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Direct interaction between XRCC1 and UNG2 facilitates rapid repair of uracil in DNA by XRCC1 complexes

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Abstract

Uracil-DNA glycosylase, UNG2, interacts with PCNA and initiates post-replicative base excision repair (BER) of uracil in DNA. The DNA repair protein XRCC1 also co-localizes and physically interacts with PCNA. However, little is known about whether UNG2 and XRCC1 directly interact and participate in a same complex for repair of uracil in replication foci. Here, we examine localization pattern of these proteins in live and fixed cells and show that UNG2 and XRCC1 are likely in a common complex in replication foci. Using pull-down experiments we demonstrate that UNG2 directly interacts with the nuclear localization signal-region (NLS) of XRCC1. Western blot and functional analysis of immunoprecipitates from whole cell extracts prepared from S-phase enriched cells demonstrate the presence of XRCC1 complexes that contain UNG2 in addition to separate XRCC1 and UNG2 associated complexes with distinct repair features. XRCC1 complexes performed complete repair of uracil with higher efficacy than UNG2 complexes. Based on these results, we propose a model for a functional role of XRCC1 in replication associated BER of uracil.

Keywords

base excision repair; XRCC1; UNG2; DNA repair complexes; replication associated repair

1. Introduction

Uracil may be introduced in DNA by two distinct mechanisms. One is by hydrolytic deamination of cytosine that generates some 70–200 uracil bases per day in DNA [1]. This deamination results in pre-mutagenic U:G mispairs, which unless repaired, give rise to C:G to T:A transitions upon replication. The other mechanism, which probably is quantitatively a more important source of uracil in DNA, is incorporation of dUMP in place of dTMP during replication. This results in U:A base pairs that can perturb transcription [2,3] or possibly be mutagenic due to erroneous BER and / or enhanced levels of AP site [4,5].

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Conflict of interest

The authors declare that there are no conflicts of interest.

Base excision repair (BER) of genomic uracil is initiated by one of four uracil-DNA glycosylases (UDG) found in mammalian cells: UNG2, SMUG1, TDG and MBD4 [6]. UNG2 removes uracil from U:A base pairs, and is the only UDG known to interact with PCNA and to localise in replication foci [7,8]. UNG deficiency perturbs normal immune response in humans [9] and mice [10] and contributes to the accumulation of mutations in mice [11].

BER is a multi-step process and can be reconstituted with a limited number of proteins. However, in recent years, a number of proteins have been identified that are not strictly required for BER *in vitro*, yet exert important effects on cellular BER effectiveness, mainly through protein-protein interactions and post-translational modification [12]. PCNA and XRCC1 are two key proteins that seemingly provide platforms for repair protein interactions. XRCC1 was originally identified as a factor involved in single-strand break DNA repair and cells deficient in XRCC1 show elevated levels of sister chromatid exchange [13]. XRCC1 interacts with a number of proteins known to participate in BER including: APE1 [14], POL β [15], Lig III [16], PCNA [17], poly (ADP-ribose) polymerase 1 (PARP-1) [18], PARP-2 [19], polynucleotide kinase (PNK) [20] and the DNA glycosylases OGG1 [21], MPG, NEIL1 and hNTH1 [22].

The DNA glycosylases MPG, UNG2 and human MutY homolog (MYH) interact with PCNA through their PCNA binding motif (PIP-box) and co-localize with PCNA in replication foci [8,23–25]. UNG2 is responsible for post-replicative removal of misincorporated dUMP during replication [8]. Similar to UNG2, MYH interacts with PCNA and initiates post-replicative repair of adenine from A:8-oxoG mispairs in newly synthesized DNA [23,26]. While these observations suggest the presence of post-replicative BER, little is known about the overall organization of BER during replication.

XRCC1 co-localizes and directly interacts with PCNA [17] and POL β and PNK co-localize with XRCC1 and PCNA in replication foci (Akbari et al 2010, submitted to DNA repair), thus, a specific function for XRCC1-BER complexes during replication is likely. Previously, UNG2 immunoprecipitates containing XRCC1 were found to be capable of complete short patch and long patch BER [27]. However, the extent of protein-protein interactions and whether XRCC1 and UNG2 directly interact or are part of a large complex (es) was not elucidated.

Our aim for conducting this study was to investigate whether repair of uracil in DNA in S-phase cells takes place via XRCC1 mediated protein complexes. We show that XRCC1 and UNG2 co-localize in replication foci and are in close proximity of each other. Using a pull-down assay we show that UNG2 directly interacts with the NLS-region of XRCC1. Extracts from synchronised cells expressing EYFP-fusions of UNG2 and XRCC1 and use of tag specific antibodies, allowed us to directly compare the biochemical activities and protein content of XRCC1 and UNG2 immunoprecipitates from S-phase cells. Our results provide evidence for the presence of XRCC1 complexes containing UNG2 in addition to separate XRCC1 and UNG2 associated complexes with distinct repair characteristics. We propose a model for a role of XRCC1 in repair of uracil in replication foci.

2. Materials and methods

2.1 Chemical and antibodies

Synthetic oligonucleotides were from Eurogentech (Belgium), [α -³³P]dTTP and [α -³³P]dCTP (3000 Ci/mmol) were from Amersham Biosciences, restriction enzymes from New England BioLabs, paramagnetic protein-A beads from Dynal (Norway). Phenylmethyl sulfonyl fluoride (PMSF) and Complete protease inhibitor were from Roche. Antibody against (α)-POL β (ab3181), α -DNA Lig I (ab615), α -DNA Lig III (ab587), α -APE-1 (ab194), α -XRCC1 (ab1838 (mouse) and abcam 11429 (rabbit)), α -PARP-1 (ab18376), α -FLAG (Sigma) and α -GFP

(ab290, Abcam, UK), α -GFP (in house, only used for IP). Both α -GFPs also recognize the other GFP variants (ECFP and EYFP). Other antibodies used were α -PNK (MAB-005, Cytostore, USA), α -PCNA (PC10, Santa Cruz, USA), α -Fen-1 (Betyl, USA), and in-house affinity purified rabbit polyclonal antibodies raised against UNG2 [28]. Secondary antibodies: polyclonal rabbit α -mouse IgG/HRP or peroxidase-labeled polyclonal swine α -rabbit IgG were from Dako Cytomation (Denmark), Alexa fluor 532 goat- α -mouse IgG and Alexa Fluor 647 goat- α -rabbit IgG (Invitrogen). IgGs were crosslinked to protein-A magnetic beads according to procedure (New England Biolabs).

2.2. Cloning of fusion constructs

pEC/YFP-PCNA, HcRed-PCNA, pXRCC1-EYFP have been described [17,29,30]. The UNG2-EYFP construct is a modified version of the ProA-UNG2-EYFP construct from which UNG2-EYFP transcription is regulated by its own promoter [7]. A 3 $\times$ FLAG sequence is cloned in front of UNG2-EYFP in the EcoRI blunted / KpnI site, thus the FLAG sequence is separated from the N-terminus of UNG2 by a 84 bp linker.

