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Cockayne Syndrome group B protein stimulates NEIL2 DNA glycosylase activity

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Abstract

Cockayne Syndrome is a segmental premature aging syndrome, which can be caused by loss of function of the CSB protein. CSB is essential for genome maintenance and has numerous interaction partners with established roles in different DNA repair pathways including transcription coupled nucleotide excision repair and base excision repair. Here, we describe a new interaction partner for CSB, the DNA glycosylase NEIL2. Using both cell extracts and recombinant proteins, CSB and NEIL2 was found to physically interact independently of DNA. We further found that CSB is able to stimulate NEIL2 glycosylase activity on a 5-hydroxyl uracil lesion in a DNA bubble structure substrate *in vitro*. A novel 4,6-diamino-5-formamidopyrimidine (FapyA) specific incision activity of NEIL2 was also stimulated by CSB. To further elucidate the biological role of the interaction, immunofluorescence studies were performed, showing an increase in cytoplasmic CSB and NEIL2 co-localization after oxidative stress. Additionally, stalling of the progression of the transcription bubble with α -amanitin resulted in increased co-localization of CSB and NEIL2. Finally, CSB knockdown resulted in reduced incision of 8-hydroxyguanine in a DNA bubble structure using whole cell extracts. Taken together, our data supports a biological role for CSB and NEIL2 in transcription associated base excision repair.

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Keywords

Cockayne Syndrome; CSB; NEIL2; base excision repair; oxidative damage

1 Introduction

Cockayne Syndrome (CS)¹ is a severe autosomal recessive disorder associated with a segmental premature aging phenotype. Patients exhibit variable characteristics. Poor growth and neurological abnormalities are required for diagnosis, while photosensitivity, retinal degeneration, optic atrophy, hearing loss, dental caries and gait disorder are often observed (Nance and Berry, 1992). The genetic cause of CS is a mutation in either of the genes *CS-B* (*ERCC6*) (in 80% of the cases) or *CS-A* (*ERCC8*) (in 20% of the cases) (Lehmann, 1982). *CS-B* encodes the CSB protein, which shows sequence homology to the SNF2-like helicase superfamily. To date no helicase activity has been demonstrated for CSB, yet the protein exhibits a double stranded DNA dependent ATPase activity, which is more pronounced with DNA bubble structures than canonical duplex DNA structures as cofactor (Berquist and Wilson, 2009; Christiansen et al., 2003; Troelstra et al., 1992).

Cell lines from CS patients are characterized by hypersensitivity towards UV irradiation due to a deficiency in transcription coupled nucleotide excision repair (TC-NER) (Venema et al., 1990) and an inability to restore RNA synthesis after UV irradiation (Mayne and Lehmann, 1982). Multiple studies also implicate CSB in transcription (Balajee et al., 1997; Kristensen et al., 2013; Kyng et al., 2003; Newman et al., 2006; Proietti-De-Santis et al., 2006; Selby and Sancar, 1997). In addition to hypersensitivity to lesions repaired by TC-NER, CSB deficient human cells also show hypersensitivity towards oxidative agents including KBrO₃, H₂O₂ and γ -irradiation, which causes damages primarily repaired by the base excision repair (BER) pathway (Nardo et al., 2009; Ropolo et al., 2007; Spivak and Hanawalt, 2006; Tuo et al., 2003; Tuo et al., 2001). In line with this, CSB deficient mouse cells are hypersensitive to γ -irradiation, paraquat and menadione, which all produce reactive oxygen species (ROS) (de Waard et al., 2004; de Waard et al., 2003; Osenbroch et al., 2009). ROS can also be formed endogenously as by-products of the normal cellular metabolism, especially as part of the mitochondrial respiration process. ROS can react with a variety of macromolecules including proteins, lipids, RNA and DNA. Attacks on DNA by ROS can result in DNA strand breaks, minor base lesions and abasic (AP) sites. Minor base lesions and AP sites are generally repaired by BER. This pathway is initiated by removal of the damaged base by a DNA glycosylase followed by cleavage of the DNA backbone. End-trimming, nucleotide incorporation and ligation complete the pathway (reviewed in (Krokan and Bjoras, 2013). Some of the most frequent oxidative lesions include 8-oxo-7,8-dihydro-2'-deoxyguanine (8oxoG), 5-hydroxyuracil (5OHU), 4,6-diamino-5-formamidopyrimidine (FapyA) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) (reviewed in (Wilson and Bohr, 2007)). A battery of specialized enzymes repairs the different oxidative lesions. Endonuclease III homolog 1 (Nth1) and oxoguanine DNA glycosylase 1 (OGG1) are primarily involved in incision of oxidized pyrimidines and purines, respectively, but several other DNA glycosylases with overlapping substrate specificity also exist (Hu et al., 2005). Three different orthologs of the *E. coli* Nei glycosylase, the Endonuclease VIII-like (NEIL) DNA glycosylases, have been identified in mammalian cells: NEIL1, NEIL2 and NEIL3.

¹Abbreviations: (5OHU) 5-hydroxyuracil; (8oxoG) 8-oxo-7,8-dihydro-2'-deoxyguanine; (AP) abasic; (APE1) AP-endonuclease-1; (B11) 11bp bubble structure; (BER) base excision repair; (co-IP) Co-immunoprecipitation; (control B11) cytosine in an 11 nucleotide bubble; (CS) Cockayne Syndrome; (ds) double stranded; (FapyA) 4,6-diamino-5-formamidopyrimidine; (FapyG) 2,6-diamino-4-hydroxy-5-formamidopyrimidine; (KD) knock down; (NEIL) endonuclease VIII-like; (NTH1) Endonuclease III homolog 1; (OGG1) oxoguanine-DNA glycosylase 1; (PLA) proximity ligation assay; (RNAPII) RNA polymerase II; (ROS) reactive oxygen species; (TC-NER) nucleotide excision repair; (WCE) whole cell extract

NEIL1 and NEIL2 carry out base incision by $\beta\delta$ -elimination, while NEIL3 mainly incises damages by β -elimination. NEIL1 deals with both oxidized purines and pyrimidines, including FapyA, FapyG, 5OHU and the hydantoin lesions spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) (Krishnamurthy et al., 2008; Zhao et al., 2010), while the preferred substrates for NEIL3 are the Sp and Gh lesions (Krokeide et al., 2013). NEIL2 activity has primarily been described for oxidation products of cytosine, including 5OHU (Dou et al., 2003). In contrast to OGG1 and NTH1, the NEIL orthologs are able to excise base lesions from single stranded and bubble substrates, which may be relevant during transcription and replication. NEIL1 is expressed in a cell cycle dependent manner and is therefore a likely candidate for elimination of potentially mutagenic lesions during replication (Dou et al., 2003; Hazra et al., 2002a; Hazra et al., 2002b; Hegde et al., 2013; Morland et al., 2002; Neurauter et al., 2012). This is further supported by specific functional interactions identified between NEIL1 and proteins involved in nuclear DNA replication including PCNA and 9-1-1 (Dou et al., 2008; Guan et al., 2007; Hegde et al., 2013). Meanwhile, NEIL2 may potentially eliminate oxidative lesions during transcription associated repair, due to its preference for single stranded/bubble structure substrates and identified protein interaction partners in transcription, including RNA polymerase II and the transcriptional regulator heterogenous nuclear ribonucleoprotein-U (hnRNP-U) (Banerjee et al., 2011; Hazra et al., 2002a; Hazra et al., 2002b).

