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TECHNICAL NOTE

Translocation of chromatin proteins to nucleoli—The influence of protein dynamics on post-fixation localization

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Abstract

It is expected that the subnuclear localization of a protein in a fixed cell, detected by microscopy, reflects its position in the living cell. We demonstrate, however, that some dynamic nuclear proteins can change their localization upon fixation by either crosslinking or non-crosslinking methods. We examined the subnuclear localization of the chromatin architectural protein HMGB1, linker histone H1, and core histone H2B in cells fixed by formaldehyde, glutaraldehyde, glyoxal, ethanol, or zinc salts. We demonstrate that some dynamic, weakly binding nuclear proteins, like HMGB1 and H1, may not only be unexpectedly lost from their original binding sites during the fixation process, but they can also diffuse through the nucleus and eventually bind in nucleoli. Such translocation to nucleoli does not occur in the case of core histone H2B, which is more stably bound to DNA and other histones. We suggest that the diminished binding of some dynamic proteins to DNA during fixation, and their subsequent translocation to nucleoli, is induced by changes of DNA structure, arising from interaction with a fixative. Detachment of dynamic proteins from chromatin can also be induced in cells already fixed by non-crosslinking methods when DNA structure is distorted by intercalating molecules. The proteins translocated during fixation from chromatin to nucleoli bind there to RNA-containing structures.

KEYWORDS

chromatin, fixative, nucleus, nucleolus, translocation

Mirosław Zarębski and Rosevalentine Bosire contributed equally to this work.

Rosevalentine Bosire is on study leave from the Technical University of Kenya, Nairobi, Kenya.

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1 | INTRODUCTION

Many nuclear proteins interact with chromatin, some very stably while others only weakly and intermittently [1]. Histones in a core of a nucleosome are an example of stable association with DNA [1]. The majority of core histones remain bound to DNA for hours, and a mobile pool (i.e., molecules momentarily not bound to DNA) of core histones diffusing in the nucleoplasm is very small. Many other proteins are dynamic, i.e. they interact with chromatin only intermittently, and their mobile pools are considerable. Linker histone H1 belongs to the group of dynamic proteins; it constantly dissociates and binds to DNA (with a residence time in the order of minutes) [2]. It has only a small mobile pool and its binding to DNA is critical for maintaining higher order chromatin structures [3,4]. HMGB1, a known chromatin architectural protein, is highly dynamic, competes with H1 for binding sites [5], and binds to DNA transiently with a residence time of less than a second [6,7]. A mobile pool of HMGB1 is significant such that the unbound molecules diffusing in the nucleoplasm appear as a readily detectable uniform signal in the whole nucleus.

When the presence and subnuclear localization of proteins is investigated by microscopy, it is reasonable to expect that once the cell is fixed by standard, generally accepted procedures, and the protein is detected by immunofluorescence, the presence and intensity of the fluorescence signal in an image reveal the original, true position and relative concentration of the protein of interest. We demonstrate here that this reasoning can be misleading, since some nuclear proteins may not only be lost from the original binding sites, but they can diffuse during the fixation process and eventually bind in an area, in which they did not reside in a living cell. By using fluorescently tagged fusion proteins we demonstrate that a dynamic protein like HMGB1 can escape non-crosslinking, and even crosslinking fixation, and diffuse out of its original positions on chromatin repositioning somewhere else. For instance, following fixation by various methods, HMGB1 can be found accumulated almost entirely in the nucleoli. We also show that the linker histone H1 translocates during fixation with ethanol from chromatin to the nucleoli, a subnuclear compartment where detectable amounts of H1 were not present prior to fixation. Further, we demonstrate that translocation of histone H1 to the nucleoli can also be induced in already fixed cells by altering the DNA structure, which occurs upon exposing them to DNA intercalators.

Such translocation to nucleoli is not induced (neither in live nor in fixed cells) in the case of the core histone H2B, a protein, which is stably bound to DNA and other histones.

Accumulation of various proteins in the nucleolus of live cells has been reported before [8–10] and led to the suggestions that the nucleolus may serve as a protein storage or a processing site. The ability of some dynamic proteins to translocate from chromatin to nucleoli during the process of cell fixation demonstrated herein suggests that binding of these proteins within nucleoli is driven by physicochemical interactions and does not require active biological processes.

2 | MATERIALS AND METHODS

REAGENTS. Formaldehyde was from (Electron Microscopy Sciences, Hatfield, PA, USA), glyoxal, glutaraldehyde, and ethanol from Sigma-Aldrich, zinc salts BD Pharmingen. Doxorubicin, daunorubicin, propidium iodide, and DAPI were from Sigma-Aldrich, DRAQ5 from Biostatus. Cell culture reagents and materials were purchased from Sigma and Invitrogen.

CELLS. Human osteosarcoma U2OS cells stably expressing GFP-tagged HMGB1 and RFP-tagged H2B were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with G418 (0.5 mg/mL) and FCS (10%), at 37°C and 5% CO₂. Before the experiment 16,000 cells were seeded onto each well of an 8-well chamber (Ibidi, Martinsried, Germany), and allowed to attach overnight. Cells were then rinsed 2x with 300 µL/well PBS without calcium and magnesium ions (150 mM NaCl, 3.3 mM KCl, and 8.6 mM disodium phosphate dodecahydrate (Na₂HPO₄·12H₂O) and 1.69 mM potassium dihydrogen phosphate (KH₂PO₄)) before application of fixative. HeLa cells were cultured in Dulbecco's MEM (low glucose), supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin), in a humidified atmosphere of 95% air and 5% CO₂, at 37°C. The cells were cultured in T-25 flasks or on Petri dishes and sub-cultured every two to seven days using 0.25% trypsin solution. For imaging experiments cells were seeded onto round coverslips (thickness 0.17 mm, diameter 18 mm; Menzel-Gläser, Braunschweig, Germany). Cells stably expressing histone H1 or H2B were used [11,12].