Hamster Xrcc1-EYFP and Xrcc1-ECFP constructs were prepared as follows: Total RNA was isolated from AA8 cells (RNeasy midi kit, Qiagen). First strand hamster Xrcc1 cDNA substrate was generated using dT-oligo and TaqMan Reverse Transcription Reagents (Applied Biosystem). PCR was carried out using AccuTaq LA DNA Polymerase (Sigma-Aldrich), and oligonucleotides

Xrcc1F-XhoI (5'-GAATTCCTCGAGGATGCCGGAGATCAGCCTCCGC-3') and Xrcc1R-XmaI (5'-GAATCCCCCGGGCTCCGGCCTGTGGCACCACCTC-3'). The PCR product was digested with XhoI/ XmaI and cloned into XhoI/ XmaI site of EYFP-N1 vector (Clontech). The deletion constructs were amplified using the following primer pairs:

For Xrcc1₁₋₃₂₅-ECFP:

Xrcc1F-XhoI (5'-GAATTCCTCGAGGATGCCGGAGATCAGCCTCCGC-3') and Xrcc1R2-XmaI (5'-GGATCCCCCGGGCCACCACACCCTGCAGAATC-3').

For Xrcc1₁₋₁₈₀-ECFP:

Xrcc1F-XhoI (5'-GAATTCCTCGAGGATGCCGGAGATCAGCCTCCGC-3') and Xrcc1R3-EcoRI (5'-GGATCCGAATTCCTTTTCACACGGAAGTGGC-3').

For Xrcc1₃₁₅₋₆₃₃-ECFP:

Xrcc1F2-EcoRI (5'-GGATCCGAATTCGGAGCTGGGGAAGATTCTG-3') and

Xrcc1R-XmaI (5'-GAATCCCCCGGGCTCCGGCCTGTGGCACCACCTC-3').

PCR products were purified, digested with appropriate restriction enzymes, and cloned into the appropriate restriction sites of pECFP-N1. The primer sequences were derived from NCBI-AF034203.

2.3. Cell cultures

Cells were transfected with the different fusion constructs using calcium phosphate (Profection Promega) or Eugene 6 (Roche). HeLa and EM9 (Chinese hamster ovary cell line) cells stably expressing human or Chinese hamster XRCC1-EYFP or deletion constructs thereof, UNG2-EYFP and EYFP were prepared by transfection followed by cell sorting or cloning by dilution and prolonged culturing in selective media (DMEM (Gibco)) containing 10% fetal calf serum, gentamycin (0.1 mg/ml, Gibco), glutamine (1 mM), fungizone (2.5 μ g/l) and geneticin (G418,

0.4 mg/ml, Invitrogen). For transient transfection the cells were examined after 16 to 24 h. Untransfected cells were cultured in the same medium without geneticin.

2.4. Proximity Ligation Assay (PLA) and immunofluorescence staining

PLA was performed to visualize UNG2 / XRCC1 interactions in vivo. Briefly, in PLA oligonucleotide conjugated probe antibodies are directed against primary antibodies. Annealing of the probes occurs when the target proteins are in close proximity, which initiates the amplification of a reporter signal [31]. Cells were grown on coverslips overnight. Cells were washed once with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 15 min on ice, washed once with PBS, permeabilized with methanol at -20°C for 30 min, washed once with PBS-FCS (2% fetal calf serum in PBS) and blocked by incubation in PBS-FCS for 30 min prior to incubation with primary antibodies diluted in PBS-FCS (mouse monoclonal α -FLAG (1:1000) and rabbit polyclonal α -XRCC1 (1:250). PLA was performed as described by the manufacturer (Olink Biosciences) using the Duolink Detection Kit with PLA PLUS and MINUS probes. For immunohistochemistry, the cells were fixed, permeabilized and blocked and incubated with primary antibodies as above. Secondary antibodies used were Alexa fluor 532 goat- α -mouse IgG and Alexa Fluor 647 goat- α -rabbit IgG.

2.5. Confocal microscopy

Fluorescent images of live cells co-transfected with ECFP, EYFP and HcRed constructs (1 μm thickness) were produced using a Zeiss LSM 510 Meta laser scanning microscope equipped with a Plan-Apochromate 63 $\times$, 1.4 oil immersion objective. Three colour images were taken using three consecutive scans and the following settings: ECFP-excitation at $\lambda = 458\text{ nm}$, detection at $\lambda = 470\text{--}500\text{ nm}$, EYFP- excitation at $\lambda = 488\text{ nm}$, at $\lambda = 530\text{--}600$ and HcRed excitation at $\lambda = 543\text{ nm}$, detection at $\lambda > 615\text{ nm}$. For immunofluorescence staining: excitation at $\lambda = 543\text{ nm}$ and $\lambda = 633\text{ nm}$ and detection at $\lambda = 560\text{--}615\text{ nm}$ and $\lambda > 650\text{ nm}$ respectively. PLA: excitation at $\lambda = 543\text{ nm}$ and detection at $\lambda = 560\text{--}615\text{ nm}$, together with EYFP excitation at $\lambda = 514\text{ nm}$ and detection at $\lambda = 560\text{--}615\text{ nm}$.

2.6. Cell synchronization

HeLa cells stably expressing XRCC1-EYFP or UNG2-EYFP or EYFP alone were synchronised by double thymidine block, which arrests the cells at the G1/S border [32]. The cells were released at mid-S-phase (3 h) [33]. Cell cycle phase was verified by flow cytometry. The DNA analysis of the cells was performed after methanol fixation (70%), RNase treatment (100 $\mu\text{g/ml}$ in PBS at 37°C for 30 min) and propidium iodide (PI) staining (50 $\mu\text{g/ml}$ in PBS at 37°C for 30 min) on a FACS Canto flow cytometer (BD-Life Science).

2.7. Formaldehyde crosslinking of proteins in intact cells and preparation of whole cell extracts

Cells were synchronised by double thymidine block [34] and analysed for cell phase by flow cytometry (BD Canto Flow) after methanol fixation, RNase treatment and propidium iodide staining. The cell fractions enriched in S- and G1-phase were harvested and washed twice with cold PBS. $5\text{--}6 \times 10^6$ cells were resuspended in 10 ml PBS containing 0, 0.125 or 0.25% formaldehyde and incubated at 37°C for 20 min. Crosslinking was stopped by adding glycine (final concentration 0.125 M) and further incubation at room temperature for 5 min. Cells (non-crosslinked and crosslinked) were collected by centrifugation and washed once in PBS, suspended in $8 \times$ packed cell volume (PCV) in buffer I (20 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 % Nonidet P-40, 1 mM DTT and Complete protease inhibitor) containing 5 μl Omnicleave Endonuclease (200 U/ μl Epicentre Technologies, WI) and sonicated. DNase and RNase cocktail I (2 μl (200 U/ μl) of

Omnicleave Endonuclease, 1 μ l Bensonase (250 U/ml, Novagene, Ge), 10 μ l RNase (10 mg/ml, Sigma-Aldrich), 1 μ l DNase (10 U/ μ l, Roche Inc.), and 1 μ l micrococcal nuclease (100–300 U/mg, Sigma-Aldrich) per 30 mg cell extract) was added to the homogenate and incubated at room temperature for 1 h and dialyzed at 4 °C overnight in buffer II (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM DTT and Complete protease inhibitor). The extracts were cleared by centrifugation at $16,200 \times g$ for 10 min and the supernatant was used for further analyses. For preparation of extracts from the chromatin enriched fraction, sonicated HeLa cells were pelleted at $16,200 \times g$. The pellet was resuspended in buffer II containing the DNase and RNase cocktail I and incubated at 37 °C for 1 h. The extract was cleared by centrifugation at $16,200 \times g$ and the supernatant was used for immunoprecipitation with anti-EYFP coupled beads. The immunoprecipitation of all samples (formaldehyde cross-linked and non-crosslinked samples and the chromatin enriched samples) was carried out in 5 ml buffer II, and the beads were washed $5 \times$ with 1 ml buffer II prior to suspension in loading buffer. For the cross-linked samples the immunoprecipitates were heated at 65 °C for 10 min to release the proteins / protein complexes from the beads. Half of the released material (after removal of beads) was further incubated at 95 °C for 30 min to reverse the crosslinks. The proteins were separated on denaturing polyacrylamide gel and analysed by Western blot.