Polymorphisms in the NEIL2 gene have been suggested to be markers for increased risk and progression of squamous cell carcinoma of the oral cavity and oropharynx (Zhai et al., 2008) as well as for cognitive performance in healthy elderly individuals (Lillenes et al., 2011). Recently, NEIL2 was found to be present in mitochondria isolated from human cells in addition to the nucleus and it may therefore also play an important role in maintaining mitochondrial genome integrity (Mandal et al., 2012).

Several results support the involvement of CSB in repair of oxidative lesions (reviewed in (Stevnsner et al., 2008)). Notably, the frequencies of FapyG, FapyA and 8oxoG lesions are increased in the genomic DNA of kidney and brain tissue from CSB deficient mice and FapyA is increased in the mitochondrial DNA in the liver of these mice (Muftuoglu et al., 2009). In addition, CSB deficient cells showed decreased repair of 8oxoG in both the nucleus and mitochondria as well as decreased nuclear repair of 7,8-dihydro-8-oxoadenine (Dianov et al., 1999; Stevnsner et al., 2002; Tuo et al., 2002a; Tuo et al., 2003; Tuo et al., 2002b; Tuo et al., 2001). The CSB protein has been found to interact with several classical genome maintenance protein such as AP-endonuclease-1 (APE1) (Wong et al., 2007). In addition, we have shown that CSB stimulates NEIL1 incision of FapyG, FapyA and 5OHU in duplex DNA structures whereas the incision of 8oxoG is not affected. However, CSB does not stimulate the NEIL1 mediated incision of 5OHU in a bubble structure, suggesting that the stimulation is substrate and structure specific (Muftuoglu et al., 2009).

Based on the fact that CSB plays a role in BER and that NEIL2 may be involved in transcription associated BER processes, we speculated whether they might act in concert. Therefore, a potential interaction between the two proteins was investigated. In this study we demonstrate that CSB stimulates NEIL2 initiated base excision activity on specific DNA substrates, and identify FapyA lesions as substrates for NEIL2. Based on our results, using various DNA substrates and the increased interaction between NEIL2 and CSB after stalling of the transcription bubble, we suggest that the context in which NEIL2 is stimulated by CSB may be associated with transcription.

2 Experimental Procedures

2.1 Recombinant proteins

Recombinant N-terminal hemagglutinin antigen and C-terminal His₆-double-tagged human CSB protein was purified from HiFive insect cells as described previously (Christiansen et al., 2003). Human NEIL2 was cloned into the pET-22b vector resulting in a recombinant C-terminal His₆-tagged human NEIL2 expression plasmid. *E. coli* transfected with the plasmid was grown at 37°C in lysogeny broth media supplemented with 500mM D-sorbitol, 1mM betaine and 100µg/mL ampicillin. Expression of the fusion protein was induced in log phase cells at OD=0.8, by addition of 0.1mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested 4h after induction by centrifugation at 6500×g and resuspended in sonication buffer (50mM Na₂HPO₄/NaH₂PO₄, pH 8.0, 300mM NaCl, 10mM β-mercaptoethanol and 10mM imidazole). The cells were then lysed by sonication and insoluble particles removed by centrifugation at 20,000×g for 20min. The lysate was applied to a Ni-column pre-equilibrated with sonication buffer. The column was washed in three volumes sonication buffer followed by two volumes of sonication buffer containing 50mM imidazole. Protein was eluted in three column volumes of sonication buffer with 300mM imidazole and collected in fractions. The fractions were analyzed on a protein gel and dialyzed against dialysis buffer (10mM Tris-HCl pH 7.0, 50mM NaCl and 10mM β-mercaptoethanol). The fractions containing the highest concentrations of NEIL2 were applied to a HiTrapSP XL 1mL column (Amersham) and eluted with a 50mM to 2M gradient of NaCl in 10mM Tris-HCl (pH 7.0) and 10mM β-mercaptoethanol. The fractions with the purest NEIL2 protein were pooled. NEIL2 protein and CSB protein concentrations were determined by SDS-PAGE using BSA as standard, followed by total protein staining with Imperial Protein Stain (Thermo Scientific). Protein size marker (Spectra multicolour, Fermentas) was run on the same gel (Supplemental Fig. 1).

2.2 Cell lines, CSB knock down and oxidative stress treatment

NEIL1 knock down (KD) and control KD HeLa cells (Muftuoglu et al., 2009) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (41966, GIBCO) with 10% Fetal Bovine Serum (FBS) (10270, GIBCO), 1% penicillin/streptomycin (15140 GIBCO) and 2µg/mL puromycin (Invitrogen). CSB siRNA KD was optimized with regard to incubation time after transfection, siRNA amount and transfection conditions. In brief, 1.1×10^6 cells grown for 24h were transfected with 900µL siRNA complexes of 110pmol either negative control siRNA (UAGCGACUAAACACAUCAAUU, UUGAUGUGUUUAGUCGCUAAUU, RiboTask) or CSB siRNA targeting exon 18 in CSB mRNA (sense GCAGUAAACUUCUAAUCGAAUU, antisense UUCGAUUAGAAGUUACUGCUU, Dharmacon) using the calcium phosphate transfection method. Forty-eight hours after transfection, cells were either treated with the indicated stressor or mock treated for 1h. For stress treatment, medium without FBS containing menadione (Sigma) or H₂O₂ at either 50µM or 200µM concentration was added to the cells. After treatment, cells were washed twice in PBS and harvested by centrifugation in cold PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 1mM dithiothreitol (DTT). The cell pellet was stored at -80°C until use.

2.3 Whole Cell Extract (WCE) Preparation

Cells were thawed on ice and resuspended in buffer 1 (10mM Tris-HCl (pH 7.8) and 200mM KCl) and sonicated in order to lyse cells and fragmentize DNA. One volume of buffer 2 (10mM Tris-HCl (pH 7.8), 200mM KCl, 2mM ethylenediaminetetraacetic acid (EDTA), 40% glycerol, 0.2% Igepal CA-630 (NP-40), 4µM DTT, 1mM PMSF, 20µM leupeptin, 3µM pepstatin) was added followed by incubation for 2h. The cell lysate was centrifuged at 16,000×g for 15min and the supernatant dialyzed overnight in dialysis buffer

(10% glycerol, 50mM KCl, 25mM Hepes-KOH (pH 7.0), 2mM EDTA, 2mM DTT). Aliquots of WCE were stored at -80°C .

2.4 Co-immunoprecipitation (co-IP)

HeLa cells were grown in 15cm^2 tissue culture plates, washed in PBS and either mock or menadione treated in PBS with or without $200\mu\text{M}$ menadione for 1h. Nuclear extract was prepared by employing nuclear extraction kit (Pierce) according to the manufacture's protocol. Co-IP was performed as described in (Muftuoglu et al., 2009) with minor modifications. Four hundred μL nuclear extract was pre-cleared using $2.5\mu\text{g}$ rabbit IgG and $30\mu\text{L}$ packed rProtein G agarose beads (Invitrogen) for 1h. The pre-cleared lysate was incubated with either $5\mu\text{g}$ rabbit IgG, rabbit anti-CSB sc-25370 (Santa Cruz), goat IgG, or goat anti-NEIL2 sc-47614 (Santa Cruz) overnight with rotation. Thirty μL packed and equilibrated Protein G agarose beads (Invitrogen) was subsequently added to the lysate with appropriate antibody and incubated for 1–2h. Beads were washed six times in ice cold lysis buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM Na_3VO_4 0.5% NP-40, 0.5% Triton X-100, 0.5mM PMSF, 1mMDTT, DNase I (10 U/mL) or EtBr (10–50 $\mu\text{g}/\text{ml}$) as indicated, 5% glycerol and 1 complete protease inhibitor tablet (Roche) per 50mL buffer. Proteins were eluted in $30\mu\text{L}$ Laemmli sample buffer (Bio-Rad). The supernatant was analyzed by western blotting. Unless otherwise stated, all steps were carried out at 4°C .