TRANSFECTION. For XRCC1 and PCNA transient transfection was used. Cells were transfected when they reached confluence of

approx. 60%. 1 h before the transfection medium was changed to Opti-MEM medium. Transfection mixture contained 200 ng of plasmid DNA per sample (XRCC1-mRFP, PCNA-eGFP), and 3.5 μ L of FuGene HD transfection reagent (PROMEGA) per 1000 ng of plasmid DNA, Optimem (no FBS added). 50 μ L of the transfection mixture was used for 1000 ng of plasmid DNA. Opti-MEM, plasmid DNA and FuGene were added to a sterile Eppendorf tube. The mixture was vortexed for 15 s and then left for 15 min. Subsequently it was added to the wells with cells on coverslips. Cells were incubated with transfection mixture for 24 h and then imaged.

FIXATION PROCEDURES. For U2OS cells expressing GFP-HMGB1 and H2B-RFP fixatives were either (i) 4% formaldehyde dissolved in PBS-EDTA, (ii) glyoxal pH 5 (4 mL glyoxal was made by mixing 2.835 mL ddH₂O, 0.789 mL absolute ethanol, 0.313 mL glyoxal [40% stock solution from Sigma-Aldrich, #128465], 0.03 mL acetic acid, pH was adjusted using 5 M NaOH), (iii) 4% formaldehyde mixed with 2% glutaraldehyde, and (iv) chilled 70% ethanol in PBS. 300 μ L/well of a given fixative was added to the cells and incubated on ice for 30 min and a further 30 min at room temperature. Formaldehyde was quenched by incubating the sample with 100 mM glycine for 20 min at room temperature. Fixed cells were then permeabilized using 0.5% Tx-100/PBS-EDTA for 10 min at room temperature. This was followed by washing three times using PBS-EDTA 300 μ L/well.

For HeLa cells expressing H1.1-GFP, H2B-GFP fixatives were (i) 4% formaldehyde ready solution (RNase free) 10 min at RT or 37°C, washed twice by PBS-A, (ii) 70% ethanol solution, -20°C or ice cold, 10 min, washed twice by PBS-A, or (iii) Zinc based fixative overnight, 4°C washed twice by PBS-A.

Fixation of XRCC1, PCNA, H1-GFP and H2B - GFP with ethanol (70%, -20°C) was done on a microscope stage.

IMMUNOFLUORESCENCE. To prevent non-specific antibody binding, samples were blocked with 0.5% BSA/PBS-EDTA for 30 min. 200 μ L of 1 μ g/mL anti-HMGB1 polyclonal antibody produced in rabbit (FineTest, Wuhan, Cat # FNab10218) was added to each well and samples incubated overnight at 4°C. Samples were then washed 3x to remove unbound primary antibody before adding 200 μ L/well of alexa-647 conjugated goat anti-rabbit antibody and incubated on ice for a further 2 h before imaging.

FLUORESCENCE IMAGING. U2OS cells expressing GFP-HMGB1 and RFP-H2B were imaged on Olympus Fluoview 1000 confocal laser scanning microscope. GFP, RFP and Alexa-647 were excited using laser lines 488, 543, and 633 nm, respectively. Emission filters 510–530, 560–600, and >650 nm were used.

Live and fixed HeLa cells were imaged at 37°C, using Leica SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63 \times HCX PL APO CS NA 1.4 oil immersion lens, argon ion (458, 488, and 514 nm) and diode lasers (405, 633, 594, and 543 nm) and a microscope stage microincubator, using standard procedures described previously [13,14]. Briefly, a coverslip with live or fixed cells was mounted in custom made steel holder and placed on a microscope stage. Live cells were imaged in DMEM/F12 medium without Phenol Red, buffered for contact with air.

For studies of XRCC1 and PCNA images of live cells were collected first. Subsequently cells were fixed on a microscope stage with ice cold (-20°C) 70% ethanol for 10 min and washed with PBS (containing Ca²⁺ and Mg²⁺). Subsequently images were collected again, using the same microscope settings. In studies of cells prior and following fixation with zinc salts live cells were imaged first, then culture medium was removed, cells were rinsed with PBS, solution of zinc salts was added and the sample was maintained at 4°C for 12 h. Subsequently the same fields of view were imaged.

IMAGE PROCESSING. Microscope images were processed using Fiji (ImageJ) software [15]. Contrast of some images was adjusted to show weak signals. Thus, fluorescence intensity profiles are shown in order to demonstrate the original signal intensities.

3 | RESULTS AND DISCUSSION

3.1 | HMGB1—Transfer to nucleoli upon fixation

In live U2OS cells, GFP-tagged HMGB1 protein exhibits a uniform distribution throughout the whole cell nucleus (including nucleoli) (Figure 1(A,C)). We investigated how various fixatives influence the final localization of the fusion and of the endogenous protein. Cells expressing GFP-HMGB1 as well as RFP-H2B core histone fusion proteins were fixed by crosslinking with formaldehyde or glyoxal. A non-crosslinking method of fixation using ethanol (which dehydrates the cells and leads to denaturation of proteins) was also tested. Subsequently HMGB1 protein was labeled by immunofluorescence, and the fusion as well as the endogenous proteins were visualized by fluorescence microscopy. Figure 1(D–K) show fluorescence images of GFP-HMGB1, H2B-RFP, and HMGB1 in cells fixed by crosslinking methods. Subnuclear distribution of GFP-HMGB1 in the fixed cells clearly does not match the original distribution in live cells (Figure 1(A, C)). In contrast to live cells, in cells fixed with formaldehyde (Figure 1(D,F)), as well as in cells fixed with glyoxal (Figure 1(H,J)), GFP-HMGB1 was concentrated in the nucleoli, while the nucleoplasmic concentration of this fusion protein was much lower. High concentration of HMGB1 in nucleoli was observed in all cells, regardless of the level of expression of this protein, and in cells with no expression (Figure 1(H) vs. Figure 1(K)). At the same time, the H2B core histone remained in the same regions of the cell nucleus following fixation by either of the two methods (Figure 1(B,E,I)). We note, that accumulation of HMGB1 in nucleoli following formaldehyde fixation was also reported in [16].

