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Regulation of oxidized base damage repair by chromatin assembly factor 1 subunit A

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ABSTRACT

Reactive oxygen species (ROS), generated both endogenously and in response to exogenous stress, induce point mutations by mis-replication of oxidized bases and other lesions in the genome. Repair of these lesions via base excision repair (BER) pathway maintains genomic fidelity. Regulation of the BER pathway for mutagenic oxidized bases, initiated by NEIL1 and other DNA glycosylases at the chromatin level remains unexplored. Whether single nucleotide (SN)-BER of a damaged base requires histone deposition or nucleosome remodeling is unknown, unlike nucleosome reassembly which is shown to be required for other DNA repair processes. Here we show that chromatin assembly factor (CAF)-1 subunit A (CHAF1A), the p150 subunit of the histone H3/H4 chaperone, and its partner anti-silencing function protein 1A (ASF1A), which we identified in human NEIL1 immunoprecipitation complex, transiently dissociate from chromatin bound NEIL1 complex in G1 cells after induction of oxidative base damage. CHAF1A inhibits NEIL1 initiated repair *in vitro*. Subsequent restoration of the chaperone-BER complex *in cell*, presumably after completion of repair, suggests that histone chaperones sequester the repair complex for oxidized bases in non-replicating chromatin, and allow repair when oxidized bases are induced in the genome.

INTRODUCTION

ROS, continuously generated in mammalian cells both endogenously and by environmental genotoxicants, induce various genomic lesions, including oxidized bases, abasic (AP) sites and single-strand breaks (SSBs). If unrepaired or mis-repaired, DNA lesions would cause mutations which may lead to cytotoxicity and cell death and also carcinogenic transformation (1). The base excision repair (BER) pathway, responsible for repair of oxidized base lesions which contribute to drug/radiation sensitivity is highly conserved from bacteria to the humans (2,3). Defects in BER have been linked to cancers, neurodegenerative diseases, immune dysfunction and aging (1,4). Oxidized base-specific BER is initiated by one of 4–5 DNA glycosylases (DGs) including NEIL1 and OGG1 that recognize and excise damaged bases and cleave the DNA strand at the damage site (2,5–9). Subsequent restoration of the original DNA sequence involves sequential steps including end processing, repair synthesis and finally strand ligation. As in most biological processes, DNA repair is coordinated via multistep signalling mechanisms, including nucleosome remodeling, which may be specific to the cell cycle phase and the chromatin state, e.g. euchromatin vs. heterochromatin region. In any event, DNA repair must be regulated at the chromatin level. *In vitro* BER with naked DNA substrates does not recapitulate *in vivo* BER with its complex regulation, which may also involve non-repair proteins and post-translational modifications in response to exogenous signaling (3,10). The intrinsic link between chromatin modifications and DNA repair has been well studied in the case of DNA double-strand breaks (DSBs) and ultraviolet (UV) ray-mediated DNA damage (11,12). However, except for few studies showing the link to histone modification and

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chromatin remodeling during BER (12–18), how chromatin factors regulate BER is unknown.

NEIL1, a well-characterized DG responsible for the repair of oxidized DNA base lesions, was shown to interact with many DNA replication proteins, and is involved in replication-associated repair (RAR) of oxidized bases (19–21). It utilizes replication proteins for long patch (LP)-BER in the S phase genome, and it also initiates single-nucleotide (SN)-BER in the quiescent cell genome (21). In this study, we identified chromatin assembly factor 1 (CAF-1) subunit 1A (CHAF1A), the p150 subunit of the histone H3/H4 chaperone (22), in the NEIL1 immunoprecipitation complex.

CHAF1A is essential for cell proliferation (23,24) and its overexpression has been linked to colon cancer and aggressive neuroblastoma (25,26). It was reported that single nucleotide polymorphisms (SNPs) in or around the CHAF1A gene is associated with increase in the risk of glioma (27). We observed CHAF1A's transient dissociation from the NEIL1 complex after ROS treatment, presumably to allow NEIL1 to initiate BER. We had expected that SN-BER, involving single nucleotide incorporation to replace the damaged base would not significantly impact the chromatin structure. This report documenting the role of histone chaperones in BER regulation suggests that nucleosome reassembly is required even after single nucleotide incorporation.

MATERIALS AND METHODS

Cell lines, plasmids and transfection

The human embryonic kidney epithelial HEK293 cell line (ATCC) was maintained in DMEM-high glucose medium (Hyclone) containing 10% fetal calf serum (Sigma) and antibiotic mixture of penicillin and streptomycin (Corning) at 37°C under 5% CO_2 and 95% relative humidity. HEK293 cells stably expressing FLAG-tagged NEIL1 (21) were maintained in zeocin (Invivogen, 100 μg/ml) supplemented medium. Hemagglutinin (HA)-tagged CHAF1A (HA-CHAF1A) construct, a kind gift from Dr Bruce Stillman (Cold Spring Harbor Laboratory), was used to generate HEK293 cells with stable expression of HA-CHAF1A by transfection using lipofectamine 2000 (Invitrogen) following manufacturer's protocol. After 48 h, cells with ectopic CHAF1A were selected in the presence of 4 μg/ml puromycin (Invivogen) for 10 days, followed by selection of puromycin-resistant colonies. HA-CHAF1A expression was confirmed by western analysis. The cells stably expressing HA-CHAF1A were maintained in DMEM supplemented with 1 μg/ml of puromycin.

PCMV5.1 recombinant plasmid encoding FLAG-tagged wild type (WT) NEIL1 (10) was used for ectopic expression of NEIL1. The NEIL1 triple mutant (K296A, K297A, K298A, abbreviated as 3KA) was generated by using Stratagene's Site Directed Mutagenesis Kit following manufacturer's protocol; these Lys residues were identified to be the primary acetyl acceptor sites in NEIL1 (unpublished study).

