Local changes of higher-order chromatin structure during DSB-repair

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Abstract. We show that double-strand breaks (DSBs) induced in DNA of human cells by γ-radiation arise mainly in active, gene-rich, decondensed chromatin. We demonstrate that DSBs show limited movement in living cells, occasionally resulting in their permanent clustering, which poses a risk of incorrect DNA rejoining. In addition, some DSBs remain unrepaired for several days after irradiation, forming lesions repairable only with difficulty which are hazardous for genome stability. These “late” DSBs colocalize with heterochromatin markers (dimethylated histone H3 at lysine 9, HP1 and CENP-A proteins), despite the low density of the surrounding chromatin. This indicates that there is epigenetic silencing of loci close to unrepaired DSBs and/or stabilization of damaged decondensed chromatin loops during repair and post-repair reconstitution of chromatin structure.

1. Introduction
Genome integrity is continuously impaired by endogenous and exogenous factors. The most serious threat is represented by double-strand breaks (DSBs), which interrupt both strands of the DNA molecule and can give rise to chromosomal exchange aberrations. If unrepaired, DSBs lead to cell death or, rarely but more importantly, the cell can survive with an unstable genome, posing a serious risk of tumorigenesis. Despite the introduction of new experimental approaches, there have been limited advances in understanding exchange aberration formation, and there remains little information on the spatiotemporal organization of DSB repair (reviewed in [1]).

The involvement of chromatin translocations in many human cancers, especially lymphomas and leukemias, was discovered many years ago, but their mechanisms of formation are still poorly understood. It is therefore crucial to broaden our understanding of double-strand break generation and repair. Our recent results [2] as well as earlier results of other authors [1, 3; 4] show that chromatin structure and dynamics must play an important role in regulation and facilitation of DNA repair.

There are contradictory data on the role of DSB mobility in formation of chromatin translocations, probably due to the different LET-characteristics of the radiation used to generate DNA lesions. With low-LET soft X-rays, stable nuclear positions of DSBs were observed [5] and only breaks generated close to one another were thought to be liable to DNA misjoining. On the other hand, movement and temporary clustering of individual DSBs was found when using high-LET α-particles [6] or lasers [3], sources which often generate clustered DNA lesions [7, 8, 9, 10]. Lisby et al [9] and Aten et al [6] interpreted the association of DSBs as relocation into special nuclear compartments or “repair factories”; however, this aggregation allows misjoining of DNA ends of previously distant DSBs. Concentration of nuclear processes into specialized nuclear subcompartments is not unprecedented, and occurs, for example, with co-regulated genes during transcription [reviewed in 11, 12, 13].
Indirect support for the dynamic nature of DSBs is also provided by the temporary centripetal shift of centromeres minutes to hours after irradiation or application of radiomimetic drugs (H₂O₂) [14, 15, 16]. However, data for DSB dynamics after the physiologically most relevant γ-irradiation (resulting in isolated, non-clustered DSB-breaks) remain poor and inconclusive.

Both major repair pathways that developed during evolution – homologous recombination (HR) and non-homologous end joining (NHEJ) [for review see 17] – process DSBs using large multiprotein complexes. Since DNA in eukaryotes is packed into compact, highly organized chromatin, the question arises of how the repair machinery gains access to free DNA ends; in particular, whether the assembly of huge repair complexes takes place at decondensed original DSB sites [5], or whether relocation of DSBs into appropriate nuclear compartments is required [9, 6]. Loizou et al [4] reviewed that chromatin remodelling enzymes colocalize with DSBs as well as DSB repair enzymes, facilitating changes in chromatin structure during DSB processing. Rapid phosphorylation of histone H2AX (an H2A variant) at sites of DSBs is an example [reviewed in 18], although the exact role in this has yet to be quantified. Theoretically, two alternative chromatin conformations may be required for DSB processing: an “open” structure, allowing access of proteins to damaged DNA and assembly of repair complexes, or a “condensed” structure stabilizing free DNA ends and preventing transcription from damaged sites [19]. In support of both possibilities, enzymes participating both in chromatin condensation and decondensation (e.g. histone acetylases and deacetylases respectively) were detected at DSB sites [discussed e.g. in 20]. Antagonistically acting enzymes thus could function sequentially and/or simultaneously [20, 21, etc.].

