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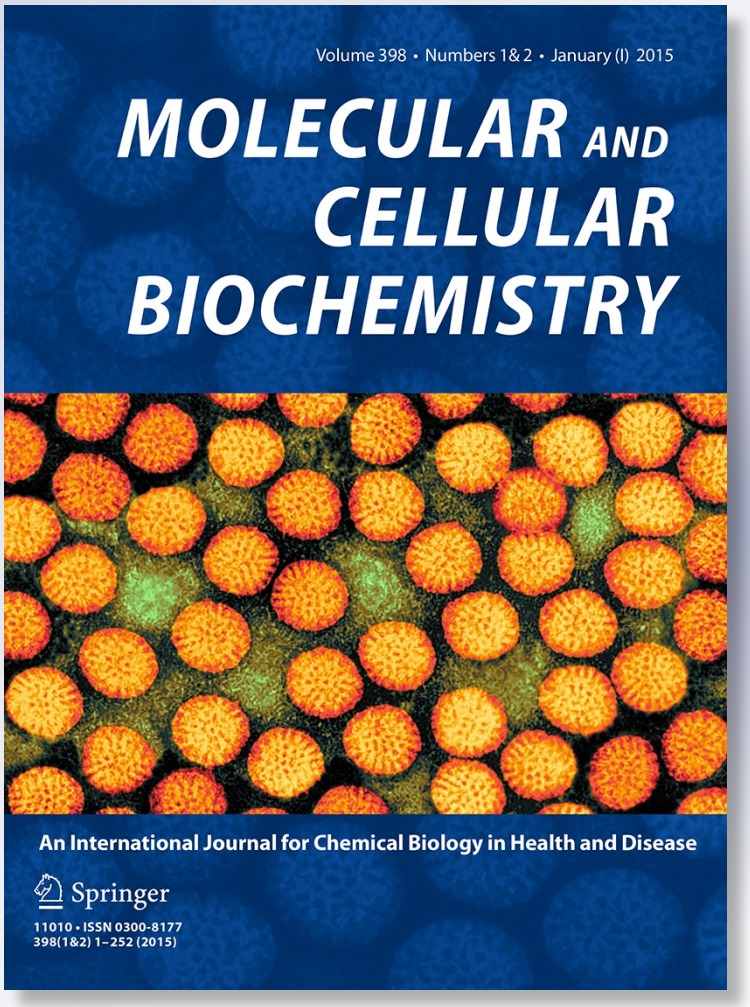

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Nuclear translocation of p19INK4d in response to oxidative DNA damage promotes chromatin relaxation

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Abstract DNA is continuously exposed to damaging agents that can lead to changes in the genetic information with adverse consequences. Nonetheless, eukaryotic cells have mechanisms such as the DNA damage response (DDR) to prevent genomic instability. The DNA of eukaryotic cells is packaged into nucleosomes, which fold the genome into highly condensed chromatin, but relatively little is known about the role of chromatin accessibility in DNA repair. p19INK4d, a cyclin-dependent kinase inhibitor, plays an important role in cell cycle regulation and cellular DDR. Extensive data indicate that p19INK4d is a critical factor in the maintenance of genomic integrity and cell survival. p19INK4d is upregulated by various genotoxics, improving the repair efficiency for a variety of DNA lesions. The evidence of p19INK4d translocation into the nucleus and its low sequence specificity in its interaction with DNA prompted us to hypothesize that p19INK4d plays a role at an early stage of cellular DDR. In the present study, we demonstrate that upon oxidative DNA damage, p19INK4d strongly binds to and relaxes chromatin. Furthermore, in vitro accessibility assays show that DNA is more accessible to a restriction enzyme when a chromatinized plasmid is incubated in the presence of a protein extract with high levels of p19INK4d. Nuclear protein extracts from cells overexpressing p19INK4d are better able to repair a chromatinized and damaged plasmid. These observations support the notion that p19INK4d would act as a chromatin accessibility factor that allows the access of the repair machinery to the DNA damage site.

Keywords DNA damage response · Chromatin relaxation · p19INK4d · Genome integrity

Abbreviations

INK4	Inhibitor of cyclin-dependent kinase 4
CDK	Cyclin-dependent kinase
MNase	Micrococcal nuclease
DDR	DNA damage response
OGG1	8-Oxoguanine DNA glycosylase 1

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Introduction

A broad spectrum of DNA lesions caused by exposure of cells to stresses present in their environment, like chemical agents, UV radiation, and ionizing radiation, or stresses inherent to their own metabolism, such as reactive oxygen species and replication stress, constantly threaten the integrity of the genome [2, 3]. If damage is left unrepaired, genomic instability can arise, compromising cell survival [4, 5]. Therefore, it is of vital importance that DNA integrity is kept well conserved and protected against

endogenous and exogenous genotoxic factors. Accordingly, diverse mechanisms exist in cells to repair different kinds of DNA lesions [6]. In addition, the signals emanating from damaged DNA activate checkpoints for delaying cell cycle progression. This activation provides more time for successful completion of DNA repair before DNA replication and chromosome segregation, or elimination of irreparably injured cells through apoptosis [7, 8].

Eukaryotic DNA and histones are assembled into nucleosomes, the main subunit of chromatin fibers. The tight DNA–histone interaction acts as a physical barrier that hinders the access of various protein machineries responsible for transcription, replication, recombination, and repair. Hence, enzymatic activities accountable for DNA repair must circumvent this natural barrier in order to repair DNA lesions. A prevailing model describing how DNA repair occurs in a chromatin environment is the 'Access–Repair–Restore' model [9, 10]. This model hypothesizes that chromatin would be locally destabilized after damage to facilitate access of the repair machinery to DNA lesions. The original chromatin organization would be restored after completion of DNA repair. Therefore, the enzymatic mechanisms responsible for DNA repair would need additional players to access the damage and restore chromatin structure after their action [11].