Generation of CHAF1A deficient cell line

Conditional depletion of CHAF1A polypeptide in human osteosarcoma U2OS cells (ATCC), grown in DMEM-high glucose medium supplemented with FBS and antibiotics) was achieved after stable transfection with lentiviral expression plasmid containing the CHAF1A targeting sequence (5′-AGGGGAAAGCCGATGACAT-3′) using a published protocol (28). Briefly, oligonucleotide sequences were subcloned into pENTR/pTER+ vector which were recombined with pLenti RNAi X2 neo plasmid to generate pLenti RNAi X2 Neo/pTER CHAF1A shRNA-1. Recombinant lentiviruses were produced by transfecting pLenti RNAi X2 Neo/pTER CHAF1A shRNA-1 plasmids with the packaging plasmids in HEK293FT cells, and were harvested 48 h post-transfection. After transduction of the U2OS cells with the recombinant lentivirus, the cells were selected with 400 μg/ml neomycin (Gibco-BRL). Depletion of CHAF1A was induced by addition of 1 μg/ml of doxycycline (Dox; Sigma) and monitored by western analysis using anti-CHAF1A antibody.

Glucose oxidase (GOx) and ionizing radiation (IR) treatment

Cells were treated for 45 min with 50 ng/ml of GOx (which generates H_2O_2) to induce oxidative stress followed by washing in phosphate-buffered saline (PBS), incubation in fresh medium and harvesting at indicated times. For IR treatment, cells were exposed to 3 Gy radiation from Rad Source RS 2000 X-ray irradiator (Rad Source Technologies) and harvested at indicated times.

Antibodies

Anti-HA (# 2367), anti-H3 (# 4499), anti-FLAG (# 2368), and anti-ASF1A (# 2990) antibodies were purchased from Cell Signaling. Anti-OGG1 (# ab124741), anti-CAF-1 p48 (# ab47456), anti-CHAF1B (# ab72520) and anti-CHAF1A (# ab126625) antibodies were bought from Abcam. Anti-APE1 (# NB100–116) antibody was bought from Novus Biologicals. Anti-PNKP antibody was a kind gift from Dr Michael Weinfeld (University of Alberta, Cross Cancer Institute) and anti-NEIL1 antibody was custom-generated (29).

Cell synchronization and cell cycle analysis

Forty percent confluent cells in 10 cm dishes were synchronized at G1 and peak S phases by double thymidine (Sigma) arrest and then release (3–4 h), respectively, according to standard protocol (21,30). Cells were harvested for FACS analysis at HMRI's Flow Cytometry Core Facility as described previously (31). Briefly, cells were washed with PBS buffer. To 1 ml of cells in PBS, 4 ml of ice cold ethanol was added gently for fixing and incubated at −20°C overnight. Fixed cells were pelleted by centrifugation, rehydrated in PBS at room temperature, centrifuged again, and the cell pellet was stained with propidium iodide (PI) staining solution (3 μM PI, 100 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40, 5 μg/μl RNaseA), and then used for FACS analysis.

Subcellular fractionation

Cell lysates were fractionated for isolating soluble nuclear and chromatin fractions (32). In brief, cells from 80% confluent 10 cm plates for each cell type were lysed in cytoplasmic lysis buffer (10 mM Tris–HCl pH 7.9, 0.34 M sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40 and protease inhibitor mixture (Roche Applied Science)) and the nuclei were pelleted by centrifugation at 3500 g for 15 min at 4°C. The nuclei were lysed in nuclear lysis buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 3 mM EDTA, 150 mM K-acetate, 10% glycerol, 0.5% NP-40 and protease inhibitor mixture), and vortexed for 15 min at 4°C followed by centrifugation at 14 000 rpm for 30 min at 4°C. The supernatant was labeled as the soluble nuclear fraction (SNF). The chromatin pellet was dissolved in chromatin lysis buffer (150 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 150 mM KOAc, 10% glycerol and protease inhibitor mixture), and incubated with 0.15 unit/μl of Benzonase (Novagen) at 37°C for 30 min, followed by centrifugation at 14 000 rpm for 30 min at 4°C. The supernatant was called the chromatin fraction (CF). The SNF and CF were stored at −80°C for later use.

Co-immunoprecipitation (Co-IP) assay

For co-IP assay, the cell lysates were immunoprecipitated for 3 h at 4°C with FLAG M2 antibody bound agarose beads (Sigma; # A2220) as described previously (33). The beads were washed five times with 1 ml of cold Tris-buffered saline (TBS) containing 1% Triton X, eluted in 40 μl Laemmli buffer. The eluate was separated by SDS-PAGE for western analysis with appropriate antibodies. For HA IP, the HA agarose beads (Thermo Scientific) were used as described previously (34).

Mass spectroscopic (MS) analysis

The lysates from chromatin fraction of cells (forty 10 cm plates at 80% confluence) with stable expression of FLAG-NEIL1 and in parallel control cells were incubated with FLAG M2 agarose beads for 3 h at 4°C. The samples were then washed five times with 1 ml TBS, eluted in 40 μl of elution buffer with 3× FLAG-peptide. The eluted proteins were analyzed by SDS-PAGE followed by silver staining. The bands which were present only in the FLAG-NEIL1 IP and not in the IP of empty vector-transfected (control) cells were sliced for MS analysis in Baylor College of Medicine's Proteomics Core Facility.

Purification of recombinant proteins

Recombinant WT NEIL1, 3KA mutant NEIL1 and WT OGG1 polypeptides were purified from *Escherichia coli* as described previously (35). His-tagged CHAF1A and CAF-1 heterotrimer were purified from Sf9 insect cells infected with the recombinant baculoviruses. Briefly, Sf9 cells were infected with baculovirus expressing 6xHis-tagged CHAF1A or MacroBac baculovirus co-expressing 6xHis-tagged CHAF1A/CHAF1B/p48 at 27°C for 70 h. Cells were resuspended in Ni buffer (5 mM Tris, 50 mM NaH_2PO_4 pH 7.5, and 300 mM NaCl), supplemented with 10 mM imidazole, 0.1% NP-40, 1 mM PMSF and EDTA-free protease inhibitor tablets, and lysed by homogenization using a Dounce homogenizer. Cell lysates were clarified by centrifugation at 13 200 rpm for 30 min at 4°C, batch bound to Ni-NTA superflow beads (Qiagen) for 1 h, washed extensively with Ni buffer supplemented with 20 mM imidazole, and eluted with Ni buffer supplemented with 300 mM imidazole. The pooled eluate was dialyzed against 25 mM Tris, 150 mM NaCl pH 7.5 and 10% glycerol, flash frozen and stored at −80°C.