As well as their role in the repair process and/or transcriptional silencing of damaged loci, chromatin modifiers may also influence the formation of translocations directly by regulating the ability of free DNA ends to fuse. In Drosophila, chromosomal ends are protected from end-to-end fusions by heterochromatin protein 1 (HP1) even when all telomere-associated sequences have been deleted [22]. Accordingly, frequent chromosomal fusions were observed in cells lacking ATM (Ataxia Telangiectasia Mutated kinase) or HP1 [22]. In mammals and other organisms that use sequence-specific binding proteins such as TRF2 to protect telomeric ends, the ATM and HP1 pathway plays a minor role in stabilization of normal chromosomal DNA ends, but may be critical in the case of telomeres that are too short to bind telomere-sequence specific TRF2 [22]. This situation could be similar to DSB repair and, not surprisingly, ATM and TRF2 were the first proteins to colocalize with newly formed DSBs [23, 24].

In this work we analyzed the movement of DSB-breaks induced by γ-rays producing isolated lesions. We show limited movement of DSBs, probably associated with decondensation and their relocation into low-density chromatin. Sometimes, clusters of DSB-foci can be seen, which may persist for several days after irradiation, and may represent sites of formation of exchange aberrations. These late lesions, which are probably difficult to repair, colocalized with dimethH3K9 and HP1, despite their location in sparse chromatin. We suggest that these proteins play an important role in transcriptional silencing of damaged loci and/or in recondensation of decondensed chromatin domains after repair. We also show that DSBs are induced preferentially in low density chromatin.

2. Materials and Methods

2.1. Cell culture and transfection
Human MCF7 mammary carcinoma cell line and human skin fibroblasts 04-147 were grown in DMEM medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells were transfected with GFP-tagged NBS1 [25], HP1β [26] and PML using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.
2.2. **Cell irradiation, fixation, permeabilization and immunostaining**

Cells grown on microscope slides were irradiated with $^{60}$Co $\gamma$-rays 24 hr after plating, and either fixed immediately or incubated for various periods of time before fixation. Doses of 1.5, 4.5 and 7 Gy (1 Gy/min) were used.

To fix the cells, the slides were immersed in 4% paraformaldehyde/2 × SSC at room temperature (RT) for 10 min, washed three times in fresh PBS (1x Phosphate buffered saline), permeabilized with 0.2% Triton X-100 in PBS for 18 min, washed 3 × 5 min in PBS, and then immunolabelled.

Primary antibodies from two different hosts (rabbit and mouse) were used to detect two different antigens in the same nucleus; incubation was overnight, followed by washing. Labelling was detected by incubation for 1 hr at RT with FITC-conjugated donkey anti-mouse, and Cy3-conjugated donkey anti-rabbit antibodies (Jackson Laboratory). Nuclei were counterstained with 1 µM TOPRO-3 (Molecular Probes).

2.3. **Image acquisition and microscopy**

An automated Leica DM RXA fluorescence microscope, equipped with a CSU10a Nipkow disc (Yokogawa, Japan) for confocal imaging, a CoolSnap HQ CCD-camera (Photometrix) and an Ar/Kr-laser (Inova 70C, Coherent), was used for image acquisition [27, 28]. Automated exposure, image quality control and other procedures were performed using FISH 2.0 software [27, 28]. The exposure time and dynamic range of the camera in the red, green and blue channels were adjusted to the same values for all slides to obtain quantitatively comparable images. Forty serial optical sections were captured at 0.3 µm intervals (along the z-axis). For observation of living cells, an iXon DV 887ECS-BV (Andor) camera was used together with the 3D viewer [29].

2.4. **Living cell observation and time-lapse microscopy**

Two types of *in vivo* observations were performed: short and medium-term. For short-term experiments, “2D” images consisting of a few (3–5) confocal slices with a z-step of 0.3-0.5 µm were acquired in a millisecond (20-500 ms) interval for a period of approximately 1.5 min. For medium-term observations, 40 optical sections were captured (3D-images) with a 0.2-0.3 µm z-step. Intervals of 50 s were allowed between individual stacks of 40 sections, and observations were continued for a total of 20 min. The light exposure was kept as low as possible to avoid phototoxic effects.

