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Running title: Chromatin remodeling at DNA lesions

ABSTRACT

Chromatin relaxation is one of the earliest cellular responses to DNA damage. However, what determines these structural changes, including their ATP requirement, is not well understood. Using live-cell imaging and laser micro-irradiation to induce DNA lesions, we show that the local chromatin relaxation at DNA damage sites is regulated by PARP1 enzymatic activity. We also report that H1 is mobilized at DNA damage sites but, since this mobilization is largely independent of poly(ADP-ribosyl)ation , it cannot solely explain the chromatin relaxation. Finally, we demonstrate the involvement of Alc1, a poly(ADP-ribose)- and ATP-dependent remodeler, in the chromatin relaxation process. Deletion of Alc1 impairs chromatin relaxation after DNA damage, while its over-expression strongly enhances relaxation. Altogether, our results identify Alc1 as an important player in the fast kinetics of the NAD^+- and ATP-dependent chromatin relaxation upon DNA damage *in vivo*.

INTRODUCTION

The complex multiscale architecture of chromatin poses a formidable challenge for the DNA repair machinery, which requires regulated access to DNA lesions. Early steps of the DNA damage response involve chromatin remodeling, leading to an increased sensitivity of chromatin to nucleases (Smerdon and Lieberman, 1978). Experiments in living cells have shown that DNA damage induced by laser micro-irradiation leads to an ATP-dependent, but ATM-independent, chromatin relaxation at sites of DNA damage (Kruhlak et al., 2006). While the dense packing of chromatin may hinder the efficiency of DNA repair (Schuster-Böckler and Lehner, 2012), recent reports also show that chromatin over-compaction at DNA lesions may also be important to inhibit transcription during repair and to keep the broken DNA ends in close proximity (Ayrapetov et al., 2014; Burgess et al., 2014).

One of the earliest events upon DNA damage is the recruitment and activation of poly(ADP-ribose) polymerase 1 (PARP1), a key regulator of chromatin structure during DNA repair and transcription (Lebeaupin et al., 2015). It is activated by DNA lesions and attaches poly(ADP-ribose) (PAR) to itself and other chromatin factors including histones. The binding of PARP1 to chromatin modifies its compaction state through multiple, sometimes opposite, mechanisms. Inactive PARP1 competes with the linker histone H1 leading to the formation of compact and transcriptionally repressed genomic regions (Kim et al., 2004). In contrast, PARylated polynucleosomes appear as a loose, beads-on-a-string fiber, on electron micrographs (Poirier et al., 1982). It was suggested that the PARylation of chromatinized H1 could counteract its ability to condense chromatin (Huletsky et al., 1989). Additionally, PARylation is also involved in the recruitment and the regulation of several chromatin remodeling enzymes whose ATP-dependent activity could promote chromatin relaxation (Chou et al., 2010; Polo et al., 2010; Smeenk et al., 2013).

In the present work, we sought to address the impact of PARP1 on chromatin structure and dynamics following DNA damage. Using photo-activated histones, live-cell imaging and laser micro-irradiation in human cells, we analyzed the contributions of PARylation, linker histone H1, ATP and the nucleosome remodeler Alc1 during the transient chromatin relaxation observed upon DNA damage.

RESULTS AND DISCUSSION

DNA damage induction by laser micro-irradiation induces a rapid chromatin relaxation at the DNA lesions

To assess large-scale chromatin reorganization at sites of DNA damage in living cells, we established an assay using human U2OS cells expressing the core histone H2B labeled with the photo-convertible dyes PAGFP or PATagRFP. By irradiating a predefined nuclear area with a 405 nm laser, we simultaneously photo-convert the tagged histones and, if cells have been Hoechst-presensitized, induce DNA lesions, allowing us to compare chromatin dynamics in the presence or absence of DNA damage (Figure 1A).

Upon micro-irradiation at 405 nm of cells expressing photoactivatable H2B and pre-sensitized with Hoechst, we observed a rapid increase of the size of the photo-converted chromatin area (Figure 1B-C), indicating chromatin relaxation at DNA damage sites, as previously reported (Kruhlak et al., 2006). However, an alternative interpretation could be the local release of photo-converted H2B through nucleosome remodeling induced upon DNA damage (Polo, 2015). To distinguish between these two possibilities, we labeled DNA by incorporating fluorescent nucleotides (Schermelleh et al., 2001). Upon irradiation, we observed the directional movement of fluorescent spots away from the irradiated line

(Figure 1D-E and supplementary movie 1), with a speed similar to the one characterizing the expansion of the H2B photo-converted area (Figure 1F). These results indicate that the changes in the size of the photo-activated H2B area upon DNA damage reflect the relaxation of chromatinized DNA, rather than the local release of photo-activated H2B. This fast initial chromatin relaxation upon DNA damage is followed by a slow recondensation with chromatin recovering a compaction state close to its pre-damage level in about 20 minutes (Figure 1G).

Chromatin relaxation at DNA damage sites is controlled by PARP1 activation

In agreement with recent reports (Khurana et al., 2014; Strickfaden et al., 2016), we observed that chromatin relaxation at DNA lesions is PARylation dependent (Figures 2A and S1A-D). Interestingly, inhibiting PARylation not only abolished chromatin relaxation at DNA damage sites but also induced a small but significant chromatin over-compaction upon laser micro-irradiation (Figure 2A). The human PARP enzyme family has multiple members and we found that PARP1, PARP2 and PARP3 are all recruited at DNA damage sites (Figure S1E). PARP1 is responsible for about 85% of PARylation activity (Woodhouse and Dianov, 2008). Therefore, to address the specific role of PARP1 in chromatin relaxation, we generated PARP1 knockout (KO) U2OS cell lines. While PARP1 was absent from these cells, we could detect similar amount of PARP2 and PARP3 as compared to wild-type (Figure S1F). Chromatin relaxation at DNA lesions was dramatically reduced in PARP1 KO cells (Figure 2A-B), a phenotype that could be partially rescued by re-expressing wild-type PARP1 (Figure 2C). Remarkably, laser irradiation in the PARP1 KOs did not lead to chromatin over-compaction, even after inhibition of PARylation (Figure 2A). Instead, we observed a residual chromatin relaxation independent of PARylation activity, suggesting that it was not triggered by the activity of other PARPs, such as PARP2 or PARP3. Altogether, since PARP inhibitors do not block the recruitment of PARP1 to DNA damage (Timinszky et al., 2009), our data suggest that chromatin over-compaction when inhibiting PARylation is due to PARP1 binding to DNA lesions, whereas its product, PAR, is responsible for chromatin relaxation. These findings reconcile oppositely reported effects of PARP1 on chromatin structure (Kim et al., 2004; Poirier et al., 1982).