2.8. Pull-down assay and Far Western analysis

Cell extracts from EM9 cells expressing constructs of hamster Xrcc1-EYFP were incubated with anti-EYFP beads overnight. The beads were washed three times in 20 mM HEPES pH 7.5, 500 mM KCl, 2 mM DTT, 5 mM PMSF, Complete protease inhibitor and 1% Tween 20 in order to wash unrelated proteins off the fusion proteins. The beads were then washed once in 20 mM HEPES pH 7.5, 100 mM KCl, 2 mM DTT, 20 mg/ml BSA 1% Tween 20, 5 mM PMSF and Complete protease inhibitor and incubated with 0.2 μ g/ml Δ 93 UNG2 in the same buffer at 4 °C for one hour under constant rotation. The beads were washed in 20 mM HEPES pH 7.5, 200 mM KCl, 2 mM DTT, 1% Tween 20, 5 mM PMSF and Complete protease inhibitor, and the pull-down material was analysed by Western blot. In the Far Western experiment, the immunoprecipitates were washed three times in 20 mM HEPES pH 7.5, 1 M KCl, 2 mM DTT, 5 mM PMSF and Complete protease inhibitor and 1% Tween 20, separated on Bis-Tris-HCl NuPAGE ready gels (4–12%) and transferred to PVDF membranes (Immobilon, Millipore). The membrane was incubated overnight in a buffer containing 10 mM Tris, pH 7.5, 0.5% BSA, 0.25% gelatin, 0.2% Triton X-100, 5 mM β -mercaptoethanol, and 150 mM NaCl. Fresh buffer containing 100 mM NaCl was added with 5 μ g of recombinant Cy-3 labelled Δ 93 UNG2 protein (Cy 3 Ab Labeling kit (PA33000), Amersham) and the membrane was incubated for a further 4–6 h, washed briefly at room temperature with buffer containing 10 mM Tris-HCl, pH 8, 0.05% Tween, and 100 mM NaCl. The membrane was dried and first analysed in a Typhoon Trio Imager (GE Healthcare), then stripped and probed with α -Xrcc1 antibody for Western analysis.

2.9. Immunoprecipitation and Western blot analysis

For immunoprecipitation, antibodies covalently linked to protein-A paramagnetic beads were added to the extracts in 5 ml buffer II and incubated at 4 °C overnight. The beads were collected from the solution, washed once in 1 ml of buffer II and thereafter three times in 10 mM Tris-HCl pH 7.5 and 50 mM KCl. The beads were used in BER assays (described below) or suspended in loading buffer, heated, separated on Bis-Tris-HCl NuPAGE ready gels and transferred to PVDF membranes. The membranes were blocked in 5% low fat dry milk in PBST (PBS with 0.1% Tween 20) and incubated with primary antibody in 1% dry milk at 4 °C overnight. Following incubation for 1 h with secondary antibody, membranes were treated with Chemiluminescence reagent (SuperSignal West Femto Maximum, PIERCE) and the proteins visualised in the Kodak Image Station 2000R.

2.10. BER assay

An illustration of DNA substrate and strategy for BER patch size analysis is shown in Figure 3. To prepare the AP site substrate, we incubated uracil-containing DNA with purified catalytic domain of UNG [28]. The BER assay was carried out essentially as described [27,35]. The purified DNA was digested with appropriate restriction enzymes for BER analysis.

2.11. UDG activity assays

UDG activity was assayed according to previously described procedures [7,36]. Briefly, UDG activity was measured in 20 μ l of assay mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5 mg / ml BSA, 1.8 μ M [3 H]dUMP-containing calf thymus DNA (specific activity 0.5 mCi / μ mol) and nuclear extracts. The mixture was incubated at 30 $^{\circ}$ C for 10 min and the amount of released uracil was measured by scintillation counting.

3. Results

3.1. XRCC1, UNG2 and PCNA co-localize in replication foci

Deamination of cytosine results in a U:G mismatch that can give rise to a C:G to T:A transition mutation if not repaired prior to replication. Previously, UNG2 was shown to localise in replication foci and to be responsible for immediate post-replicative removal of misincorporated uracil in DNA [8]. In view of the proposed role of XRCC1 in organising DNA repair ahead of replication [17], we first examined whether UNG2 and XRCC1 are simultaneously present in replication foci during S-phase. Using HeLa cells transiently transfected with tagged proteins, we found that the PCNA foci contained both UNG2 and XRCC1 (Fig. 1A, white spots), while several XRCC1 foci in S-phase cells and all XRCC1 foci in non S-phase cells, contained neither UNG2 nor PCNA (Fig. 1A and B, red spots).

Next, we employed in situ Proximity Ligation Assay (PLA), a technique that combines dual recognition of target proteins using secondary antibodies with attached DNA strands as proximity probes, forming templates for rolling circle amplification for detecting protein interactions and / or proximity of proteins [31]. Two proteins can appear to co-localise in confocal microscopy even being up to 20 folds further apart [37] as compared to PLA [31]. So, PLA provides stronger indications for in situ protein-protein interactions. Expression of UNG2 is strictly cell cycle regulated, i.e. it increases in late G1, achieves maximal expression in mid S-phase and becomes degraded in G2/M [33]. Thus, correct intra-cellular localisation of UNG2 is likely to be tightly associated with its level of expression. We prepared cells stably expressing 3 $\times$ FLAG-UNG2-EYFP (UNG2-EYFP) containing the native UNG2 promoter [7, 38] and used this cell line for PLA analysis of UNG2 and XRCC1. For primary antibodies we used a monoclonal anti-FLAG antibody (α -FLAG) for UNG2 detection and a polyclonal antibody for XRCC1 detection (α -XRCC1). The specificity of these proteins was verified in HeLa cells transfected with 3 $\times$ FLAG-UNG2-EYFP (UNG2-EYFP) and Xrcc1-deficient (EM9) Chinese hamster ovary (CHO) cells (CHO-EM9) transfected with XRCC1-EYFP. Non-transfected cells, did not stain with these antibodies (Figure 1B, see arrows). Similar to the co-localization seen in live cells (Fig. 1A), staining of stable UNG2-EYFP expressing cells with α -FLAG and α -XRCC1 antibodies identified nuclear foci containing both UNG2 and XRCC1 (Fig. 1B, lower row). By using both EYFP and Flag tagged UNG2, we could examine co-localization of PLA foci with UNG2 foci by detection of EYFP (UNG2-EYFP) as an extra quality control. When using these antibodies in the PLA system, we detected specific nuclear foci that co-localized with UNG2-EYFP foci (Fig. 1C, upper panel). In a control experiment, using PLA with only α -XRCC1, we detected a few foci (Fig. 1C, lower panel, PLA control), but less than 10% of these foci co-localized with UNG2-EYFP foci suggesting that they were unspecific. In contrast, more than 90% of the PLA foci using both α -FLAG and α -XRCC1 antibodies co-localized with UNG2-EYFP (Fig. 1C, upper panel, PLA). The overview images

(Fig. 1C, left) show additional representative cells where the nuclei is outlined. In conclusion, the results of PLA assay show that UNG2 and XRCC1 are sufficiently close to each other in S-phase foci to suggest a direct interaction between the proteins.