2.5. **Analysis of experimental data and motion of loci**

The analysis method used was described in [2]. Briefly, the off-line image analysis and tracking (2D, 3D) of fluorescence signals were done with the FISH 2.0 software and a 3D image viewer [28, 29]. Coordinates were taken at the centre of gravity of the visualized objects, and corrected for rotation of the cell nucleus and drift of the images during longer time-lapse observations. The objects were traced in the time-lapse series on the basis of matching algorithms. In 2D, the distances between two signals were calculated using the equation: $d = \sqrt{(x_1-x_n)^2 + (y_1-y_n)^2}$; or in 3D: $d = \sqrt{(x_1-x_n)^2 + (y_1-y_n)^2 + (z_1-z_n)^2}$, where $x_1$, $y_1$ and $z_1$ ($x_n$, $y_n$ and $z_n$) were coordinates for the first measurement and the n-th measurement of the same object. The mean $d^2$ was calculated from individual $d^2$ values of all possible signal pairs at the particular time point. The mean difference of $d^2$ (mean $\Delta d^2$) was calculated at each time point (t) as $\Delta d^2 = (d_t - d_{t+\Delta t})^2$, where $\Delta t$ was the time interval between measurements.

Evaluation of data and statistical analyses were performed using the Sigma Plot statistical package (Jandel Scientific). When required, measured distances were normalized to the nuclear radius [% of R] to allow comparison between nuclei. Intensity profiles of R-G-B channels were obtained using Andor iQ 1.2.0 Software (Andor Technology).

2.6. **SDS-PAGE and Western analysis**

Cell suspensions were washed twice with PBS, centrifuged for 3 min at 1000 × g, cooled on ice to 2°C and resuspended in lysis buffer (50 mM TrisHCl pH 7.5, 120 mM NaCl, 0.5% Nonidet P40 and 1 mM phenylmethylsulfonyl fluoride (PMSF)) by vigorous vortexing for 1 min, and left on ice for 30 min
with intermittent homogenization with a plastic pestle, briefly (10 sec) sonicated, and spun down (14,000 rpm for 15 min). The supernatant was transferred into a new tube and kept on ice. Samples containing proteins and chromatin were diluted to the same value of A$_{260}$ absorbance, boiled in SDS loading buffer, and loaded on the gel. Proteins were separated in 15% polyacrylamide gels. After electrophoresis, the gels were either stained with Coomassie blue R-250 (Sigma) or electrotransferred to a nitrocellulose membrane in Tris-glycine buffer containing 10% methanol. The membranes were blocked, incubated with anti-HP1β (Upstate) (dilution 1:100) and anti-PML (Santa Cruz) (dilution 1:10) antibodies, and then treated with peroxidase-conjugated anti-mouse antibodies and detected with an ECL detection system (Amersham Corp.). The intensity of the protein bands was compared after ECL detection using the Vilber Lourmat Photodocumentation and Imaging System.

3. Results

3.1. Nuclear distribution of DSBs induced by γ-rays relative to functionally different chromatin domains

We analyzed the nuclear distribution of double-strand breaks (DSBs) immediately (2 min) post irradiation (PI). DSBs induced by a single exposure to 1.5, 4.5 or 7 Gy of γ-rays were immunocytochemically visualized in situ in spatially fixed human fibroblasts, using antibody against the DSB-specific marker histone H2AX phosphorylated at serine 139 (γH2AX). Except in S-phase nuclei, γH2AX foci were found in only a small proportion of non-irradiated cells (the mean was about 0.4 γH2AX foci per non S-phase nucleus). On average, 38 DSBs per nucleus per Gy appeared rapidly (2-5 min) after exposure to 1.5 and 4.5 Gy; after 7 Gy a large number of DSBs was produced which were difficult to count. In all experiments, most breaks (about 70%) were formed in low-density chromatin, identified as weak TOPRO-3 staining (“chromatin holes”) (Fig. 1A). It is important to note that rest of the breaks were in medium or intensely stained nuclear areas (Fig. 1A). In this figure, the localization of two representative DSBs in sparse (left) and dense chromatin (right) is clearly shown by the intensity profiles of the R-G-B channels.

