights into how the participation of RPA in pre- and post-incision stages of NER regulates incision, and how impaired gap filling/sealing leads to an overall defect in photolesion repair. We show that in chromatin of UV-irradiated cells a complex of all preincision factors without RPA can be formed. In addition, our data are more consistent with a release of all preincision factors including XPC upon damage incision in vivo, contrary to reported in vitro data. We propose a model wherein RPA regulates NER by allowing initiation of new repair events only after completion of ongoing repair synthesis (Fig. 9). According to this model, preincision 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 preincision factors. Under normal conditions, the released RPA is able to associate with and subsequently stabilize and activate newly formed preincision complexes, enabling further incision events and continuation of repair. In contrast, the persistent association of RPA in post-incision complexes when repair is incomplete 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.

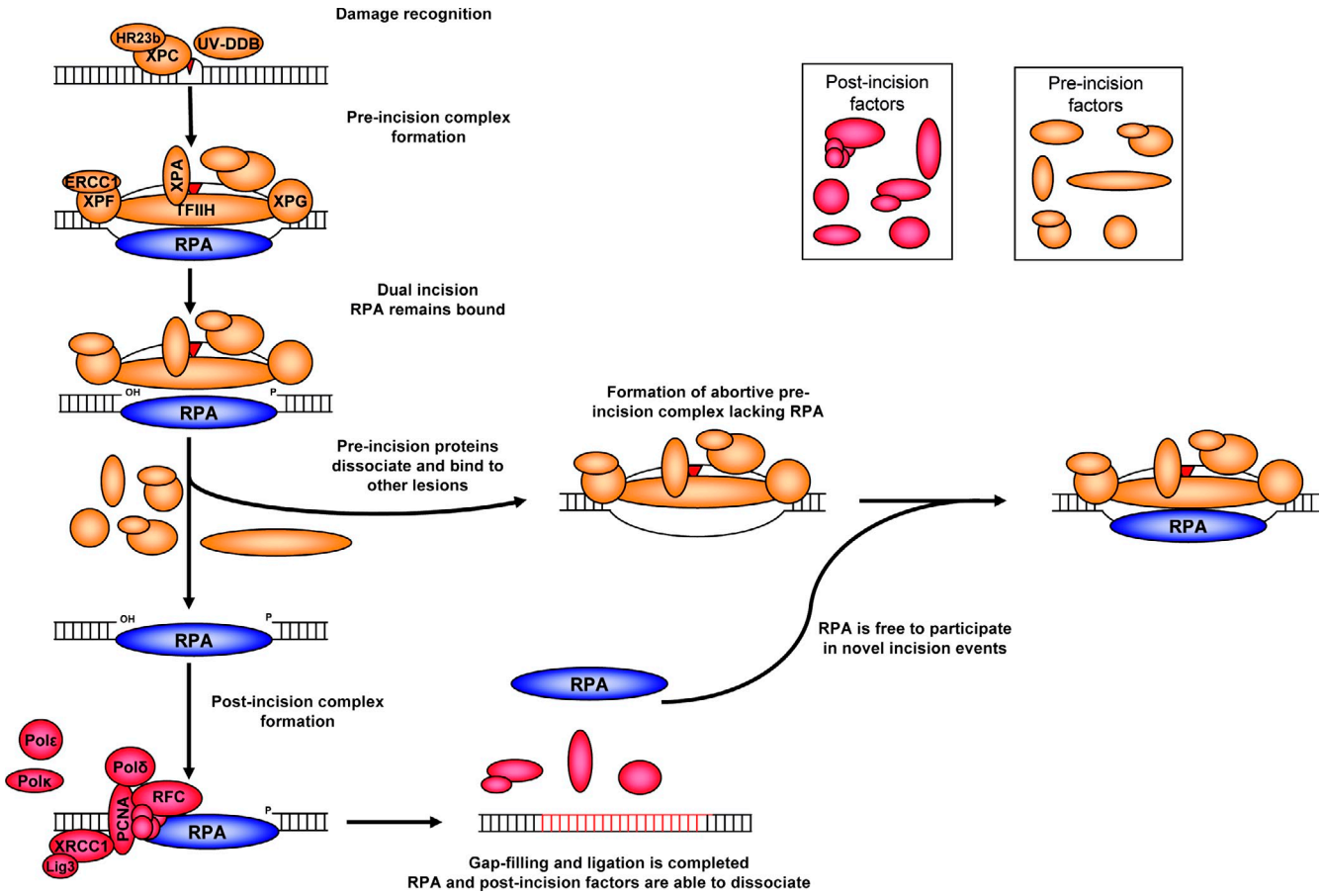


Figure 9. **Schematic depiction of regulation of NER-mediated incision events in vivo.** Upon lesion recognition by UV-DDB and XPC-hHR23B, local opening of DNA by TFIIH provides access to the core GG-NER machinery, i.e., XPA, RPA, XPG, and XPF/ERCC1. RPA binds to the undamaged single-stranded DNA stabilizing 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, which remains bound to the repair site, probably on the undamaged single-stranded DNA. The later stages of repair are performed by RFC stable 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 and UV irradiation

Cells used in this study have been grown in DME supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% $CO_2$ 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). In addition, SV40-transformed XP-G and XP-F cells, complemented with catalytically dead XPG and XPF, respectively, were used (Staresincic et al., 2009).

