

Oxidative Damage to RPA Limits the Nucleotide Excision Repair Capacity of Human Cells

Melisa Guven¹, Reto Brem¹, Peter Macpherson¹, Matthew Peacock¹ and Peter Karran¹

Nucleotide excision repair (NER) protects against sunlight-induced skin cancer. Defective NER is associated with photosensitivity and a high skin cancer incidence. Some clinical treatments that cause photosensitivity can also increase skin cancer risk. Among these, the immunosuppressant azathioprine and the fluoroquinolone antibiotics ciprofloxacin and ofloxacin interact with UVA radiation to generate reactive oxygen species that diminish NER capacity by causing protein damage. The replication protein A (RPA) DNA-binding protein has a pivotal role in DNA metabolism and is an essential component of NER. The relationship between protein oxidation and NER inhibition was investigated in cultured human cells expressing different levels of RPA. We show here that RPA is limiting for NER and that oxidative damage to RPA compromises NER capability. Our findings reveal that cellular RPA is surprisingly vulnerable to oxidation, and we identify oxidized forms of RPA that are associated with impaired NER. The vulnerability of NER to inhibition by oxidation provides a connection between cutaneous photosensitivity, protein damage, and increased skin cancer risk. Our findings emphasize that damage to DNA repair proteins, as well as to DNA itself, is likely to be an important contributor to skin cancer risk.

Journal of Investigative Dermatology (2015) **135**, 2834–2841; doi:10.1038/jid.2015.255; published online 6 August 2015

INTRODUCTION

Skin cancer is one of the few human cancers that are associated with a well-defined and pervasive mutagen. Non-melanoma skin cancer genomes are dominated by the mutational signatures of sunlight-induced DNA lesions (Alexandrov *et al.*, 2013; Jayaraman *et al.*, 2014; South *et al.*, 2014). The nucleotide excision repair (NER) system provides an important protection against skin cancer by removing potentially mutagenic DNA cyclobutane pyrimidine dimer (CPD) and pyrimidine (6-4) pyrimidone (6-4Py:Py) photolesions induced by solar UVB radiation. Inefficient NER in the genetic disorder xeroderma pigmentosum is associated with photosensitivity and a hugely increased skin cancer risk (Friedberg *et al.*, 2006).

Photosensitivity and an increased susceptibility to sunlight-related malignancy are side effects of some medications. For example, patients prescribed azathioprine for inflammatory bowel disorders or to prevent organ transplant rejection are UVA photosensitive and have high rates of non-melanoma skin cancer (Euvrard *et al.*, 2003; Peyrin-Biroulet *et al.*, 2011; Ramiscal and Brewer, 2013). Azathioprine metabolism

culminates in the incorporation of 6-thioguanine (6-TG) into patients' DNA where it acts as a potent UVA photosensitizer. In cultured human cells, the combination of DNA 6-TG and UVA generates reactive oxygen species. These cause widespread DNA and protein damage that is associated with inhibition of DNA repair (Gueranger *et al.*, 2014). Other photosensitizing drugs include the fluoroquinolone antibiotics that are photocarcinogens in mice (Itoh *et al.*, 2005) and may increase skin cancer risk in patients (Traianou *et al.*, 2012). Combinations of UVA and the fluoroquinolones ciprofloxacin or ofloxacin duplicate many of the effects of DNA 6-TG/UVA in cultured human cells. In particular, fluoroquinolone/UVA treatment causes DNA and protein damage and inhibits DNA repair, including NER (Peacock *et al.*, 2014).

Effective NER requires the coordinated activities of numerous proteins to recognize and process DNA lesions. The human replication protein A (RPA) is an abundant DNA-binding protein complex that participates in almost all aspects of DNA metabolism and is essential for NER. Its high-affinity binding protects single-stranded DNA against nucleases and facilitates the correct processing of unpaired DNA regions generated during replication and repair (Binz *et al.*, 2004). Recruitment of specific proteins to DNA-bound RPA is important in the activation of the cellular checkpoints that maintain genome stability in the face of arrested replication (Friedel *et al.*, 2009). Consistent with its pivotal role in protecting DNA, genetic inactivation of murine RPA is lethal, and haploinsufficiency is associated with cancer proneness and reduced life span (Wang *et al.*, 2005). Reduced RPA expression in cultured human cells results in spontaneous

¹Francis Crick Institute, Clare Hall Laboratory, South Mimms, Herts, UK

Correspondence: Peter Karran, Francis Crick Institute, Clare Hall Laboratory, Herts EN6 3LD, UK. E-mail: peter.karran@crick.ac.uk

Abbreviations: 6-4Py:Py, pyrimidine (6-4) pyrimidone; 6-TG, 6-thioguanine; CPD, cyclobutane pyrimidine dimer; NER, nucleotide excision repair; RPA, replication protein A

Received 20 April 2015; revised 1 June 2015; accepted 18 June 2015; accepted article preview online 2 July 2015; published online 6 August 2015

DNA damage and activation of DNA damage-related checkpoints (Dodson *et al.*, 2004). RFA, the yeast RPA homolog, is limiting for UV protection, and hypomorphic RFA mutations confer UVC sensitivity (Umezu *et al.*, 1998) and a mutator phenotype (Chen *et al.*, 1998).

RPA is a heterotrimer (Wold, 1997). Its subunits of 70, 32, and 14 kDa (RPA70, RPA32 & RPA14) contain several DNA- and protein-interacting domains. The most important DNA-binding motifs lie in the RPA70 subunit. Protein-interacting domains are located in the N terminus of RPA70 and the C terminus of RPA32 (Binz *et al.*, 2004). An N-terminal RPA32 domain also contains multiple conserved phosphorylation sites that control RPA function. RPA32 phosphorylation triggered by the presence of long single-stranded DNA tracts at arrested replication forks may serve to regulate the distribution of limiting RPA resources between replication and repair (reviewed in Binz *et al.* (2004)). The role of RPA14 appears to be largely to stabilize the heterotrimer.

We previously showed that protein oxidation is associated with NER inhibition in human cells treated with 6-TG/UVA or fluoroquinolone/UVA (Gueranger *et al.*, 2014; Peacock *et al.*, 2014). Our results suggested that RPA damage might underlie

the reduced NER capability. Here we demonstrate that the vulnerability of RPA to oxidation confers a risk of suboptimal NER under oxidative stress conditions. We describe oxidative RPA lesions in cultured human cells exposed to conditions that simulate events in the sun-exposed skin of patients taking azathioprine or fluoroquinolones. Our findings emphasize the importance of protein oxidation as a determinant of DNA repair efficiency and skin cancer risk in patients taking photosensitizing drugs.