This model is supported by recent data that have shed light on factors involved in chromatin destabilization and restoration in response to genotoxic treatments. For example, the mammalian TATA-binding protein-free TAF complex (TFTC), which contains the Gcn5 histone acetyltransferase, binds to UV-irradiated DNA and acetylates histone H3 in nucleosomes [12]. Acetylation of histone H4 is also thought to be essential for DNA repair. For example, expression of dominant-negative Tip60 H4 histone acetyltransferases [13] abrogates the repair of double-strand breaks (DSBs) in mammalian cells, whereas depletion of the histone acetyltransferase cofactor TRRAP reduces UV-induced hyperacetylation of histone H4 and the recruitment of repair proteins to sites of DSBs [14, 15]. It has also been suggested that p53 plays a cooperative role with p300 in inducing histone H3 acetylation and chromatin relaxation after UV irradiation [16]. More recently, it has been reported that ING1b associates with chromatin in an UV-inducible manner and facilitates DNA access to the nucleotide excision repair factor XPA [17].

Inhibitor of cyclin-dependent kinase 4 (INK4) proteins are a family of cell cycle inhibitors that bind to CDK4/6 kinases and thus block the G1 to S phase transition of the cell cycle [18, 19]. However, novel cell cycle-independent functions have been recently described for some of them [20]. Interestingly, p16INK4a and p19INK4d (hereafter referred to as p19) have been linked to the cellular response to genotoxic agents [21–23]. In particular, extensive data indicate that p19 is a critical factor in the maintenance of genomic integrity and cell survival. It has been reported that UV light, cisplatin, and β-amyloid peptide promote p19 transcriptional induction and nuclear translocation [23]. Despite these findings, precisely how p19 contributes to DNA damage response (DDR) remains unknown.

The fact that p19 is transcriptionally upregulated by various genotoxics, improving the repair of different types of DNA damage, coupled with the evidence of nucleus translocation of p19, and its low sequence specificity-interaction with DNA, prompted us to hypothesize that p19 plays a role at an early stage of cellular DDR, perhaps in chromatin structure reorganization.

We demonstrate here that upon an oxidative DNA damage p19 strongly binds to and relaxes the chromatin structure. Furthermore, in vitro assays confirmed that the presence of p19 facilitates the repair of a plasmid DNA in a chromatin environment. These observations support the notion that p19 would act as a chromatin accessibility factor that allows the access of the repair machinery to the DNA damage site.

Materials and methods

Plasmid construction, cell cultures, and treatments

To construct the p19-GFP fusion protein, the open reading frame of human p19 was inserted into the pEGFP-C2 vector (Clontech) at *Bgl*II/*Eco*RI sites. Transient transfections were performed with Effectene Transfection Reagent (Quiagen) according to the manufacturer's instructions. HeLa (ATCC-CCL-2), non-SV40-transformed HEK293 (ATCC-CRL-1573), and BHK-21 (ATCC-CCL-10) cell lines were grown in DMEM (Invitrogen) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine at 37 °C in a 5 % CO_2 humidified atmosphere. Stable cell lines, BHK21-p19S and BHK21-p19AS, were performed as previously described [23]. To induce the metallothionein promoter, cells were treated with 75 μM $ZnSO_4$ for at least 5 h. Cells at about 60 % of confluence were treated with 20 mM $KBrO_3$ (Sigma) diluted in D-PBS, for 30 min at 37 °C. Cells were then allowed to recover in DMEM for the indicated periods of time before fixation or extraction.

RNA extraction and Northern blot analysis

RNA extraction and Northern blot analysis were carried out as previously described [24]. Briefly, total RNA was denatured, electrophoresed in 1 % glyoxal-agarose gels, and transferred to nylon membranes (Hybond N, Amersham). Membranes were sequentially hybridized with the

indicated [^{32}P]-labeled probes, and radioactivity was detected using a PhosphorImager (FujiFilm BAS-1800II) and quantified using ImageJ software.

Total cell extracts, nuclear extracts, and chromatin isolation

To prepare total cell extracts, cultured cells were harvested by centrifugation, washed in PBS, and directly resuspended in Laemmli buffer, followed by sonication for 15 s at 25 % amplitude. Proteins were resolved in 15 % polyacrylamide gels and analyzed by immunoblotting using monoclonal anti-human anti-p19 (P0999-55A USBiological), or anti-mouse anti-p19 (37-8700 Invitrogen), anti-αHP1 (1H5 Euromedex), anti-β-actin (sc-47778 Santa Cruz Biotechnology), anti-Histone 3 (sc-8654-R Santa Cruz Biotechnology), anti-RNA polymerase II (H5 Eurogentec), and anti-GAPDH (sc-32233 Santa Cruz Biotechnology) antibodies.

Chromatin was isolated as previously described [25]. To verify the correct separation of each fraction, anti-GAPDH was used as cytoplasm marker and anti-histone H3 (sc-8654-R, Santa Cruz Biotechnology) as chromatin marker.

Heterochromatin and euchromatin fractionation

Sub-nuclear fractions were obtained according to Frenster et al. [26]. Total cell extract, heterochromatin, and euchromatin were directly resuspended in the same volume of Laemmli buffer 1× before proceeding to Western blot analysis. The correct separation was verified using anti-α-HP1 as a heterochromatin marker and RNAPolII as a euchromatin marker.

Histone salt extraction analyses and micrococcal nuclease digestion

Salt extraction of histones was analyzed as described previously [27]. Extracted histones were subjected to SDS-PAGE and analyzed by Western blot.