Interestingly, in formaldehyde-fixed cells, we failed to detect accumulation of HMGB1 in the nucleoli by immunofluorescence (Figure 1(G)) despite the clear presence of GFP-HMGB1 there (Figure 1(D,F)). This failure of immunofluorescence, which qualifies as a false negative, is most likely due to tight crosslinking of various proteins in the nucleolus, reducing its accessibility to antibodies [17]. In line with this interpretation, in cells fixed by glyoxal (which is known to create less and shorter crosslinks than formaldehyde [18,19] HMGB1 was readily detected in the nucleoli by immunofluorescence

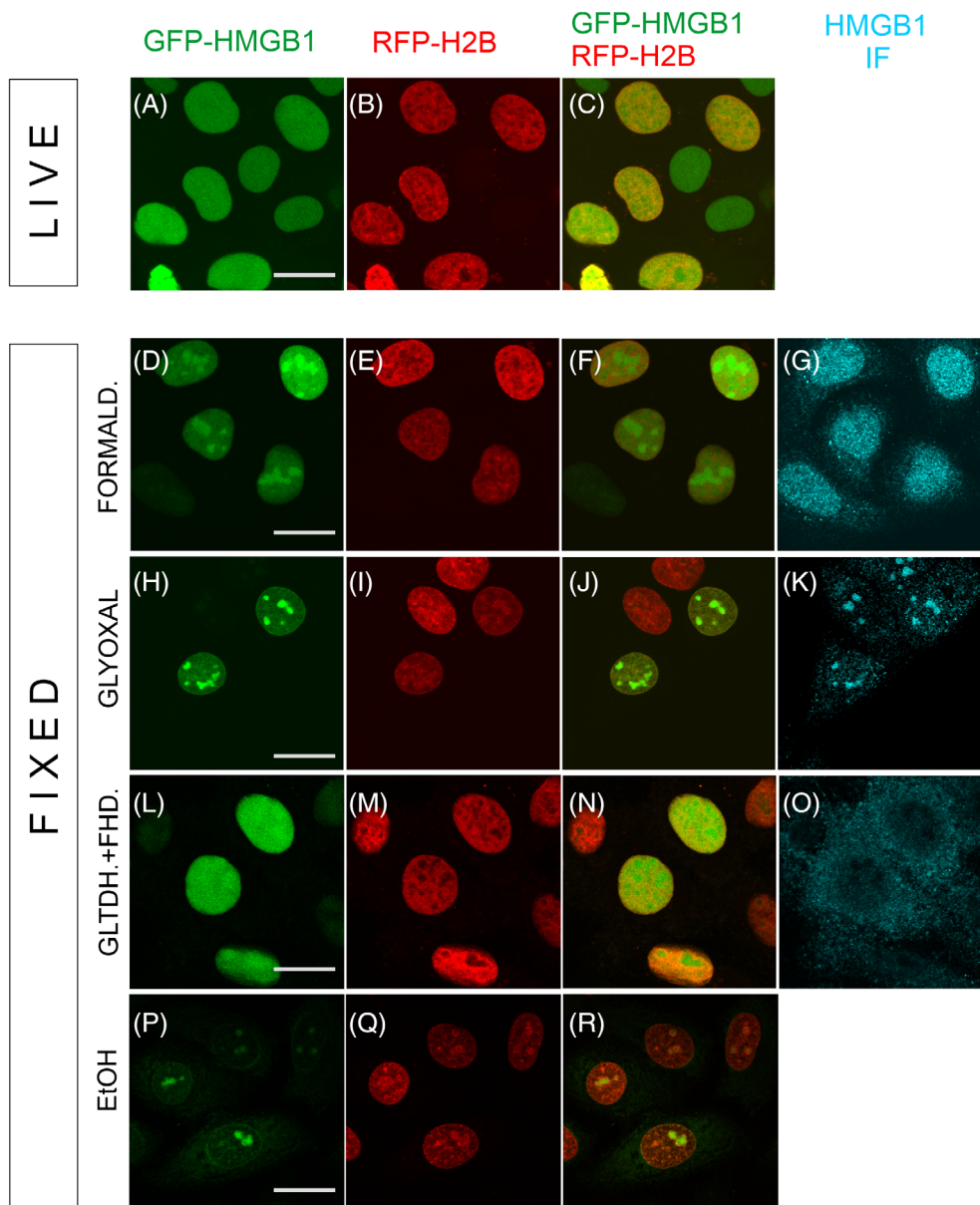


FIGURE 1 Images of HMGB1 and H2B core histone in live U2OS cells, and in cells fixed by various crosslinking and non-crosslinking methods, showing changes of subnuclear localization of HMGB1, but not H2B: (A–C) live cells, (D–G) fixed by formaldehyde, (G) immunofluorescence, (H–K) fixed by glyoxal, (K) immunofluorescence, (L–N) fixed by glutaraldehyde with formaldehyde (note that addition of formaldehyde largely eliminated glutaraldehyde autofluorescence in the green channel), (O) immunofluorescence, (P–R) fixed by ethanol. Scale bars in all panels 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 1(K)). These data confirm that HMGB1 molecules are free to diffuse and translocate from their original binding sites on chromatin to nucleoli even during the process of fixation.

3.2 | The high mobility of HMGB1 facilitates its translocation to the nucleolus during fixation

The apparent translocation of HMGB1 from chromatin to the nucleoli is most likely facilitated both by its fast diffusion and by the absence of strong, lasting interactions between the protein and the DNA. These features make it possible for HMGB1 to diffuse within the nucleus and escape fixation even during the formation of formaldehyde or glyoxal crosslinks, since such processes may last for minutes or longer [18,20]. In the case of ethanol fixation, since no crosslinks are formed, HMGB1 readily diffuses away from chromatin and

accumulates in the nucleoli (Figure 1(P)), while the core histones remain in their original position in the cell nucleus (Figure 1(Q)). This observation is in agreement with an increase of protein content in nucleoli upon fixation with ethanol observed by Raman spectroscopy [21].