Chromatin relaxation at DNA lesions is not directly triggered by the mobilization of linker histone H1

In vitro studies identified the linker histone H1 to be crucial for the formation of compact chromatin (Thoma et al., 1979). Since H1 is a substrate of PARP1, PARylation of H1 could trigger its dissociation from chromatin, as shown for regulated transcription (Ju et al., 2006), and lead to chromatin relaxation. To test this hypothesis, we analyzed H1 (H1.1 variant) dynamics at DNA lesions in cells co-expressing H2B-PATagRFP and H1-PAGFP, allowing us to simultaneously label the damaged chromatin area and follow the dynamics of the H1 proteins localized within this area at the time of irradiation (Figure 3A and supplemental movie 2). To quantify the redistribution of photo-activated H1 from the irradiated area independently of the co-occurring chromatin relaxation process, the integrated fluorescence signal for H1 was measured within the irradiated area defined by the segmentation of the H2B channel (Figure 3B).

We found that H1 initially localized within the irradiated area is released faster in the presence of DNA damage (Figure 3B-C). Knowing that most H1 molecules are bound to chromatin at any given time (Beaudouin et al., 2006), this can only reflect impaired binding to chromatin. Once the photoconverted H1 proteins are redistributed over the entire nucleus, the DNA damage area appears to be depleted for H1 (Figure S1G). This depletion progressively disappears as chromatin slowly recondenses. At the same time, we observed no significant release of the core histone H2B from the irradiated region (Figure S1H).

Inhibiting PARylation reduced H1 dynamics both in the presence and absence of DNA damage, while deleting PARP1 only slowed down H1 dynamics in the presence of DNA damage (Figure. 3C). These data are consistent with the observation that the PARylation of H1 increases its dynamics (Ju et al., 2006). Nevertheless, even in the presence of PARP inhibitor or in the PARP1 KO cells, H1 dynamics were always much faster after DNA damage as compared to the dynamics observed when no damage was induced (Figure 3C). Since, at the same time, chromatin relaxation was abolished in cells treated with PARP inhibitors and strongly reduced in the PARP1 KOs (Figure 2A), it indicates that chromatin loosening at DNA lesions is not the direct consequence of PAR-driven H1 mobilization at DNA lesions. This result contrasts with a recent report which correlates H1 eviction and PAR-dependent chromatin relaxation at DNA lesions (Strickfaden et al., 2016). The discrepancy with our findings may arise from the difference in laser irradiation methods, which could lead to different DNA damage (Kong et al., 2009). Nevertheless, we cannot exclude that the PARylation-dependent chromatin relaxation requires concomitant H1 mobilization, that is always observed upon DNA damage independently of PARP1 activation. Furthermore, it is possible that the DNA damage-induced H1 mobilization accounts for the observed residual chromatin relaxation observed in the PARP1 KOs.

Contribution of ATP-dependent processes in chromatin relaxation at DNA lesions

In vitro PARP1 activation results in chromatin loosening in the absence of ATP (Poirier et al., 1982), whereas ATP depletion abolishes chromatin relaxation at DNA lesions in live cells (Kruhlak et al., 2006; Luijsterburg et al., 2012). In order to establish the role of ATP in our assays, we quantified chromatin relaxation and PARylation levels upon laser micro-irradiation in cells depleted for ATP. We found that ATP depletion significantly impaired chromatin relaxation upon DNA damage (Figure 4A) while not affecting the level of PARylation at the lesions, as shown by the similar accumulation of the PAR-binder WWE domain of RNF146 at DNA damage sites (Figure 4B) (Wang et al., 2012). Nevertheless, ATP depletion did not fully abolish chromatin relaxation, its amplitude corresponding to approximately half of the control situation. This result suggests that PARylation may act on chromatin in both ATP-dependent and -independent ways but it may also be due to only partial depletion of ATP. A confounding effect of ATP inhibition is chromatin hyper-condensation (Figure 4C). To test if chromatin hyper-condensation could explain the inhibition of chromatin relaxation seen upon ATP depletion, we induced chromatin tightening in another way. Cells were bathed in hypertonic media to induce a shrinking of the nuclear volume (Figure 4D-E) which, in turn, leads to chromatin hyper-condensation. The chromatin patterns in hypertonic cells visually resembled those obtained after ATP-depletion (Figure 4C). The hypertonic treatment itself does not activate the PARylation signaling pathway (Figure 4F-G). In hypertonic cells, chromatin loosening upon DNA damage was slightly increased as compared to isotonic controls (Figure 4H), while PARylation at the site of damage was unchanged (Figure 4I). Thus, the reduction of chromatin relaxation at DNA lesions observed upon ATP depletion does not seem to be the mere consequence of a tighter chromatin packing prior to damage induction.

The ATP-dependent remodeler Alc1 contributes to chromatin relaxation at DNA damage sites

Several ATP-dependent chromatin-remodeling enzymes have been shown to be regulated by PARP activation (Chou et al., 2010; Polo et al., 2010; Smeenk et al., 2013). However, the only chromatin-remodeling enzyme with an ADP-ribose-binding domain that actively remodels nucleosomes upon PARP1 activation is Alc1 (Ahel et al., 2009; Gottschalk et al., 2009; Pines et al., 2012). To address the role of Alc1, also known as CHD1L, in chromatin relaxation, we generated an Alc1 KO U2OS cell line (Figure S2A). By co-expressing a fluorescently tagged version of Alc1 together with H2B-PAGFP in these cells, we followed the recruitment of this protein at DNA damage sites together with the relaxation process (Figure 5A-B). The fast accumulation of Alc1 observed at the site of DNA damage, with a maximum recruitment a few seconds after laser micro-irradiation, is compatible with a role for Alc1 in chromatin relaxation at DNA breaks, a process that lasts approximately 60s. Moreover, the recruitment of Alc1 at DNA damage sites was abolished by PARP inhibitor treatment or for Alc1 lacking the PAR-

binding macrodomain (Figure S2B-C), indicating that Alc1 recruitment, similar to chromatin relaxation, is fully controlled by PARP1 activation at DNA lesions.

The loss of Alc1 had no detectable effect on chromatin architecture in the absence of DNA damage (Figures 5C and S2D-E) but led to impaired chromatin relaxation upon laser irradiation (Figures 5D and S2F-G). Expression of wild-type Alc1 in Alc1 KOs fully restored chromatin relaxation at DNA lesions in contrast to the expression of ATPase-dead Alc1 mutants (Alc1-E175Q or Alc1-K77R) despite their efficient recruitment at DNA damage sites (Figure S2B,H). Cells depleted for Alc1 using RNAi behaved in a similar manner (Figure S2I-J). While ATP depletion only slightly reduced chromatin relaxation in the Alc1 KO cells (Figure 5E), the inhibition of PARylation completely suppressed the relaxation process (Figure 5F) suggesting that the remaining chromatin relaxation observed at DNA damage sites in the absence of Alc1 is mediated mainly by the ATP-independent loosening effect of PARylation. Importantly, the over-expression of Alc1 in wild-type cells strongly increased chromatin relaxation at DNA lesions, while over-expressing the ATPase-dead Alc1-E175Q had no effect (Figure 5G). Altogether, these results identify Alc1 as a mediator of PARylation-dependent chromatin relaxation through its ATP-dependent remodeling activity. A recent publication also reported the role of the remodeler CHD2 in the chromatin relaxation at DNA lesions (Luijsterburg et al., 2016). Since CHD2 appears to be recruited at the DNA damage sites slightly later than Alc1, the two remodelers may act sequentially to allow chromatin loosening. Further work is required to understand how the activities of these two remodelers are coordinated.