Nuclease digestion was performed as previously described [1]. Briefly, cells were resuspended in 1 ml of lysis buffer (10 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 0.5 % NP-40) and incubated on ice for 5 min. Nuclear pellet was obtained by centrifugation and resuspended in digestion buffer (15 mM Tris–HCl pH 7.4, 60 mM KCl, 5 mM NaCl, 0.25 M sucrose, 1 mM $CaCl_2$, and 0.5 mM DTT). Reaction volumes of 100 µl were pre-incubated at 32 °C with 0.2 U/µl of Micrococcal Nuclease (MNase). DNA was purified by standard procedures, and electrophoresis was carried out in 1 % agarose gels.

Immunofluorescence and microscopy

Immunofluorescence was carried out as previously described [22, 28]. Cells were previously extracted for 5 min on ice with cytoskeleton (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5 % Triton X-100, and protease inhibitors) before fixation with 4 % paraformaldehyde for 20 min at room temperature. Nuclear DNA was counterstained with 4,6 diamidino-2-phenylindole (DAPI, 2 µg/µl). Image acquisition was performed with a Leica confocal microscope SPE (Wetzlar, Germany). Images were treated and analyzed with Leica and ImageJ softwares. GFP intensity levels were obtained using CellProfiler cell image analysis software. For each condition, GFP intensity was measured in about 250–500 cells.

Preparation of a *Drosophila melanogaster* embryonic nuclear extracts

Extracts for efficient chromatin assembly were prepared from *Drosophila* preblastoderm embryos. Preblastoderm embryos were harvested from a healthy population of *Drosophila melanogaster* as described previously [29, 30] and resuspended in EW solution (0.7 % NaCl, 0.05 % Triton X-100). The suspension was treated with hypochlorous acid for 3 min (3 % v/v final concentration). Dechorionated embryos were separated by filtration and carefully washed with cold water. Only settled embryos were transferred to a 500-ml flask and successively washed with EW solution, 0.7 % NaCl and EX buffer (10 mM Hepes–KOH pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA-KOH pH 8.0, 10 % glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). Embryos were resuspended in 5 ml EX buffer and transferred to a Potter–Elvehjem homogenizer vessel on ice and allowed them to settle for at least 15 min. The EX buffer was aspirated and embryos homogenized with one stroke at 3,000 rpm and six strokes at 1,500 rpm. $MgCl_2$ was added to the homogenate to a 6.5 mM final concentration, and an additional homogenization stroke at 1,500 rpm was carried out. The homogenate was centrifuged for 5 min at 17,000*g* at 4 °C and the resulting cytoplasmic extract was centrifuged at 1,90,000*g* for 2 h. The clear extract was isolated, and 200-µl aliquots were immediately frozen in liquid N_2 and stored at −80 °C.

Chromatin assembly

Chromatin assembly reactions were performed as described by Bonte and Becker [31] using covalently closed circular DNA of the pET-CREB plasmid that had been previously

relaxed with recombinant *Drosophila* topoisomerase I. A standard reaction contained 1 μg relaxed covalently closed circular DNA, 10 μg of *Drosophila* extract, and 5 μl of a 10× energy supplier mix (30 mM Mg_2Cl, 10 mM DTT, 300 mM creatine phosphate, 30 mM ATP, and 1 μg creatine phosphokinase per 100 μl), and EX buffer in a final volume of 50 μl. Incubation was carried out at 26 °C for 5 h.

Preparation of nuclear extracts

Exponentially growing BHK21-p19S and BHK21-p19AS cells were trypsinized and seeded at 50–60 % confluence. Twenty hours after plating, cells were irradiated with 20 J/m^2 UV (range 240–280 nm) 4 h after metallothionein promoter induction with 75 μM $ZnSO_4$. Four hours later, the medium was changed, $ZnSO_4$ removed, and cells harvested for nuclear extract preparations. Nuclear extracts from UV-irradiated BHK21-p19S, and BHK21-p19AS cells were prepared as described by Digman and Roeder [32].

Restriction enzyme accessibility assay

The linearized pET-CREB plasmid was subjected to chromatin assembly by incubation with extracts of *Drosophila* preblastoderm embryos, as described above. Briefly, 2 μg of chromatinized or naked DNA was incubated with 10 μg nuclear extract from BHK21-p19S or BHK21-p19AS cells, 5 μl of the 10× energy supplier mix, and 30 μl of buffer A (40 mM Hepes–KOH ph 7.8, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.4 μg/μl bovine serum albumin) in a final volume of 60 μl and incubated at 37 °C. Four hours later, 2 U *Xba*I were added to the reaction mixture and incubated for different times. DNA was extracted, purified, and electrophoresed in 1 % agarose gels.

In vitro plasmid DNA repair

The pET-CREB plasmid was irradiated with UV 100 J/m^2 and subjected to chromatin assembly by incubation with extracts of *Drosophila* preblastoderm embryos as described above. In vitro DNA repair was assessed as described by Wood et al. [33]. Briefly, 10 μg of chromatinized or naked UV-irradiated plasmid DNA was incubated with 5 μl of the 10× energy supplier mix, 50 μCi (α-^{32}P)dCTP, 10 μl of a 5× reaction buffer (550 mM Hepes–KOH pH 7.6, 200 mM $MgCl_2$, 500 mM dithiothreitol, 25 mM dATP, 25 mM dGTP, 25 mM dTTP, 25 mM dCTP, and 10 μg/μl bovine serum albumin), and 10 μg nuclear extract from BHK21-p19S or BHK21-p19AS cells in a final volume of 50 μl and incubated at 37 °C for 2 h. DNA was extracted, purified, and electrophoresed in 1 % agarose gels.