2.5 Western Blot

Proteins were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in TBS-T (20mM Tris (pH 8.0), 137mM NaCl, 0.05% Tween) with 5% dried non-fat milk followed by exposure to primary antibody. After incubating with secondary antibody, the membrane was developed with ECL+ (Amersham Bioscience). Primary antibodies employed: rabbit anti CSB (H300 sc-25370, Santa Cruz) 1:1000; goat anti Lamin B (Santa Cruz) 1:500; goat anti NEIL2 (P-19 sc-47614, Santa Cruz); mouse anti NEIL2 (2626C2a, sc-81566 Santa Cruz); mouse NEIL2 (1B7, Sigma); TFIIH p89 (S-19, sc-293, Santa Cruz).

Secondary antibodies employed: anti-rabbit whole IgG peroxidase linked (#A0545 Sigma) 1:40,000, anti-goat (Amersham) 1:5000, anti-goat peroxidase linked (Santa Cruz), anti-rabbit and anti-mouse provided in chemiluminescence kit (Pierce).

2.6 Far western

NEIL2 protein and control protein (BSA) (800ng each) were separated on a 10% SDS-PAGE and transferred to a PVDF membrane for far western blotting. The membrane was stained with $1\times$ Amido Black (Sigma-Aldrich) and a picture was taken. The PVDF membrane was then blocked in PBS with 5% non-fat dried milk and 0.1% Tween20. Recombinant CSB protein or control protein BSA ($4\mu\text{g}$) was dissolved in PBS with 0.25% non-fat dried milk, 0.1% Tween20, 1mM DTT and 1mM PMSF and incubated for 1h at room temperature. The membrane was washed in PBS with 0.25% milk and 0.1% Tween20 followed by blocking in PBS with 5% non-fat dried milk and 0.1% Tween20. Primary antibody goat anti-CSB (sc-10459, Santa Cruz) was added in 1:1000 dilutions and incubated overnight at 4°C . The membrane was washed, incubated with secondary rabbit anti-goat IgG peroxidase conjugate 1:5000 (A-5420, Sigma), and washed in $1\times$ PBS, 0.25% milk. The membrane was exposed to chemiluminescent substrate (West Femto, Pierce), according to the manufacture's protocol.

2.7 Dot blot

CSB (400ng), BSA (100ng) and NEIL2 (10–20ng) were blotted on PVDF (Invitrogen) membranes. The membrane was blocked with TBS-T with 5% non-fat dried milk for 1h at 4°C. Purified NEIL2 (1µg) was added in PBS and incubated overnight at 4°C. The membrane was washed in TBS-T. Primary antibody, mouse anti-NEIL2 (sc-81566), was added in TBS-T with 5% non-fat dried milk and incubated for 1h at room temperature. The membrane was washed in TBS-T followed by incubation with secondary antibody, anti-mouse IgG peroxidase conjugate (A9044, Sigma), in TBS-T with 5% non-fat dried milk. The membrane was then washed and developed with ECL kit (GE health Sciences) according to the manufacture's protocol.

2.8 Oligodeoxynucleotides

Oligodeoxynucleotides employed in this study (for sequences see Table 1) were purchased from DNA Technology, Denmark except for the FapyA containing oligodeoxynucleotide, which was a kind gift from Marc Greenberg (Jiang et al., 2005). The oligodeoxynucleotides were 5'-end labeled using T4 oligonucleotide kinase (Fermentas) and ^{32}P - γ -ATP (Perkin Elmer) as described before (Souza-Pinto et al., 1999). The labeled oligodeoxynucleotides were annealed to a completely complementary strand resulting in a double stranded structure or to a partially complementary oligodeoxynucleotide resulting in an 11nt bubble structure (B11), with the lesion positioned in center of the bubble. Annealing of the two complementary DNA strands was performed in 100mM KCl by heating for 5min at 90°C and slowly cooling to room temperature. AP site containing oligodeoxynucleotides were generated by UNG processing of uracil for 10 min.

2.9 Mung Bean nuclease digestion

To investigate the structure of hybridized DNA substrates 20nM 5OHU B11 substrate, 20nM fully hybridized 5OHU substrate, 5'-end labeled in either the damaged or undamaged strand, respectively, and 20 nM 5OHU 5'-end labeled single stranded oligodeoxynucleotides were incubated with 1U Mung Bean Nuclease (New England Biolabs) for 10min at 37°C. Mung Bean nuclease digested and undigested substrates were subsequently separated by denaturing PAGE and visualized by phosphorimaging by Personal Molecular Imaging (Bio Rad)

2.9 DNA glycosylase assays with recombinant proteins

Incision activity assays were performed as described in (Muftuoglu et al., 2009) with minor modifications. Incision of 5OHU and cytosine (lesion free control) in an 11nt bubble or in a was performed by incubating the indicated amount of recombinant human NEIL2 protein with the relevant oligodeoxynucleotide in a 10µL reaction volume with 1× reaction buffer (40mM Hepes pH 7.4, 50mM KCl, 100µg/mL BSA, 5% glycerol, 1mM EDTA and 2mM DTT) at 37°C for 15min or 30min for 5OHU B11 or fully annealed 5OHU, respectively. For kinetic studies, 100nM 5OHU B11 and 10nM NEIL2 was incubated with or without 10nM CSB in 1× reaction buffer for 0, 1, 2, 5 or 10min. Initial reaction velocity (rate of catalysis) was determined as the slope of best linear trend line for percent incision as a function of time in minutes for the time points 0, 1 and 2min. For incision assay of 8oxoG in an 11nt bubble 1nM substrate was used and reactions incubated for 2h at 37°C. For incision of FapyA, 1nM substrate and 100ng tRNA was included and the incubation time was 20min at 37°C. When investigating stimulation of NEIL2 by CSB protein increasing amounts (as indicated) of CSB protein were included in the reaction. Reactions were stopped by adding 10µl 2× formamide loading dye (70 % formamide with 100mM NaOH) to cleave any residual AP sites. Substrates and products were separated on a 20% denaturing

polyacrylamide gel, visualized and quantified using Personal Molecular Imager (Bio-Rad) and Image lab software or QuantityOne (Bio-Rad), respectively.

2.10 DNA trapping assay

DNA trapping assays were performed as described for the glycosylase assay, with indicated enzymes, and with addition of 50mM freshly made NaCNBH_3 or NaBH_4 , as indicated, from the start of the reactions. The reactions were stopped and analyzed as described in (Jensen et al., 2003).

2.11 Incision assays with whole cell extract

WCE from mock, menadione and H_2O_2 treated cells were analyzed with regard to 5OHU B11 and 8oxoG B11 incision capacity. In order to compare the effect of CSB on the incision capacity, WCE from NEIL1/CSB siRNA KD and NEIL1/control siRNA KD cells treated the same way (either mock, menadione or H_2O_2) were compared.

For optimization increasing amounts of WCE were incubated with 0.5nM oligonucleotide containing 5OHU, 8oxoG or cytosine in an 11nt bubble in 20 μL reaction volume of 1 $\times$ reaction buffer (112.5mM KCl, 19mM Hepes KOH pH 7.0, 7.5% glycerol, 1.5mM EDTA, 1.5mM DTT and 2.5mM MgCl_2) and incubated at 37°C. The reactions were stopped by addition of 1 $\times$ volume of 70% formamide with 130mM NaOH and incubated for 15min at 37°C to cleave AP-sites and thereby avoid differences due to difference in APE1 activity.