3.2. UNG2 binds to the NLS region of XRCC1

One of the first indications for a direct interaction between XRCC1 and UNG2 arose when we attempted to purify human XRCC1 from *E. coli* extract. Bacterial *E. coli* Ung, which has high sequence homology (55.7 % identity and 73.3 % similarity when considering conserved residues) to the catalytic domain of human UNG2 [39], was readily co-purified with full length human XRCC1 (data not shown). Thus, we examined for an interaction of purified catalytic domain of human UNG2 with different constructs of *Cricetulus griseus* Xrcc1 fused with ECFP (illustrated in Fig. 2C), using a combination of immunoprecipitation, pull-down assay and Far Western. The constructs were expressed in CHO-EM9 (Xrcc1^{-/-}) cells to avoid interaction with endogenous Xrcc1 and to allow posttranslational modification which may be important for protein interactions. Total cell extracts (DNase and RNase treated, see Materials and Methods) from the plasmid transfected CHO cells were incubated with α -GFP (capable of capturing EGFP, ECFP and EYFP tags) coupled beads. The beads were washed extensively with high salt wash buffer and then incubated with the catalytic domain of UNG2 (Δ 93 UNG2) [28] for pull down analysis or separated by gel electrophoresis and transferred to a membrane for Far Western analysis.

Western blot analysis of the pull-down material showed that the fusion proteins containing the NLS region of Xrcc1 as well as full length Xrcc1-ECFP were able to pull down Δ 93 UNG2 (Fig. 2A). This result suggests that UNG2 directly interacts with Xrcc1 at the NLS region of Xrcc1.

To further verify a direct interaction between UNG2 and Xrcc1, and exclude the possibility that this binding is mediated via a common binding protein such as PCNA, we performed Far Western analysis incubating the membrane containing the different fusions proteins immunoprecipitated from transiently transfected CHO-EM9 cells with Cy-3 labelled Δ 93 UNG2. Figure 2B shows specific Cy-3 bands corresponding in size to full length Xrcc1, Xrcc1₁₋₃₂₅ and Xrcc1 _{Δ 428-560} fusion proteins (lanes 6–8 and 1–3 respectively, white arrows). No specific bands for UNG2 can be detected for the Xrcc1₁₋₁₈₀ and Xrcc1₃₁₅₋₆₃₃-fusion proteins (lanes 9–10, and 4–5, respectively, red arrows). Some of the degraded fusion proteins in lane 1–3 were also able to interact with Cy-3 labelled UNG2 (lanes 6–8). In summary, these results clearly demonstrate a direct interaction between Xrcc1 and UNG2. Based on the results shown in Figures 2–5 (see also below), only a sub-fraction of Xrcc1 binds UNG2, possibly mediated by a posttranslational modification on Xrcc1.

3.3. XRCC1 immunoprecipitates carry out UNG specific uracil-BER

To examine whether XRCC1-EYFP immunoprecipitates were proficient in uracil-BER we carried out functional analysis of the immunoprecipitates using a closed circular DNA containing uracil at a defined position (Fig. 3, left panel). Compared to the robust capacity for complete BER of AP sites (Fig. 3, right panel, lanes 7 to 9), we detected a low, but significant, level of uracil-BER activity in the immunoprecipitates (lanes 1 and 4). The latter activity was completely inhibited by neutralising α -UNG [28] (lanes 2 and 5) or Ugi [40] (lanes 3 and 6). These results indicate that XRCC1-EYFP immunoprecipitates contain functional repair complexes with a capacity for UNG2 specific uracil-BER.

3.4. UNG2 and XRCC1 are part of a common complex as well as distinct repair complexes in S-phase cells

UNG2 co-localized with XRCC1 in replication foci (Fig. 1A), thus we next examined whether XRCC1 and UNG2 are present in a common or distinct replication associated complexes. For this we used 3×FLAG-UNG2-EYFP expressing cells, cells expressing XRCC1-EYFP (the XRCC1-EYFP expressing cell line is described in the accompanying paper, Akbari et al., 2009) and cells only expressing EYFP. We employed *in vivo* formaldehyde crosslinking and immunoprecipitation with antibodies against the EYFP tag to analyze the protein composition of the immunoprecipitates. Whole cell extracts were prepared from formaldehyde crosslinked and non-crosslinked cells enriched at the G1/S-boarder and mid S-phase (double thymidine block synchronization). Immunoprecipitates from EYFP expressing cells were used as the control. Importantly, the experiments were carried out under identical conditions with the same antibody.

Formaldehyde crosslinking is a sensitive method that needs to be optimized to avoid excessive crosslinking. To examine the extent of crosslinking, we carried out BER analysis of XRCC1-EYFP immunoprecipitates from crosslinked cell extracts. Since the immunoprecipitates showed detectable uracil and AP site BER activity (Fig. 4A), a considerable number of proteins in the immunoprecipitates were functional, illustrating that the crosslinking was mild under our experimental conditions.

For Western analysis, the immunoprecipitates were suspended in loading buffer and heated at 65 °C for 10 min to release proteins from the beads (most crosslinks still present). Half of the eluted material (after removal of the beads) was further heated at 95 °C for 30 min to reverse the crosslinks. The results show that close to equal amounts of XRCC1-EYFP and UNG2-EYFP were immunoprecipitated from the crosslinked and non-crosslinked samples (Fig. 4B, compare fusion proteins at different formaldehyde concentrations). This further supports that under our experimental conditions, the crosslinking was mild, as excessive crosslinking would have likely resulted in a considerable reduction in the amount of immunoprecipitated fusion protein from the samples due to loss of available epitopes. We detected high molecular weight proteins or protein complexes (>200 kDa) in the immunoprecipitates from the crosslinked extracts containing XRCC1 and POL δ (Fig. 4B, first and third column, marked with red asterisk). Much less of these bands were detected after reversal of the crosslinks by heating at 95 °C, whereas the bands for XRCC1 and POL δ became correspondingly more intense (Fig. 4B, second and fourth column). Notably, the high molecular weight complexes appeared to be more pronounced in the XRCC1-EYFP immunoprecipitates from the mid S-phase extracts compared to the G1/S extracts, suggesting an increase in the amount of large XRCC1-EYFP complexes in S-phase. In agreement with the confocal images, in which we identified co-localization of XRCC1 and UNG2 only in replication foci (Fig. 1A), we detected more UNG2 and PCNA in immunoprecipitates from mid S-phase XRCC1-EYFP extracts, as well as higher amounts of XRCC1 and PCNA in immunoprecipitates from mid S-phase UNG2-EYFP extracts, as compared to immunoprecipitates from G1/S boarder cell extracts (Fig. 4B, second and fourth column). Western analysis of immunoprecipitates from the control EYFP expressing cells was negative for XRCC1, UNG2, PCNA, POL δ , Lig III, Lig I, PARP-1, PNK and APE-1 (data not shown), demonstrating that non-specific binding to beads and excessive non-specific crosslinking of proteins *in vivo* was not a problem under the conditions employed. This observation is in agreement with a recent study showing that immunoprecipitation of tagged proteins with magnetic beads display a low level of unspecific binding to the immunoprecipitates compared to several available methods [41].