The notion that the high rate of binding and dissociation of HMGB1 to and from DNA facilitates an escape of this protein from fixation (by formaldehyde or ethanol) is supported by an experiment, in which cells were fixed by a fixative containing glutaraldehyde supplemented with formaldehyde, and subsequently imaged. Glutaraldehyde forms crosslinks rapidly, much faster than formaldehyde (formaldehyde helps here to minimize green fluorescence arising from glutaraldehyde fixation). In this case, almost all the HMGB1 was fixed in the nucleoplasm (Figure 1(L–N)), that is in a manner observed in live cells. Immunofluorescence again failed to detect HMGB1 in fixed cells (Figure 1(O)).

These observations raise a question as to why proteins like HMGB1 (or H1, see below) exhibit preference for binding to or within nucleoli in fixed cells, but do not show this tendency in live cells.

3.3 | Protein binding in nucleoli of fixed cells

It might be suspected that protein accumulation in nucleoli of fixed cells is an artifact in some way arising from the presence of a fluorescent protein tag. In order to examine this hypothesis we followed the fate of several dynamic nuclear proteins during fixation by ethanol. XRCC1, a component of base-excision repair (BER), and PCNA, a critical factor in DNA replication and repair, were imaged in live, and subsequently in ethanol-fixed cells.

Typically, XRCC1 is represented by a large, uniformly distributed mobile fraction, and several foci in the nucleus. It has been demonstrated by FCS that RFP-XRCC1 has some weak binding sites distributed throughout the nucleus (Berniak et al., in preparation). Following fixation by ethanol both populations, the one which was evenly distributed, and the one concentrated in foci, were still present. Despite

its ability to diffuse and the presence of a fluorescent protein tag, no RFP-XRCC1 was accumulated in the nucleoli (Figure 2(A,B)).

Similarly, no accumulation of PCNA was observed in the nucleoli (Figure 2(C-F)). This protein has a large mobile fraction in non-replicating cells (Figure 2(C)), while in S-phase most of the available PCNA is concentrated in replication foci (Figure 2(E)). Following ethanol fixation, we observed a generalized reduction in fluorescence signifying removal of the entire mobile fraction, while a large proportion of the DNA bound GFP-PCNA remained fixed in replication foci (Figure 2(D-F)). The latter is expected since unloading of the PCNA trimer from DNA in human cells is tightly controlled and requires enzymatic activity [22].

From these observations, we conclude that the presence of a GFP or RFP tag does not in itself direct the dynamic proteins to nucleoli upon fixation by a non-crosslinking method. Accumulation in nucleoli must be, at least to some extent, specific, since, as shown by the example of PCNA and XRCC1, it is not observed for all dynamic nuclear proteins. It is likely that two types of binding sites for HMGB1 exist—the ones on DNA, and much weaker ones in or on nucleoli. Such binding sites may be existing in the live cells as lower affinity site

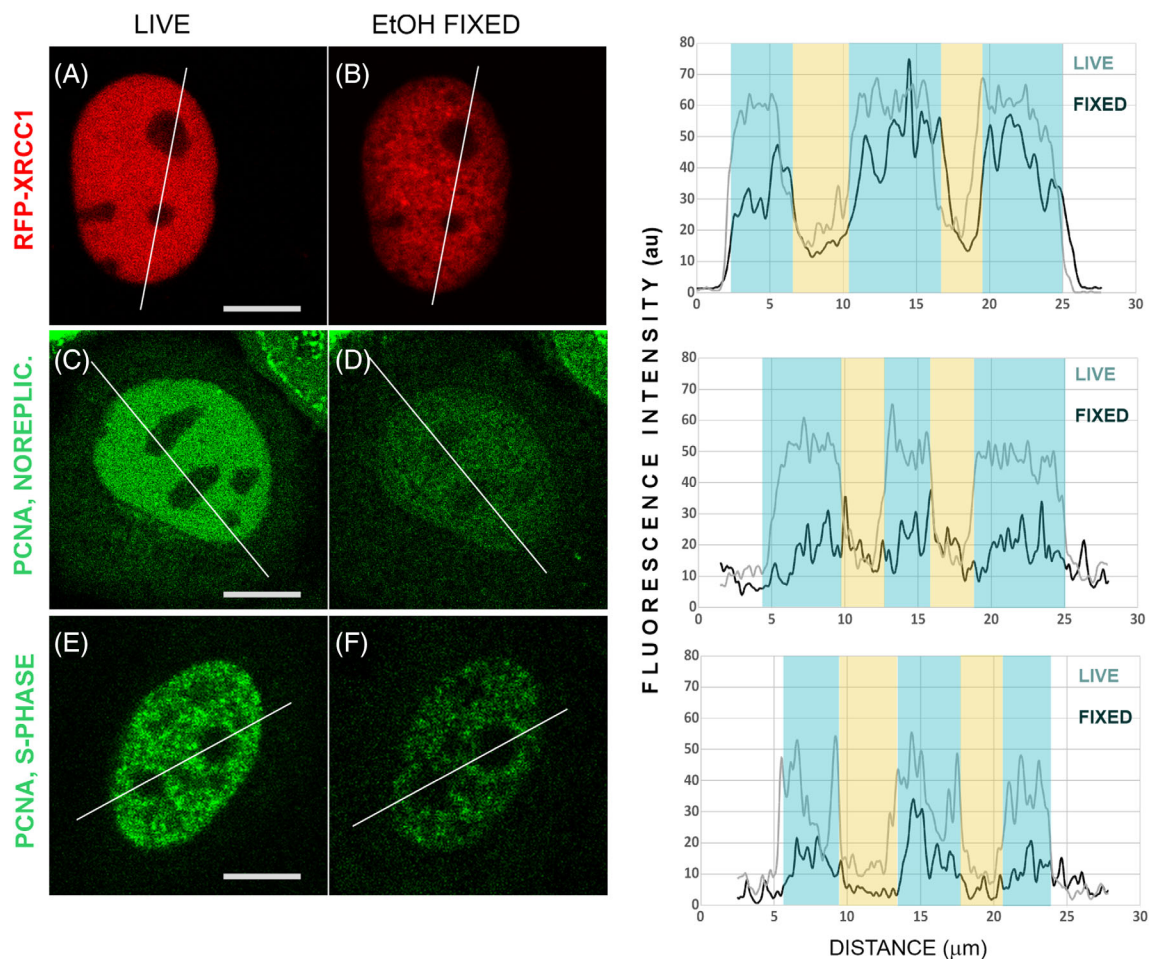


FIGURE 2 Two dynamic nuclear proteins, XRCC1 and PCNA, in live cells, and in cells fixed by ethanol. Scale bars 5 μm. Fluorescence intensity profiles, recorded along white dotted lines in the images of live and fixed cells, are shown. Blue background corresponds to chromatin regions and yellow to nucleoli [Color figure can be viewed at wileyonlinelibrary.com]