Results and discussion

p19 is induced in response to oxidative DNA damage

To explore whether p19 is induced in response to oxidative DNA damage, we used $KBrO_3$, a carcinogenic agent known to induce oxidative stress in eukaryotic cells. The genotoxicity of $KBrO_3$ requires reduction of bromate by thiols (as glutathione or reduced cysteines) and, while it generates strand breaks, it induces predominantly 8-oxoG lesions in DNA [34]. The oxidized base is recognized and excised by a specific glycosylase that initiate the base excision repair (BER) [35, 36]. To select the $KBrO_3$ dose to be used, we first performed a dose-curve response of $KBrO_3$ in HEK293 cells and examined p19 transcript and protein levels by Northern and Western blot, respectively. We observed that $KBrO_3$ induced p19 expression in a dose-dependent manner, reaching a maximum at 20 mM (Fig. 1a, b). Next, HEK293 cells were treated at different times with 20 mM $KBrO_3$, and p19 protein levels were examined by Western blot. The levels of p19 increased after 30 min of $KBrO_3$ treatment, reaching a maximum at 1 h and returned to basal levels at 8 h of recovery (Fig. 1c). Next, we checked the genotoxic effect of $KBrO_3$ treatment by detecting the recruitment to chromatin of 8-oxoguanine DNA glycosylase 1 (OGG1) [34], the DNA glycosylase that recognizes the 8-oxo-G oxidized base and initiates its BER [37], and the phosphorylation of the H2AX histone. $KBrO_3$-treated cells showed a strong fluorescent signal for both OGG1 (upper panel) and γH2AX (lower panel) compared to non-treated cells (Fig. 1d).

It has been reported that p19 is rapidly induced following different types of DNA damage such as those caused by UV light, cisplatin, β-amyloid, neocarzinostatin, and camptothecin in several cell lines [23, 38, 39]. DNA lesions induced by UV light and the chemotherapeutic agent cisplatin are mainly removed by the nucleotide excision repair (NER) [40], while neocarzinostatin and camptothecin activate DSBs repair mechanisms [41–44]. The data presented above show that p19 is also induced in response to $KBrO_3$ treatment. These results confirm that p19 is induced in response to different types of DNA damage, independently of the DNA repair mechanism triggered, suggesting that p19 plays a role in DDR at an early step.

p19 translocates to the nucleus and localizes to euchromatin regions upon $KBrO_3$ treatment

It has been previously reported that p19 translocates from the cytoplasm to the nucleus following diverse genotoxic insults [22, 38]. To determine p19 localization after DNA damage with $KBrO_3$, HeLa cells were