The results thus far suggested that UNG2 and XRCC1 are likely part of a common multiprotein complex in replication foci. However, the overall composition of XRCC1-EYFP and UNG2-EYFP associated proteins varied significantly with respect to the amount of Lig III, Lig I,

PARP-1 and PNK in the immunoprecipitates (Fig. 4B, mid panel). For example, the XRCC1 partner, Lig III [16], was readily detected in the XRCC1-EYFP immunoprecipitates both without and after crosslinking (Fig. 4B, second column), whereas in the UNG2-EYFP immunoprecipitates, the amount of Lig III followed the amounts of XRCC1, i.e. more was detected in mid S-phase extracts (Fig. 4B, fourth column). Lig I was barely visible in any XRCC1-EYFP immunoprecipitates, while it was readily detected in all UNG2-EYFP immunoprecipitates, with more in the mid S-phase immunoprecipitates (Fig. 4B, mid panel, second and fourth column). Thus, XRCC1 and UNG2 seem to be part of separate complexes, as well as a common complex, during S-phase.

Interestingly, the amounts of PARP-1, POL β and Lig III were higher in the immunoprecipitates from non-crosslinked XRCC1-EYFP extracts than from the crosslinked extracts (Fig. 4B, mid and lower panel). This may be due to protein interactions that take place in the extracts before or during immunoprecipitation, which may otherwise occur at a lower extent in situ. On the other hand, APE1 was only detected in the immunoprecipitates from the crosslinked cells (Fig. 4B, lower panel), suggesting that APE1 associates with XRCC1 and UNG2 with low affinity, so most of it may fall off during immunoprecipitation. This seemingly agrees with the disparate reports on the interaction of APE1 with XRCC1 at different experimental conditions [14,17]. However, immunoprecipitated XRCC1-EYFP complexes clearly contained AP-endonuclease activity (Fig. 3 and Fig. 4A), indicating the presence of APE1, even though it was not detected by Western analysis from non-crosslinked extract.

3.5. XRCC1 and UNG2 are part of a common complex in chromatin

Because the confocal studies showed that XRCC1 and UNG2 co-localize in replication foci and Western analysis suggested that these two proteins are present in a common, higher order complex during S-phase, we fractionated HeLa cells and carried out immunoprecipitation of a chromatin enriched fraction, which likely contains replication associated complexes. We used antibodies against endogenous UNG2 and XRCC1 for immunoprecipitation. Fig. 4C shows that similar to the results from the crosslinked samples, Lig III and PNK were readily detected in the XRCC1 immunoprecipitates, while Lig I was only detected in UNG2 immunoprecipitates. A low amount of UNG2 was detected in the XRCC1 immunoprecipitate and a low amount of XRCC1 was detected in the UNG2 immunoprecipitate. These data support the notion that XRCC1 and UNG2 are part of distinct as well as shared complexes, where only a fraction of XRCC1 associated complexes contain UNG2 and vice versa. These results also show that the overall organisation of protein-protein interactions within immunoprecipitates of fusion proteins using tag specific antibodies is largely identical to those detected in immunoprecipitates of corresponding endogenous XRCC1 or UNG2 proteins.

3.6. XRCC1 complexes immunoprecipitated from S-phase cells perform complete uracil and AP site repair

Next, we carried out comparative BER analysis of the immunoprecipitated XRCC1-EYFP and UNG2-EYFP from mid S-phase enriched cells for repair of uracil and AP site in DNA (U:A and U:G). We found that UNG2-EYFP immunoprecipitates were unable to efficiently generate the final ligated product with any of the substrates (Fig. 5A, upper panel, lanes 1, 2 and 8), whereas complete BER was carried out by the XRCC1-EYFP immunoprecipitates at the same time (15 min) (Fig. 5A, lower panel, same lanes). To examine whether the levels of APE1 or a DNA ligase were responsible for the observed deficiency in repair activity of the UNG2-EYFP immunoprecipitates, we carried out BER analysis in the presence of supplemented APE1 or T4 DNA ligase. Addition of APE1 had no detectable effect on the amount of incorporated nucleotide during BER, indicating that sufficient AP-endonuclease activity was present in the UNG2-EYFP immunoprecipitates (Fig. 5A, upper panel, lanes 3, 4 and 9). In contrast, addition of T4 DNA ligase increased the amount of ligated products significantly (Fig. 5A, upper panel,

lanes 5, 6 and 10), indicating that the ligation step was slow despite the presence of Lig I in similar immunoprecipitates (Fig. 4B and C). On the other hand, the XRCC1-EYFP immunoprecipitates performed complete uracil and AP site BER, and addition of recombinant APE1 and T4 DNA ligase had no detectable effect on total BER (Fig. 5A, lower panel, lanes 3–6 and 9 and 10).

Next, we carried out time-course kinetic analysis of uracil-BER by the different immunoprecipitates and found that the amount of ligated products by UNG2 immunoprecipitates increased with prolonged incubation, but the unligated products were still more abundant (Fig. 5B, lane 7). The XRCC1-EYFP immunoprecipitates on the other hand, appeared to complete the repair pathway rapidly, and no unligated repair intermediates could be detected at any time point (Fig. 5B, lanes 2, 5 and 8). To examine whether the low level of ligated products in the UNG2 immunoprecipitates was due to unligatable DNA repair intermediates, we carried out uracil-BER of UNG2 immunoprecipitates for 60 min, and then incubated half of the purified DNA with excess T4 DNA ligase (see Material and Methods), which is expected to seal all ligatable ends [42]. DNA was then analysed for ligated and unligated repair products. The amount of ligated products increased, similar to what was found after addition of T4 DNA ligase in Fig. 5A (lane 10). However approximately 45% of the DNA was still unligated (Fig. 5C). This result indicates that also DNA end-trimming was less efficient in UNG2 immunoprecipitates as compared to XRCC1 immunoprecipitates. In summary, we have identified two functionally different S-phase associated BER complexes based on immunoprecipitation of UNG2 and XRCC1.

3.7. XRCC1 affects the rate of uracil-BER

We next examined if absence of XRCC1 could have any effect on repair of uracil by cell extracts. We examined the repair efficiency of cell extracts from wild type (AA8) and *Xrcc1* deficient (EM9) cell lines, as well as an EM9 cell line expressing *Cricetulus griseus* *Xrcc1*-EYFP fusion protein. *Xrcc1*-EYFP was functional because it restored DNA repair capacity of EM9 cells following hydrogen peroxide challenge as assessed by the Comet assay (data not shown). We could not include the *Xrcc1* deletion mutants used for pull-down experiments in this experiment, because we failed to establish EM9 cells stably expressing the respective constructs.

In order to distinguish between UNG2 and the other uracil-DNA glycosylases (SMUG1, TDG and MBD4) present in the cell extract, and thereby to focus on a biological role of the *Xrcc1*-UNG2 interaction, we used a U:A containing substrate which is strictly processed by UNG2 [7,43]. The U:G substrate, arising following deamination of cytosine, on the other hand, is efficiently corrected by SMUG1, TDG, MBD4 as well as UNG2 [44]. BER analysis for repair of U:A showed that after 5 min the level of BER was considerably lower in the *Xrcc1* deficient extract, as compared to extracts from the wild type or *Xrcc1*-EYFP expressing cells (Fig. 5D, upper panel, lanes 4–6). However, this was not seen for AP site repair under identical conditions (Fig. 5D, lower panel, lanes 4–6), indicating that the slower uracil-BER in the absence of *Xrcc1* was unlikely due to overall reduced stability of POL β or Lig III in these cells [45,46]. To rule out that the observed differences in U:A BER were related to varying ability of the extracts for uracil excision (UDG) from the U:A context, we performed a standard UDG activity assay [36]. We found that extracts from the *Xrcc1* deficient cells had 20% higher UDG activity compared to the wild type cell extract, indicating that the slower U:A BER in the *Xrcc1* deficient cell extracts was not simply related to UDG capacity of the extract, but more likely due to a role for XRCC1 in coordinating the repair of uracil.