For concentration dependence studies of 5OHU B11 incision, 0.1 – 1 μg WCE were incubated with 0.5nM oligodeoxynucleotide in 20 μL reaction buffer for 1h at 37°C. For time dependence studies of incision capacity, 1 μg WCE were incubated with 0.5nM oligodeoxynucleotide in 20 μL reaction buffer at 37°C for 0–1h as indicated. Reactions were stopped and analyzed as for incision assays with recombinant proteins.

For concentration dependence studies of 8oxoG B11 incision, 0.2 – 2 μg WCE were incubated with 0.5nM oligodeoxynucleotide in 20 μL reaction buffer for 3h at 37°C. For time dependence studies of incision capacity, 0.75 μg WCE were incubated with 0.5nM oligodeoxynucleotide in 20 μL reaction buffer at 37°C for 0–3h as indicated in figures. Reactions were stopped and analyzed as for incision assays with recombinant proteins.

2.12 Immunofluorescence

Cells were grown, mock or menadione treated, fixed, permeabilized and stained as described in (Aamann et al., 2010) with minor modifications. Cells were fixed for 10min at room temperature in 4% formaldehyde in PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5min at room temperature and blocked for unspecific protein binding using 5% FBS in PBS for 1h at room temperature. Primary antibodies for NEIL2 (1B7 Mouse monoclonal, Sigma, 1:75) and CSB (H-300, rabbit polyclonal, Santa Cruz, 1:300) or COX IV (Goat Polyclonal, Santa Cruz) and NEIL2 (1B7 Mouse monoclonal, Sigma, 1:75) were added and incubated overnight at 4 °C. The slides were washed three times in PBS and stained with fluorescent-labelled secondary antibodies for 1h at room temperature (Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 647 donkey anti-rabbit and Alexa Fluor 647 donkey anti-goat, all 1:1000) followed by four times washes in PBS (Muftuoglu et al., 2009). Cells were mounted with hard set mounting media with DAPI (Vector-Shield) and the antibodies were checked for cross-reaction background and bleed-through between the two channels as described in (Muftuoglu et al., 2009; Aamann et al., 2010). Pictures were acquired on a Nikon Eclipse TE-2000e confocal microscope controlled by Volocity software 5.0 (Improvision/PerkinElmer, Coventry, UK) using the 40 $\times$ objective lens, 0.2- μm z-stacks throughout the cells. Co-localization was measured as overlap between the selected NEIL2

and CSB signals that was bigger than 1.5 voxels. An average of three experiments (total of 80–100 cells counted) is presented with the respective representative pictures.

2.13 In situ proximity ligation assay

Interaction of CSB and NEIL2 was visualized through the Duolink in situ proximity ligation assay (PLA) (Duolink II, Olink Bioscience). HeLa cells grown over night were either mock or α -amanitin treated. They were fixed in 4% formaldehyde and permeabilized in 0.25% Triton-X followed by blocking and incubation with primary antibodies CSB (rabbit) (H-300, Santa Cruz) and NEIL2 (mouse) ((2626C2a, Santa Cruz) in dilutions 1:500, as indicated. Proximity of the bound antibodies was detected by the PLA kit, according to the manufacturers manual. Nuclei were counterstained with DAPI. The PLA signal, indicating CSB-NEIL2 interaction was visualized using a Zeiss axiovert 200m microscope with a plan apochromatic 63 $\times$ 1.4 NA objective, and a CoolSNAP-HQ camera (Roper Scientific), operated by the MetaMorph[®] software.

2.14 Statistics

All statistical comparisons were performed using Student's *t* test. The minimum level of statistical significance was $P = 0.05$.

3 Results

3.1 Complex formation between CSB and NEIL2

CSB has numerous interaction partners with functions throughout the genomic maintenance machinery, including partners involved in BER (Aamann et al., 2013). As such, multiple studies have demonstrated that CSB is important for efficacy and efficiency of the BER process (reviewed in (Stevnsner et al., 2008)). Recently, the CSB protein was found to functionally interact with the DNA glycosylase NEIL1 (Muftuoglu et al., 2009). This prompted us to explore if an interaction existed between CSB and the NEIL1 orthologue NEIL2. Co-immunoprecipitation (co-IP) experiments were performed on nuclear extracts from HeLa cells, which were either mock treated or treated with the oxidizing agent menadione (Fig. 1A). The results showed that a NEIL2 specific antibody was able to pull down CSB protein specifically, indicating that CSB is present in a NEIL2 containing complex in vivo (Fig. 1A, top panel). This was supported by reciprocal co-IP using CSB antibody and subsequent detection of NEIL2 protein (Fig. 1A, bottom panel). Importantly, we observed an increased amount of co-immunoprecipitated NEIL2 and CSB, respectively, after menadione treatment (Fig. 1A) indicating that the two proteins increase their complex formation after oxidative stress.

In order to analyze the NEIL2 and CSB interaction *in vitro*, recombinant CSB and NEIL2 proteins were expressed and purified (Supplemental Fig. 1A). Dot blot analysis was positive for a NEIL2-CSB interaction, and no signal was observed when BSA was used as a control (Fig. 1B). Thus, *in vitro*, the two proteins interact directly, independent of DNA. The interaction was further verified by far western blotting (Fig. 1C). Collectively, these data indicate that CSB and Neil2 interact directly both *in vitro* and *in vivo*.

Deficiency in functional Xeroderma Pigmentosum group B protein (XPB alias p89), renders cells more sensitive to the genotoxic effects of oxidative stress (Soerensen et al., 2010). CSB has previously been demonstrated to be in complex with p89 (Bradsher et al., 2002). Notably, p89 was observed in the complexes pulled down with NEIL2 and CSB, respectively. (Fig. 1A).

3.2 Co-localization of NEIL2 and CSB

We have previously seen that CSB re-localizes to the mitochondria after menadione treatment (Aamann et al., 2010). The NEIL2 protein has been shown to be present in nuclei, in the cytoplasm (Hazra et al., 2002b) and in mitochondria (Mandal et al., 2012). In order to verify the presence of *in vivo* complexes of CSB and NEIL2, we analysed the cellular localization of the two proteins, by immunofluorescence microscopy. Localization of NEIL2 was found to be both nuclear and cytoplasmic with some co-localization with the mitochondrial marker, Cox IV (Fig. 2A). The specificity of the monoclonal NEIL2 antibody used for immunofluorescence was tested by IP with NEIL2 goat antibody and western blotting using the monoclonal NEIL2 antibody (Supplemental Fig. 2). To investigate if CSB and NEIL2 co-localize in cells, and if a possible co-localization is affected by oxidative stress, we co-stained mock and menadione treated cells for NEIL2 and CSB. The results demonstrated that CSB and NEIL2 partly co-localized in the cytoplasm after menadione treatment (Fig. 2B), with a Pearson's correlation coefficient of 0.6–0.7, indicating that it is significant.