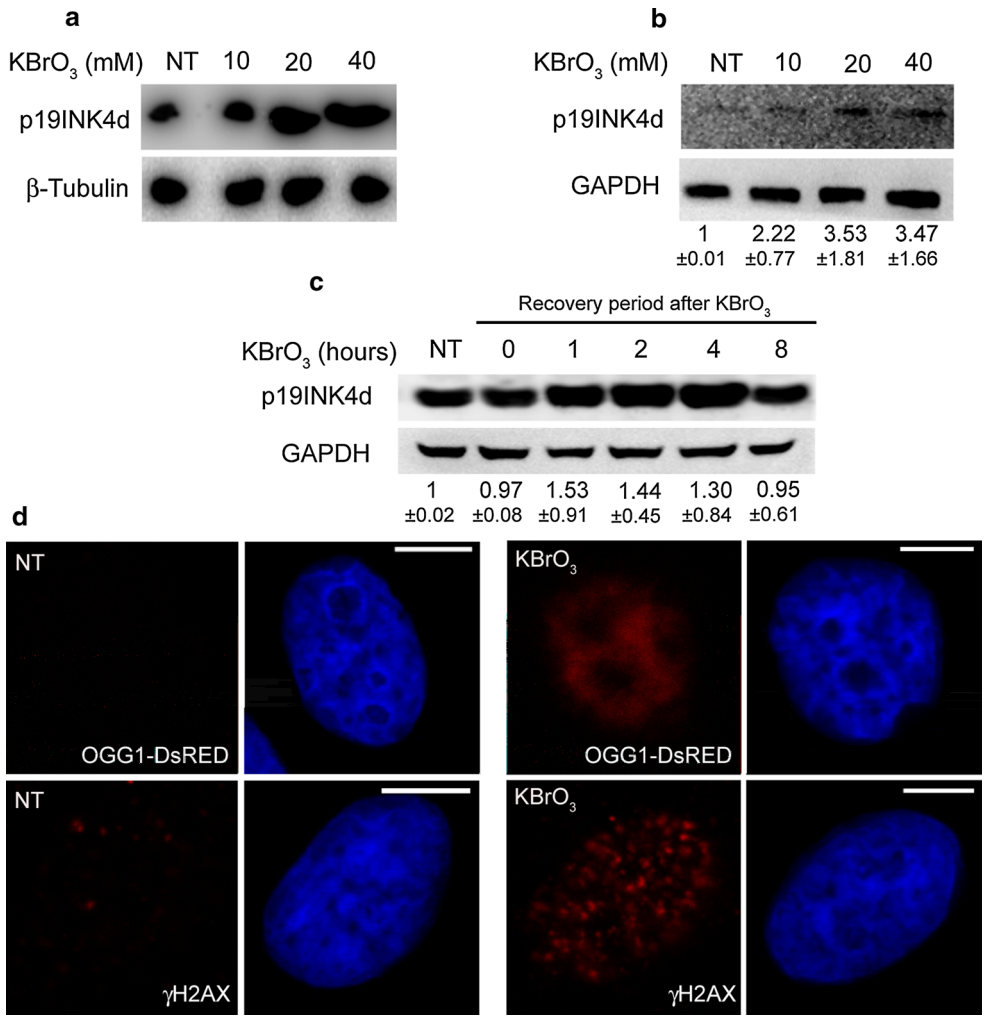

Fig. 1 p19 is induced in response to $KBrO_3$ treatment. **a**, **b** HEK293 cells were treated for 30 min with different doses of $KBrO_3$ after which cells were allowed to recover for 4 h in fresh medium. **a** Total RNA (20 μg) was subjected to Northern blot analysis with the (^{32}P)-labeled probes indicated at the *left margin*. **b** Equal amounts of protein from cell lysates were subjected to 15 % SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western blot with p19 antibody. **c** HEK293 cells were exposed to 20 mM $KBrO_3$ for 30 min, after which cells were allowed to recover in fresh medium. Cell samples were taken at various times and lysates were analyzed by Western blot with p19 antibody. **b–c** Densitometric analysis of p19 is represented in the *lower panels*. Data represent the mean ± SD of three experiments **d** HeLa cells, transiently transfected with the fusion protein OGG1-DsRED, were treated with 20 mM $KBrO_3$ for 30 min, after which cells were allowed to recover in fresh medium. Localization of OGG1-DsRED (*upper panel*) and γH2AX (*lower panel*) were assessed through immunofluorescence (*scale bar* 5 μm)

transfected with GFP-p19 and treated with a detergent-containing buffer at different times in order to remove soluble proteins. After 30 min of $KBrO_3$ treatment, nuclear p19 signal increased significantly, reaching a maximum at 1 h of recovery (Fig. 2a, b), indicating not only p19 nuclear translocation but also a strong interaction with an insoluble structure.

The above results confirm that p19, predominantly cytoplasmic, translocates into the nucleus in response to DNA damage. p19 does not contain the standard basic monopartite or bipartite nuclear localization signal (NLS) [45]. However, the direct phosphorylation of transported proteins can constitute a regulatory mechanism for cellular localization of proteins [46, 47]. In this regard, it has been demonstrated that the function of p19 in DNA repair and cell survival is modulated by its sequential CDK2- and PKA-dependent phosphorylation at serine 76 and threonine 141 and that nuclear translocation of p19 induced by DNA damage is dependent on serine 76 phosphorylation [38].

In view of this result, we performed a subcellular fractionation analysis to evaluate whether, after DNA damage, nuclear p19 interacts with chromatin. We observed an increase in p19 levels in the chromatin fraction of $KBrO_3$-treated cells (Fig. 3a). After subsequent fractionation, p19 was predominantly detected in the RNA polymerase II-rich

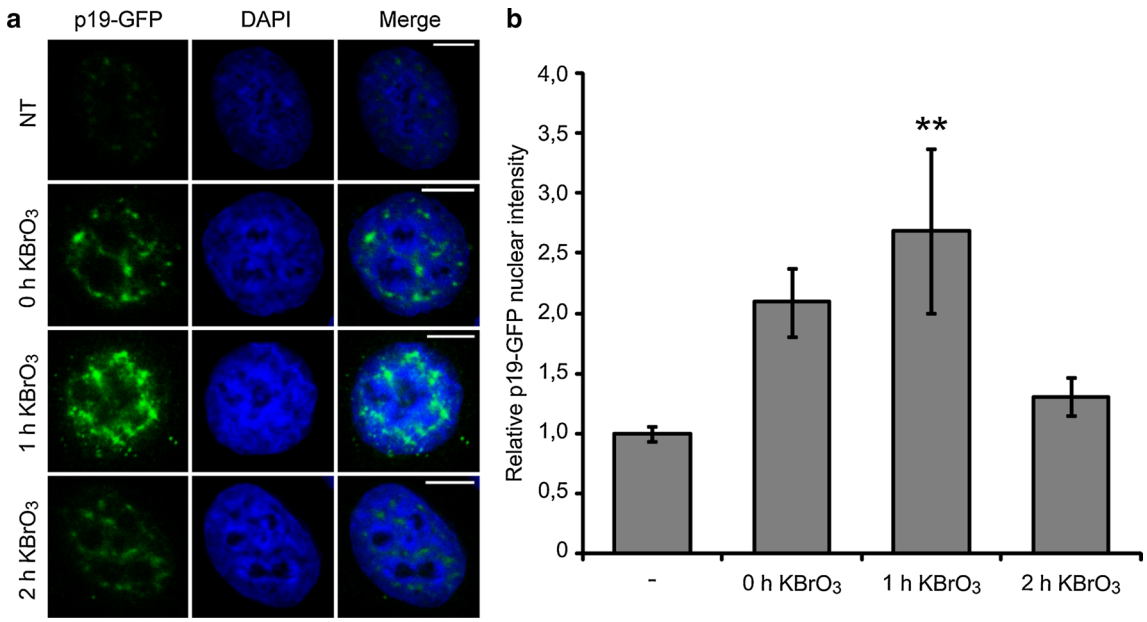

Fig. 2 p19 translocates to the nucleus following $KBrO_3$ treatment. **a** HeLa cells, transiently transfected with p19-GFP, were treated with 20 mM $KBrO_3$ for 30 min, after which cells were allowed to recover in fresh medium. Cell samples were taken at various times and, prior to fixation, soluble proteins were removed with CSK-0.5 % Triton X-100. Localization of p19-GFP was assessed through confocal fluorescence microscopy. *Scale bar* 5 μm. **b** Quantification of p19-GFP nuclear intensity relative to the untreated control (NT) cells, which represent 10 % of the maximum intensity detected. Data represent the mean ± SEM of at least three independent experiments, in which 20–50 cells were analyzed for each condition. *p* values were obtained by one-way ANOVA followed by Dunnett's post-test: $^{**}p < 0.01$