In vitro pulldown assay

For the His-affinity pulldown analysis, (i) His-tagged CHAF1A or CAF-1 trimer were bound to 20 μl suspension of Ni-NTA beads, mixed with either non-tagged WT NEIL1 or 3KA mutant NEIL1 in 0.5 ml TBS buffer or (ii) His-tagged NEIL1 were bound to 20 μl of Ni-NTA beads, mixed with either FLAG-tagged CHAF1A or CHAF1B (Origene) in 0.5 ml TBS buffer, and incubated for 20 min with constant rocking at 4°C. After washing the beads five times with 0.5 ml TBS buffer, the bound proteins were eluted with Laemmli buffer and fractionated by SDS/PAGE followed by western analysis.

Analysis of NEIL1 and OGG1 activity

The DNA glycosylase activities of NEIL1 and OGG1 were assessed using 5'-^{32}P-labeled 5-hydroxyuracil (5-OHU) and 8-oxoguanine (8-OxoG) lesion containing oligonucleotide substrates, respectively, as previously described (10,21,33).

In vitro repair assay

NEIL1-initiated repair assay using a 5-OHU-containing duplex oligonucleotide substrate was described previously (10,20). The nuclear extracts or FLAG-NEIL1 IP from control or CHAF1A downregulated cells, untreated or treated with GOx, were used. For FLAG-NEIL1 IP, nuclear extracts from control or CHAF1A downregulated cells were incubated with FLAG agarose beads and eluted with 3× FLAG peptide, and normalized for the FLAG-NEIL1 levels before the assay. For SN-BER reaction, the nuclear extracts or IP eluates were incubated in 20 μl of the repair reaction mixture containing 1 mM of ATP, 2 μCi of [α-^{32}P] dCTP and 25 μM unlabelled dCTP for 30 min at 37°C. The LP-BER reaction included 1 mM of ATP, 2 μCi of [α-^{32}P] dTTP and 25 μM of unlabelled dNTPs. The products were then analyzed by electrophoresis in 20% polyacrylamide denaturing gel.

Comet assay

Trevigen's Fpg FLARE (Fragment Length Analysis using Repair Enzymes) Comet assay kit (# 4040-100-FK) was used following manufacturer's protocol. Briefly, control, GOx treated cells and cells after recovery were lysed on Comet slides in low melting agarose followed by incubation with Fpg before alkaline electrophoresis. DNA in the nucleoid was visualized by SYBR Gold staining in a fluorescence microscope (EVOS FL auto, Life Technologies). Data