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

### Immunofluorescence

Immunofluorescent labeling of proteins or UV photolesions (6-4PP) was performed essentially as described previously (Volker et al., 2001; Moser et al., 2007). In brief, cells were 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. After fixation, cells were washed with cold PBS and incubated with 5% bovine albumin in PBS for 30 min at RT. The cells were subsequently incubated with primary antibodies and diluted in wash buffer (WB; PBS containing 0.5% bovine serum albumin and 0.05% Tween 20) for 2 h at RT. The cells were washed three times with WB and thereafter incubated with secondary antibody for 1 h at RT. Cells were mounted in Aqua/Polymount (Polysciences, Inc.) containing 1.5 µg/ml DAPI. Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2005). In brief, images were captured with a microscope (Axioplan2; Carl Zeiss, Inc.) equipped with a camera (Axiocam MRm; Carl Zeiss, Inc.) using either a Plan-NeoFluar 40/1.30 or 63/1.25 objective. Images were taken with equal exposure times and the total fluorescence per nuclei was measured for 50–100 nuclei per point per experiment (Axiovision software).

### Antibodies

The following primary antibodies were used in this study: rabbit polyclonal α-XPA, α-p89 (XPB), α-ERCC1 and mouse monoclonal α-DNA Polδ (Santa Cruz Biotechnology, Inc.); mouse monoclonal α-PCNA (PC10), α-XRCC1, and α-XPA (Abcam); mouse monoclonal α-DNA ligase 3α (Genetex); mouse monoclonal α-XPG (8H7, Invitrogen); mouse monoclonal α-RPAp70 (Ab-1, Oncogene); and 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, 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 were 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 were UV irradiated (20 J/$m^2$) and incubated at 37°C for 40 min before in vivo cross-linking. In vivo cross-linking and purification of the cross-linked DNA–protein complexes was performed as described previously (Fousteri et al., 2006; Moser et al., 2007). All procedures were performed at 4°C. In brief, cells were cross-linked with 1% formaldehyde for 11 min and collected by scraping in cold PBS, after the addition of 0.125 M glycine solution. The cells were lysed in lysis buffer (50 mM Hepes, pH 7.8, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% Triton X-100, and 10% glycerol) with a 10-min rotation. The soluble supernatant fraction was removed after centrifugation (1,300 rpm, 5 min), whereas the pellet was further washed with wash buffer (0.01 M Tris-HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, and 1 mM PMSF) and finally resuspended in 1x RIPA buffer. The soluble chromatin was sonicated on iced water (nine times) using a Bioruptor (Diagenode) in 30-s bursts followed by 1 min of cooling. Samples were spun down (13,200 rpm, 15 min) and the fractionated chromatin was stored at −80°C.

For each ChIP reaction, an equal amount of precleared cross-linked chromatin from UV- and nonirradiated cells was incubated with up to 1 µg of the specific antibody in 1x RIPA at 4°C overnight. The immunocomplexes were collected by adsorption (3 h) to precleared protein A or G Sepharose beads (Millipore) in the presence of 0.1 mg/ml sonicated salmon sperm DNA (ssDNA) and 100 µg BSA. After five sequential washes with 1x RIPA with increasing salt concentrations, the beads were washed with 20 vol of LiCl buffer.

For Western blot analysis, one beads volume of 2x Laemmli SDS sample buffer was added to each reaction and the beads were incubated at 95°C for 30 min before gel loading. Western blot analysis was performed as described previously (Fousteri et al., 2006) and protein bands were visualized via chemiluminescence (ECL-Plus; GE Healthcare) using horseradish peroxidase–conjugated secondary antibodies and exposure to ECL-Hyperfilms (GE Healthcare). Alternatively, fluorescently labeled secondary antibodies were scanned by the Odyssey infrared imaging system (LI-COR Biosciences).

**RNA interference**

SmartPool siRNA oligos (Thermo Fisher Scientific) were used for all experiments unless otherwise noted. siRNA transfection was performed using HiPerfect (QIAGEN) transfection reagent according to the manufacturer's instructions. In typical experiments, 5 nM of siRNA oligos were transfected in suspension, termed "reverse transfection", and followed by one additional transfection cycle 24 h after the first transfection (double transfection). Experiments were performed 48 h after the first siRNA transfection. Knockdown efficiencies were confirmed by Western blotting and immunofluorescence.

**Online supplemental material**

Fig. S1 illustrates the disassembly kinetics of NER subcomplexes from sites of UV-induced DNA damage. Fig. S2 shows that dissociation of preincision factors from NER sites requires incision, whereas dissociation of RPA requires DNA synthesis. Fig. S3 confirms that post-incision factors and RPA cannot dissociate from sites of ongoing repair and associate with unprocessed UV lesions when DNA synthesis is impaired. It further demonstrates that release of XPC from NER sites in vivo is dependent on a functional preincision complex assembly and incision. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201006011/DC1.

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