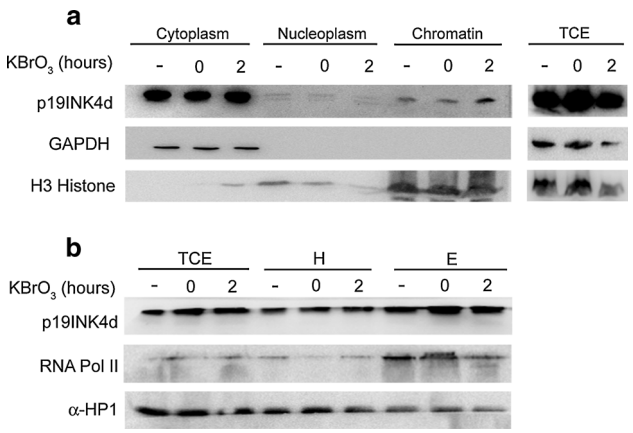

Fig. 3 p19 binds to euchromatin after $KBrO_3$ treatment. **a**, **b** HEK293 cells were treated with 20 mM of $KBrO_3$ for 30 min, after which cells were allowed to recover in fresh medium. Cell samples were taken at different times and subjected to subcellular fractionation. **a** The levels of the indicated proteins were assessed by Western blot in the different fractions. GAPDH and H3 histone were used as markers of cytoplasm and chromatin fraction, respectively. **b** The levels of the indicated proteins were assayed by Western blot; α-HP1 and RNAPolII were used as markers of heterochromatin and euchromatin fraction, respectively. Total cell extract (TCE), heterochromatin (H) and euchromatin (E)

fraction corresponding to euchromatin, thus indicating a preferential recruitment of p19 to open chromatin regions (Fig. 3b).

Expression of p19 protein leads to a more relaxed chromatin after DNA damage

Chromatin remodeling is a prerequisite for both DNA damage signaling and DNA repair and represents an early step in DDR [48]. In the light of the above results, we next examined whether p19 belongs to a protein network responsible for chromatin remodeling in response to DNA damage. To test this, BHK-21 cells stably transfected with p19 cDNA in a sense (BHK21-p19S) or antisense (BHK21-p19AS) orientation driven by a metallothionein promoter were treated with $KBrO_3$ (Fig. 4a). We then examined the potential role of p19 in the global chromatin structural change looking at the in vivo MNase accessibility. Interestingly, DNA obtained from the chromatin fraction isolated from $KBrO_3$-treated BHK-21p19AS cells was less prone to digestion than that from BHK-21WT cells. In contrast, the chromatin fraction from BHK-21p19S cells was more sensitive to MNase digestion than that from BHK-21WT cells (Fig. 4b). This suggests that the chromatin is less condensed when p19 is overexpressed. Next, we examined the electrostatic interaction between histones and chromatin DNA by extraction of histones with buffers containing different concentrations of NaCl (0.25–1 M). The extraction of histone 3 (H3) was much lower in BHK-21p19AS than in BHK-21WT cells at 0.5 M of NaCl