or may arise as a result of the altered structure of RNA and proteins induced by fixatives. They may also be sites that become available following loss of nucleolar components during the fixation procedure. While this possibility cannot be discounted on the basis of the data presented here, the fact that only some proteins accumulate in nucleoli during a fixation points to a specificity of this binding. Further the fact that daunomycin evicted histone H1 accumulates in nucleoli in live cells, [14] speak against the formation nonspecific binding sites by non-crosslinking fixation procedure.

We next investigated what role dynamic protein binding to DNA might play in the relocation phenomena. By virtue of creating protein-DNA and protein-protein bonds [23], crosslinking fixation causes permanent coating of DNA with various proteins and decreases the accessibility of DNA for dynamic DNA-binding proteins. As a result, most of the molecules of the mobile pools of highly dynamic proteins like HMGB1, which are not bound to DNA at the onset of the process of fixation, are no longer able to access and bind to the DNA. The equilibrium between the DNA-bound and the mobile fraction shifts towards the latter. These unbound molecules are free to diffuse throughout the nucleus and are readily trapped by their weak binding sites in nucleoli. The shielding of binding sites on DNA by crosslinking fixation was confirmed in an experiment where cells fixed with formaldehyde and subsequently permeabilized with Triton X-100 took up much less ethidium than cells, which were subjected to Triton X-100 before formaldehyde fixation (Data S1, Figure S1). The lower amount of ethidium taken up by DNA in formaldehyde-fixed cells is most likely due to the limited accessibility of DNA coated with cross-linked proteins. Less protein-DNA crosslinking and thus less shielding of DNA occurs in cells exposed to Triton X-100 without prior fixation due to the loss of a fraction of the proteins, thus the number of accessible binding sites for EB in such cells is higher.

3.4 | Transfer of histone H1 to the nucleolus during ethanol fixation

Exposing cells to formaldehyde resulted in fixation of the linker histone on DNA (Figure 3(A,B)). In contrast, upon fixation with ethanol, we observed accumulation of H1 in nucleoli (Figure 3(C,D)). These observations suggest that histone H1, which is less dynamic than HMGB1, can be fixed onto DNA by formaldehyde, unlike HMGB1. However, when the cell is fixed in ethanol, which alters the DNA structure, H1 detaches and diffuses away from DNA. In this case the next best binding sites for H1 molecules appear to be located in nucleoli. Neither of these fixation methods changed the chromatin localisation of histone H2B (Figure 3(E,F), see also Figure 1(E,I,M,Q)).

3.5 | Mechanism of ethanol-induced detachment of the linker histone from DNA

In order to shed some light on the mechanism of ethanol-induced removal of histone H1 from DNA we examined the results of fixing

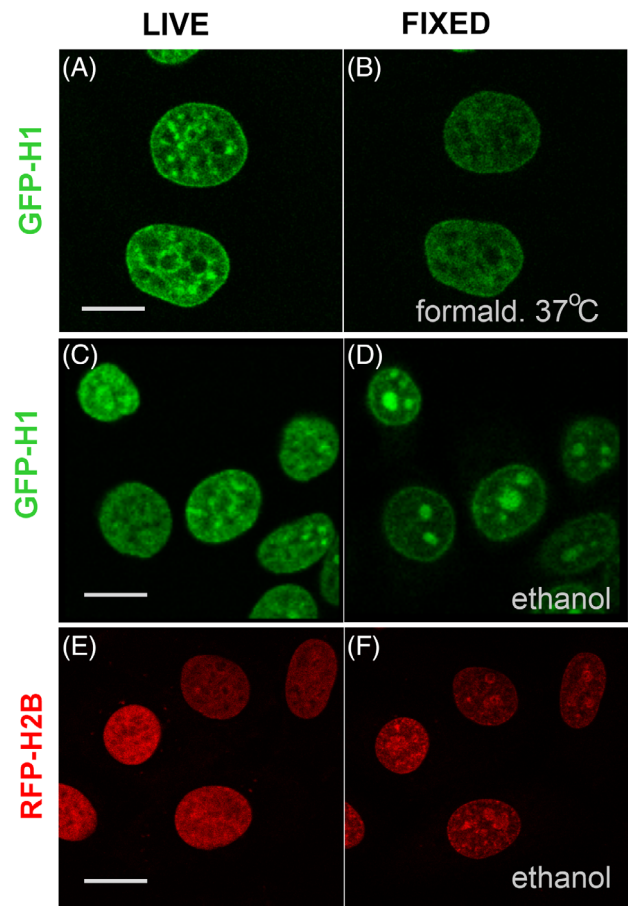


FIGURE 3 Subnuclear localization of H1 linker histone in live cells, and in cells fixed by formaldehyde or ethanol (with translocation to nucleoli), and H2B core histone in live cells and cells fixed by ethanol (with no change of subnuclear localization). Scale bar 10 μm in (A,B) and 20 μm in (C–F) [Color figure can be viewed at wileyonlinelibrary.com]

cells by zinc salts. This is also a non-crosslinking fixation method, but, unlike ethanol, does not cause sample dehydration and protein denaturation. Moreover, in contrast to protein denaturing and crosslinking fixatives like formaldehyde, zinc salts were shown (by indirect methods) to exert only minor influence on DNA structure [24]. Moreover, in contrast with protein denaturing and crosslinking fixatives like formaldehyde, zinc salts were shown to exert only minor influence on DNA structure [24]. Indeed, fixation by zinc salts did not remove either histone H1 or H2B from DNA (Figure 4(A–J)), nor did it, by itself, result in translocation of these histones from DNA to nucleoli. This suggests that dissociation of histone H1 from DNA observed during fixation by ethanol may be caused by fixative-induced distortion of the DNA double helix rather than denaturation of the H1 protein. In support for this notion, H1 attached to DNA in cells fixed by zinc salts was readily evicted from DNA-binding by altering the DNA conformation by the intercalating molecule daunomycin. We observed a similar phenomenon in live cells expressing GFP-H1.1 [4,14]. When daunomycin (used at a concentration in the range of 1–3 μM) entered live cells and intercalated into the DNA double helix, histone H1.1