In conclusion, our present work extends our understanding of the contribution of the PARylation signaling pathway in the early chromatin remodeling at DNA lesions. We demonstrate the dual impact of PARP1 on the chromatin structure. In line with in-vitro observations (Kim et al., 2004), our data in living cells indicate that PARP1 binding to DNA breaks leads to chromatin over-compaction while the formation of PAR chains due to PARP1 activity triggers its relaxation, in agreement with a recent report (Strickfaden et al., 2016). Moreover, our data shows for the first time the direct contribution of the ATP-dependent chromatin remodeling activity of the remodeler Alc1 in the rapid chromatin relaxation observed upon DNA damage induction. We also found that the absence of either PARP1 or Alc1 reduces cell survival capacity upon X-ray irradiation (Figure 5H). This result, that corroborates other reports showing that several members of the PARylation signaling pathway are important for efficient DNA repair (Khurana et al., 2014; Nagy et al., 2016), argues for a key regulatory role of the PARylation-dependent modulation of the chromatin compaction state during the DNA damage response. In addition, we propose that the dramatic increase in chromatin relaxation together with the cell hypersensitivity to X-ray irradiation observed in case of Alc1 over-expression (Figure 5G-H) might underlie the oncogenic potential of this remodeler, which has been shown to promote cancer progression and metastasis (Cheng et al., 2013).

MATERIAL AND METHODS

Plasmids

The core histone H2B, subcloned from the pH2B-mCherry vector (gift from J. Ellenberg, Neumann et al., 2010, Euroscarf accession number P30632), was cloned into pPATagRFP-N1 using NdeI and BamHI restriction sites. pPATagRFP-N1 was a gift from V. Verkhusha (Subach et al., 2010), Addgene plasmid # 31941). The histone H2B-PAGFP and histone H1.1-PAGFP were gifts from J. Ellenberg (Beaudouin et al., 2006), Euroscarf accession number P30499 and P30503, respectively. Another construct of H1.1-

PAGFP was produced with the PAGFP tag on the other side of the protein to ensure that similar results could be obtained with both constructs (Hutchinson et al., 2015). H1.1 was PCR amplified from the H1.1-PAGFP plasmid and subcloned into pmEGFP-N1 using BglII and ApaI to obtain the H1.1-EGFP construct. Wild type Alc1 and E175Q Alc1 mutant fused to the C-terminus of EGFP or mCherry were obtained by exchanging YFP for the respective fluorescent protein in the constructs described previously (Gottschalk et al., 2009). The Alc1-Δmacro mutant fused to YFP was described previously (Gottschalk et al., 2009).The Alc1-K77R construct fused to mCherry was obtained by first mutating a wild type ALC1 construct fused to YFP (Gottschalk et al., 2009) using Quikchange *in-vitro* mutagenesis (Agilent), and then exchanging YFP for mCherry. The WWE domain of RNF146 (amino acids 99-183) was cloned into pmEGFP-C1 using BglII and EcoRI by PCR amplifying it from a cDNA library. PARP1-mCherry was described previously (Timinszky et al., 2009). This construct was also used to generate PARP1-EGFPby exchanging mCherry with EGFP. PARP2-EGFP was generated by PCR cut with NheI/SmaI into pmEGFPC1 with NheI/SmaI. PARP3-EGFP (short isoform) is a gift from C. Prigent (Rouleau et al., 2007). Mammalian expression was under the control of CMV promoter. All constructs were sequence verified.

Cell culture, inhibitor treatments, and osmotic shocks

Cells used for this work were wild-type U2OS cells or knockout cells made from parental U2OS cells. Cells were routinely cultured in Dulbecco's modified Eagle's medium (with 4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/mL penicillin, 100 U/mL streptomycin in 5% CO_2 at 37 °C. For microscopy, cells were plated on Lab-Tek II chambered coverglass (Thermo Scientific). Pre-sensitization was achieved by bathing cells for 1h in culture medium containing 0.3 µg/mL Hoechst 33342 (Life Technologies). Immediately before imaging, the growth medium was replaced by Leibovitz's L-15 medium (Life Technologies) supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 µg/mL penicillin and 100 U/mL streptomycin. The PARP1 inhibitors AG14361 and Olaparib (Euromedex) were used at 30 µM and 50 µM, respectively. ATP depletion was achieved as described in (Platani et al., 2002). The osmotic shock procedure was previously described in (Walter et al., 2013). All experiments presented in this work were performed on unsynchronized cells.

Live cell DNA labeling with fluorescent nucleotides

U2OS cells expressing H2B-PATagRFP were synchronized at the G1/S phase transition by treating the cells with aphidicolin (Sigma) at 5 µg/mL for 18h. After aphidicolin release, the cell layer, bathed with growing medium containing 10 µM of dUTP-ATTO633 (Jena-Bioscience), was scraped using a silicon stick to allow nucleotide loading and integration to the DNA during replication (Schermelleh et al., 2001).

Transfections and generation of stable and knockout cell lines

Transient transfections were performed 12-24h after plating cells using XtremeGENE HP (Roche) or JetPRIME (Polyplus Transfection) according to manufacturer's instructions. Cells were imaged 48 to 72h after transfection.

To establish cell lines stably expressing H2B-PATagRFP or Alc1-YFP (construct described in Gottschalk et al., 2009), wild-type U2OS cells were transfected with the appropriate DNA construct and grown in culture medium containing Geneticin (PAA) for selection. Clones with stably integrated H2B-PATagRFP or Alc1-YFP were picked after two weeks of Geneticin selection. Once selected, these cells were cultured in normal medium supplemented with 500 µg/mL Geneticin (PAA).

The knockout cell lines were made according to the protocol described by the Zhang lab (Ran et al., 2013). The target sequence for *ALC1* (5'-GACTTCCCTCAAGTACGTTAG-3') and PARP1 (5'-GTCCAACAGAAGTACGTGCAA-3') was chosen according to the web-based CRISPR design tool from

Zhang lab (http://www.genome-engineering.org). The sgRNA oligos were introduced into pX458 expressing Cas9 nuclease fused to GFP (Addgene #48138). pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138). We transfected the plasmids using the transfection reagent XtremeGENE HP (Roche) according to manufacturer's protocol Single GFP positive cells were sorted into 96-well plates using FACS. The knockout cell lines grown up from the single cells were identified by western blot using specific antibodies against PARP1 or Alc1.

siRNA knock down

For RNAi-mediated knockdown, we used Silencer® Select Negative Control No. 2 (ref 4390846) and siRNA against Alc1 (CHD1L) (ref s18358) from Ambion (Thermo Fisher Scientific). Cells grown in normal culture medium were transfected with 500 nM siRNA using Oligofectamine (Life Technologies) according to the manufacturer's instructions. After 48h, cells were used for imaging or harvested for protein analysis.