RESULTS

RPA expression, NER, and toxicity

Treatment of human cells with 6-TG/UVA inhibits NER (Gueranger *et al.*, 2014). To investigate how RPA influences NER efficiency, we compared the removal of UVC-induced 6-4 Py:Py by U2OS and the related U2OS-RPA21 cells in which RPA is overexpressed (Toledo *et al.*, 2013). Western blotting confirmed an approximately 2-fold RPA overexpression in U2OS-RPA21 cells (Figure 1a). Increased RPA protected NER against inhibition by 6-TG/UVA. Whereas 6-TG/UVA-treated U2OS cells excised <20% of UVC-induced 6-4 Py:Py in 3 hours, similarly damaged U2OS-RPA21 cells

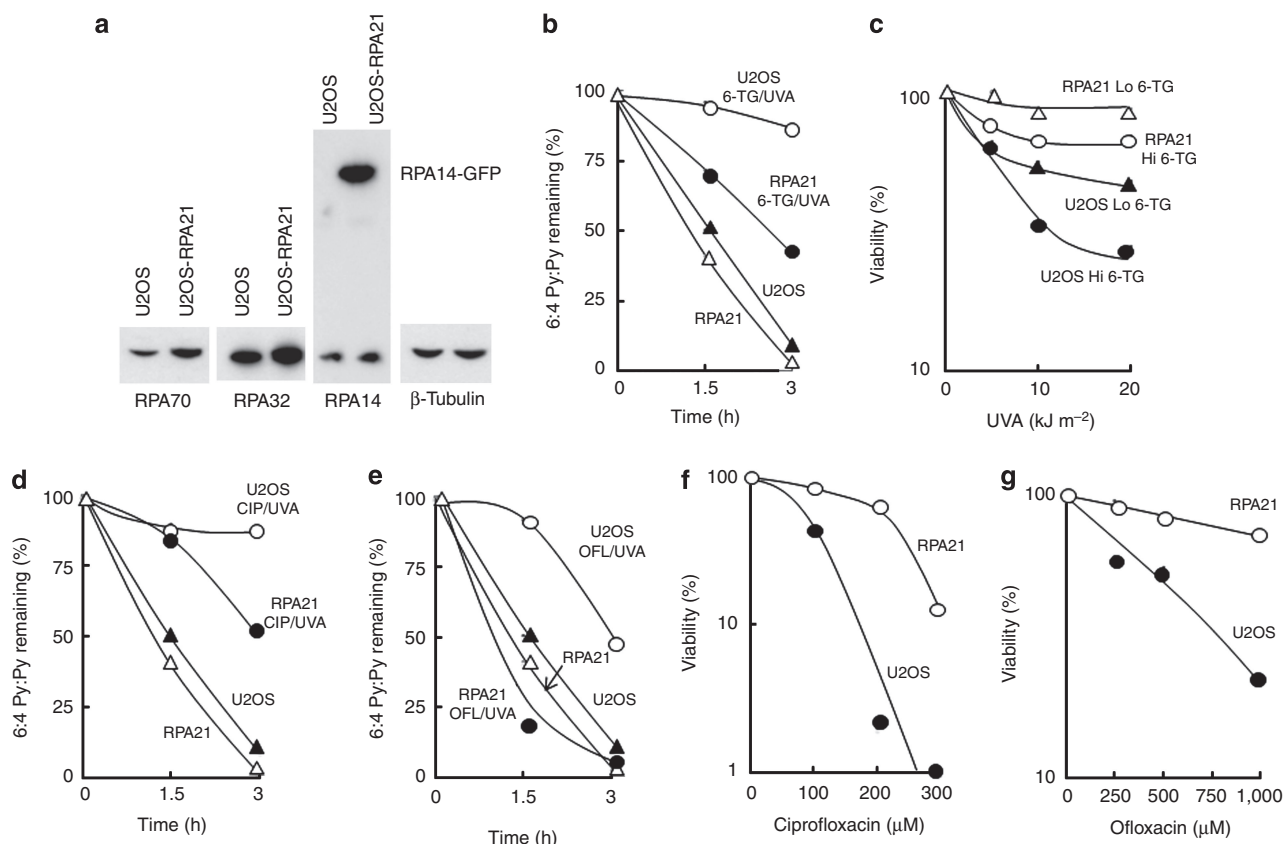


Figure 1. Replication protein A (RPA) overexpression, nucleotide excision repair (NER), and sensitivity. (a) Western blotting for RPA14, RPA32, and RPA70. (b) 6:4 Py:Py ELISA of 6-TG-treated U2OS and U2OS-RPA21 cells irradiated with UVA (20 kJ m^{-2})+ 10 J m^{-2} UVC. (Δ \blacktriangle) 10 J m^{-2} UVC alone. Mean values from ≥ 3 experiments. (c) Viability of UVA-irradiated cells with Lo ($\sim 0.06\%$) or Hi ($\sim 0.14\%$) DNA 6-thioguanine (6-TG). Mean values from two experiments. (d) 6:4 Py:Py ELISA of cells treated with ciprofloxacin (CIP; 1 hour; 0.5 mM)+UVA (20 kJ m^{-2})+UVC (10 J m^{-2}). Mean values from ≥ 3 experiments. (e) As in d, cells treated with 1 mM ofloxacin, UVA+UVC. (f) Ciprofloxacin/UVA toxicity. Viability of cells treated for 1 hour with ciprofloxacin and UVA (20 kJ m^{-2}). Mean values from ≥ 3 experiments. (g) Ofloxacin/UVA toxicity. As in f, cells were treated with ofloxacin and UVA.

removed ~60% of these photoproducts in the same period (Figure 1b). U2OS and U2OS-RPA21 cells irradiated with UVC alone removed >95% of 6-4 Py:Py in 3 hours.

Increased RPA expression also conferred resistance to the toxicity of 6-TG/UVA. MTT assays revealed a UVA dose-dependent decrease in U2OS viability at two levels of DNA 6-TG substitution. At the higher DNA 6-TG level, approximately <20% of U2OS cells survived after 20 kJ m^{-2} UVA. In contrast, DNA 6-TG-containing U2OS-RPA21 cells were largely insensitive to UVA, and >80% survived even at the highest UVA dose (Figure 1c). Treatment with 6-TG or UVA alone reduced viability by <10%.