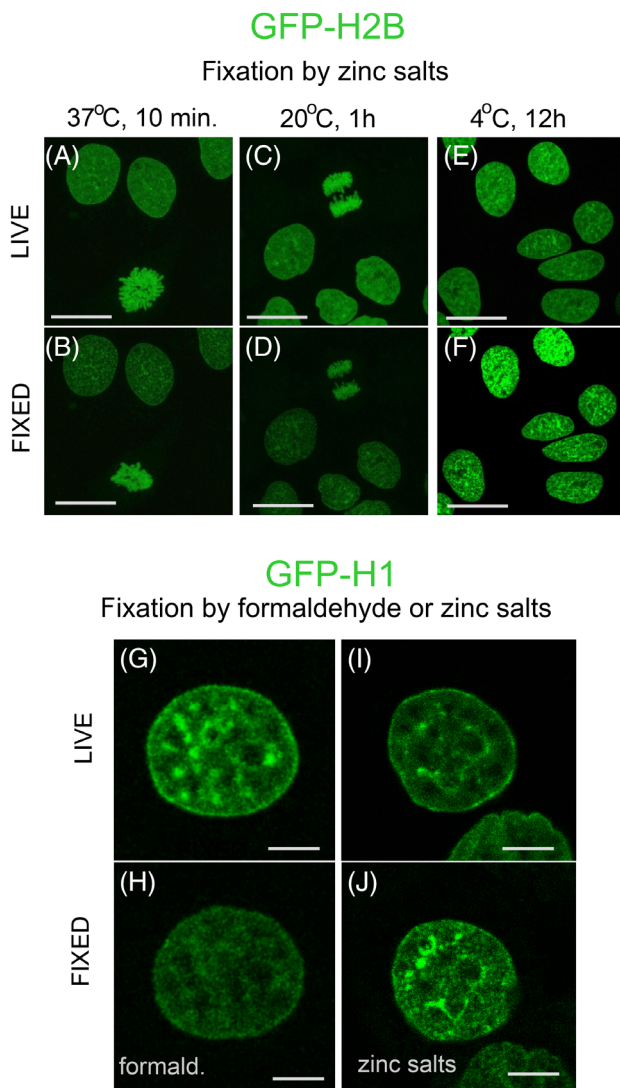


FIGURE 4 Fixation of H1 and H2B histones by zinc salts, showing no accumulation in nucleoli. Images of H2B in cells fixed by zinc salts demonstrate that (in contrast to formaldehyde) this method of fixation does not fully preserve the nuclear structure. Aggregation of chromatin is seen regardless of the temperature used during fixation by zinc salts. The architecture of mitotic plates is not fully preserved. Scale bars 20 μm in (A–F) and 5 μm in (G–J) [Color figure can be viewed at wileyonlinelibrary.com]

was evicted from the DNA and subsequently accumulated in nucleoli. We now demonstrate that when cells are first fixed by zinc salts and subsequently exposed to a DNA intercalator doxorubicin (another anthracycline drug, which is structurally very similar to daunomycin), histone H1.1 can still be evicted from the original position on DNA, and translocated to nucleoli (Figure 5(A,B), and Data S2 (Movie S2)). Our early observations of eviction of histone H1.1 from DNA of live cells by daunomycin were interpreted as a consequence of a change in DNA structure caused by the intercalation of the planar molecules of daunomycin, leading to weakening of the binding of histone H1.1 to DNA, and subsequent trapping of the liberated histone in nucleoli. It was unclear at that point if this transition could be explained in

terms of a biological mechanism or described as a molecular process governed by physicochemical phenomena. Now, with the experiments described above, it becomes clear that fixative- or drug-induced detachment of H1.1 from DNA, and its binding in nucleoli, is a physical process, which is induced by distortion of the DNA structure, and does not require active cellular metabolism.

In order to assess the type and degree of distortion of DNA helix which leads to detachment of histone H1, we exposed cells fixed by zinc salts to another intercalator, propidium anion which also binds to dsRNA in the nucleoli, and to mixed mode DNA binders DRAQ5 and DAPI. All three compounds, when added to cells fixed by zinc salts, caused concentration-dependent relocation of histone H1.1 to nucleoli, with a minor pool of histone molecules remaining in heterochromatic regions at the nuclear envelope (Figure 5(A–F,I,J)). The propidium anion caused translocation of H1.1 to the nucleolus, and binding of PI to dsRNA in the nucleoli did not prevent this process. DRAQ5, which binds by positioning itself in the minor groove and by intercalation of its terminal heterocyclic rings between the DNA bases, caused eviction of H1.1 (Figure 5(E,F)). DAPI did not induce H1 detachment at the concentration when it binds to the DNA minor groove (Figure 5(G,H)). Such binding is expected to stiffen the DNA but should not result in major changes of the double helix structure. However, at higher concentrations, intercalation also occurs [25] and under such conditions H1.1 was detached from DNA (Figure 5(I,J)). These observations confirm that DNA helix distortion causes detachment of histone H1, and are in agreement with previously reported higher mobility of the linker histone H1.1 induced by exposure to daunomycin [14].