Western blot

Cell lysates were separated using SDS-PAGE, and transferred to nitrocellulose membranes (GE Healthcare) and blocked in 5% (w/v) milk powder in 0.05% (v/v) PBS-Tween 20 at room temperature. The primary antibodies were diluted in 5% (w/v) milk powder in 0.05% (v/v) PBS-Tween 20 and used at the following concentrations: affinity purified anti-Alc1 rabbit polyclonal, 1:2500; anti-Actin (Sigma-Aldrich, A5060), 1:1000; anti-PARP1 rabbit polyclonal, 1:10000; anti-PARP2 polyclonal rabbit antibody (Active Motif, #39743), 1:1000; anti-PARP3 polyclonal rabbit (Thermo Fisher, PA5-21478), 2 µg/ml and the mouse monoclonal (DM1A) anti-tubulin (Sigma-Aldrich T9026), 1:20000. To detect primary antibodies, HRP-conjugated secondary antibodies were used. The HRP-conjugated anti-rabbit IgG and anti-mouse IgG antibodies (Bio-Rad) were used at 1:10000 and the blot was developed using the ELC reagent (Merck Millipore).

Immunofluorescence staining

Cells were washed once in PBS and incubated in serum free DMEM containing DMSO, Olaparib (50 µM) or AG14631 (30 µM) for one hour. Cells were then exposed to H2O2 (0.5 mM) in serum free DMEM for 10 min. Cells were fixed in ice-cold methanol:acetone (1:1) for 10 min. Cells were washed once with PBS and then blocked for 1 h (5% milk in PBS + 0.05% Tween-20). Cells were incubated with anti-poly-ADPr mouse monoclonal 10H antibody (ascites) diluted (1:800) in blocking buffer overnight at 4°C. Cells were washed three times with PBS + 0.1% Triton X-100 before being incubated with Alexa Flour 488 anti-mouse IgG (Life Technologies) (4 µg/ml) in blocking buffer for 1 hr. Cells were washed three times with PBS + 0.1% Triton X-100 and nuclei were stained using Hoescht (1 µg/mL) for 10 min. Cells were washed twice with PBS + 0.1% Triton X-100 before imaging.

Quantification of cell viability upon X-ray irradiation

Cells were seeded at a density of 500 cells per well in a 12-well plate. Plates were immediately treated with X-ray irradiation (1 or 2 Gray, Fixatron) and returned to the incubator for 11 days to allow colony formation. Cells were washed once with PBS before being fixed and stained for 30 min with a 4% paraformaldehyde and 0.5% crystal violet solution. Staining solution was removed and plates were immersed in water to remove excess stain. The colony area percentage was calculated using the ColonyArea plugin for ImageJ according to Guzmán et al., 2014. Average colony area percentage was normalised to an untreated control.

Live cell imaging and laser micro-irradiation

Live cell imaging was performed on an inverted confocal spinning disk (imaging scan head CSU-X1 from Yokogawa and microscope body Ti-E from Nikon) equipped with a single-point scanning head to allow laser micro-irradiation and local photoactivation using a 405 nm laser. We used a Plan APO 63x oil immersion objective lens (O.N. 1.4) and a sCMOS ORCA Flash 4.0 camera (Hamamatsu) for imaging the cells. The pixel resolution at the object plane was 108 nm. The fluorescence of EGFP and the activated form of PAGFP was excited with a laser at 488 nm and the one of mCherry and the activated form of PATagRFP was excited with a laser at 561 nm. For fluorescence detection, we used band pass filters adapted to the fluorophores. Laser powers were adjusted to minimize bleaching during the time-lapse acquisitions. Photoactivation and DNA damage were induced simultaneously using a 405 nm laser. The power of the 405 nm laser used for photoactivation and, for cells pre-sensitized with Hoechst, induction of DNA lesions, was set to 125 µW at the sample level, unless stated otherwise. Cells were irradiated along a 16 µm-long line crossing the nucleus. The microscope is equipped with a heating chamber to maintain cells at 37 °C during the imaging experiments. When performing long timelapse experiments of 30 to 60 minutes to study chromatin remodeling in response to DNA damage, premature cell death that would indicate a phototoxic effect due to imaging was never observed.

Images shown on Figure S1A,C were taken on an inverted AxioObserver Z1 confocal spinning-disk microscope (Zeiss) equipped with a single-point scanning head for laser micro-irradiation and local photoactivation using a 405 nm laser (Rapp OptoElectronic). We used a C-Apo 63× water immersion objective lens (O.N. 1.2) and the images were acquired on a AxioCam HRm CCD camera (Zeiss). The pixel resolution at the object plane was 171 nm. The fluorescence of EGFP and YFP was excited with a laser at 488 nm and the one of the activated form of PATagRFP was excited with a laser at 561 nm. For fluorescence detection, we used band pass filters adapted to the fluorophores. The micro-irradiation conditions at 405 nm were adjusted to obtain amplitudes of the chromatin relaxation at DNA lesions which were similar to those obtained with the system described above. The cells were maintained at 37°C using a heating chamber.

Image analysis

The time-lapse sequences were analyzed automatically using custom-made routines written in MatLab (MathWorks) to quantify chromatin relaxation at DNA lesions. The chromatin area micro-irradiated at 405 nm and tagged with photoactivatable H2B were segmented by k-means segmentation. An ellipsoid was fitted to the segmented area and its minor axis length was used to estimate the width of the micro-irradiated chromatin area and thus assess changes in the chromatin compaction level.

To analyze the release of the photoactivatable H1 proteins from the area irradiated at 405 nm, the H1 integrated intensity was measured inside the segmented micro-irradiated chromatin area in cells co-expressing H1 and H2B tagged with two different photoactivatable dyes. This intensity was divided by the H1 intensity integrated over the whole nucleus to correct for bleaching and small focus drifts. For this step, the whole nuclei were segmented using the low fluorescence signal coming from the non-activated tagged H2B proteins. The same approach was used to analyze the release of the H2B proteins from DNA lesions and to characterize Alc1 recruitment kinetics.

When necessary, nuclei movements occurring during the timelapse experiments were corrected using the ImageJ plugin StackReg (Thévenaz et al., 1998).

The accumulation of the fluorescently tagged WWE domain of RNF146 at the DNA lesions was quantified as follows. By manual segmentation, the mean fluorescence intensity in three areas was

estimated: at the site of DNA damage (I_d), in a region of the nucleus not subjected to laser irradiation (I_{nd}) and outside the cells (I_{bg}). The accumulation of the WWE domain at the DNA lesions A_{WWE} was then calculated as:

$$A_{WWE} = \frac{I_d - I_{bg}}{I_{nd} - I_{bg}}$$

For quantifying the immunofluorescence staining with anti-PAR (10H) antibody, the nuclei were segmented using Hoechst staining and the mean fluorescence intensity for the anti-PAR antibody was measured inside each nucleus after background subtraction.