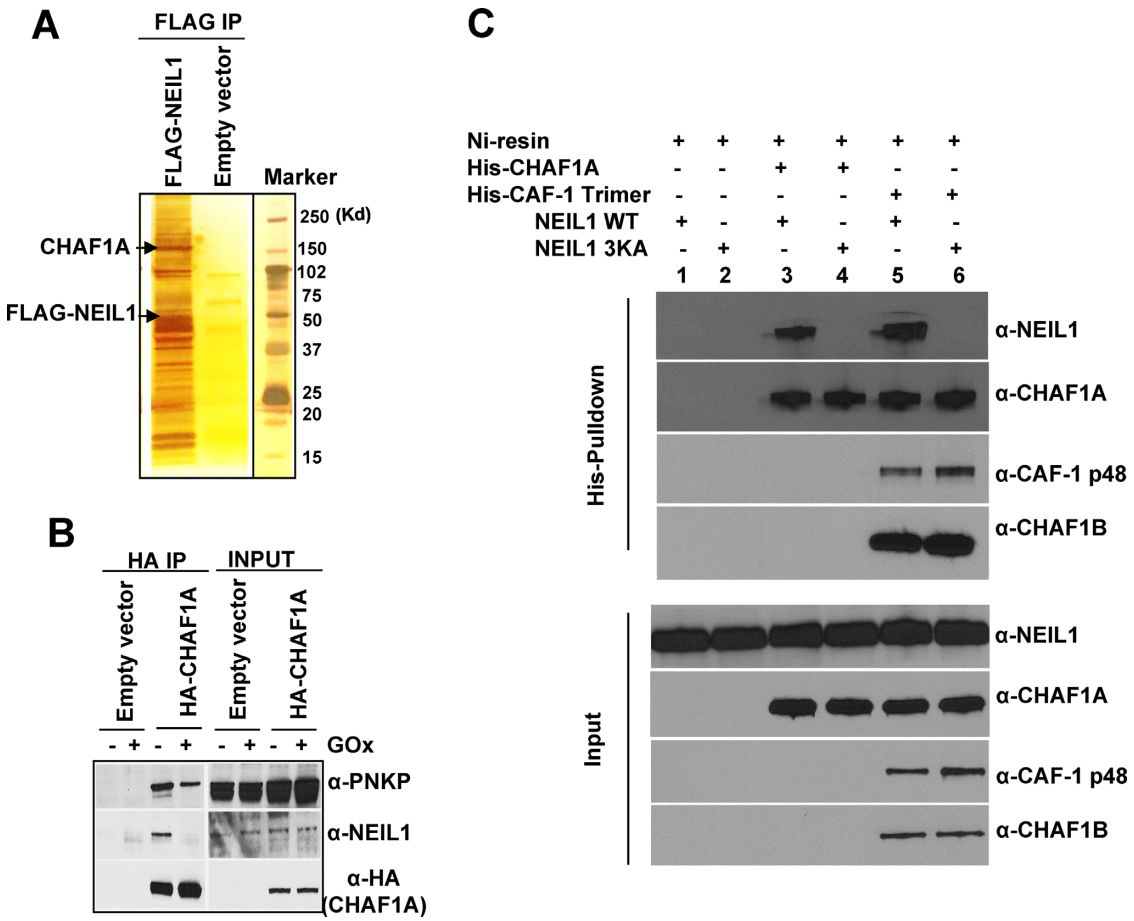

Figure 1. Characterization of CHAF1A's interaction with NEIL1. (**A**) Identification of CHAF1A in NEIL1 IP complex. FLAG-NEIL1 IP was isolated from the CF of HEK293 cells stably expressing FLAG-NEIL1. After separation of protein bands by SDS-PAGE and silver staining, the 150 kD band was identified as CHAF1A by mass spectroscopy. (**B**) Detection of NEIL1 and PNKP in HA-CHAF1A IP isolated from HEK293 cells pretreated with GOx, as described in Materials and Methods. (**C**) *In vitro* interaction of CHAF1A with NEIL1. Ni column-bound His-CHAF1A or His-CAF-1 heterotrimer was incubated with WT NEIL1 or the 3KA mutant. After washing off unbound proteins, as described in Materials and Methods, the bound proteins were eluted and analyzed by Western blotting.

analysis was performed using Open Comet of Image J program (NIH) with 50 randomly selected cells, and plotted as mean tail moment from two to three independent sets.

RESULTS AND DISCUSSION

CHAF1A stably associates with NEIL1 in chromatin

In order to elucidate the involvement of chromatin factors in NEIL1-initiated BER, we tested interaction of NEIL1 with chromatin components. MS analysis of NEIL1′s interacting partners was performed in the immunoprecipitation (IP) complex of ectopic FLAG-NEIL1 isolated from the CF of HEK293 cells which stably express FLAG-NEIL1 (21). CHAF1A, the largest subunit (p150) of CAF-1 (22,25,26,36–38), was identified in the NEIL1 IP from untreated cells (Figure 1A). To confirm *in cell* association of NEIL1 and CHAF1A, we generated HEK293 cells stably expressing HA-tagged CHAF1A and observed NEIL1 in the HA-CHAF1A IP (Figure 1B). Furthermore, *in vitro* co-elution analysis using purified histidine-tagged CHAF1A (His-CHAF1A) or CAF-1 (His-CAF-1) trimer and non-tagged WT NEIL1 recombinant proteins established their direct, binary interaction (Figure 1C, lanes 3 and 5). We also detected polynucleotide kinase/phosphatase (PNKP), which is downstream to NEIL1 in the same BER subpathway (39) interacting with CHAF1A as well (Figure 1B), suggesting that CHAF1A is involved in NEIL1-initiated BER. This was subsequently confirmed (Figure 4C). Interestingly, oxidative stress induced by GOx caused dissociation of both NEIL1 and PNKP from the CHAF1A IP (Figure 1B).

To confirm the specificity of NEIL1/CHAF1A or NEIL1/CAF-1 association, we included mutant NEIL1 recombinant protein (K296A, K297A and K298A; 3KA) as a control in the *in vitro* binding assay. In an independent study, we had discovered NEIL1′s acetylation in cells and identified K296, K297, K298 as the major acetyl acceptor residues. Acetylation is not required for NEIL1′s glycosylase activity *in vitro* but essential for its translocation to chromatin (unpublished results). We observed that neither CHAF1A and nor the other CAF-1 trimer interacts with the mutant NEIL1 (Figure 1C, lanes 4 and 6). CAF-1 was shown earlier to stably interact with proteins involved in DSB repair, nucleotide excision repair (NER) and mismatch