3.6 | Mechanism of accumulation of H1 in nucleoli

Accumulation of the H1.1 linker histone in nucleoli in cells fixed by non-crosslinking methods indicates that nucleoli in such cells contain some binding sites for this histone. Assuming that rRNAs might serve as such binding sites, we exposed cells to RNase A, which led to a partial removal of H1.1 from the nucleoli along with a loss of the PI signal (Figure 6). Some histone H1.1 still remained bound in the perinucleolar heterochromatin and under the nuclear envelope. These remnants of H1.1 were readily removed by rinsing the sample with ethanol, demonstrating that these remaining H1.1 molecules were only weakly bound. We interpret this observation as evidence that histone H1.1 was bound to RNA in the nucleoli, possibly to double stranded regions of rRNA, or to some molecular complex of which RNA was an important structural component.

It is important to note that the two non-crosslinking methods, which we used, did not fully preserve the architecture of chromatin. Fixation by zinc salts resulted in chromatin condensation, seen best in cells with H2B-GFP at all temperatures used during the fixation process (Figure 4). Both methods weaken the binding of histone H1 to DNA, or even cause dissociation of a large proportion of the H1 pool as in the case of ethanol. Since histone H1 is a key factor in maintaining higher order chromatin structures [3] the well-known lack of preservation of nuclear structure by ethanol and zinc salts fixation

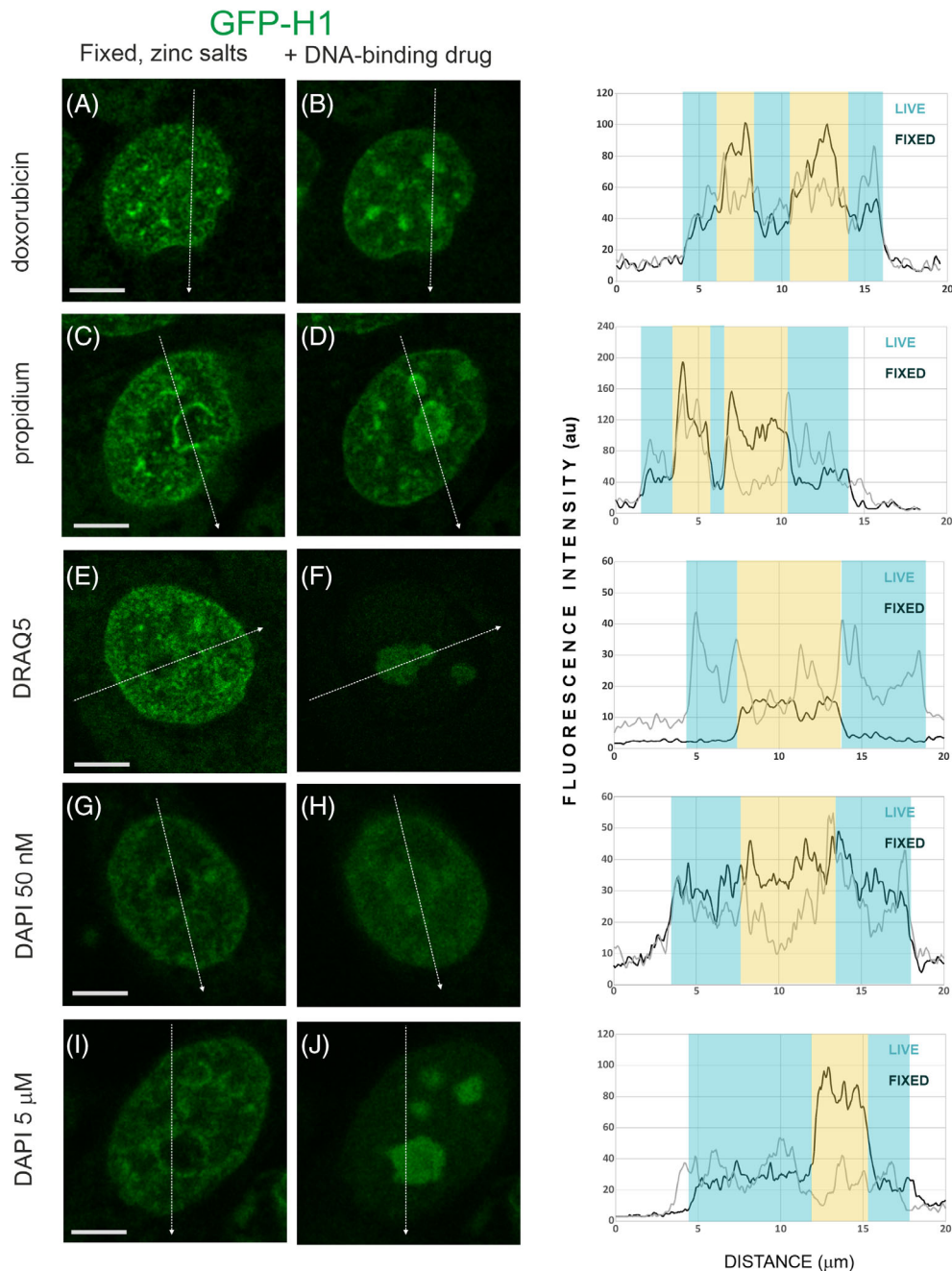


FIGURE 5 Eviction of histone H1 from chromatin to nucleoli in zinc-fixed cells by DNA-binding molecules (doxorubicin, propidium anion, DRAQ5, and DAPI), upon intercalation and the resulting change of DNA structure. Fluorescence intensity profiles, recorded along white dotted lines in the images of live and fixed cells are shown. Blue background corresponds to chromatin regions and yellow to nucleoli. Scale bars 5 μm [Color figure can be viewed at wileyonlinelibrary.com]

is most likely the result of weakening of the bonds and loss of this histone from DNA.

3.7 | Protein dynamics, fixation, and translocation—No transfer of core histones to nucleolus

The data presented above suggest that rapid dynamic exchange, and the presence of large mobile protein pools, facilitate escape of certain proteins from the crosslinks formed by a crosslinking fixative, and may allow translocation of such proteins to new regions in the cell. Although penetration of formaldehyde into a cell may be fast, the

process of fixation by crosslinking may take many minutes. In the case of zinc salts fixation may even take hours. Therefore, it is reasonable to expect that highly dynamic proteins are likely to evade fixation not only by non-crosslinking but also by crosslinking agents. This may explain the ease with which fixation with formaldehyde or glyoxal results in a large amount of HMGB1 binding to nucleoli. A less dynamic protein, like histone H1, which resides on DNA for relatively long times, is more likely to be fixed at its original localization.