Regions of chromatin not stained by TOPRO-3 (“holes”) were distinguished from nucleoli by anti-fibrillarin antibody. Although nucleoli are free of DSBs, holes of sparse chromatin contain many breaks (Fig. 1B). DSBs did not directly colocalize with histone H4 acetylated at lysine 12 (acetylH4K12), the characteristic marker of active chromatin, but were close to it (Fig. 2A, B). Acetylated histone H4K12 usually formed a linker between the γH2AX and denser chromatin stained by TOPRO-3 (Fig. 2B). In the case of DSBs located in dense chromatin, acetylH4K12 signals were usually absent (Fig. 2A, bottom RGB profile). Changes of histone modifications (acetylH4K12, acetylH4K5) at sites of DSB during the PI time and colocalization of DSBs with Tip60 histone acetylase were described in [2] and showed progressive chromatin decondensation up to 20min PI.

3.2. Nuclear dynamics of DSBs during the post-irradiation period

At later post-irradiation (PI) times (30 min, 120 min and 240 min), rapid phosphorylation of H2AX led to the progressive growth of γH2AX foci and their protrusion into the TOPRO-3 “holes” (Fig. 2B). Unlike early breaks located either in sparse or dense (Fig. 1A, 3A), later DSBs associated with the MRN-complex, marked here by phosphorylated NBS protein (NBSp), were located almost exclusively in TOPRO-3 “holes” (Fig. 3B). Moreover, accumulation of γH2AX + NBSp foci in the restricted space of the chromatin “holes” occasionally resulted in clustering of two or more foci (Fig. 3C). The number of these clusters increased with the PI time and correlated with the increasing percentage of γH2AX foci localized in sparse chromatin.
Figure 1. Localization of DSBs in functionally different chromatin domains. A: Localization of early DSBs (5 min PI) in weakly stained sparse chromatin and intensely stained dense chromatin (γH2AX foci intersected by yellow lines 1 and 2 respectively) is demonstrated on the central slice (1 µm thick) through the nucleus of a γ-irradiated (1.5 Gy) human fibroblast and by the intensity profiles of RGB channels (R = red, NBSp; G = green, γH2AX foci; B = blue, TOPRO-3 stained chromatin) along the yellow lines (x-axis: relative fluorescence, y-axis: relative distance along the indicated path). B: DSBs are absent from nucleoli (red: antifibrillarin antibody; green: HP1β, blue: TOPRO-3) but were detected in most TOPRO-3 unstained chromatin “holes” (Fig. A).

To exclude the possibility that the clustering and changes in the higher-order chromatin structure are only due to growth of γH2AX foci, we analysed nuclear dynamics of DSBs in living human cells after irradiation with 3.0 Gy. Expression of GFP-tagged NBS protein was observed both in transiently
Figure 2. Mutually exclusive localizations of DSBs (γH2AX foci, green) and an active chromatin marker (histone H4 acetylated at lysine 12, acetylH4K12, red). Maximal images together with RGB profiles intersecting three randomly selected γH2AX foci are shown at Fig. A (x-axis: relative fluorescence, y-axis: relative distance along the indicated path), the x-y, y-z and x-z slices throughout indicated (arrow) γH2AX focus at Fig. B.
Figure 3. Nuclear movement and clustering of DSBs. A and B compares nuclear localization of γH2AX (green) and γH2AX + NBSp (green + red) foci in 3D-fixed human fibroblasts γ-irradiated with 1.5 Gy. Right panels: intensity profiles of RGB channels (R: NBSp; G: γH2AX; B: TOPRO-3; x-axis: relative fluorescence, y-axis: relative distance along the nuclear path) for the indicated DSBs (arrows at left panels). C: Clustering of actively repaired DSBs 10-20 min PI in the same cells (3D projections with optical slices 0.3 µm, γH2AX foci -green, NBSp - red, chromatin counterstaining with TOPRO-3 - blue).
Figure 4. Left panel: Nuclear positions of GFP-NBS foci in irradiated (γ, 1.5 Gy) MCF7 cells during the PI time. The “M” column shows superimposed images from the “T1” and “T2” columns. Right panel: An example of DSB cluster formation from spatially distinct DSBs (GFP-NBS foci) monitored in 3D + time in living MCF-7 cells during a 40 min interval PI.
transfected MCF7 control and irradiated cells, but larger NBS foci appeared in the latter as well as small signals. NBS foci in irradiated cells were also more frequent than in the controls.