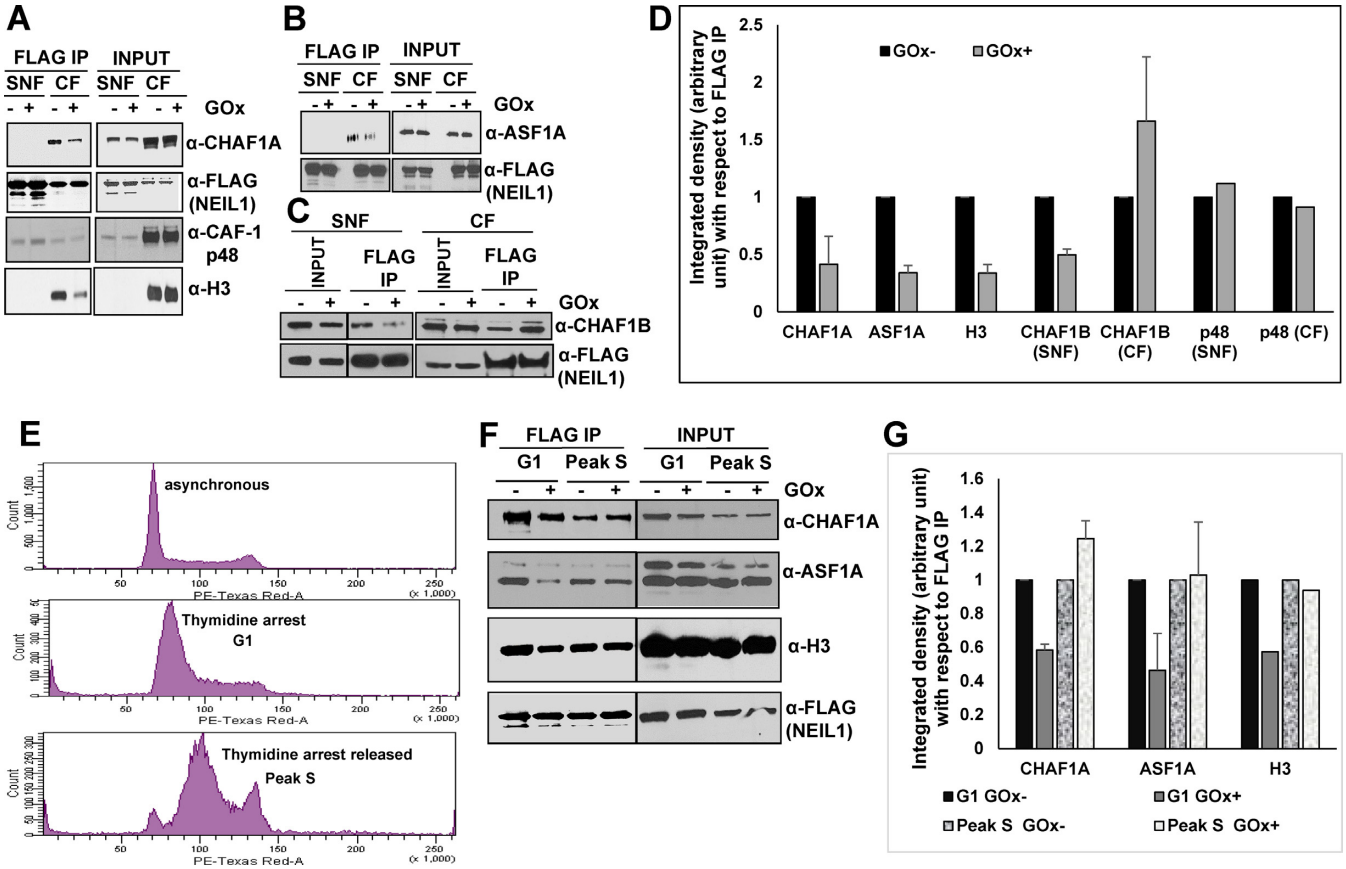

Figure 2. CHAF1A associates with NEIL1 only in chromatin and dissociates after ROS treatment. (**A–C**) Western analysis for (A) CHAF1A, p48 and H3, (B) ASF1A and (C) CHAF1B in FLAG-NEIL1 IP isolated from the SNF or CF of FLAG-NEIL1 expressing HEK293 cells after GOx treatment. (**D**) Histogram showing relative amount (arbitrary unit) of CHAF1A, ASF1A, H3, CHAF1B and p48 in FLAG-NEIL1 IP from control vs. GOx treated cells using Image J analysis tool. Untreated samples were used as reference, error bar is based on ±SD from two to three biological replicates. (**E**) FACS analysis of asynchronous FLAG-NEIL1 expressing HEK293 cells stained with propidium iodide, after double thymidine block (G1 phase), followed by release at 3–4 h after thymidine arrest (peak S phase). (**F**) Cell cycle specific association of FLAG-NEIL1 with CHAF1A, ASF1A and H3. Dissociation of CHAF1A, ASF1A and H3 from the chromatin bound NEIL1 complex occurs only in G1 phase cells after GOx treatment. (**G**) Histogram showing relative levels of CHAF1A, ASF1A and H3 in FLAG-NEIL1 IP from G1 vs. S phase cells after GOx treatment.

repair (MMR) machineries (40–44). However, CAF-1′s involvement in oxidized base damage repair has not been reported so far. Here, for the first time, we demonstrate that CHAF1A is involved in oxidized base repair in chromatin by interacting with BER enzymes.

Transient dissociation of chaperone/NEIL1 complex in chromatin by oxidative stress

The presence of CHAF1A in the NEIL1 IP complex was observed only in the CF but not in the SNF (Figure 2A). Anti-silencing function protein 1 A (ASF1A), another H3/H4 chaperone which functions together with CAF-1 in assembling nucleosomes on nascent DNA (45,46), shows similar association pattern with NEIL1, again only in CF (Figure 2B). Remarkably, in cells exposed to oxidative stress, CHAF1A, ASF1A and histone H3 dissociate from the NEIL1 IP complex (Figure 2A, B and D). This suggests that the association of NEIL1 with CHAF1A and ASF1A in chromatin is disrupted after ROS induced oxidative genome damage. Such damage including single strand breaks was confirmed by Fpg FLARE alkaline Comet assay (Supplementary Figure S1), as was also shown earlier by our group (47,48).

Unlike CHAF1A, the p48 and p60 (CHAF1B) subunits of CAF-1 were found to be present in the NEIL1 complex isolated from both CF and SNF (Figure 2A and C). *In vitro* analysis using recombinant FLAG-CHAF1B and His-NEIL1 shows their binary interaction (Supplementary Figure S2). Interestingly, the CHAF1B level increased in the chromatin bound NEIL1 IP complex, accompanied by its reduction in the IP from SNF after induced oxidative stress (Figure 2C and D). On the other hand, the level of p48 in the NEIL1 IP complex did not change significantly after GOx treatment (Figure 2A and D). These data suggest a unique role of CHAF1A and ASF1A in BER which is not shared by CHAF1B. It should be mentioned here that CHAF1A appears to have independent functions. For example, CHAF1A alone is involved in replication of pericentric heterochromatin containing HP1 (49), and was recently shown to regulate nucleolar maintenance of chromosomes (50).

We analyzed NEIL1′s interaction with these chaperones in G1 vs. S phase cells, which were synchronized via double