For chromatin texture analysis, wild-type and Alc1 knockout U2OS cells were plated on Lab-TekII-chambered coverglass, fixed with 4% paraformaldehyde for 10 min at room temperature and stained with Hoechst 33342 (1 µg/mL) for 1h. Confocal images were captured on a Leica SP8 confocal microscope using a Plan APO 63x oil immersion objective lens (O.N. 1.4). Hoechst staining was excited with a 405-nm laser and the emission band was chosen to optimize fluorescence collection. The pinhole was set to one Airy unit and we used a pixel size of 60 nm. To analyze chromatin texture, the GLCM ImageJ texture plugin written by Julio E. Cabrera was used. The correlation and contrast parameters were chosen to characterize chromatin texture using a pixel-to-pixel distance of 7 pixels, which allowed maximizing the differences measured between the cells bathed with the isotonic medium and those subjected to osmotic stress.

In cells labeled with fluorescent nucleotides, the chromatin dynamics was assessed by tracking the fluorescently labeled DNA replication foci using the plugin Particle Tracker from Image J (Sbalzarini and Koumoutsakos, 2005).

Statistics

In the figure legends, n refers to the number of cells used for a given experimental condition. Boxplots were generated using a web-tool developed by the Tyers and Rappsilber labs (http://boxplot.tyerslab.com/). The box limits correspond to the 25th and 75th percentiles and the bold line indicates the median value. The whiskers extend 1.5 times the interquartile range and outliers are shown by dots. The numbers in parentheses refers to the number of cells for each condition. Unless stated otherwise, p values were calculated using unpaired Student's t-test assuming unequal variances. On the boxplots, * refers to $p<0.05$, ** to $p<0.01$, *** to $p<0.001$, **** to $p<0.0001$ and n.s. to non significant.

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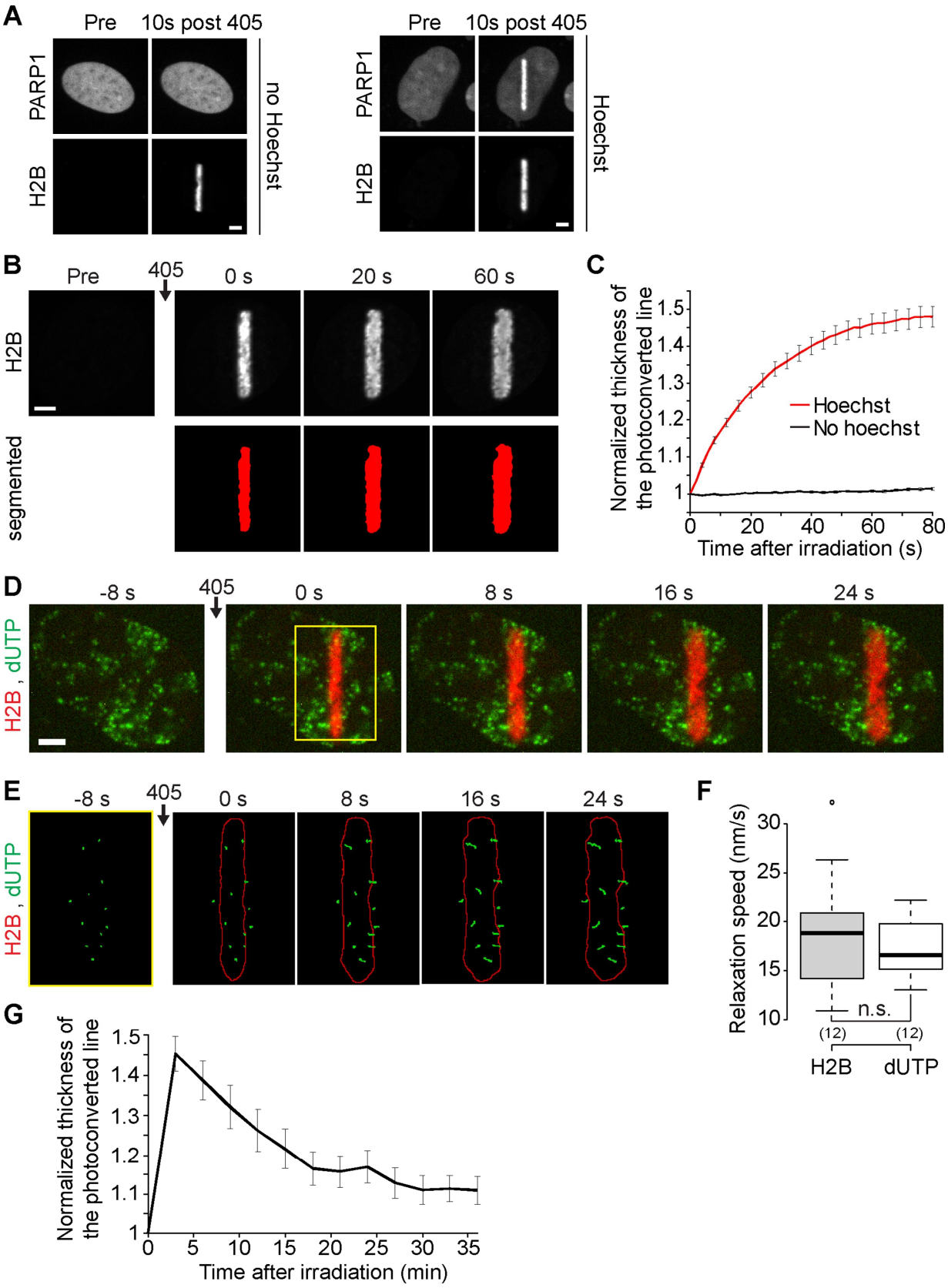
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PARP1
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B
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Normalized thickness of the photoconverted line
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Time after irradiation (s)
D
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16 s
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Relaxation speed (nm/s)
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H2B
dUTP
G
Time after irradiation (min)