Measurements of DSB (NBS) movement were therefore derived from changes in the distances between all possible pairs of large NBS foci and were described in [2]. A very short period of 20-500 ms between slices was used to eliminate “shivering” of the whole nucleus. The mean Δd2 calculated revealed similar mobility of NBS (0.025 µm²/min) as for chromatin [2] (Fig. 4). However, for a small proportion of individual NBS foci, noticeable movement sometimes resulting in stable or temporal fusion of signals was observed (Fig. 4, right). Using CENP-A immunostaining, some extent of movement was observed also for centromeres, which showed a centripetal shift 30 min after exposure to 1.5 Gy. Histograms of DSBs radial-distance distributions ([locus]-to-[nuclear centre] distances) in control (87.534 ± 0.466%R) and irradiated (83.276 ± 0.487%R) fibroblasts are compared in Fig. 5.

![Figure 5](image)

Figure 5. Radial distributions ([locus]-to-[nuclear centre] distances normalized to the nuclear radius [R], expressed as [%R]) of centromeres (CENP-A protein) in control (dashed bars, n = 558) and γ-irradiated (1.5 Gy) (grey bars, n = 719) human fibroblasts.

Double-labelling experiments immunostaining simultaneously γH2AX and NBS or Mre11 proteins, both representing members of the MRN complex, revealed that localization of DSBs (γH2AX foci) in sparse chromatin (chromatin holes) increases with continually growing colocalization of this foci with mentioned repair proteins (Fig. 6). In other words, DSBs located in decondensed sparse chromatin already colocalized with MRN complex, whereas DSBs in dense chromatins domain either colocalized or not. Only 41% and 24% of γH2AX foci colocalized on average with NBSp and Mre11, respectively, immediately (2 min) PI. During the PI period the size and frequency of colocalizing γH2AX foci increased up to 2 h (76% for NBSp and 73% for Mre11) [2] and than started to decrease; Large γH2AX foci however still persisted in a subgroup of nuclei, colocalizing with NBSp and MRE11 (Fig. 6). Increasing localization of γH2AX foci in low-density chromatin was associated with a higher probability of clustering (Fig. 3C, 6). Clustered DSB lesions started to appear within minutes PI (Fig. 3C, 6) and their number increased slightly with time.

3.3. Late DSBs and clustered lesions

Long-time persisting foci frequently corresponded with clustered lesions. These complex lesions usually colocalized with the MRN-complex but without being repaired, indicating a lower repair efficiency of clustered γH2AX. Despite the presence of unpaired DSBs, some cells have divided, as shown by the presence of adjacent micronuclei, sometimes containing double-minute DSB lesions.
Chromatin containing DSBs was either deleted from the genome and excluded in micronuclei, or persisted unrepaired in the next cell generation.

Figure 6. DSB repair and late DSBs. Development of γH2AX foci (green) and their colocalization with MRN-complex participants (NBSp protein, red) during a long PI period (5 min to 3 days). Human fibroblasts irradiated with 1.5 Gy, chromatin counterstaining by TOPRO-3 (blue).

3.4. Late DSBs show many features of transcriptionally silenced heterochromatin
As described above, DSBs were located preferentially in decondensed chromatin, adjacent to, but not colocalizing with, acetylated H4K12, with a peak 30 min PI (Fig. 7A). On the other hand, late γH2AX foci colocalized with heterochromatin markers: dimethylated histone H3 at lysine 9 (dimetH3K9) [2], HP1β protein, and in some cases centromeric heterochromatin (CENP-A protein) (Fig. 7B). The frequency of colocalization with dimetH3K9 and HP1β increased significantly with PI time, and late DSBs in particular showed almost complete colocalization with HP1β (Fig. 8A) despite the constant nuclear level of this protein, which was not affected by irradiation as determined by Western blotting (Fig. 9). A similar tendency was shown for large γH2AX foci and centromeres (Fig. 8A), but when all γH2AX foci were included in the analysis, colocalization started to decrease slightly 2 h PI (Fig. 8A). Simultaneous visualization of γH2AX, NBSp and HP1β revealed that 50% (at 2 h PI) of the γH2AX foci colocalize with both the proteins during repair (in other words, about 77% of γH2AX foci colocalizing with NBSp colocalized with HP1β). From 2 to 4 h PI, the number of [γH2AX+NBSp+HP1β] foci decreased slightly, in accordance with the decreasing and stable number of [γH2AX+NBSp] and [γH2AX+HP1β] foci respectively (Fig. 8B). A positive correlation between the proportion of γH2AX colocalizing with HP1β and the PI time was confirmed in living MCF7 cells transfected with NBSp protein, γ-irradiated with 1.5 Gy, and subsequently immunoassayed with HP1β antibody (Fig. 10A, B).