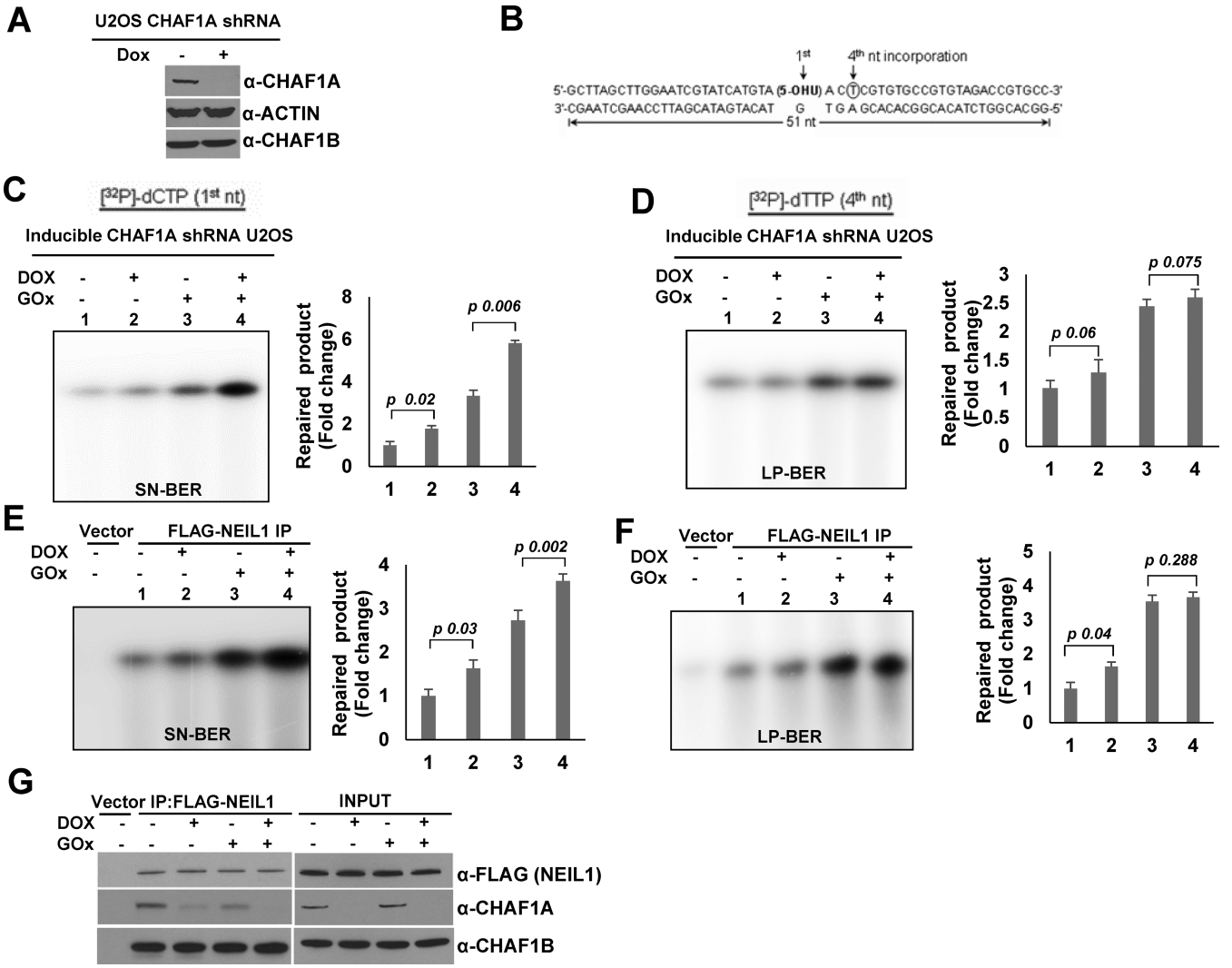

Figure 3. Depletion of CHAF1A enhances NEIL1 initiated SN-BER but not LP-BER. (**A**) Western analysis of CHAF1A and CHAF1B in doxycycline (Dox) inducible CHAF1A shRNA expressing U2OS cells. (**B**) Schematic representation of the substrate oligonucleotide indicating incorporation of radiolabelled [α-^{32}P]dCMP reflecting SN-BER, while incorporation of [α-^{32}P]dTMP indicates LP-BER resulting from strand displacement 3′ to the lesion site (5-OHU) with replicated patch of four nucleotides (10,20). (**C**) Effect of CHAF1A on NEIL1 initiated SN-BER. NE from control and CHAF1A downregulated cells with or without GOx treatment were isolated for the assay, as described in Materials and Methods. (**D**) Effect of CHAF1A on NEIL1 initiated LP-BER as in C. (**E**) SN-BER activity in FLAG-NEIL1 IP complex isolated from control and CHAF1A downregulated cells. Other experimental details are described in Materials and Methods. (**F**) LP-BER assay with FLAG-NEIL1 IP complex. Relative quantification of repaired product are shown (with *P* values; Student's *t* Test). (**G**) Western analysis of FLAG (NEIL1), CHAF1A and CHAF1B levels in the FLAG IPs of cell lysates from control and CHAF1A downregulated cells.

thymidine block (21). The cell cycle phase was confirmed by FACS analysis (Figure 2E). ROS-induced dissociation of CHAF1A, ASF1A and H3 from the NEIL1 complex was observed only in G1 but not in the S phase cells (Figure 2F and G). This suggests that CHAF1A and ASF1A together with associated H3 (and presumably H4 as well) regulate NEIL1-mediated SN-BER in G1 phase cells, and that their dissociation from the NEIL1 complex could be temporally linked to SN-BER. Moreover, because we used predominantly G1 cells in most experiments where oxidized bases are repaired via SN-BER (1,5,51), it appears likely that CHAF1A alone, without involving other CAF-1 subunits, regulates SN-BER in the chromatin.

To further test the hypothesis that CHAF1A regulates NEIL1-initiated SN-BER and not LP-BER (in S phase cells), we established a CHAF1A shRNA inducible cell line. After doxycycline induction, most of CHAF1A was depleted, while the CHAF1B level did not change (Figure 3A). We then analyzed SN-BER of 5-OHU-containing DNA duplex oligonucleotide substrate (Figure 3B) using nuclear extract (NE) from control and CHAF1A downregulated cells (Figure 3C). Complete repair activity in NE isolated from CHAF1A downregulated cells was higher than that from the control cells (Figure 3C, lane 1 versus 2). About 2-fold increase in SN-BER was observed with NE of GOx-treated CHAF1A downregulated cells, compared to the NE from the control cells (Figure 3C, lane 3 vs. 4).