3.3 NEIL2 incises FapyA and is stimulated by CSB

FapyA lesions are very stable and occur frequently in the mammalian genome where they can give rise to AT to TA transitions. We recently found elevated levels of oxidative FapyA lesions in DNA from CSB knock out mouse brain, liver and kidney tissue compared to wild type. Moreover, CSB was found to stimulate NEIL1 dependent removal of these lesions (Muftuoglu et al., 2009). Given the direct protein-protein interaction and co-localization of NEIL2 and CSB and the known substrate redundancy of DNA glycosylases we speculated that CSB and NEIL2 might cooperate in the repair of these highly mutagenic lesions. We therefore examined NEIL2 activity on FapyA in a duplex structure in a DNA glycosylase assay as described in Experimental procedures (Table 1 and Fig. 3A). The results identify FapyA as a substrate for NEIL2, as increasing amounts of recombinant NEIL2 protein causes an increase of incised product (Fig. 3B). No incision activity was observed on a control oligodeoxynucleotide with identical sequence without base damage (data not shown).

We next examined whether NEIL2 and CSB functionally interact in the repair of FapyA lesions in duplex structures, by assaying the incision capacity of NEIL2 in the presence of increasing amounts of CSB. We observed up to 4-fold increase in product formation in the presence of a 1:5 molar ratio of NEIL2 to CSB (Fig. 3C lanes 4–7 and 3D). The presence of excess BSA in the reaction excluded that the stimulation was a consequence of increased protein concentration. Furthermore, CSB alone (Fig. 3C, lane 2) did not result in product formation indicating that the increase in glycosylase activity was due to stimulation of NEIL2 activity by the CSB protein. Thus our data indicate that FapyA lesions are substrates for the NEIL2 glycosylase and that this activity is increased in the presence of recombinant CSB, suggesting that the two proteins can cooperate in the repair of oxidative lesions.

3.4 CSB stimulates NEIL2 incision of 5OHU in a bubble substrate

We next explored if the stimulatory effect of CSB on NEIL2 was also present on classical NEIL2 substrates. NEIL2 has previously been reported to have a high affinity for 5OHU in DNA bubble structures (Dou et al., 2003). The cytosine derivative has a high mutagenic potential resulting in GC to AT transitions if left unrepaired (Tremblay and Wagner, 2008). Initially, we confirmed that 5OHU in an 11nt bubble structure (5OHU B11) is a substrate for NEIL2 (Fig. 4A and B). The position and size of the bubble in the substrate was verified by digestion with the single strand specific Mung Bean nuclease (Supplemental Fig. 3). We next examined whether CSB was able to stimulate NEIL2. Recombinant NEIL2 was incubated with 5OHU B11 substrate and increasing amounts of CSB. We found that CSB

was able to stimulate the activity of NEIL2 by more than 3-fold increase, when used at molar ratios of up to 1:1 of CSB to NEIL2. Additional CSB did not further stimulate the NEIL2 glycosylase activity, suggesting that the proteins interact in a 1 to 1 ratio (Fig. 4C, lanes 3–7 and 4D). All tested CSB concentrations led to statistically significant increases in product formation compared to NEIL2 alone (p-values <0.01). The observed glycosylase activity of NEIL2 was DNA damage specific as no product was observed with a control oligodeoxynucleotide containing an 11nt bubble without base lesion (data not shown). No incision was observed with CSB alone (Fig. 4C, lanes 8), indicating that the stimulation was not due to contaminants. Excess BSA in the reaction excluded that stimulation of the NEIL2 glycosylase activity was a cause of increased protein concentration. In order to investigate how CSB affects the kinetics of NEIL2, we analyzed product formation as a function of time for NEIL2 alone or NEIL2 and CSB in a 1:1 molar ratio within the first 10min of reaction (Fig. 4E). With the higher substrate and enzyme concentration used in the experiments in order to investigate the initial product formation, we observed that the reaction is linear only within the first two minutes. This is possibly due to enzyme instability or product inhibition. The initial velocities (V_0) of the reactions with or without CSB were calculated as the slope of best linear trend line within the first two minutes of the reaction. The ratio V_0 with CSB to V_0 without CSB was close to 1.6 in two independent experiments. This supports our observation that CSB increases the product formation catalysed by NEIL2 on a 5OHU B11 substrate.

3.5 CSB does not stimulate NEIL2 incision of 5OHU in a duplex substrate

Next, we examined whether CSB stimulated NEIL2 glycosylase activity on 5OHU in a canonical duplex DNA substrate (Fig. 5A). The *in vitro* affinity of NEIL2 to 5OHU in duplex substrates is substantially lower than for a bubble structure (Dou et al., 2003; Katafuchi et al., 2004). In order to detect incision activity on dsDNA with NEIL2 alone, we increased the concentration of NEIL2 protein substantially, relative to experiments with the bubble structured DNA substrate. In contrast to the bubble substrate results, we did not see any stimulation by CSB on NEIL2 activity on duplex DNA at any of the tested CSB concentrations (Fig. 5B and C). We detected a decrease in activity at the highest CSB concentration. This is likely due to unspecific binding of CSB protein to the DNA substrate, preventing NEIL2 from binding to 5OHU.

3.6 CSB does not stimulate NEIL2 incision of a bubble substrate containing an 8oxoG lesion

Next, we used another known bubble substrate of NEIL2, 8oxoG in an 11nt bubble structure (8oxoG B11) (Table 1 and Fig. 6A). 8oxoG adducts are common products of attacks on DNA by ROS. If left unrepaired they can give rise to GC to TA transversions in the genome (Pastoriza-Gallego et al., 2007). An 8oxoG B11 targeted glycosylase activity of NEIL2 previously reported in (Dou et al., 2003) was verified (Fig 6B). To examine a potential effect of CSB on this activity, recombinant NEIL2 protein was incubated with 8oxoG B11 substrate and increasing amounts of CSB. No change in glycosylase activity was detected when up to a 1:2 molar ratio of NEIL2 and CSB was incubated with this substrate (Fig. 6C and D). Again, a decreased glycosylase activity was observed when CSB was added in large excess. Thus, we conclude that, under our experimental conditions, CSB does not stimulate NEIL2 glycosylase activity on all of its known bubble structure substrates.

3.7 CSB may stimulate NEIL2 by increasing the dissociation from product

NEIL2 is a bifunctional glycosylase, which catalyzes the cleavage of AP-sites by $\beta\delta$ -elimination via the AP-lyase activity. This reaction involves formation of a transient Schiff base between the substrate and NEIL2 (Hazra et al., 2002b), which can be trapped by

sodium borohydride (NaBH₄). In order to examine whether CSB stimulates NEIL2 substrate association and thereby Schiff base formation, we first incubated the 5OHU B11 DNA substrate with NEIL2 in the presence of NaBH₄. This resulted in a trapped transient intermediate, whereas no trapped intermediate was observed in the absence of NEIL2 or NaBH₄ (Fig. 7A, compare lane 3 with lane 1 and 8). No substrate was trapped when control (lesion free) B11 substrate was used instead of 5OHU B11, showing that substrate trapping is dependent on the presence of a lesion (Fig. 7A, lane 9 and 10). In order to examine whether CSB stimulates NEIL2 substrate association, increasing amounts of CSB were added to the trapping reaction. We did not observe any significant changes in the fraction of trapped NEIL2-DNA complexes upon addition of CSB (Fig. 7A, compare lane 3 with lanes 4–7 and Fig. 7B), suggesting that CSB does not stimulate substrate binding by NEIL2. The observed stimulation of NEIL2 by CSB may therefore be caused by increased NEIL2 dissociation from the substrate.