We note that mislocalisation of mannosidase I from the endoplasmic reticulum membrane to the Golgi was also observed during a process of fixation and permeabilization of cells [26], an effect that was largely alleviated by mild fixation (0.1% formaldehyde) without permeabilization. Similarly, replacement of Triton-X-100 with saponin

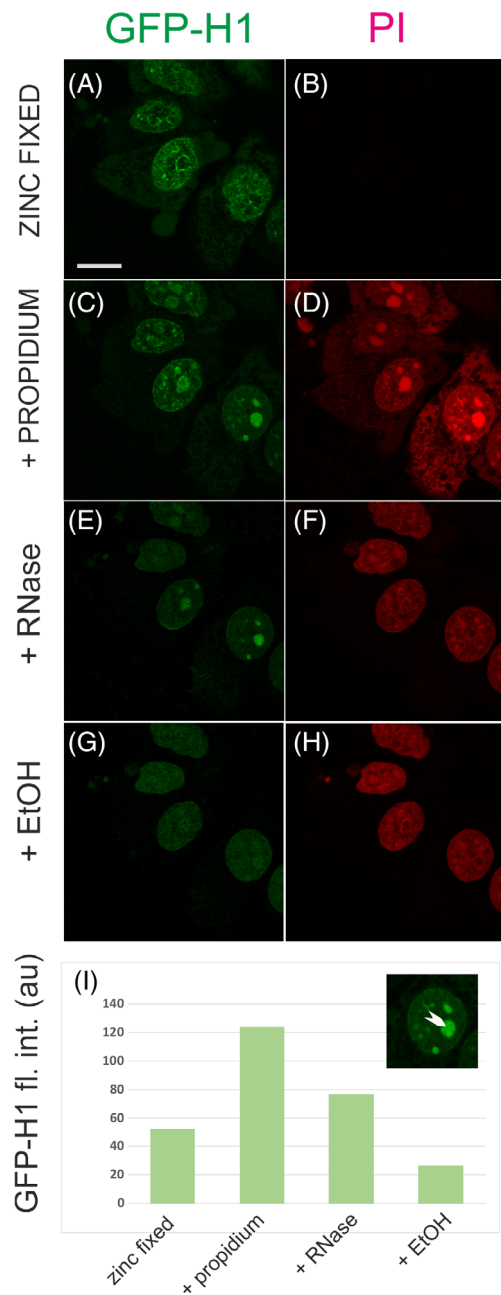


FIGURE 6 The role of RNA in accumulation of histone H1 in nucleoli of cells fixed by zinc salts. Images (scale bar 10 μ m) show GFP-H1 (green) and propidium anion bound to DNA and RNA (red): (A,B) H1 in cells fixed by zinc salts; (C,D) GFP-H1.1 after adding propidium iodide (0.5 mg/mL); (E,F) after 30 min with RNase, showing removal of most H1; (G,H) after rinsing with ethanol, showing complete removal of H1; (I) relative intensities of GFP-H1 fluorescence in the nucleolus, which is marked with an arrowhead [Color figure can be viewed at wileyonlinelibrary.com]

as well as gradual addition of formaldehyde to cells still in medium led to better visualization of late endosomes and actin filaments by immunofluorescence [27]. This suggests that fixation artifacts are not limited to chromatin proteins only, and that they can be alleviated by prudent optimization of fixation and permeabilization procedures, and

supplementing immunofluorescence data with live-cell imaging of fusion proteins.

The advent of super-resolution fluorescence imaging makes optimization of sample fixation and permeabilization procedures ever more important. Super-resolution techniques are mostly incompatible with live cells and thus require sample fixation. However, since even subtle changes in a protocol can influence the positions of structures and the quality of a final high resolution image [18,28], the advantage of increased spatial resolution is lost if fixation does not preserve the structure well. Further, artifacts that would otherwise go undetected by conventional confocal microscopy due to low resolution are likely to show up as “actual structures” due to the increased resolution. The problem may be amplified if the structures under study have not been previously studied and therefore there are no other images for comparison. It is therefore prudent to optimize currently available protocols to suit the molecule under study as well as the available microscopy technique.

4 | CONCLUSIONS

During the process of crosslinking fixation by formaldehyde, glutaraldehyde or glyoxal, and non-crosslinking fixation by methods employing ethanol, or zinc salts, dynamic nuclear DNA-binding proteins can detach from DNA, diffuse across the nuclei, and accumulate in nucleoli. The more dynamic the protein is, the more likely it is to evade fixation onto the site of its residence. Detachment of a protein from DNA can be induced by distorting the DNA helix by interaction with the fixative or a DNA-binding molecule. The loss of dynamic proteins during fixation and their translocation to nucleoli may severely complicate assessment of the amounts and subcellular localization of nuclear proteins by cytometric methods, confocal and super-resolution imaging. We also note that the loss of dynamic proteins from their binding sites on DNA may also influence chromatin immunoprecipitation data, a possibility, which remains to be examined.

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AUTHOR CONTRIBUTIONS

Mirosław Zarebski: Conceptualization; data curation; formal analysis; investigation; methodology; writing-review & editing. **Rosevalentine Bosire:** Conceptualization; data curation; formal analysis; investigation; methodology; writing-review & editing. **Julita Wesolowska:** Data curation; formal analysis. **Oskar Szelest:** Data curation; formal analysis. **Ahmed Eatmann:** Data curation; formal analysis. **Katarzyna Jasińska-Konior:** Data curation; formal analysis. **Oliver Kepp:** Methodology. **Guido Kroemer:** Methodology. **Gabor Szabo:** Conceptualization; resources; supervision; writing-review & editing. **Jurek Dobrucki:** Conceptualization; resources; supervision; writing-original draft; writing-review & editing.

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SUPPORTING INFORMATION

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