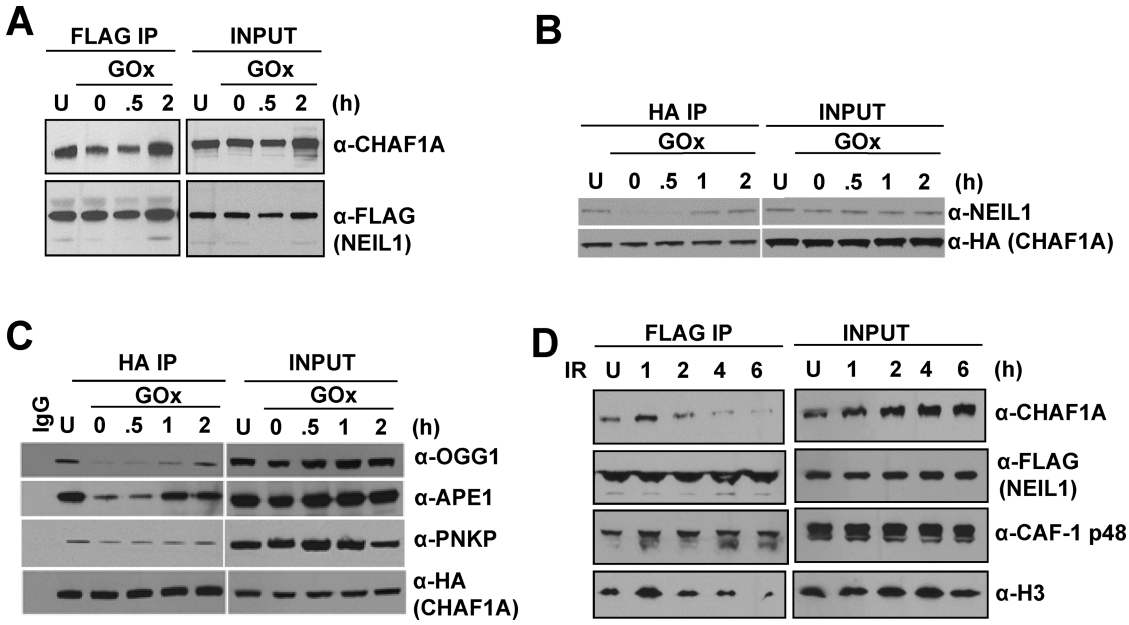

Figure 4. Effect of oxidative stress on CHAF1A/BER complex in chromatin. (**A**) Kinetics of CHAF1A's dissociation from FLAG-NEIL1 IP complex after GOx treatment. Western analysis of CHAF1A in FLAG IP isolated from CF of FLAG-NEIL1 expressing HEK293 cells at various times after GOx treatment. U: untreated control. (**B** and **C**) Kinetics of association of core BER enzymes (NEIL1, OGG1, APE1 and PNKP) in HA-CHAF1A IP from CF after GOx treatment. (**D**) Effect of ionizing radiation (IR; 3 Gy) on association of CHAF1A, p48 and H3 with FLAG-NEIL1.

We then analyzed LP-BER using these NEs; no significant difference in LP-BER activity was observed between the NEs from control vs. CHAF1 downregulated cells (Figure 3D). We also performed SN-BER and LP-BER assays with FLAG-NEIL1 IP complex (Figure 3G) isolated from both control and CHAF1A downregulated cells, and with or without GOx treatment. CHAF1A was not present in the NEIL1 IP complex isolated from CHAF1A downregulated cells, as expected; however CHAF1B was present in the NEIL1 IP complex isolated from both cell types (Figure 3G). Similarly, only SN-BER (Figure 3E) and not LP-BER (Figure 3F) was increased in CHAF1A downregulated cells after GOx treatment. Together, our data thus show that CHAF1A alone is involved in the regulation of SN-BER in G1 cell chromatin.

Constitutively bound CHAF1A is released from the BER complex to allow repair of induced oxidized bases

We analyzed the kinetics of CHAF1A's binding to the chromatin bound NEIL1 repair complex after oxidative stress and observed its transient dissociation followed by reassociation within 2 h (Figure 4A, Supplementary Figure S3A). Generation of ROS induced damage and its repair was confirmed by Fpg FLARE alkaline Comet assay (Supplementary Figure S1).

We also carried out reciprocal studies of NEIL1′s association to and dissociation from the HA-tagged CHAF1A IP complex and observed similar kinetics of binding and release (Figure 4B). Similar results were obtained for other BER proteins including OGG1, APE1 and PNKP (Figure 4C). We thus generalize the concept that CHAF1A sequesters BER complexes in chromatin of unstressed G1 cells, and releases them after induced oxidative damage, in order to allow repair.

Ionizing radiation (IR) induces clusters of genome damages including DSBs, SSBs, and oxidative DNA lesions (52). Based on published reports by others and more recently by us (53,54), we propose a hierarchical order in repair of these diverse damages involving multiple pathways, where repair of the DSBs precede that of oxidative base damage. The kinetics of CHAF1A-NEIL1 association after IR exposure demonstrates that CHAF1A does not dissociate from the NEIL1 complex at early times (Figure 4D, Supplementary Figure S3B) when DSBs are repaired, in contrast to the situation after GOx treatment. This result is supported by our recent finding that NEIL1 (and other DGs) are sequestered early after irradiation to prevent repair of oxidized bases during DSB repair (54). Consistent with this scenario, CHAF1A dissociated from the NEIL1 complex at a later time (after 2 h; Figure 4D, Supplementary Figure S3B) post irradiation, presumably after completion of DSB repair and initiation of BER.

We decided to explore whether negative regulation of oxidized base repair by CHAF1A observed *in vitro* impacts *in cell* repair of oxidative genome damage. We attempted to analyze ROS toxicity in CHAF1A depleted cells. Unfortunately, these cells could not survive for clonogenic assay even in the absence of oxidative stress, presumably because of CAF-1′s essentiality in depositing histone H3/H4 on nascent DNA in replicating cells (23,24). Hence, we used the Fpg FLARE alkaline Comet assay to monitor oxidative damage repair. We observed higher endogenous genome damage (comprising single strand breaks, double strand breaks and oxidized bases) after CHAF1A downregulation (Supplementary Figure S4A). Interestingly, when the cells were exposed to oxidative stress using GOx, the CHAF1A