3.8 Transcription stalling increases CSB and NEIL2 co-localization

Our observations of functional interactions of CSB and NEIL2 specifically on the transcription mimicking DNA substrate 5OHU B11 prompted us to investigate CSB and NEIL2 cofunction in the cellular context of transcribing and stalled RNAP II. α -amanitin specifically stalls RNA polymerase II (RNAPII), over RNA polymerase I and RNA polymerase III, and thereby halts the progression of transcription bubbles. HeLa cells, grown overnight were treated with α -amanitin for 3hrs to stall transcription. The cells were then fixed, permeabilized and probed with antibodies for CSB (rabbit) and NEIL2 (mouse). Co-localization of CSB and NEIL2 was then visualized using Duolink in situ proximity ligation assay (PLA). Each PLA foci denote a site of bound primary antibodies specific for CSB and NEIL2 no more than 40nm apart, and are thus indicative of direct protein-protein interactions. We observed a baseline number of foci, well above the level of unspecific signal, in mock treated cells (Fig. 8), supporting the *in vivo* co-localization observed by immunocytochemistry (Fig. 2). Interestingly, we observed a substantial increase in the number of CSB and NEIL2 co-localized specific foci in α -amanitin treated cells compared to mock treated cells (Fig. 8A, compare upper and lower rows). This suggests that CSB and NEIL2 interact to a larger extent upon stalling of the progression of the transcription bubble.

3.9 CSB affects 8oxoG incision by NEIL2 in whole cell extracts

Next, we investigated the potential *in vivo* consequences of the identified protein-protein and functional interactions of CSB and NEIL2. To examine the glycosylase activity of NEIL2 we used a previously established NEIL1 KD cell line, which displayed a near complete knock down of NEIL1 (Muftuoglu et al., 2009). Since NEIL1 and 2 are the only glycosylases known to have specific activity on 8oxoG and 5OHU lesions in bubble structures the NEIL1 KD cell line allowed us to study the activity of NEIL2 on these substrates specifically. Additionally, a transient knock down of CSB was made in the NEIL1 KD cells, creating a double protein knock down. Near complete knock down of CSB was ensured (Supplemental Fig. 4A). Based on the observed increase in co-localization of NEIL2 and CSB (Fig. 2B) and multiple studies demonstrating re-localization of CSB after oxidative stress (Imam et al., 2007; Thorslund et al., 2005; Aamann et al., 2010) cells were treated with menadione or H₂O₂ to study this effect as well.

We then compared the glycosylase activity on 8oxoG B11 substrates in WCE from the NEIL1/CSB double KD and NEIL1 KD/control siRNA cells using an undamaged DNA substrate as negative control (Fig. 9A). No product was observed when incubating WCE with an undamaged control substrate. (Fig. 9B, lanes 12–14). Quantifications of the results from three independent experiments showed a small but statistically significant decrease in incision capacity for 8oxoG B11 in WCE from CSB/NEIL1 double KD cells when

compared to NEIL1 KD cells after both mock and 50 μ M H₂O₂ treatment (Fig 9B–E). For both treatments an average of 15% to 20% increase in incision capacity was observed in WCE from NEIL1 deficient cells compared to cells lacking both NEIL1 and CSB (Fig. 9C and E). The same pattern was observed in experiments with increasing concentrations of WCE (data not shown). The results were confirmed using independently produced WCEs. These results demonstrate that CSB increases 8oxoG B11 incision capacity in NEIL1 KD WCEs and indicates that CSB affects NEIL2 glycosylase activity *in vivo*. Equal amounts of protein in all reactions were verified by Coomassie staining (Supplemental Fig. 4B).

No significant difference in 8oxoG B11 incision capacity between NEIL1/CSB KD and NEIL1 KD cells was observed when comparing WCE from cells treated with menadione or WCE from cells treated with 200 μ M H₂O₂ (Supplemental Fig. 5). Comparing 5OHU B11 incision capacity revealed no difference between NEIL1/CSB KD and NEIL1 KD cells (Supplemental Fig. 6).

4 Discussion

Results supporting a role for CSB in BER have emerged over the last two decades (reviewed in (Khobta and Epe, 2013; Menoni et al., 2012; Stevnsner et al., 2008)). Here we report that CSB stimulates NEIL2 glycosylase activity on 5OHU in a DNA bubble structure. No stimulatory effect was observed on incision activity on 5OHU in a fully hybridized substrate. As the trapping assay did not show increased Schiff-base formation of NEIL2 with 5OHU B11 in the presence of CSB, we speculate that the *in vitro* mechanism of stimulation could be dissociation of NEIL2 from the product. The initial velocity of product formation showed increased initial enzymatic velocity of NEIL2 by a factor 1.6 in the presence of CSB. This suggests that in the presence of CSB, NEIL2 efficiency increases by approximately 60%.

We have previously reported that another Endonuclease VIII-like DNA glycosylase, NEIL1, is stimulated by CSB when acting on 5OHU in a canonical duplex structure, but not when acting on a 5OHU in a bubble structure (Muftuoglu et al., 2009). This could provide insight into the different cellular roles of the NEIL1 and NEIL2 proteins *in vivo*. NEIL1 and NEIL2 have been suggested to be involved in DNA repair during replication and transcription, respectively (Banerjee et al., 2011; Dou et al., 2008; Hazra et al., 2002a; Hazra et al., 2002b; Hegde et al., 2013). Thus, we speculate that CSB has divergent roles in BER and is involved in repair of 5OHU together with NEIL2 during transcription but with NEIL1 during replication.

An association of NEIL2 and RNAPII and preferential repair of transcribed genes was recently described by Mitra and co-workers (Banerjee et al., 2011). The 11nt bubble used in our *in vitro* experiments resembles that of transcription bubbles (Gamper and Hearst, 1983). The observed stimulation of NEIL2 activity on the 5OHU B11 substrate by CSB prompted us to speculate whether the biological relevance for the interaction is related to transcription. This was further supported by the *in situ* PLA studies, which showed an increased interaction of CSB and NEIL2 when progression of the transcription bubble was halted by α -amanitin induced stalling of RNAPII. Therefore, it is likely that the context in which NEIL2 is stimulated by CSB is associated with the transcription process. The possible coupling of transcription and repair of BER specific lesions are supported in a number of recent studies, describing NEIL2 and CSB as essential for the removal of 5OHU and 8oxoG in transcribed strand, respectively (Banerjee et al., 2011; Guo et al., 2013). The cellular relevance of a role for CSB and NEIL2 in such a pathway might be to ensure repair of oxidative lesions before they introduce transcriptional mutations. Hereby, CSB could be involved in preventing production of dysfunctional proteins.

The mutagenic formamidopyrimidine lesion, FapyA, which is proposed to be more duplex destabilizing than other oxidative lesions (Delaney et al., 2002), occur at similar or even greater frequency than 5OHU and 8oxoG *in vivo* (Bukowy et al., 2008; Mozrzymas et al., 2011). It is therefore important that this lesion is removed efficiently. We show for the first time that NEIL2 has activity towards FapyA lesions in a duplex structure and that CSB stimulates the incision of this lesion. The involvement of CSB in repair of FapyA is biologically relevant since accumulation of FapyA is observed in brain, liver and kidney DNA as well as in liver mitochondrial DNA from CSB deficient mice (Muftuoglu et al., 2009). CSB appears to stimulate both NEIL1 (Muftuoglu et al., 2009) and NEIL2 in their repair of FapyA. Thus, CSB seems to stimulate these enzymes that apparently backup each other in the repair of these frequently occurring lesions.