Figure 1. DNA damage induced by laser microirradiation induces transient chromatin relaxation.(A) Recruitment of PARP1 at the micro-irradiated area in cells co-expressing PARP1-mCherry and H2B-PAGFP. Bar = 4 μm. In cells non pre-sensitized with Hoechst, the 405 nm irradiation induces local photoactivation of the H2B-PAGFP but no recruitment of PARP1-mCherry. In contrast, in case of Hoechst pre-sensitization, the 405 nm irradiation induces both photoactivation of the H2B-PAGFP and a marked recruitment of PARP1-mCherry, indicating the presence of DNA lesions. Similarly, we observed the recruitment of 53BP1 only in cells pre-sensitized with Hoechst (data not shown).(B) Confocal image sequence of a human U2OS nucleus expressing H2B-PAGFP. Bar = 4 μm. The automatic segmentation of the histone H2B channel is shown in red below the raw images. The average thickness of the segmented line can be plotted as a function of time after irradiation, as shown in (C) for cells pre-sensitized (n=17) or not (n=23) with Hoechst (mean ± SEM). Based on this analysis, the ratio between the thicknesses of the photo-converted line at time = 60s and time = 0s can be calculated to estimate the relative relaxation of the irradiated region. (D) Confocal image sequence of a U2OS cell expressing H2B-PATagRFP (red) and labeled with fluorescent nucleotides dUTP-ATTO633 (green). Bar = 4 μm. (E) Enlarged view of the region overlaid in yellow on the previous panel. On the images are shown the segmentation of the photo-converted chromatin area (red outline) and trajectories of individual foci labeled with fluorescent nucleotides (green). For this experiment, the power of the 405 nm laser used for simultaneous photo-activation and micro-irradiation was set to 250 μW at the sample level, instead of 125 μW, to induce an enhanced chromatin relaxation allowing an easier identification of the phase of directed motion for the dUTP-labeled foci. (F) Comparison between the speed at which the width of the H2B labeled region is growing and the speed of the dUTP-labeled foci perpendicular to the irradiation line. We show the average speed for the 30s subsequent to laser micro-irradiation. p values were calculated by paired t-test. (G) Dynamics of the chromatin compaction state at DNA damage sites over long time scales measured in wild type U2OS cell expressing H2B-PATagRFP (mean ± SEM, n=16).

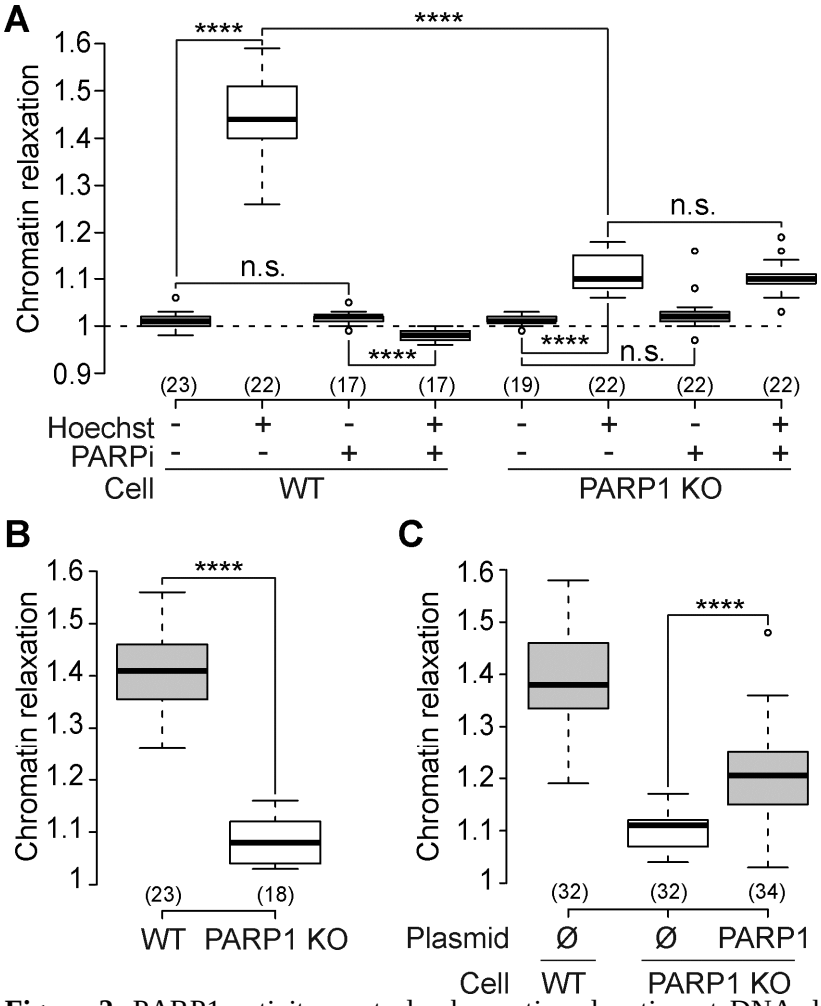


Figure 2. PARP1 activity controls chromatin relaxation at DNA damage sites. (A) Relative chromatin relaxation at 60s after laser micro-irradiation in wild type and PARP1 knockout cells (clone C8) transfected with H2B-PAGFP and treated or not with the PARP inhibitor AG14361 (30 µM, 1h).(B) Similar results were obtained with a second PARP1 knockout cell clone (clone C12).(C) Partial rescue of the impairment of chromatin relaxation in the PARP1 knockout cells (clone C8) by re-expression of wild-type PARP1 fused to mCherry