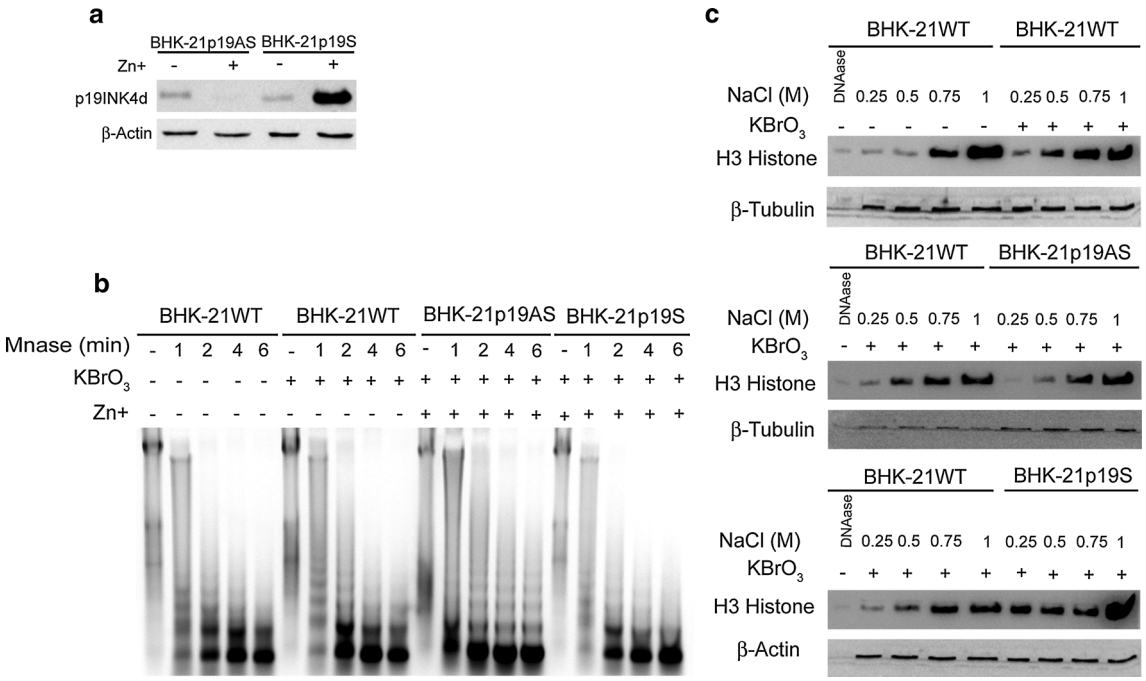

Fig. 4 p19 increases chromatin relaxation in $KBrO_3$-treated cells. **a** BHK-21 cells were stably transfected with an expression vector encoding sense (BHK-21p19S) or antisense (BHK-21p19AS) p19 cDNA under the regulation of the metallothionein promoter. Cell lines were incubated in the presence or in the absence of 75 μM ZnSO4 for 5 h and equal amounts of cell extract were analyzed by 15 % SDS-PAGE and subjected to immunoblotting with p19 antibody. **b–c** BHK-21WT and BHK-21p19S or BHK21-p19AS cells were pre-incubated with 75 μM $ZnSO_4$ for 5 h and treated with 20 mM $KBrO_3$ for 30 min. Following 1 h of recovery (**b**) nuclei were isolated and incubated with MNase (0.2 U/μl) at different times, after which equal amounts of DNA from each sample were subjected to agarose gel electrophoresis, **c** nuclei from BHK-21WT (*upper panel*), BHK-21p19AS (*middle panel*) and BHK-21p19S (*lower panel*) were isolated and assayed for histone core extraction with different NaCl concentrations. H3 histone was detected by western blot

(Fig. 4c). In addition, the amount of H3 extracted at 0.25 M of NaCl was greater in BHK-21p19S than in BHK-21WT. Together, these results suggest that electrostatic relaxation of chromatin is modulated by the p19-expression level.

p19 facilitates chromatin accessibility in vitro after DNA damage

In view of the above results, we next examined whether the presence of p19 would facilitate the access of a protein with enzymatic activity to its DNA target sequence in an in vitro chromatin assay. To do this, the linearized pET-CREB plasmid, containing a single digestion site for the *Xba*I restriction enzyme, was incubated with an extract from *D. melanogaster* preblastoderm embryos to obtain a chromatinized DNA structure. Then, both the chromatinized and the naked plasmid were incubated with a nuclear extract from BHK21-p19S or BHK21-p19AS cells previously irradiated with 20 J/m^2 UV and, after 1 h, *Xba*I was added to the incubations. DNA was extracted at different times, purified, and electrophoresed in agarose gels. While incubation with either BHK21-p19S or BHK21-p19AS extracts did not interfere with the exhaustive digestion of the naked plasmid, chromatinized DNA was digested, albeit poorly, by *Xba*I only when previously incubated with the BHK21-p19S extract (Fig. 5a). These results show that restriction enzyme accessibility to the chromatinized plasmid is facilitated by the presence of p19 in the nuclear extract.

We have previously demonstrated that p19 improves the repair of different types of DNA damage [22, 23]. Furthermore, the above results point to the chromatin structure as a target for p19 action. In order to confront our hypothesis, we performed an in vitro nucleotide excision repair assay using both naked and chromatinized DNA-damaged plasmid. The rationale was that if p19 exerts its action on DNA repair by rendering a more relaxed chromatin, the presence of p19 should lead to an improved DNA repair on the chromatinized plasmid but not on the naked one.

The in vivo MNase assay showed that, as well as in the case of $KBrO_3$ as DNA damager, chromatin from UV-treated BHK-21p19S cells was more susceptible to MNase digestion, demonstrating that p19 action is independent of the genotoxic used (Fig. 5b). Next, we carried out the in vitro DNA repair. To do this, the pET-CREB plasmid was UV-irradiated and either chromatinized or not, as