To investigate a potential effect of CSB on NEIL2 activity *in vivo* we used a previously established cell line with a stable KD of NEIL1. This setup allowed us to exclude potential contributions of NEIL1 glycosylase activity. We found a robust incision activity toward 5OHU and 8oxoG in 11nt bubble structures using WCE from the NEIL1 deficient cell line. This suggests that NEIL2 plays an important role in the repair of these lesions in DNA bubble structures *in vivo*. Transient knock down of CSB in the NEIL1 KD cells allowed us to measure the effect of CSB on the endogenous activity of NEIL2. We investigated the effect of CSB on the incision capacity of NEIL2 in WCE toward 5OHU B11 or 8oxoG B11. Importantly, we found 15–20% higher incision capacity of 8oxoG in a bubble in WCE from NEIL1 KD cells compared to NEIL1/CSB double knock down cells under both mock and 50µM H₂O₂ treatments. These results show that CSB increases the incision activity on 8oxoG in bubble DNA structures in a NEIL1 independent manner. NEIL1 and NEIL2 are the only known glycosylases to have an incision activity on bubble structures (Dou et al., 2003). Furthermore, NEIL1 has very poor activity toward 8oxoG B11 compared to 8oxoG:C and 8oxoG single stranded substrates, whereas NEIL2 prefers 8oxoG B11 over 8oxoG:C and 8oxoG single stranded substrates (Dou et al., 2003). This eliminates the possibility that small amounts of residual NEIL1 protein remaining in the extracts could give rise to 8oxoG cleavage in the bubble structure. In addition, CSB has not been found to stimulate NEIL1 activity on a bubble structure. The effect seen here is therefore most likely due to a stimulation of NEIL2 by CSB. The decrease in incision activity observed in CSB deficient extracts was modest but the results are supported by our findings of *in vitro* functional and physical interaction and *in vivo* physical interaction between NEIL2 and CSB. Additionally, the increased co-localization between CSB and NEIL2 after α-amanitin treatment further supports a biological relevance for the physical and functional interaction between NEIL2 and CSB. Since there are a number of compensatory pathways to BER, knock down of accessory proteins, such as CSB, cannot be expected to result in drastic changes in DNA repair activity in WCE. In particular, Y-box binding protein (YB-1) stimulates the NEIL2 incision of 8oxoG B11 *in vitro* (Das et al., 2007). Hence, the depletion of cellular CSB will not eliminate all stimulation of NEIL2 incision of 8oxoG B11. It might be difficult to detect an effect of CSB on NEIL2 incision of 5OHU B11 *in vivo* due to additional proteins stimulating NEIL2 on this substrate (Banerjee et al., 2011; Das et al., 2007).

We observed an *in vitro* and *in vivo* interaction of NEIL2 and CSB. The latter was shown by both immunocytochemistry and by the stringent PLA method (Fig 2, and 8A). By immunocytochemistry, NEIL2 was found to be mainly cytoplasmic with partial co-localization with the mitochondrial marker COX IV. This is in agreement with the recent report from Hazra and co-workers showing a mitochondrial localization and function of NEIL2 (Mandal et al., 2012). We observed that increasing amounts of cytoplasmic NEIL2 and CSB protein co-localized after menadione treatment. We have previously reported that menadione treatment results in partial re-localization of CSB to the mitochondria (Aamann et al., 2010) and this observation is supported by another report on mitochondrial CSB

localization after oxidative stress (Kamenisch et al., 2010). Additionally, we have previously shown that FapyA lesions are increased in mitochondrial DNA from CSB deficient mice (Muftuoglu et al., 2009) and we here show that NEIL2 removes this lesion and that the removal is stimulated by CSB. It is therefore tempting to suggest that the co-localization is partly mitochondrial and that the CSB-NEIL2 functional interaction could be relevant in both nuclear and mitochondrial BER.

5 Conclusion

We report that CSB stimulates the 5OHU glycosylase capacity of NEIL2 when the lesion is localized in an 11nt DNA bubble structure. Trapping of the transient Schiff base formed during NEIL2 removal of 5OHU in the bubble structure indicates that CSB affects the dissociation of the glycosylase from the product. Additionally, we show that NEIL2 has activity toward FapyA in a duplex structure and that CSB stimulates NEIL2 in removing this lesion. We find that CSB directly interacts with NEIL2 and is in the same complex as NEIL2 in nuclear extracts. DNA glycosylase incision assays using WCE from NEIL1 KD and NEIL1-CSB double KD cells showed that the repair capacity for 8oxoG in an 11nt bubble structure decreased upon CSB knock down suggesting a biological relevance for CSB stimulation of NEIL2. This is supported by the physical and functional NEIL2-CSB interaction. Upon menadione treatment, we find that cytoplasmic CSB and NEIL2 co-localization increases. Furthermore, we observe increased co-localization of NEIL2 and CSB in response to α -amanitin treatment, suggesting increased localization in response to stalled RNAPII transcription bubbles. Together, these results suggest that CSB stimulates NEIL2 incision capacity *in vitro* and *in vivo* with a possible biological relevance for transcription associated BER.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

CSB physically interacts with NEIL2

CSB affects NEIL2 incision activity in vitro

Stalling of transcription bubble progression increases NEIL2 and CSB co-localization

CSB positively affects incision of 8oxoG by NEIL2 in a DNA bubble structure in vivo

FIGURE 1. Physical interaction between NEIL2 and CSB

A, Nuclear extracts from mock or menadione treated (200 μ M) HeLa cells were used for co-IP with NEIL2, CSB or IgG antibodies. The immunoprecipitated complexes and 5% input were run on SDS-PAGE and blotted against NEIL2, CSB or XPB (p89) protein. **B**, Dot blot of NEIL2 CSB interaction. Purified recombinant CSB, NEIL2 and control protein, BSA, were blotted on PVDF membranes and incubated with purified NEIL2. The membrane was then incubated with primary mouse anti-NEIL2 antibody followed by secondary anti-mouse IgG peroxidase conjugated antibody. **C**, Far Western of NEIL2 and CSB interaction. Recombinant NEIL2 and BSA protein separated on a 10% SDS-PAGE gel and transferred to PVDF membrane followed by incubation with purified CSB protein. Bound CSB protein was detected with CSB antibody.

A

B

FIGURE 2. Co-localization of NEIL2 and CSB

A, Cellular distribution of NEIL2. HeLa cells were fixed and stained for NEIL2 (Mouse monoclonal, Sigma) and COX IV (Goat Polyclonal, Santa Cruz). COX IV was used for mitochondrial staining. *B*, Co-localization of NEIL2 and CSB. HeLa cells were treated with 200µM menadione, fixed and stained for NEIL2 (Sigma) and CSB (Santa Cruz), respectively.

FIGURE 3. Stimulation of NEIL2 incision activity on FapyA substrate with CSB

A, Substrate containing FapyA lesion. **B**, Activity of NEIL2 on FapyA substrate. 1nM FapyA substrate was incubated with increasing amounts of purified recombinant NEIL2 protein. *Lane 1*; 1nM FapyA substrate, no NEIL2. *Lanes 2–6*; 10, 20, 50, 100 and 150nM NEIL2. **C**, Representative autoradiogram showing stimulation of NEIL2 incision activity on FapyA substrate by CSB. *Lane 1*; 1nM FapyA substrate alone. *Lane 2*; 200nM CSB alone. *Lane 3*; 40nM NEIL2 alone, *Lanes 4–7*; 40nM NEIL2 with increasing concentrations of CSB; 40, 80, 120 and 200nM respectively. **D**, Stimulation of NEIL2 (40nM) incision activity on 1nM FapyA substrate with 0, 40, 80, 120 and 200nM CSB. The diagram shows quantification and average of three independent experiments. Error bars represent standard deviations.