We also studied the colocalization of γH2AX foci with two other human HP1 isoforms (α, γ). Stably high colocalization (about 80%) of both proteins was observed independently of PI time, unlike the gradually increasing colocalization with HP1β and dimetH3K9 (Fig. 11A). However, minor
Figure 7. Colocalization of DSBs with heterochromatin markers. A: Localization of γH2AX foci (green) exclusively to transcriptionally active domains characterized by H4 histone acetylated at lysine 12 (acetylH4K12, red) during the PI period. Human fibroblasts, central sections (0.3 µm); chromatin counterstained with TOPRO-3 (for all images of Fig. 4). Inserted graph shows the proportion of γH2AX foci colocalizing with acetylH4K12 against PI time. B: Colocalization of γH2AX (green) with dimetH3K9, HP1β and CENP-A proteins respectively (all red) during the PI period. Human fibroblasts, central sections (0.9 µm).
Figure 8.

A. Proportion of $\gamma$H2AX foci colocalizing with hetero-chromatin markers HP1$\beta$, dimet-H3K9 and CENP-A in relation to PI time (human fibroblasts exposed to 1.5 or 4.5 Gy $\gamma$-rays, as indicated). Left: number of $\gamma$H2AX foci (black circles) observed at the PI time indicated, and the number of $\gamma$H2AX foci colocalizing with the heterochromatin marker (green circles). Right: percentage of colocalized $\gamma$H2AX foci. Inserted graphs show the same distributions when only large $\gamma$H2AX foci were scored. B. Colocalization of $\gamma$H2AX, NBSp and HP1$\beta$ proteins during the PI period. Human fibroblasts $\gamma$-irradiated with 1.5 Gy; red circles: colocalization of all three proteins, black circles: $\gamma$H2AX without any colocalization; small black circles: $\gamma$H2AX + NBSp; dashed line: $\gamma$H2AX + HP1$\beta$. 
Figure 9. Western blot comparison of the amount of HP1β in control (lane 4) and γ-irradiated (1.5 Gy, 1 h PI) (lane 5) human fibroblasts. Half sample concentrations (relative to lanes 4, 5) were independently loaded in lanes 7, 8 for more precise evaluation. Quantification of PML protein (lane 1: non-irradiated; lane 2: irradiated cells) was used as a control with unchanged expression (previously confirmed for PI times up to 1 h, see also [30]). Results of densitometric and software analysis of the protein bands are summarized in the associated table.

(20-30%) and “moderate” (40-50%) colocalization was detected for HP1α and γ respectively only when HP1 signals significantly protruding into γH2AX foci were scored (Fig. 11A, inserted graph, 11B), except the higher values (about 45 and 65% respectively) measured 3 days PI. Unlike HP1γ and especially HP1β and dimethH3K9 signals, which were usually located inside the γH2AX territories, colocalizing signals of HP1α formed often bridges between γH2AX foci and the TOPRO-3 stained rim of chromatin “holes” (Fig. 11C-J). Optical slices intersecting the γH2AX foci in two planes (x-z, y-z) clearly demonstrate colocalization of HP1β and dimethH3K9 with γH2AX and the absence of those proteins in the vicinity of γH2AX (Fig. 11G, I), proving that the gradual increase of colocalization with PI time is not simply due to a simultaneously growing volume of γH2AX foci. Moreover, the increase of γH2AX colocalization with HP1β and dimethH3K9 (Fig. 11A, B) does not correspond to the increase of the mean γH2AX volume during the PI period (Fig. 11B, inserted graph).