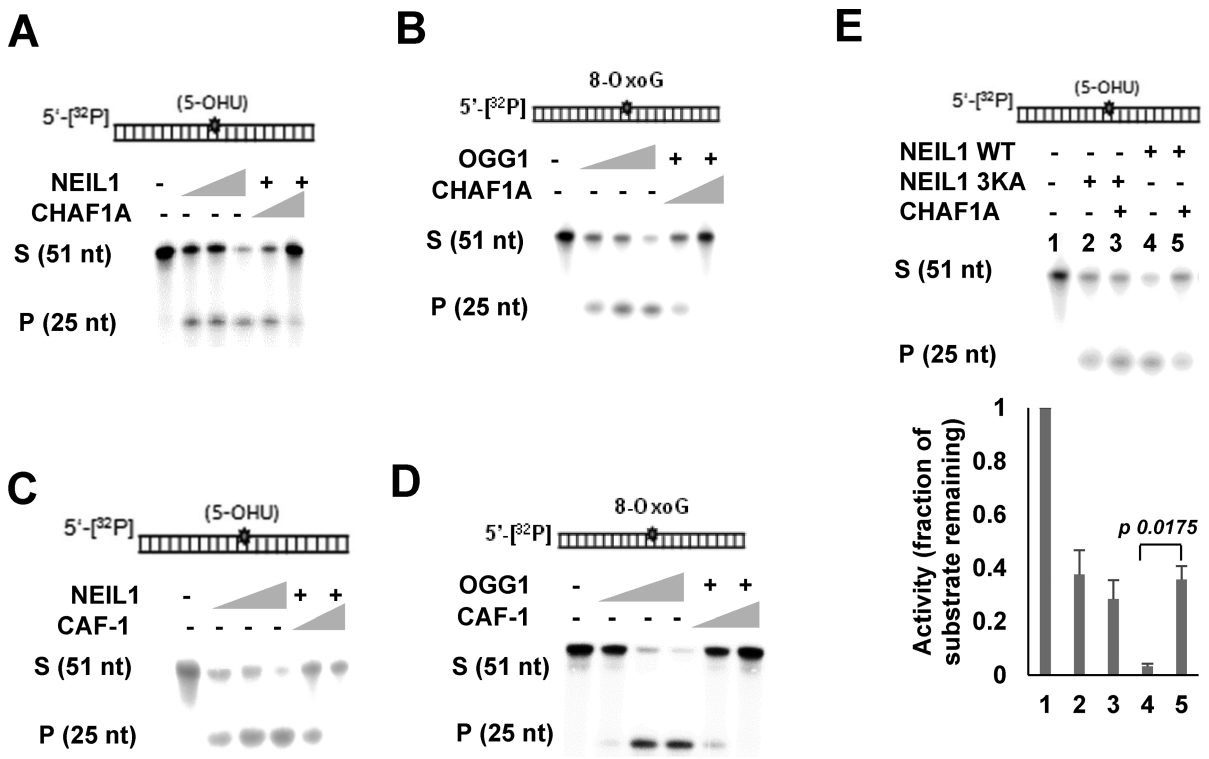

Figure 5. CHAF1A and CAF-1 inhibit base excision activity of NEIL1 and OGG1 *in vitro*. (**A**) Dose-dependent inhibition of DG activity of NEIL1 (5, 10 and 20 nM) by CHAF1A (5 and 20 nM) as determined by using a ^{32}P-labeled 5-OHU lesion-containing oligonucleotide substrate (schematically shown above the figure). Other experimental details are described in Materials and Methods. (**B**) Inhibition of OGG1′s (5, 10 and 20 nM) activity using a ^{32}P-labeled 8-OxoG lesion-containing duplex oligonucleotide substrate by CHAF1A as in A. (**C** and **D**) Inhibition of DG activity of NEIL1 (C) and of OGG1 (D) by CAF-1 trimer. (**E**) Inhibition of DG activity of WT NEIL1 but not of the 3KA mutant NEIL1 by CHAF1A. Bottom panel shows relative quantification of activity (with *P* value; Student's *t*-test).

downregulated cells (mostly G1) showed enhanced repair of oxidized bases within 2 h (Supplementary Figure S4B). These results are consistent with our conclusion about the negative regulatory function of CHAF1A in base damage repair in the nonreplicating cells.

CHAF1A inhibits DNA glycosylases *in vitro*

Our data so far are consistent with the scenario that CHAF1A temporally dissociates from the NEIL1 repair complex following ROS exposure, in order to allow repair of oxidized bases, which implies that CHAF1A inhibits NEIL1′s DG activity. We tested this directly by analyzing the effect of purified CHAF1A on the DG activities of NEIL1 and OGG1 *in vitro,* using ^{32}P-labeled duplex oligonucleotide substrates containing either 5-OHU for NEIL1 or 8-OxoG for OGG1 (21,33). Both NEIL1 (Figure 5A) and OGG1 (Figure 5B) were inhibited by CHAF1A in a dose-dependent manner. We also observed that the CAF-1 heterotrimer like the CHAF1A subunit inhibited base excision activities of NEIL1 (Figure 5C) and OGG1 (Figure 5D). This suggests that the release of CHAF1A from the BER complex after oxidative damage restores base excision activity of NEIL1 and OGG1 for initiating repair, which are constitutively sequestered by histone chaperone complexes in nonreplicating cells. We further observed that CHAF1A, which did not bind to the 3KA mutant NEIL1 (Figure 1C), did not inhibit the activity of the mutant enzyme (Figure 5E); underscoring our notion that CHAF1A inhibits NEIL1′s DG activity via direct interaction. Surprisingly, CHAF1B did not inhibit NEIL1′s DG activity (Supplementary Figure S5), even though it was also bound to the enzyme (Supplementary Figure S2). This suggests different modes of binding of the two CAF-1 subunits to NEIL1 which warrants further studies.

In summary, this study documents for the first time that chromatin factors regulate BER in nonreplicating cells and highlights the need for chromatin remodeling and nucleosome deposition even in the case of SN-BER. Recently, the roles of CAF-1 and ASF1 were documented in nucleosome remodeling during MMR in human genome (44). However, unlike for SN-BER, MMR involves extensive DNA synthesis. This stresses the complexity in regulation of oxidized base damage repair when it involves replacement of only a single base.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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