FIGURE 4. Stimulation of NEIL2 glycosylase activity on 5OHU in a bubble structure by CSB
A, 5-Hydroxyuracil substrate in an 11nt bubble (5OHU B11) used for incision experiments.
B, Increasing amounts of NEIL2 protein were incubated with 5OHU B11. Lane 1–9; 0.0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.5, 1 and 2nM NEIL2 respectively with 0.2nM 5OHU B11. **C**, Representative autoradiogram showing stimulation of NEIL2 by CSB. Lane 1; substrate alone. Lane 2–7; 0.05nM NEIL2 and 0.0, 0.025, 0.05, 0.1, 0.25 and 0.5nM CSB, respectively. Lane 8; 0.5nM CSB. **D**, Average of three independent DNA glycosylase experiments with 0.05nM NEIL2 protein, 0.2nM 5OHU B11 substrate and 0.0, 0.025, 0.05, 0.1 and 0.25nM CSB, respectively. **E**, Graph for time dependent 5OHU B11 incision

activity of NEIL2 alone or NEIL2 and CSB in a 1:1 molar ratio at time 0, 1, 2, 5, and 10min using 100nM 5OHU B11 and 10nM NEIL2 with or without 10nM CSB. Representative for two independent experiments. Error bars represent standard deviation.

FIGURE 5. NEIL2 glycosylase activity on 5OHU in a DNA duplex in the presence of CSB
A, 5OHU substrate in fully annealed DNA used for incision experiment. *B*, Representative gel for NEIL2 incision with CSB. *Lane 1*; Substrate alone. *Lane 2–7*; 4nM NEIL2 and 0, 2, 3, 4, 8 and 16nM CSB, respectively. *C*, Average of three independent DNA glycosylase experiments with 4nM NEIL2 protein, 0.2nM 5OHU duplex substrate, and 0, 2, 3, 4, 8 and 16nM CSB, respectively. Error bars represent standard deviations.

FIGURE 6. NEIL2 glycosylase activity on 8oxoG in bubble structure in presence of CSB
A, 8oxoG substrate in an 11nt bubble (8oxoG B11) used for incision experiment. *B*, Increasing amounts of NEIL2 protein were incubated with an 8oxoG B11 substrate. *Lane 1–9*; 0.0, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2 and 5nM NEIL2 respectively with 1nM 8oxoG B11. *C*, Representative gel for NEIL2 incision with CSB. *Lane 1*; Substrate alone. *Lane 2–4*; 1nM NEIL2, and 0, 1 and 2nM CSB, respectively. *D*, Average of three independent DNA glycosylase experiments with 1nM NEIL2 protein, 8oxoG B11 substrate (1nM) and 0, 1, 2, and 5nM CSB, respectively. Error bars represent standard deviations.

FIGURE 7. Molecular mechanism for CSB stimulation of NEIL2 on 5OHU B11 substrate by trapping transient Schiff-base intermediate during incision

A, Representative autoradiogram of NEIL2 forming Schiff base with the substrate. *Lane 1*; no protein. *Lane 2*; 0.25 nM CSB. *Lane 3–7*; 0.05nM NEIL2 with increasing amounts of CSB (0.0, 0.025, 0.05, 0.1, 0.25nM, respectively). *Lane 8*; 0.05nM NEIL2 and 0.25nM CSB, no NaBH₄. *Lane 9*; control B11 substrate with 0.05nM NEIL2. *Lane 10*; control B11 with 0.05nM NEIL2 and 0.25nM CSB. B, Quantification of three independent experiments with 5OHU B11, 0.05nM NEIL2 and 0.0, 0.025, 0.05, 0.1, 0.25nM CSB, respectively, together with NaBH₄. Error bars indicate standard deviations.

A

NEIL2 CSB

Mock

Mock

α -amanitin α -amanitin

FITC

Merge

B

IgG Control

CSB IgG

NEIL2 IgG

Merge

FIGURE 8. Detection of NEIL2 and CSB by in situ PLA

A, In situ PLA (Duolink) demonstration of the NEIL2 and CSB association in mock (upper row) and α -amanitin (lower row) treated HeLa cells. Left side, PLA specific signals detected by FITC. Right side, merge of FITC, DAPI and DIC signals. B, Merge of FITC, DAPI and DIC from PLA with CSB Ab (rabbit) and NEIL2 ab (mouse) vs. IgG, respectively which served as controls for unspecific PLA signals.

FIGURE 9. 8oxoG B11 incision capacity in WCE from mock and H₂O₂ treated cells

A; 8oxoG substrate in an 11nt bubble (8oxoG B11) and control substrate, respectively, used for incision experiments. **B**, Representative gel for 8oxoG B11 and control B11 incision reactions with WCE from mock treated NEIL1/CSB KD and NEIL1/control KD cells. *Lane 1*; single stranded oligonucleotide marker. *Lane 2–6*; 8oxoG B11 incision with WCE from NEIL1/CSB KD cells. *Lane 7–11*; 8oxoG B11 incision with WCE from NEIL1/control KD cells. Reactions were stopped after 0, 30, 60, 120 and 180min, respectively. *Lane 12–14*; control B11 substrate with WCE from NEIL1/CSB KD cells stopped at 180, 120 and 0min, respectively **D**, Representative gel for 8oxoG B11 incision reaction with WCE from 50μM H₂O₂ treated NEIL1/CSB KD and NEIL1/control KD cells. *Lane 1*; single stranded oligonucleotide marker. *Lane 2–7*; 8oxoG B11 incision with WCE from NEIL1/CSB KD cells. *Lane 8–13*; 8oxoG B11 incision with WCE from NEIL1/control KD cells. Reactions were stopped at 0, 10, 30, 60, 120 and 180min respectively. **C and E**: Quantification of three independent experiments as shown in **B** and **D** respectively. Error bars indicate standard

deviation. * indicates $P < 0.05$ in Student t-test for difference between control siRNA and CSB siRNA KD WCE.

Table 1

Oligonucleotide sequences used in the study

Name	Sequence
5OHU	GCT TAG CTT GGA ATC GTA TCA TGT AXA CTC GTG TGC CGT GTA GAC CGT GCC X= 5OHU
8oxoG	GCT TAG CTT GGA ATC GTA TCA TGT AXA CTC GTG TGC CGT GTA GAC CGT GCC X= 8oxoG
Control	GCT TAG CTT GGA ATC GTA TCA TGT ACA CTC GTG TGC CGT GTA GAC CGT GCC
Bubble complementary strand	GGC ACG GTC TAC ACG GCA CAA ACA GCC CAC GGA TAC GAT TCC AAG CTA AGC
Duplex complementary strand	GGC ACG GTC TAC ACG GCA CAC GAG TGT ACA TGA TAC GAT TCC AAG CTA AGC
FapyA	CGT TCA ACG TGC ACT XAC AGC ACG TCC CAT X=FapyA
Complementary strand for FapyA	GCA AGT TGC ACG TGA TTG TCG TGC AGG GTA
AP	ATA TAC CGC GCX CGG CCG ATC AAG CTT ATT X=Uracil, processed by UNG
Complementary strand for AP	TAT ATG GCG CGG GCC GGC TAG TTC GAA TAA

Oligodeoxynucleotides used for incision assays. 5OHU, 8oxoG and control can all be annealed to Bubble complementary strand resulting in an 11bp bubble with a center located lesion. Alternatively, annealing to Duplex complementary strand creates canonical DNA structures. FapyA can be annealed to complementary strand for FapyA resulting in a double stranded substrate.