6-TG is an atypical photosensitizer in that it is DNA embedded. The UVA absorbing fluoroquinolone antibiotics ciprofloxacin and ofloxacin replicate many of the photosensitizing effects of DNA 6-TG without incorporation into DNA. In particular, ciprofloxacin/UVA and ofloxacin/UVA induce oxidative stress and cause widespread protein damage that compromises NER (Peacock *et al.*, 2014). 6-4Py:Py ELISA confirmed that ciprofloxacin/UVA and ofloxacin/UVA also inhibited NER in U2OS cells. Increased RPA expression in U2OS-RPA21 protected against inhibition. Figure 1d shows that, whereas $500 \mu\text{M}$ ciprofloxacin/ 20 kJ m^{-2} UVA almost completely abolished 6-4Py:Py excision in U2OS cells, U2OS-RPA21 cells removed around 50% of these photoproducts within 3 hours under the same conditions. Higher RPA expression also protected against NER inhibition by ofloxacin/UVA (Figure 1e). 1 mM ofloxacin/ 20 kJ m^{-2} UVA that caused a significant inhibition of 6-4Py:Py removal in U2OS cells did not detectably affect excision by U2OS-RPA21 cells. Repair inhibition was the result of UVA/drug interaction, and ciprofloxacin, ofloxacin, or these low UVA doses alone were without effect.

Increased RPA expression also protected against fluoroquinolone/UVA toxicity. Figure 1f shows that the viability of UVA-irradiated (20 kJ m^{-2}) U2OS cells was compromised by ciprofloxacin, and <2% survived treatment with $200 \mu\text{M}$. In contrast, >80% of irradiated U2OS-RPA21 cells survived treatment with $200 \mu\text{M}$ ciprofloxacin. Ofloxacin/UVA was significantly less toxic to both U2OS and U2OS-RPA21 cells. More than 20% of U2OS cells remained viable following $1,000 \mu\text{M}/20 \text{ kJ m}^{-2}$, and the effect of RPA overexpression on U2OS-RPA21 viability was correspondingly more modest (Figure 1g).

RPA chromatinization

6-TG/UVA generates DNA lesions that are powerful replication blocks. UVA activation of certain fluoroquinolones induces $T < > T$ CPDs (Lhiaubet-Vallet *et al.*, 2009) that also arrest replication. As RPA can become sequestered at arrested replication forks and no longer available for NER, we investigated recruitment of RPA to chromatin. 6-TG/UVA or ciprofloxacin/UVA reduced recovery of RPA in U2OS cell extracts (Figure 2a and b). Consistent with RPA chromatinization and the presence of replication-arresting DNA lesions, the missing RPA was recovered by digestion of the extracts with benzonase (Supplementary Figure S2 online). RPA recovery from U2OS-RPA21 cells was less affected owing to

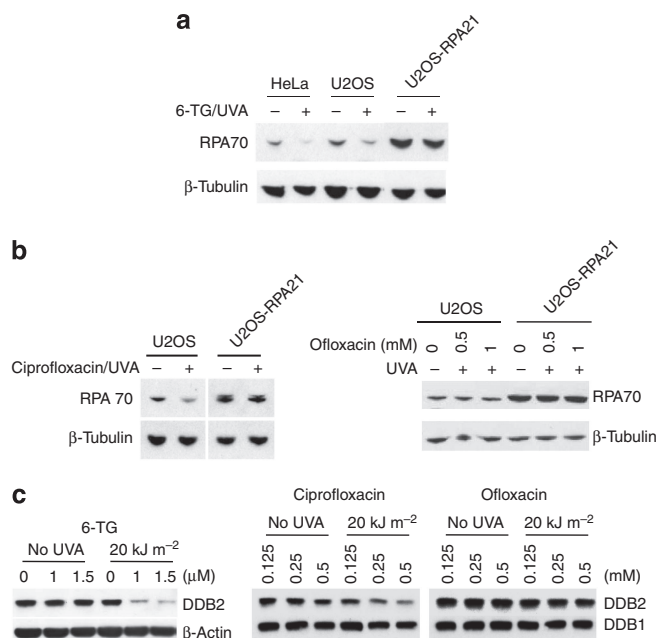


Figure 2. Replication protein A (RPA) and DDB2 chromatinization following treatment. (a) 6-TG/UVA. HeLa, U2OS, or U2OS-RPA21 cells grown for 24 hours in 1, 4, or $10 \mu\text{M}$ 6-TG, respectively, to ensure equivalent DNA substitution were UVA irradiated (20 kJ m^{-2}). Whole-cell extracts were analyzed by western blotting for RPA70. (b) Ciprofloxacin/UVA and Ofloxacin/UVA. U2OS or U2OS-RPA21 cells were treated with 0.5 mM ciprofloxacin or ofloxacin as indicated and irradiated with UVA (20 kJ m^{-2}). Whole-cell extracts were analyzed as in a. (c) DDB2. U2OS cells treated with 6-thioguanine (6-TG) for 24 hours or fluoroquinolone for 1 hour at the concentrations shown were UVA irradiated as indicated. Whole-cell extracts prepared immediately after irradiation were analyzed by western blotting for DDB2 or DDB1/β-actin as loading controls.

their higher RPA levels. As expected, ofloxacin/UVA did not detectably affect RPA recovery in U2OS extracts, as it is a poor source of CPDs (Peacock *et al.*, 2014; Figure 2b).

Chromatinization of the DDB2 component of the DDB1: DDB2 (DNA Damage Binding 1:DNA Damage Binding 2) DNA damage recognition complex is another marker for the presence of NER substrates (Otrin *et al.*, 1997). Figure 2c shows that 6-TG/UVA and ciprofloxacin/UVA but not ofloxacin/UVA provoked immediate relocalization of a substantial fraction of DDB2 in U2OS. This observation provides further confirmation that 6-TG/UVA and ciprofloxacin/UVA induce DNA damage that includes potential substrates for NER. Ofloxacin/UVA is, however, a poor source of such lesions.