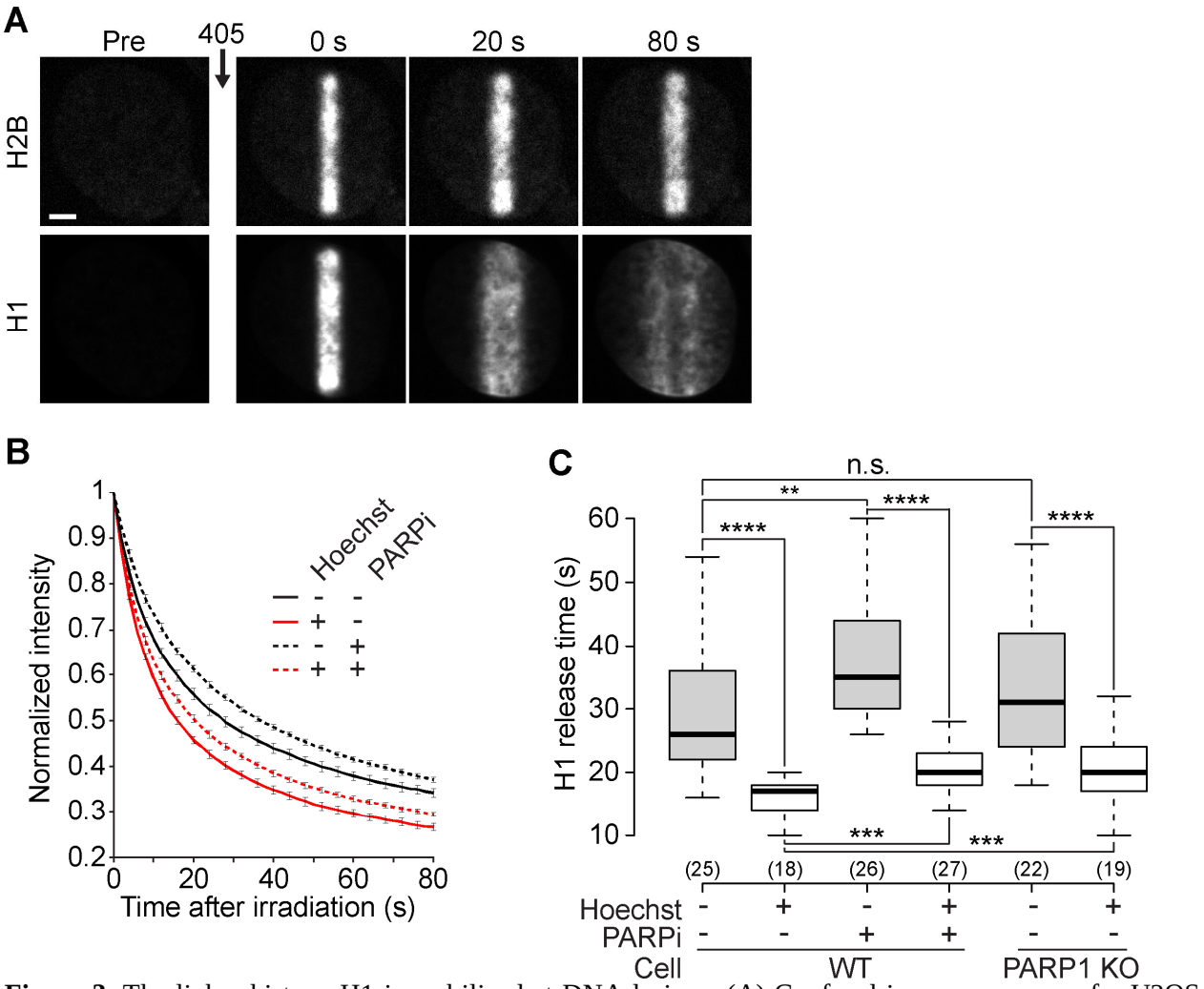
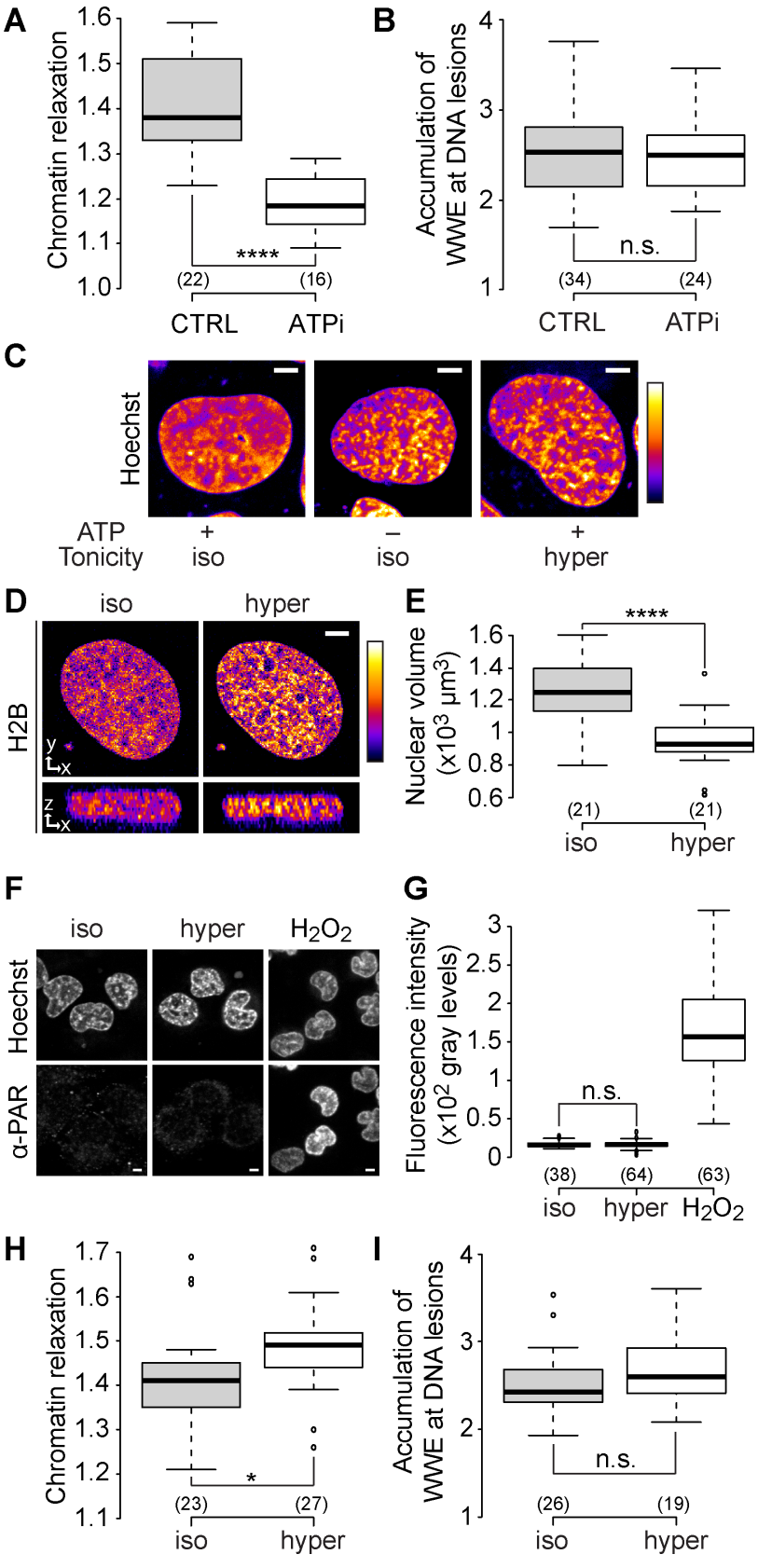


Figure 3. The linker histone H1 is mobilized at DNA lesions. (A) Confocal image sequence of a U2OS nucleus co-expressing H2B-PATagRFP and H1.1-PAGFP. For the H1 channel, the image contrast was enhanced to allow the visualization of H1 redistribution over the entire nucleus following laser micro-irradiation. This led to an apparent saturation of the image at time = 0s. Bar = 4 µm. (B) Kinetics of the release of the H1 proteins localized at the DNA lesions at the time of laser micro-irradiation in wild-type cells co-expressing H2B-PATagRFP and H1.1-PAGFP, pre-sensitized or not with Hoechst and treated or not with the PARP1 inhibitor AG14361 (30 µM, 1h) (mean ± SEM, for each condition, $17<n<28$). (C) Characteristic release time for H1, measured at half fluorescence decay, in wild type and PARP1 knockout cells.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(22)
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Accumulation of WWE at DNA lesions
4
3
2
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+
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Nuclear volume (x10³ µm³)
1.6
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Fluorescence intensity (x10² gray levels)
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2
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Chromatin relaxation
1.7
1.6
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1.4
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1.2
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(23)
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iso
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I
Accumulation of WWE at DNA lesions
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3
2
1
n.s.
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Figure 4. Chromatin relaxation at DNA damage sites partially depends on ATP.(A) Relative chromatin relaxation at 60s after laser micro-irradiation in wild type cells expressing H2B-PAGFP and depleted or not for ATP. (B) Accumulation of the WWE domain of RNF146 at the DNA lesions estimated 60s after laser micro-irradiation in wild type cells expressing an EGFP tagged version of WWE and depleted or not for ATP. (C) Confocal image of U2OS cell nuclei stained with Hoechst and left untreated, depleted for ATP or bathed with hypertonic medium. Bar = 4 μm. (D) Middle xy and xz sections of raw confocal image stacks of a U2OS cell expressing H2B-EGFP before and after the change of the bathing medium from isotonic to hypertonic. Bar = 4 μm. For panels (C) and (D), fluorescence signals are pseudocolored using the lookup table shown on the right of the images. (E) Change in nuclear volumes of U2OS cells expressing H2B-EGFP upon hypertonic treatment. The nuclear volumes were estimated by automatic segmentation of confocal image stacks. (F-G) Images and quantification of immunofluorescence staining with anti-PAR (10H) antibody performed in U2OS cells left untreated, subjected to hypertonic shock or treated with H2O2 (1 mM in PBS for 10 min). (H) Relative chromatin relaxation at 60s after laser micro-irradiation in wild type cells expressing H2B-PAGFP and bathed in isotonic or hypertonic media. (I) Accumulation of the WWE domain of RNF146 at the DNA lesions estimated 60s after laser micro-irradiation in wild type cells bathed in isotonic or hypertonic media.