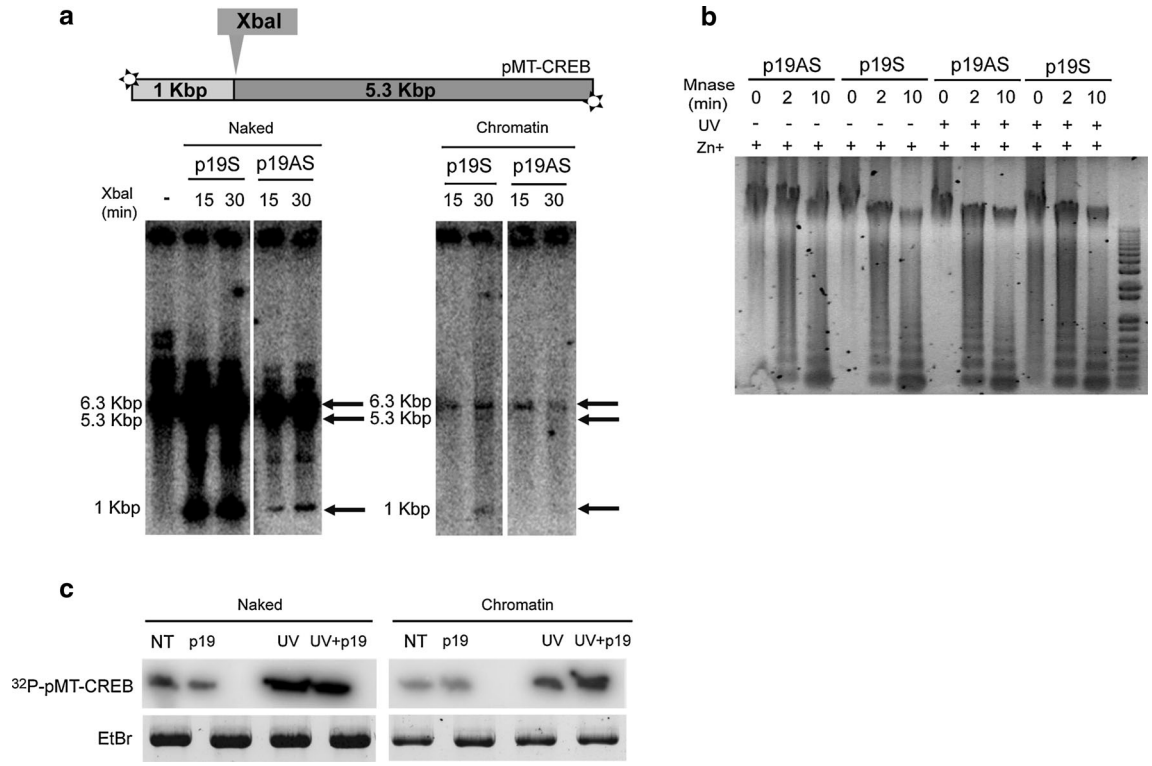

Fig. 5 p19 overexpression increases chromatin accessibility and DNA repair of a chromatinized plasmid in vitro. **a** The plasmid pET-CREB was linearized and incubated or not with an extract from *D. melanogaster* preblastoderm embryos. The naked and chromatinized plasmids were incubated with a nuclear extract from BHK-21p19S or BHK21-p19AS cells previously incubated with 75 μM $ZnSO_4$ for 5 h and irradiated with 20 J/m^2 UV. Then, both plasmids were incubated with *Xba*I for the indicated times and DNA was extracted, purified, and electrophoresed in agarose gels. **b** BHK-21p19S or BHK21-p19AS cells were pre-incubated with 75 μM $ZnSO_4$ for 5 h and irradiated with 20 J/m^2 UV. Nuclei were isolated and incubated with MNase (0.2 U/μl) at different times, after which equal amounts of DNA from each sample were subjected to agarose gel electrophoresis. **c** The naked and chromatinized pET-CREB plasmids were irradiated with 100 J/m^2 UV. Irradiated plasmids were treated with a reaction mix containing (α-^{32}P)dCTP and incubated with a nuclear extract from BHK-21p19S or BHK21-p19AS cells previously incubated with 75 μM $ZnSO_4$ for 5 h and irradiated with 20 J/m^2 UV. In vitro DNA repair was assessed and DNA reaction products were extracted, purified, and electrophoresed in agarose gels and analyzed by autoradiography

described above. Then, the plasmids were treated with a reaction mix containing (α-^{32}P)dCTP and incubated with a nuclear extract from BHK21-p19S or BHK21-p19AS cells previously irradiated with 20 J/m^2 UV. DNA reaction products were purified, electrophoresed in agarose gels, and analyzed by autoradiography. The extent of nucleotide excision repair of the UV-irradiated naked plasmid was similar in both extracts, regardless of the presence of p19. Conversely, DNA repair of the chromatinized plasmid was greater when the reaction mixture contained nuclear extracts from BHK-21p19S cells than when it contained those from BHK-21p19AS cells (Fig. 5c). Taken together, these results indicate that overexpression of p19 increases chromatin accessibility and thereby improves DNA repair in vitro.

DNA damage is linked to multiple human diseases, such as cancer and neurodegeneration, as well as to aging [49]. Nonetheless, relatively little is known about the role of chromatin accessibility in DNA repair. Eukaryotic DNA is packaged within nucleosomes, which represents an additional physical barrier for DDR factors to access damaged DNA [50, 51]. DDR factors are confronted with DNA packaged into nucleosomes, which fold the genome into highly condensed chromatin. Consequently, DNA repair mechanisms have to overcome this natural barrier, which restricts access to their substrate [52]. Studies on transcription have shown that this can be achieved in two ways; either by post-translational modification of histone tail residues (phosphorylation, acetylation, methylation or ubiquitination) or by chromatin remodeling through the action of large ATP-dependent complexes [53]. ATP-dependent chromatin remodeling is one of the fundamental mechanisms used by cells to relax chromatin in DNA repair. Numerous chromatin-remodeling factors, especially those from the INO80 and SWI2 subfamilies, are recruited to DSBs in response to DDR [54, 55]. Histone deacetylase

NuRD complex [56] and sirtuin 6 (SIRT6) [50] have been shown to be recruited to damage sites, suggesting the need of chromatin relaxation and remodeling to allow repair [49]. The histone acetyl transferase [13] Tip60 plays a critical role in DDR by interacting with the transcription factor E2F1 to promote its acetylation in response to DNA damage by cisplatin [57]. The ataxia telangiectasia mutant (ATM) protein kinase, which phosphorylates proteins involved in cell cycle checkpoints and DNA repair, is regulated by the HAT Tip60 in response to DNA damage [58].

Precisely how p19 contributes to DDR remains to be determined. Our observations indicate that p19 is tightly attached to chromatin, specifically to the euchromatin fraction. Our in vitro experiments showed that p19 increases chromatin accessibility and thus improves DNA repair. This suggests not only its association with chromatin but also its involvement in chromatin relaxation to perform its function in DNA repair.

One possibility is that p19 interacts with a chromatin-remodeling factor, altering its activity to promote chromatin relaxation. A yeast two-hybrid screen performed in our lab identified BRD7 as a potential p19-binding partner. BRD7 is a bromodomain containing protein, which is a subunit of PBAF-specific Swi/Snf chromatin remodeling complexes [59]. Drost et al. have recently demonstrated that BRD7 is required for efficient p53-mediated transcription of a subset of target genes. BRD7 interacts with p53 and p300 and is recruited to target gene promoters, affecting histone acetylation. Thus, BRD7 suppresses tumorigenicity by serving as a p53 cofactor [60]. BRD7 interacts physically not only with p53 and the acetylase p300 but also with TRIM24 [13] and BRCA1, thereby regulating genes involved in DNA repair [59]. Thus, it is possible that p19 interacts through BRD7 with these chromatin remodeling complexes, promoting histone acetylation and thereby inducing chromatin relaxation.

In summary, our results indicate that p19 plays an important role at early stages of cellular DDR and would belong to a protein network that would integrate chromatin relaxation and DNA repair to maintain genomic integrity.

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