In summary, we have identified two functionally different S-phase associated BER complexes based on immunoprecipitation of UNG2 and XRCC1. A sub-fraction of the XRCC1 complex also contained UNG2, probably mediated through a direct binding between the NLS region of

XRCC1 and catalytic domain of UNG2. We have presented data indicating reduced repair of uracil in cell extracts from *Xrcc1*^{-/-} cells as compared to wild-type cells and cells reconstituted with *Xrcc1*-EYFP, suggesting a functional implication of the XRCC1-UNG2 interaction in vivo.

4. Discussion

XRCC1 interacts with a number DNA repair proteins [12] and thus may provide a platform for high-order BER complexes (Akbari et al. accompanying paper, 2009). In addition, both UNG2 and XRCC1 interact with PCNA in replication foci [8,17,47]. These observations prompted us to examine whether XRCC1 and UNG2 directly interact and may be part of a complex involved in repair of uracil. Here we show that XRCC1 and UNG2 co-localize at sites of DNA replication and likely physically interact with each other at these sites. We show a direct interaction between the catalytic domain of UNG2 and the NLS region of XRCC1. In agreement with this, the results of BER assay analysis showed that XRCC1-EYFP immunoprecipitates from S-phase cell extracts contained UNG2-specific repair activity. Furthermore, we show that *Xrcc1* deficient cells have reduced initial UNG2 specific uracil (U:A) repair.

To compare the composition of the XRCC1 and UNG2 associated complexes, we analysed complexes isolated from G1/S and mid S-phase cells. We designed our study in a way that allowed us to immunoprecipitate target proteins from different cell lines using the same tag-specific antibody, thus avoiding variations due to different degrees of antibody specificity. Moreover, this strategy permitted us to avoid the potential problem of competition between antibody and proteins for binding to the same site on the target protein. Stringent washing of immunoprecipitates can reduce the level of non-specific protein-antibody interactions, yet some weakly bound, but physiologically relevant, proteins may be disrupted. To circumvent this problem, we included in vivo formaldehyde crosslinking of proteins, enabling us to observe proteins not detectable in non-crosslinked samples under otherwise identical conditions. Moreover, formaldehyde crosslinking of proteins in intact cells stabilizes protein-protein interactions as they take place “in situ”. The results supported the presence of UNG2 in an S-phase XRCC1 complex. Furthermore, the data supported specific interaction of Lig III, POL β and PNK with XRCC1 and specific interaction of Lig I with UNG2, indicating the presence of separate UNG2- and XRCC1-associated complexes.

Two important functional differences were detected between the immunoprecipitated XRCC1 and UNG2 complexes: (1) XRCC1 complexes carried out fast and complete BER (including ligation) and (2) UNG2 complexes did not perform efficient ligation. This was unexpected because the immunoprecipitates contained FEN-1 and Lig I. However, this could be explained by differences in organisation of the complexes as discussed below and illustrated in Figure 6.

Because UNG2 and XRCC1 only co-localize in replication foci during S-phase, and not in other non- S-phase XRCC1 foci, it is tempting to speculate that the XRCC1-UNG2 complex have a specific repair function during replication. UNG2 is the only UDG known to be localised in replication foci [44]. We have previously suggested the presence of a pre-replicative XRCC1-SSBR complex [17], and based on the data presented in this study as well as identification of POL β and PNK in replication foci with XRCC1 (Akari et al., 2009, accompanying paper), we now extend this model to include pre-replicative BER. In this model, pre-replicative XRCC1-UNG2 complexes may facilitate efficient repair of mutagenic U:G mismatches ahead of replication. We also suggest that pre-replicative XRCC1-BER complexes may be important for the repair of other mutagenic base lesions such as AP sites and 3-methyladenine (3meA). 3meA is removed from DNA by the MPG DNA-glycosylase, which

interacts with XRCC1 [22] as well as PCNA [24]. Lack of XRCC1 may therefore at least in part explain the high sensitivity of XRCC1 deficient cells to MMS [48].

Previous data have shown that UNG2 rapidly removes mis-incorporated dUMP during replication [8]. During replication, DNA synthesis on the leading and lagging strands may involve different forms of protein interactions and complexes. Thus, whereas continuous DNA synthesis on the leading strand is rapid and processive, and does not normally involve FEN-I and Lig I, DNA replication on the lagging strand requires rapid engagement of proteins also involved in long patch BER, such as POL δ , FEN1 and Lig I. Since the majority of the UNG2 associated complexes isolated from S-phase were inefficient in the ligation step of BER, post-replicative repair of uracil [8] may be organized into different forms on the leading and lagging strands. Thus, in order to retain the continuity of DNA replication on the leading strand, removal of uracil from DNA by UNG2 [8] may not be coupled to the later repair steps, and the following AP site may be rapidly repaired by post-replicative XRCC1 complexes. However, on the lagging strand, UNG2 may be part of a replication / repair complex that carries out uracil removal followed by AP site incision and repair synthesis probably in the form of long patch BER. Our proposed model for replication associated BER is shown in Fig. 6.

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References

1. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 1993;362:709–715. [PubMed: 8469282]
2. Verri A, Mazzarello P, Biamonti G, Spadari S, Focher F. The specific binding of nuclear protein(s) to the cAMP responsive element (CRE) sequence (TGACGTCA) is reduced by the misincorporation of U and increased by the deamination of C. *Nucleic Acids Res* 1990;18:5775–5780. [PubMed: 1977136]
3. Viswanathan A, You HJ, Doetsch PW. Phenotypic change caused by transcriptional bypass of uracil in nondividing cells. *Science* 1999;284:159–162. [PubMed: 10102819]
4. Akbari M, Pena-Diaz J, Andersen S, Liabakk NB, Otterlei M, Krokan HE. Extracts of proliferating and non-proliferating human cells display different base excision pathways and repair fidelity. *DNA Repair (Amst.)* 2009;8:834–843. [PubMed: 19442590]
5. Sanderson RJ, Mosbaugh DW. Fidelity and mutational specificity of uracil-initiated base excision DNA repair synthesis in human glioblastoma cell extracts. *J. Biol. Chem* 1998;273:24822–24831. [PubMed: 9733786]
6. Krokan HE, Drablos F, Slupphaug G. Uracil in DNA--occurrence, consequences and repair. *Oncogene* 2002;21:8935–8948. [PubMed: 12483510]
7. Kavli B, Sundheim O, Akbari M, Otterlei M, Nilsen H, Skorpen F, Aas PA, Hagen L, Krokan HE, Slupphaug G. hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup. *J. Biol. Chem* 2002;277:39926–39936. [PubMed: 12161446]
8. Otterlei M, Warbrick E, Nagelhus TA, Haug T, Slupphaug G, Akbari M, Aas PA, Steinsbekk K, Bakke O, Krokan HE. Post-replicative base excision repair in replication foci. *EMBO J* 1999;18:3834–3844. [PubMed: 10393198]
9. Imai K, Slupphaug G, Lee WI, Revy P, Nonoyama S, Catalan N, Yel L, Forveille M, Kavli B, Krokan HE, Ochs HD, Fischer A, Durandy A. Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. *Nat. Immunol* 2003;4:1023–1028. [PubMed: 12958596]