RPA damage

All three photosensitizers cause protein oxidation (Peacock *et al.*, 2014). As their effects on NER and survival did not correlate with the induction of DNA lesions, we examined whether RPA itself was damaged. Western blotting of extracts from 6-TG/UVA-treated U2OS cells revealed an RPA32 species of ~100 kDa (Figure 3a) that was absent from untreated cells. Western analysis of RPA32 in U2OS-RPA21 cells was complicated by the presence of additional

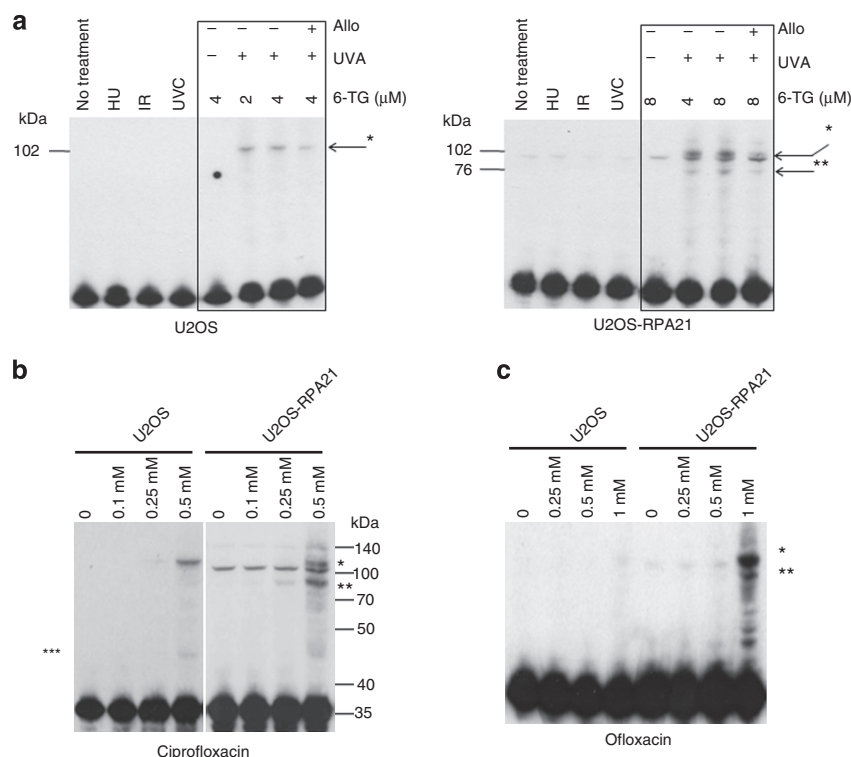


Figure 3. Formation of RPA32 complexes. (a, Left panel). RPA32 western blots of extracts from U2OS cells either untreated or treated with hydroxyurea (HU; 3 mM, 3 hours), ionizing radiation (IR; 20 Gy), UVC (100 J m^{-2}), or 6-thioguanine (6-TG)/UVA (2 or 4 μM 6-TG, 24 h/20 kJ m^{-2}). (Right panel) RPA32 western blots of U2OS-RPA21 cells treated identically, except that 6-TG concentrations were 4 or 8 μM 6-TG to ensure similar levels of DNA 6-TG. Allopurinol (1 mM) was included during 6-TG treatment as indicated. RPA32 complexes are indicated (* and **). (b) RPA32 western blots of extracts from U2OS and U2OS-RPA21 cells treated for 1 hour with ciprofloxacin/UVA (20 kJ m^{-2}) as indicated. MW markers and RPA32 complexes (*, **, and ***) are indicated. (c) As in b. Cells treated with ofloxacin/UVA (20 kJ m^{-2}).

constitutive RPA forms generated by incomplete processing of the full-length GFP-RPA polypeptide encoded by the construct (Supplementary Figure S1 online). In addition to the three correctly processed RPA proteins, U2OS-RPA21 extracts contained low but detectable levels of polypeptides corresponding to full-length GFP/RPA14-RPA70-RPA32 (143 kDa) and RPA32-RPA70 fusion polypeptides (102 kDa; Supplementary Figure S1 online). 6-TG/UVA-treated U2OS-RPA21 extracts contained an RPA32 species, apparently identical to that induced in U2OS cells. This migrated slightly behind the incompletely processed constitutive 102 kDa RPA32-RPA70 product (Figure 3a). These extracts also contained an additional RPA32-reactive polypeptide of 70–100 kDa that was not present in extracts from untreated cells (Figure 3a). The antioxidant allopurinol suppressed the formation of both 100 and 70–100 kDa complexes, consistent with oxidation-dependent formation. Neither complex was detected after exposure to IR, UVC, or hydroxyurea, all of which inhibited DNA replication by >90% indicating that they are not generated in response to extensive DNA damage or replication arrest. Ciprofloxacin/UVA (Figure 3b) and ofloxacin/UVA (Figure 3c) generated an apparently identical 100 kDa RPA32 species. Consistent with the induction of a lower level of overall protein oxidation (Peacock et al., 2014), this complex was less prominent following ofloxacin/UVA treatment. The

enrichment provided by nuclear extracts (see Supplementary Figures S3 and 4 online) confirmed its presence in ofloxacin/UVA-treated cells. Additional complexes with apparent masses between 40 and 50 kDa and 70–100 kDa were observed in extracts from ciprofloxacin/UVA-treated U2OS and U2OS-RPA21 cells, respectively. These changes in RPA32 were not restricted to U2OS osteosarcoma cells, and both ciprofloxacin/UVA and ofloxacin/UVA induced the 100 kDa and 40–50 kDa RPA species in untransformed GM8339 human fibroblasts (Supplementary Figure S3 online).

The sizes of the RPA photoproducts are consistent with covalent cross-linking of RPA32 to its RPA neighbors. The 100-kDa species is a potential RPA32:RPA70 dimer. RPA32-RPA14 dimerization is consistent with the 40–50 kDa species observed in U2OS and GM8339 cells. In U2OS-RPA21 cells, the GFP protein fused to RPA14 would contribute an additional 27 kDa to an RPA32:RPA14 complex to generate the 70–100 kDa species. To address these possibilities, RPA32 immunoprecipitated from ciprofloxacin/UVA-treated U2OS-RPA21 cells was probed for the presence of RPA70 by western blotting. Consistent with the presence of a partially processed GFP-RPA polypeptide, the material immunoprecipitated from untreated cells included an antiRPA70-reactive species of ~100 kDa (Figure 4a). Ciprofloxacin/UVA increased the amount of antiRPA70 reactive material at