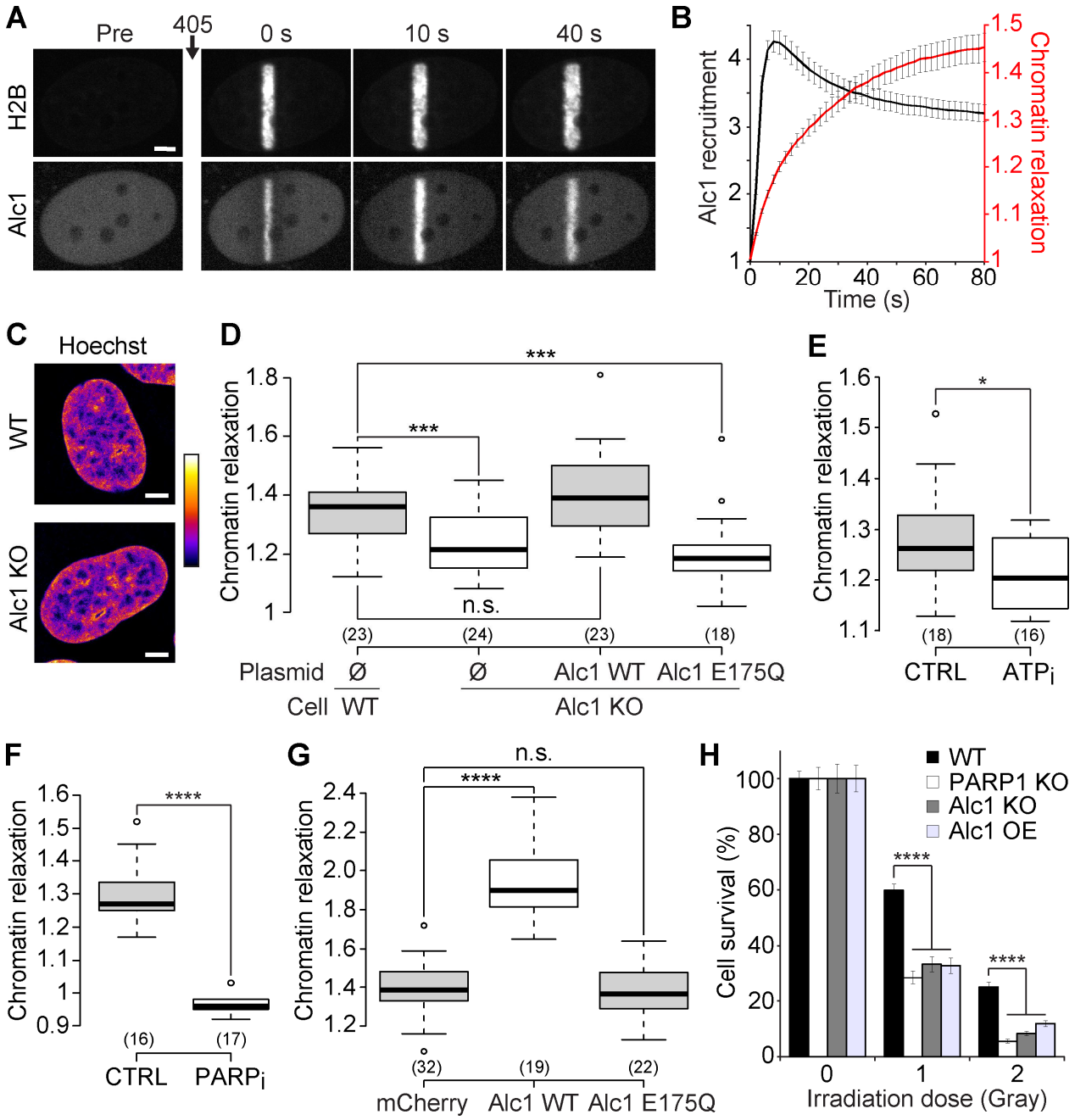

Figure 5. The chromatin remodeler Alc1 contributes to chromatin relaxation upon DNA damage. (A) Confocal image sequence of a U2OS nucleus co-expressing H2B-PAGFP and Alc1-mCherry. Bar = 4 µm. (B) Relative kinetics of Alc1 recruitment and chromatin relaxation at the DNA lesions measured in Alc1 knockout cells co-expressing H2B-PAGFP and Alc1-mCherry (mean ± SEM, *n*=20). (C) Confocal images of wild-type and Alc knockout U2OS cells labeled with Hoechst. Bar = 4 µm. Fluorescence signals are pseudocolored using the lookup table shown on the right of the images. (D) Relative chromatin relaxation at 60s after laser micro-irradiation for wild type cells versus Alc1 knockout cells co-transfected with H2B-PAGFP and an empty plasmid (Ø), wild type Alc1 or the catalytic-dead mutant Alc1 E175Q, both fused to mCherry. Cells with comparable expression levels of the wild-type or mutant Alc1 constructs were chosen, as assessed by similar fluorescence signals in the mCherry channel. (E) Relative chromatin relaxation at 60s after laser micro-irradiation in Alc1 knockout cells

expressing H2B-PAGFP and depleted or not for ATP. (F) Relative chromatin relaxation at 60s after laser micro-irradiation in Alc1 knockout cells expressing H2B-PAGFP and treated or not with the PARP1 inhibitor AG14361 (30 μM, 1h) (G) Relative chromatin relaxation at 60s after laser micro-irradiation for wild type cells expressing H2B-PAGFP and transfected with uncoupled mCherry, wild type Alc1 fused to mCherry or the catalytic-dead mutant Alc1 E175Q fused to mCherry. Cells with comparable expression levels of the transfected constructs were chosen, as assessed by similar fluorescence signals in the mCherry channel. (H) Clonogenic survival after different doses of X-ray irradiation for wild-type U2OS cells, knockouts for Alc1 and PARP1 and wild-type cells over-expressing Alc1 fused to YFP.