10. Rada C, Williams GT, Nilsen H, Barnes DE, Lindahl T, Neuberger MS. Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. *Curr. Biol* 2002;12:1748–1755. [PubMed: 12401169]
11. An Q, Robins P, Lindahl T, Barnes DE. C → T mutagenesis and gamma-radiation sensitivity due to deficiency in the Smug1 and Ung DNA glycosylases. *EMBO J* 2005;24:2205–2213. [PubMed: 15902269]
12. Fan J, Wilson DM 3rd. Protein-protein interactions and posttranslational modifications in mammalian base excision repair. *Free Radic. Biol. Med* 2005;38:1121–1138. [PubMed: 15808410]
13. Thompson LH, Brookman KW, Jones NJ, Allen SA, Carrano AV. Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. *Mol. Cell Biol* 1990;10:6160–6171. [PubMed: 2247054]
14. Vidal AE, Boiteux S, Hickson ID, Radicella JP. XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions. *EMBO J* 2001;20:6530–6539. [PubMed: 11707423]
15. Caldecott KW, Aoufouchi S, Johnson P, Shall S. XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. *Nucleic Acids Res* 1996;24:4387–4394. [PubMed: 8948628]
16. Caldecott KW, McKeown CK, Tucker JD, Ljungquist S, Thompson LH. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. *Mol. Cell Biol* 1994;14:68–76. [PubMed: 8264637]
17. Fan J, Otterlei M, Wong HK, Tomkinson AE, Wilson DM 3rd. XRCC1 co-localizes and physically interacts with PCNA. *Nucleic Acids Res* 2004;32:2193–2201. [PubMed: 15107487]
18. Masson M, Niedergang C, Schreiber V, Muller S, Menissier-de Murcia J, de Murcia G. XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. *Mol. Cell Biol* 1998;18:3563–3571. [PubMed: 9584196]
19. Schreiber V, Ame JC, Dolle P, Schultz I, Rinaldi B, Fraulob V, Menissier-de Murcia J, de Murcia G. Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. *J. Biol. Chem* 2002;277:23028–23036. [PubMed: 11948190]
20. Whitehouse CJ, Taylor RM, Thistlethwaite A, Zhang H, Karimi-Busheri F, Lasko DD, Weinfeld M, Caldecott KW. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. *Cell* 2001;104:107–117. [PubMed: 11163244]
21. Marsin S, Vidal AE, Sossou M, Menissier-de Murcia J, Le Page F, Boiteux S, de Murcia G, Radicella JP. Role of XRCC1 in the coordination and stimulation of oxidative DNA damage repair initiated by the DNA glycosylase hOGG1. *J. Biol. Chem* 2003;278:44068–44074. [PubMed: 12933815]
22. Campalans A, Marsin S, Nakabeppu Y, O'Connor TR, Boiteux S, Radicella JP. XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair. *DNA Repair (Amst.)* 2005;4:826–835. [PubMed: 15927541]
23. Boldogh I, Milligan D, Lee MS, Bassett H, Lloyd RS, McCullough AK. hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs. *Nucleic Acids Res* 2001;29:2802–2809. [PubMed: 11433026]
24. Xia L, Zheng L, Lee HW, Bates SE, Federico L, Shen B, O'Connor TR. Human 3-methyladenine-DNA glycosylase: effect of sequence context on excision, association with PCNA, and stimulation by AP endonuclease. *J. Mol. Biol* 2005;346:1259–1274. [PubMed: 15713479]
25. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. *Cell* 2007;129:665–679. [PubMed: 17512402]
26. Parker A, Gu Y, Mahoney W, Lee SH, Singh KK, Lu AL. Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair. *J. Biol. Chem* 2001;276:5547–5555. [PubMed: 11092888]
27. Akbari M, Otterlei M, Pena-Diaz J, Aas PA, Kavli B, Liabakk NB, Hagen L, Imai K, Durandy A, Slupphaug G, Krokan HE. Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells. *Nucleic Acids Res* 2004;32:5486–5498. [PubMed: 15479784]

28. Slupphaug G, Eftedal I, Kavli B, Bharati S, Helle NM, Haug T, Levine DW, Krokan HE. Properties of a recombinant human uracil-DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil-DNA glycosylase. *Biochemistry* 1995;34:128–138. [PubMed: 7819187]
29. Aas PA, Otterlei M, Falnes PO, Vagbo CB, Skorpen F, Akbari M, Sundheim O, Bjoras M, Slupphaug G, Seeberg E, Krokan HE. Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. *Nature* 2003;421:859–863. [PubMed: 12594517]
30. Opreško PL, Otterlei M, Graakjaer J, Bruheim P, Dawut L, Kolvraa S, May A, Seidman MM, Bohr VA. The Werner Syndrome Helicase and Exonuclease Cooperate to Resolve Telomeric D Loops in a Manner Regulated by TRF1 and TRF2. *Mol Cell* 2004;14:763–774. [PubMed: 15200954]
31. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, Landegren U. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* 2006;3:995–1000. [PubMed: 17072308]
32. Richardson RT, Batova IN, Widgren EE, Zheng LX, Whitfield M, Marzluff WF, O'Rand MG. Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *J Biol Chem* 2000;275:30378–30386. [PubMed: 10893414]
33. Hagen L, Kavli B, Sousa MM, Torseth K, Liabakk NB, Sundheim O, Pena-Diaz J, Otterlei M, Horning O, Jensen ON, Krokan HE, Slupphaug G. Cell cycle-specific UNG2 phosphorylations regulate protein turnover, activity and association with RPA. *EMBO J* 2008;27:51–61. [PubMed: 18079698]
34. Richardson RT, Batova IN, Widgren EE, Zheng LX, Whitfield M, Marzluff WF, O'Rand MG. Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *J. Biol. Chem* 2000;275:30378–30386. [PubMed: 10893414]
35. Frosina G, Cappelli E, Fortini P, Dogliotti E. In vitro base excision repair assay using mammalian cell extracts. *Methods Mol. Biol* 1999;113:301–315. [PubMed: 10443429]
36. Krokan H, Wittwer CU. Uracil DNA-glycosylase from HeLa cells: general properties, substrate specificity and effect of uracil analogs. *Nucleic Acids Res* 1981;9:2599–2613. [PubMed: 7279657]
37. Frohn JT, Knapp HF, Stemmer A. True optical resolution beyond the Rayleigh limit achieved by standing wave illumination. *Proc Natl Acad Sci U S A* 2000;97:7232–7236. [PubMed: 10840057]
38. Haug T, Skorpen F, Aas PA, Malm V, Skjelbred C, Krokan HE. Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase. *Nucleic Acids Res* 1998;26:1449–1457. [PubMed: 9490791]
39. Olsen LC, Aasland R, Wittwer CU, Krokan HE, Helland DE. Molecular cloning of human uracil-DNA glycosylase, a highly conserved DNA repair enzyme. *EMBO J* 1989;8:3121–3125. [PubMed: 2555154]
40. Wang Z, Mosbaugh DW. Uracil-DNA glycosylase inhibitor gene of bacteriophage PBS2 encodes a binding protein specific for uracil-DNA glycosylase. *J. Biol. Chem* 1989;264:1163–1171. [PubMed: 2492016]
41. Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, Morrice NA, Swift S, Rothbauer U, Leonhardt H, Lamond A. Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J. Cell Biol* 2008;183:223–239. [PubMed: 18936248]
42. Akbari M, Visnes T, Krokan HE, Otterlei M. Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. *DNA Repair (Amst.)* 2008;7:605–616. [PubMed: 18295553]
43. Kavli B, Otterlei M, Slupphaug G, Krokan HE. Uracil in DNA--general mutagen, but normal intermediate in acquired immunity. *DNA Repair (Amst.)* 2007;6:505–516. [PubMed: 17116429]
44. Visnes T, Akbari M, Hagen L, Slupphaug G, Krokan HE. The rate of base excision repair of uracil is controlled by the initiating glycosylase. *DNA Repair (Amst.)* 2008;7:1869–1881. [PubMed: 18721906]
45. Tomkinson AE, Levin DS. Mammalian DNA ligases. *Bioessays* 1997;19:893–901. [PubMed: 9363683]
46. Parsons JL, Tait PS, Finch D, Dianova, Allinson SL, Dianov GL. CHIP-mediated degradation and DNA damage-dependent stabilization regulate base excision repair proteins. *Mol Cell* 2008;29:477–487. [PubMed: 18313385]