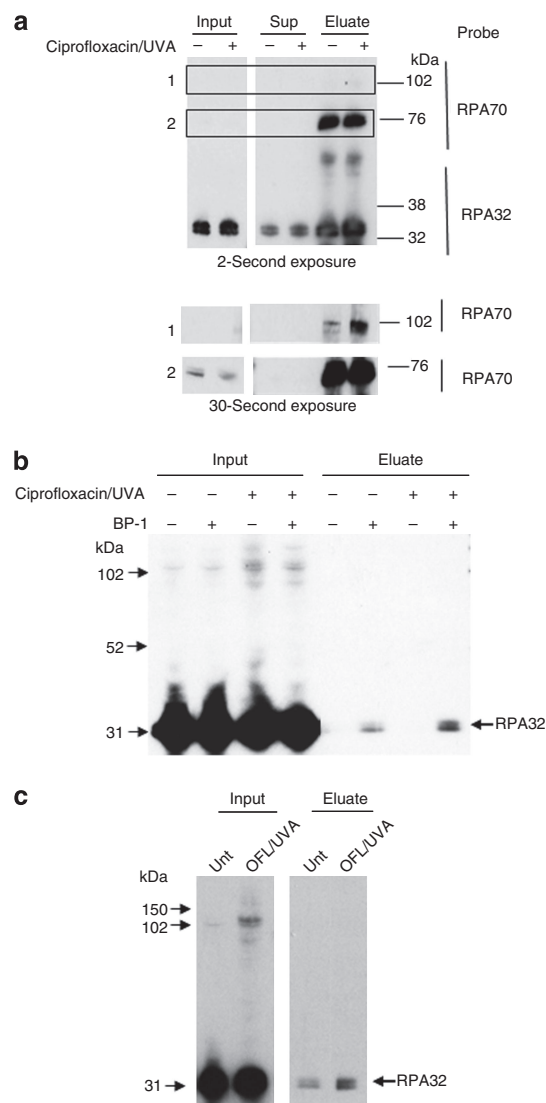


Figure 4. Identity of replication protein A (RPA) oxidation products. (a) Immunoprecipitation. Anti-RPA32 immunoprecipitates from U2OS-RPA21 cells treated with ciprofloxacin (0.5 mM, 1 hour) and UVA (20 kJ m⁻²) were analyzed by western blotting for RPA32 and RPA70. The two lower panels represent longer exposure of the boxed areas 1 and 2. Positions of molecular weight markers are indicated. (b) RPA32 cysteine sulfenates. U2OS-RPA21 cells that had been treated with ciprofloxacin (250 μM, 1 hour) and UVA (20 kJ m⁻²) were lysed in the presence of the BP-1 biotinylated sulfenylated probe. Derivatized proteins were recovered from streptavidin-linked beads (Eluate) and analyzed by western blotting for RPA32. Samples before streptavidin enrichment (Input) were also analyzed. (c) Extracts from U2OS-RPA21 cells were treated with ofloxacin (OFL 500 μM, 1 hour) and UVA (20 kJ m⁻²) and analyzed as in b.

around 100 kDa, consistent with the formation of an RPA32:70 dimer. In Figure 4a, this photoproduct is not resolved from the endogenous unprocessed material. We conclude that one of the oxidation products of these photosensitizers is a covalent complex of RPA32 and RPA70 subunits.

Cysteine residues in RPA have important structural and functional roles. We therefore examined RPA cysteine thiol

(-SH) oxidation to cysteine sulfenates (Cys-SOH). To do this, protein sulfenates in extracts of treated U2OS-RPA21 cells were selectively derivatized with a probe (BP-1), which comprises a sulfenylated group attached to biotin (Qian *et al.*, 2011), to enable their recovery via affinity binding to streptavidin. Western blotting confirmed that ciprofloxacin/UVA and ofloxacin/UVA induce the ~100 kDa RPA32:70 complex in U2OS-RPA21 cells (Input, Figure 4b and c). BP-1 derivatized RPA32 was present in untreated and treated U2OS-RPA21 cells, and the level was increased by both ciprofloxacin/UVA and ofloxacin/UVA (Figure 4b and c). These findings demonstrate that fluoroquinolone/UVA treatment causes RPA cysteine thiol oxidation. They also suggest that some RPA may contain Cys-SOH under normal oxoic growth conditions, at least when it is overexpressed. Both intersubunit cross-linking and cysteine sulfenation are potential contributors to RPA inactivation and NER inhibition in oxidatively stressed cells.

RPA damage and *in vitro* NER

Protein damage underlies the inhibitory effects of fluoroquinolone/UVA on NER (Peacock *et al.*, 2014). Consistent with previous observations, *in vitro* assays revealed that extracts from ciprofloxacin/UVA- or ofloxacin/UVA-treated U2OS cells were defective in NER. Similar treatments caused a more modest reduction in NER capacity of nuclear extracts from U2OS-RPA21 cells (Figure 5). Probing the extracts for RPA32 revealed that impaired NER activity coincided with the appearance of an RPA32 species of ~100 kDa.

The relationship between the 100-kDa RPA32 species and NER capacity was investigated further in extracts of CCRF-CEM cells. NER inhibition by ciprofloxacin/UVA and ofloxacin UVA in CCRF-CEM cells was demonstrated previously (Peacock *et al.*, 2014; Supplementary Figure S4 online upper panels). In agreement with the observations with U2OS, examination of NER-negative extracts from ciprofloxacin/UVA- or ofloxacin/UVA-treated CCRF-CEM cells revealed novel RPA32 species of ~100 kDa and 40–50 kDa (Supplementary Figure S4 online lower panels). Neither RPA32 complex was observed in NER-competent extracts from untreated, UVA-irradiated, or drug-treated cells.

We conclude that these fluoroquinolone/UVA combinations cause sufficient oxidative RPA damage to compromise NER. Damage includes RPA subunit cross-linking and thiol oxidation. Modest RPA overexpression provides sufficient RPA to prevent NER becoming compromised.

DISCUSSION

Our observations indicate that, despite its manifest importance in DNA processing, RPA is limiting for NER under oxidative stress conditions. Oxidative RPA damage compromises NER, and modest RPA overexpression is sufficient to prevent this. RPA, and therefore NER, was shown to be surprisingly susceptible to inhibition under the oxidative stress conditions induced by photosensitizers. Diminished NER capacity was associated with readily detectable forms of damaged RPA.

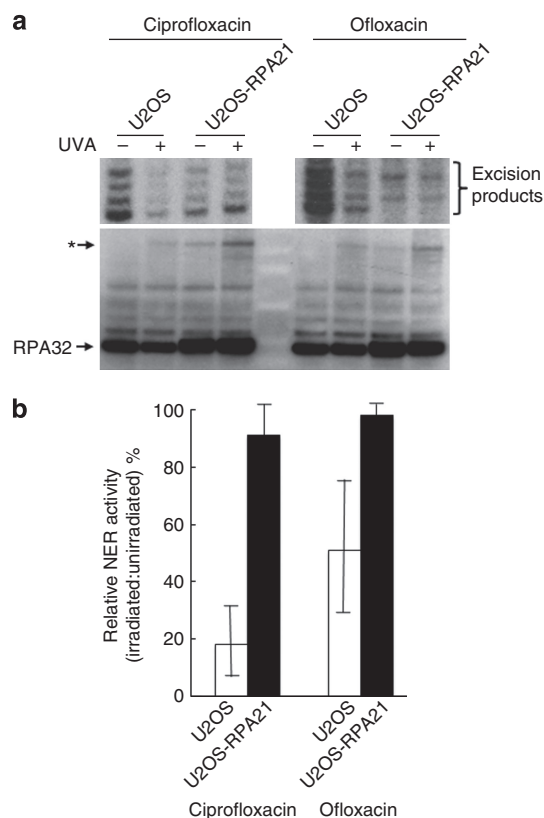


Figure 5. Nucleotide excision repair (NER) *in vitro*. (a) NER assays of nuclear extracts from U2OS or U2OS-RPA21 cells treated (1 hour) with ciprofloxacin (0.5 mM) or ofloxacin (1 mM) and irradiated with 20 kJ m⁻² UVA as indicated. Excision products (indicated) were radiolabeled and analyzed by urea gel electrophoresis (upper panels). The same nuclear extracts were analyzed by western blotting (lower panel). The position of the treatment-related RPA32 complex is indicated as *. (b) Excision products were quantitated by GelDoc, and NER efficiency by extracts from drug/UVA-treated cells is expressed relative to that of extracts from cells treated with drug alone. Means of three to five assays of independently prepared extracts.

RPA is essential for the NER of the UV-induced photolésions that are a major cause of skin cancer. In addition, it is also recruited to the extensive regions of exposed single-stranded DNA at replication forks stalled by UV-induced lesions. Cells exposed to UV radiation therefore experience conflicting demands on a limiting RPA pool. In conditions of extreme replication stress and in the absence of a functional S phase checkpoint to prevent replicon firing, RPA levels are insufficient to protect stalled replication forks from collapse and DNA breakage ensues (Toledo *et al.*, 2013). This observation has prompted the suggestion that NER capacity is reduced by RPA sequestration at arrested replication forks (Tsaalbi-Shtylik *et al.*, 2014) even in cells with a functional checkpoint. Although this appears to be the case under conditions of extreme replication stress induced by hydroxyurea, the extent to which these events occur following replication arrest by DNA photolésions is unclear.

Ciprofloxacin/UVA and 6-TG/UVA inhibit NER (Peacock *et al.*, 2014). Consistent with enhanced removal of potentially lethal DNA lesions, improved NER in RPA-overexpressing

U2OS-RPA21 cells decreased their sensitivity to killing by ciprofloxacin/UVA and 6-TG/UVA. Ciprofloxacin/UVA is a source of replication-arresting T<>T CPDs that are canonical NER substrates (Lhiaubet-Vallet *et al.*, 2009). Chromatinization of both RPA and DDB2 (Otrin *et al.*, 1997) after ciprofloxacin/UVA treatment is consistent with the presence of CPDs. 6-TG/UVA also induced RPA and DDB2 relocation, indicating that it too generates replication-blocking NER substrates. Depletion of RPA by sequestration at arrested replication forks and competition by ciprofloxacin/UVA- and 6-TG/UVA-induced photolésions for NER proteins are therefore both potential contributors to a reduced NER capacity.

Several observations indicate that neither RPA sequestration nor the presence of competing substrates fully accounts for NER inhibition. First, our measure of NER activity, 6-4Py:Py removal, is largely independent of DDB2 and proceeds much more rapidly compared with T<>T CPD excision (Hwang *et al.*, 1999). DDB-recruiting lesions induced by 6-TG/UVA and ciprofloxacin/UVA-induced T<>Ts are therefore unlikely to be effective competitors of 6-4Py:Py NER. Second, RPA sequestration following replication arrest is S phase specific. In our experiments, both 6-TG/UVA and ciprofloxacin/UVA effectively abolished 6-4Py:Py excision, indicating that their inhibitory effects are not confined to S phase. Most pertinently, ofloxacin/UVA also inhibited NER, and increased RPA expression prevented this inhibition. Ofloxacin/UVA is a very weak source of T<>T CPDs (Lhiaubet-Vallet *et al.*, 2009; Peacock *et al.*, 2014). Under conditions of NER inhibition it is relatively non-toxic, and the induction of potentially lethal, replication-arresting DNA lesions cannot explain NER inhibition by ofloxacin/UVA. Thus, although these data do not exclude a contribution of DNA damage to NER inhibition, the effects of fluoroquinolone/UVA and 6-TG/UVA in U2OS and U2OS-RPA21 cells indicate additional or alternative events that compromise RPA function.

Our findings identify oxidation of RPA itself as an important determinant of NER efficiency both *in vivo* and *in vitro*. Inhibitory 6-TG/UVA, ciprofloxacin/UVA, and ofloxacin/UVA treatments that severely compromised NER in U2OS cells induced covalent cross-linking between RPA subunits to generate RPA32:70 and most likely RPA32:14 dimers. RPA cross-linking was oxygen dependent and occurred together with RPA cysteine oxidation to Cys-SOH. Cross-linked RPA was present in all NER-negative extracts of ciprofloxacin/UVA and ofloxacin/UVA-treated U2OS, U2OS-RPA21, and CCRF-CEM cells but was not detected in NER-competent extracts. RPA is limiting for NER by cell extracts (Coverley *et al.*, 1991), and our findings indicate that even the modest overexpression in U2OS-RPA21 cells provides significant protection against NER inhibition. Only a small fraction of the total RPA is present in cross-linked or sulfenated forms in NER-negative extracts. Although this is consistent with a powerful dominant negative effect, it seems more likely that the altered RPA species are markers for more widespread RPA damage.