47. Zolghadr K, Mortusewicz O, Rothbauer U, Kleinhans R, Goehler H, Wanker EE, Cardoso MC, Leonhardt H. A fluorescent two-hybrid (F2H) assay for direct visualization of protein interactions in living cells. *Mol. Cell Proteomics*. 2008
48. Thompson LH, Brookman KW, Dillehay LE, Carrano AV, Mazrimas JA, Mooney CL, Minkler JL. A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. *Mutat. Res* 1982;95:427–440. [PubMed: 6889677]

Fig. 1. Intracellular localisation of XRCC1 and UNG2. (A) Confocal microscopy analysis of co-localisation of XRCC1-ECFP, UNG2-EYFP and Hc-Red-PCNA in S-phase (upper row) and non-S-phase cells (lower row). (B) Immunohistochemistry. Upper row: HeLa cells expressing 3 × FLAG-UNG2-EYFP were fixed and incubated with a monoclonal α-FLAG antibody. Mid row: CHO EM9 cells were transiently transfected with XRCC1-EYFP, fixed and stained with a polyclonal α-XRCC1 antibody. Lower row: HeLa cells expressing 3 × FLAG-UNG2-EYFP were fixed and incubated with a monoclonal α-FLAG antibody and a polyclonal α-XRCC1 antibody. Detection was performed using Alexa Fluor (532 and 647) labelled secondary antibodies. Arrows show non-transfected cells that did not stain with the antibodies. (C) In situ

Proximity Ligation Assay (PLA). Upper row: HeLa cells expressing $3 \times \text{FLAG-UNG2-EYFP}$ (UNG2-EYFP) were fixed and incubated with a monoclonal $\alpha\text{-FLAG}$ antibody and a polyclonal $\alpha\text{-XRCC1}$ antibody followed by detection of PLA-foci using oligonucleotide conjugated probe antibodies directed against the primary antibodies. Annealing of the probes occurs when the target proteins are in close proximity, which initiates the amplification of a reporter signal and appears as foci (PLA, $\alpha\text{-Flag}$ and $\alpha\text{-XRCC1}$). An overview of several cells after PLA (Overview, PLA)(far left, the nuclei are enclosed) and a merged image of PLA and UNG2-EYFP are shown (Merged)(far right). Lower row: same as upper row but the cells were not stained with the $\alpha\text{-Flag}$ antibody (PLA ctr). Bar, 5 μm .

Fig. 2. Pull-down and Far Western analysis of Xrcc1 and UNG2 interaction. The indicated constructs of *Cricetulus griseus* Xrcc1 fused with ECFP were transiently expressed in Xrcc1 deficient Chinese hamster ovary cell line EM9. Cells expressing only EYFP were used as control. The fusion proteins were immunoprecipitated with paramagnetic beads covalently coupled with α -GFP. (A) The immunoprecipitates were incubated with the catalytic domain of UNG2 (Δ 93UNG2). The beads were suspended in loading buffer and subjected to Western analysis. Lane 7, the beads were only incubated with recombinant UNG. The membrane was incubated with α -GFP antibody (upper panel) and α -UNG antibody (lower panel). (B) Far Western analysis. The immunoprecipitates were separated on gel and transferred to a PDF membrane

and refolded. The membrane was first incubated with Cy-3 labelled $\Delta 93$ UNG2 then stripped and probed with α -XRCC1. White arrows shows binding of $\Delta 93$ UNG2 to XRCC1-fusion construct, while red arrows indicate no binding. (C) Schematic overview of the XRCC1 deletion constructs applied and their ability to bind to recombinant $\Delta 93$ UNG2.

Fig. 3. BER analysis of XRCC1-EYFP immunoprecipitates from whole cell extract. Left panel: schematic illustration of U:G and U:A DNA substrate for BER analysis. AP site DNA was prepared by incubation of uracil-containing DNA with recombinant UNG. Right panel: lanes 1 to 6, uracil-BER analysis of XRCC1-EYFP complexes in the absence or presence of UNG2 neutralising antibodies (α -UNG2) or Ugi. Lanes 7 to 9, analysis of AP site BER. Lane 10 shows BER analysis of the control immunoprecipitates from EYFP-expressing cells. The repair reaction was carried out at 32 °C for 15 min. The repaired DNA was digested with XbaI / HindIII to get information about the ligated and unligated repair products as shown.

Fig. 4. Comparative Western blot analysis of XRCC1-EYFP and UNG2-EYFP immunoprecipitates from extracts of mid S- and G1/S-phase enriched cells. (A) BER activity of immunoprecipitated XRCC1-EYFP from crosslinked and non-crosslinked cell extracts. BER was carried out at 32 °C for 15 min. The repaired DNA was digested with XbaI and HindIII to get information about the ligated and unligated repair products. (B) Western analysis of immunoprecipitates from formaldehyde crosslinked and non-crosslinked XRCC1-EYFP and UNG2-EYFP expressing HeLa cell extracts. Cells were treated with formaldehyde (0, 0.125 or 0.25%) prior to whole cell extract preparation. Western blot analysis of the immunoprecipitates before (65 °C) and after (95 °C) reversal of the crosslinks is shown. (C) Western analysis of immunoprecipitates

from insoluble chromatin-bound fraction of freely cycling HeLa cells using anti-XRCC1 (lane 1) or anti-UNG (lane 2) antibodies. One representative gel from three experiments is shown.

Fig. 5. BER analysis of UNG2-EYFP and XRCC1-EYFP immunoprecipitates from mid S-phase enriched cells and BER analysis of cell extracts in the absence or presence of Xrcc1. (A) BER activity of the immunoprecipitated UNG2-EYFP and XRCC1-EYFP complexes was analyzed using U:A, U:G and AP:G DNA substrates with or without additional recombinant APE1 and T4 DNA ligase at 32 °C for 15 min. Lanes 7 and 11 show repair activity of the immunoprecipitates from EYFP-expressing cell extracts as control. (B) Time-course BER analysis of the immunoprecipitated UNG2-EYFP and XRCC1-EYFP complexes using U:G substrate. (C) End-trimming and DNA ligase activity of UNG2-EYFP immunoprecipitates. After 1 h incubation, half of the repaired DNA was further incubated with T4 DNA ligase (lane

2). (D) Time-course BER analysis of extracts from AA8 (Xrcc1^{+/+}), EM9 (Xrcc1^{-/-}) and EM9 cells expressing Xrcc1-EYFP (EM9-Xrcc1-EYFP) on a U:A and AP:A substrate. All DNA samples were digested with XbaI and HindIII to get information about DNA ligase and end-trimming activity of the immunoprecipitates.

Fig. 6.

A model for repair of uracil in DNA by XRCC1 and UNG2 complexes. UNG2 is linked to replication through its interaction with XRCC1 and PCNA, both of which interact with a number of proteins involved in DNA replication and repair. We suggest a model for how these interactions organize efficient pre-replicative repair of U:G mismatch and post-replicative repair of mis-incorporated uracil (U:A) in DNA, during replication.