RPA is redox sensitive and presents a significant target for oxidation (Park *et al.*, 1999; Men *et al.*, 2007). Eleven of its

fifteen cysteines are located in the 70-kDa subunit. Four in the C-terminal region form a zinc finger essential for DNA replication but apparently dispensable for NER (Lin *et al.*, 1998). These two pairs of RPA70 cysteines are particularly sensitive to oxidant-induced disulfide formation (Men *et al.*, 2007). The remaining 11 cysteines were unaffected, and no cysteine sulfinic or sulfonic acid residues were observed. In our experiments, fluoroquinolone/UVA treatment increased the levels of RPA Cys-SOH—a reactive cysteine oxidation product and a marker for protein oxidation. Protein Cys-SOH can act as a redox switch to regulate redox homeostasis and signaling pathways (Groth and Jakob, 2014). These metastable intermediates can be reduced back to cysteine or undergo further and largely irreversible oxidation to more stable cysteine sulfinic and sulfonic acids. Importantly, they can react with other protein functional groups including amino groups to form sulfenamide cross-links. The RPA32 complexes were resistant to the reducing conditions associated with western blotting, consistent with a cross-link of this nature. In addition to its two cysteines, RPA32 is rather methionine rich, and these residues are also plausible candidates for oxidation to sulfoxide (Men *et al.*, 2007). Any of these oxidative changes to RPA might adversely affect its structure and are potentially deleterious for its function. In summary, RPA is particularly susceptible to oxidation damage, and it is noteworthy in this regard that screens for protein carbonyls (Peacock, 2014) and DNA–protein cross-links (M. Guven and PK, unpublished observations) both identified cellular RPA as a potential target for oxidative damage by 6-TG/UVA.

RPA is of critical importance in almost all DNA transactions. It is therefore surprising that RPA levels are limiting and that its susceptibility to oxidative stress can compromise NER. Perhaps there are constraints on RPA expression. On the basis of the oxidation sensitivity of RPA both *in vitro* (Men *et al.*, 2007) and *in vivo* as described here, it is tempting to speculate that a sensitivity to oxidative damage constrains its expression level. Protein oxidation is an unavoidable consequence of aerobic metabolism, and oxidized proteins are generally detrimental to cellular fitness. If the multiple functions of RPA require it to be constructed in a way that renders it particularly vulnerable to oxidation damage, this requirement may determine its safe expression level. Limiting RPA may simply represent a trade-off between function and avoidance of unacceptable levels of protein oxidation.

The photosensitizers we use are associated with clinical photosensitivity. They amplify the effects of solar UVA, which is an acknowledged generator of cellular reactive oxygen species. Our demonstration that RPA is particularly susceptible to oxidation damage and that this compromises NER serves to emphasize the importance of protecting the skin against the effects of the full spectrum of UV wavelengths in sunlight.

MATERIALS AND METHODS

Chemicals

6-TG, ciprofloxacin, and ofloxacin were obtained from Sigma-Aldrich.

Cells and UV irradiation

HeLa, U2OS, and U2OS-RPA21 cells (Toledo *et al.*, 2013), kindly provided by Dr J. Lucas, were grown in Dulbecco's MEM supplemented with 10% fetal calf serum. UVA irradiation was by a UVH 253 lamp (UV Light Technology Limited) with maximum emission at 365 nm. The dose rate was $0.1 \text{ kJ m}^{-2} \text{ s}^{-1}$. UVC (254 nm) was delivered by a Stratalinker UV Cross-linker (Stratagene) at a dose rate of $10 \text{ J m}^{-2} \text{ s}^{-1}$. For experiments that combined drug/UVA treatment with UVC radiation, drug/UVA treatment preceded UVC irradiation.

Cell viability was determined by the formazan/tetrazolium (MTT) cell proliferation assay on triplicate samples. All experiments were repeated at least twice.

6-TG determination

For initial incorporation measurements, HeLa, U2OS, and U2OS-RPA21 cells were grown for 48 hours in a medium containing 6-TG. DNA 6-TG was measured as previously described (Zhang *et al.*, 2007). Initial determinations indicated that the more slowly growing U2OS-RPA21 cells incorporated two or three times less 6-TG at the same external 6-TG concentration. In subsequent experiments, 6-TG concentrations were adjusted to achieve similar 6-TG incorporation levels during 24-hour growth. DNA 6-TG values (as % DNA G) were measured in each experiment to ensure comparability.

UVC 6:4 Py:Py measurements

DNA extracted by the QIAamp DNA mini kit (Qiagen) from drug/UVA and UVC-treated cells was analyzed by ELISA according to the supplier's instructions (Cosmo Bio Co). These experiments confirmed that none of the drug/UVA treatments induces detectable 6:4 Py:Py (Peacock *et al.*, 2014), and initial values for UVC-induced 6:4 Py:Py were in agreement within 10% independently of prior drug/UVA treatment. Repair data were therefore expressed as a percentage of the initial lesions at various times after irradiation.

NER assays *in vitro*

NER by nuclear extracts of treated cells was assayed as described previously (Gueranger *et al.*, 2014). Each assay was performed with at least three independent extracts. Excision products were quantified by summing band intensities by GelDoc (Bio-Rad). NER efficiency was calculated relative to extracts from cells treated with drug alone.

Immunoblotting

Whole-cell extracts were prepared with radioimmunoprecipitation assay buffer. Proteins (20 μg) were separated on 10% polyacrylamide gels (Invitrogen). After transfer, membranes were probed with antibodies against RPA32, RPA70, DDB1, or DDB2 (Abcam). Antigen–antibody complexes were visualized using ECL blotting detection agent (GE Healthcare).

Immunoprecipitation

Cells were lysed in non-denaturing lysis buffer. RPA32 antibody was incubated with Dynabeads ProteinG (Invitrogen) before addition of protein extract (500 μg).

Sulfenate labeling and detection

Treated cells were lysed with radioimmunoprecipitation assay buffer containing 1 mM biotin-1,3-cyclopentanedione (BP-1) probe (Kera-FAST). *N*-Ethylmaleimide (20 mM; Sigma) was immediately added

and proteins precipitated with acetone. The dried protein pellet was dissolved in 2% SDS. Protein concentrations were determined by the BCA assay (Thermo Scientific). Solubilized proteins were mixed with M280 Streptavidin Dynabeads (Invitrogen), which were washed three times with 4 volumes of 0.1% SDS. Following rotation overnight at 4 °C beads were washed sequentially (30 minutes each) with 2 M urea, 1 M NaCl, 0.1% SDS/10 mM DTT, and PBS. BP-1-derivatized proteins were recovered by boiling and analyzed by western blotting.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Drs L. Toledo and J. Lucas for providing U2OS and U2OS-RPA21 cells and to Professor C. Furdai for advice on BP-1 derivatization. The assistance of The Francis Crick Institute Cell Services department is gratefully acknowledged. Funding was provided by the Cancer Research UK.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Alexandrov LB, Nik-Zainal S, Wedge DC *et al.* (2013) Signatures of mutational processes in human cancer. *Nature* 500:415–21
- Binz SK, Sheehan AM, Wold MS (2004) Replication protein A phosphorylation and the cellular response to DNA damage. *DNA Repair* 3:1015–24
- Chen C, Umez K, Kolodner RD (1998) Chromosomal rearrangements occur in *S. cerevisiae rfa1* mutator mutants due to mutagenic lesions processed by double-strand-break repair. *Mol Cell* 2:9–22
- Coverley D, Kenny MK, Munn M *et al.* (1991) Requirement for the replication protein SSB in human DNA excision repair. *Nature* 349:538–41
- Dodson GE, Shi Y, Tibbetts RS (2004) DNA replication defects, spontaneous DNA damage, and ATM-dependent checkpoint activation in replication protein A-deficient cells. *J Biol Chem* 279:34010–4
- Euvrard S, Kanitakis J, Claudy A (2003) Skin cancers after organ transplantation. *N Engl J Med* 348:1681–91
- Friedberg EC, Walker GC, Siede W *et al.* (2006) *DNA Repair and Mutagenesis*. Washington: ASM Press
- Friedel AM, Pike BL, Gasser SM (2009) ATR/Mec1: coordinating fork stability and repair. *Curr Opin Cell Biol* 21:237–44
- Groth B, Jakob U (2014) Thiol-based redox switches. *Biochim Biophys Acta* 1844:1335–43
- Gueranger Q, Li F, Peacock M *et al.* (2014) Protein oxidation and DNA repair inhibition by 6-thioguanine and UVA radiation. *J Invest Dermatol* 134:1408–17
- Hwang BJ, Ford JM, Hanawalt PC *et al.* (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. *Proc Natl Acad Sci USA* 96:424–8
- Itoh T, Miyauchi-Hashimoto H, Sugihara A *et al.* (2005) The photocarcinogenesis of antibiotic lomefloxacin and UVA radiation is enhanced in xeroderma pigmentosum group A gene-deficient mice. *J Invest Dermatol* 125:554–9
- Jayaraman SS, Rayhan DJ, Hazany S *et al.* (2014) Mutational landscape of basal cell carcinomas by whole-exome sequencing. *J Invest Dermatol* 134:213–20
- Lhiaubet-Vallet V, Bosca F, Miranda MA (2009) Photosensitized DNA damage: The case of fluoroquinolones. *Photochem Photobiol* 85:861–8
- Lin Y-L, Shivji MKK, Chen C *et al.* (1998) The evolutionarily conserved zinc finger motif in the largest subunit of human replication protein A is required for DNA replication and mismatch repair but not for nucleotide excision repair. *J Biol Chem* 273:1453–61
- Men L, Roginskaya M, Zou Y *et al.* (2007) Redox-dependent formation of disulfide bonds in replication protein A. *Rapid Commun Mass Spectrometry* 21:2743–9
- Otrin VR, McLenigan M, Takao M *et al.* (1997) Translocation of a UV-damaged DNA binding protein into a tight association with chromatin after treatment of mammalian cells with UV light. *J Cell Sci* 110:1159–68
- Park J-S, Wang M, Park S-J *et al.* (1999) Zinc finger of replication protein A, a non-DNA binding element, regulates its DNA binding activity through redox. *J Biol Chem* 274:29075–80
- Peacock M (2014) *UVA photosensitizers, protein oxidation and DNA repair*. PhD Thesis University College London
- Peacock M, Brem R, Macpherson P *et al.* (2014) DNA repair inhibition by UVA photoactivated fluoroquinolones and vemurafenib. *Nucleic Acids Res* 42:13714–22
- Peyrin-Biroulet L, Khosrotehrani K, Carrat F *et al.* (2011) Increased risk for nonmelanoma skin cancers in patients who receive thiopurines for inflammatory bowel disease. *Gastroenterology* 141:1621–8
- Qian J, Klomsiri C, Wright MW *et al.* (2011) Simple synthesis of 1,3-cyclopentanedione derived probes for labeling sulfenic acid proteins. *ChemComm* 47:9203–5
- Ramiscal JAB, Brewer JD (2013) Thiopurines and risk of nonmelanoma skin cancer in inflammatory bowel disease. *JAMA Dermatol* 149:92–4
- South AP, Purdie KJ, Watt SA *et al.* (2014) *NOTCH1* mutations occur early during cutaneous squamous cell carcinogenesis. *J Invest Dermatol* 134:2630–8
- Toledo LI, Altmeyer M, Rask M-B *et al.* (2013) ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* 155:1088–103
- Traianou A, Ulrich M, Apalla Z *et al.* (2012) Risk factors for actinic keratosis in eight European centres: a case-control study. *Br J Dermatol* 167(Suppl. 2):36–42
- Tsaalbi-Shtylik A, Moser J, Mullenders LHF *et al.* (2014) Persistently stalled replication forks inhibit nucleotide excision repair *in trans* by sequestering replication protein A. *Nucleic Acids Res* 42:4406–13
- Umez K, Sugawara N, Chen C *et al.* (1998) Genetic analysis of yeast RPA1 reveals its multiple functions in DNA metabolism. *Genetics* 148:989–1005
- Wang Y, Putnam CD, Kane MF *et al.* (2005) Mutation in *Rpa1* results in defective DNA double-strand break repair, chromosomal instability and cancer in mice. *Nat Genetics* 37:750–5
- Wold MS (1997) Replication protein A: a heterotrimeric, single stranded DNA-binding protein required for eukaryotic DNA metabolism. *Ann Rev Biochem* 66:61–92
- Zhang X, Jeffs G, Ren X *et al.* (2007) Novel DNA lesions generated by the interaction between therapeutic thiopurines and UVA light. *DNA Repair* 6:344–54