4. Discussion
In our contribution we show that DSB repair is accompanied by specific sequence of epigenetic changes of chromatin structure, starting with decondensation that enables assembly of huge repair complexes and following with condensation that mediates silencing of loci close to unrepaired DSBs and/or stabilization of decondensed chromatin loops during repair and post-repair reconstitution of chromatin structure. In addition, we have observed and quantified [2] limited nuclear movement of DSBs (γH2AX foci), similar to that described by Kruhlak et al [3]. Unlike Aten et al [6], we have demonstrated that most DSBs do not move over longer distances and are repaired individually wherever they form. However, we identified a subpopulation of DSBs with significantly higher mobility than average. We also found that early DSBs (γH2AX foci) generated by γ-irradiation appear both in weak- and dense chromatin, but that later DSBs (2 h–4 days PI), represented by γH2AX foci colocalizing with the MRN-complex marked by NBSp, were mostly located in low-density chromatin. What causes changes in chromatin density in the region of DSBs persisting beyond 30 min PI? Does it reflect de-condensation of damaged heterochromatin, or looping-out of heterochromatic DSBs to less condensed areas where large repair complexes can assemble? To discriminate between these two models is extremely difficult. Chromatin decondensation is supported by continuous spreading of histone H2AX phosphorylation throughout the damaged region during the PI period and increased histone H4 acetylation in the vicinity of DSBs until about 20-30 min PI. On the other hand, occasional movement and clustering of some DSBs were also observed in living and 3D-fixed cells during the PI period, presenting the risk of chromatin exchange during the repair process. The frequency of clusters...
Figure 10. A Confirmation of the relation between the proportion of γH2AX colocalizing with HP1β and PI time, in living human MCF-7 cells γ-irradiated with 1.5 Gy. Upper graph: total numbers of γH2AX foci (black dashed line) observed at the PI time indicated, and the number of γH2AX foci colocalizing with HP1β (red line). Bottom graph: percentage of colocalized γH2AX foci.

B: Distributions of nuclei showing percentage of γH2AX foci colocalizing with HP1β protein, measured in living MCF-7 cells 5, 30 or 120 min after exposure; x-axis: % of colocalizing γH2AX foci; y-axis: number of nuclei.

increased with the absorbed dose, correlating with a higher number and density of DSBs rendering a growing probability of association of adjacent DSBs. Clustering may also appear to result from overlapping of enlarging γH2AX foci during the PI period. However, DSBs are already clustered several minutes PI, when γH2AX foci are still very small. Moreover, unlike the temporarily formed clusters described by Kruhlak et al [3], at least some of the clusters in living cells were stable. Some DSB clusters observed in low-density chromatin remained unrepaired even several days after irradiation, indicating that these lesions were difficult to repair and could represent an increased risk of chromatin exchange. The fraction of late-foci-positive cells and the average number of foci per nucleus increased significantly with the γ-dose absorbed. In some cases, cells probably divided despite containing late DSBs (not only DSB clusters) that persisted in the divided cells. Some of these divided cells were accompanied by micronuclei that sometimes also contained lesions.
Figure 11. Colocalization of γH2AX with different isoforms of HP1 protein. A: Proportion of γH2AX foci adjacent to HP1α (dashed black line), HP1β (green line), HP1γ (black line), and dimethH3K9 (red line), against PI time (human fibroblasts exposed to 1.5 Gy of γ-rays). Inserted graph (A) shows the percentage from colocalizing signals that “touched” or protruded significantly into γH2AX foci. B: as A, but only signals at least partially overlapping γH2AX foci were scored. Inserted graph (B) shows the increase in the mean γH2AX volume with PI time. C-F: Maximal images of γH2AX foci (green) colocalizing with (red) dimetH3K9 (C), HP1α (D), HP1β (E) and HP1γ (F) respectively, and G-J: x-z and y-z sections (0.3 µm) through γH2AX foci (green) colocalizing with indicated proteins (human fibroblasts γ-irradiated with 1.5 Gy).
Our results show the immediate appearance of γH2AX foci in irradiated nuclei and their colocalization with MRN-complex several minutes PI, and thus indicate that the most prominent changes in chromatin structure and nuclear topology (associated with the recognition and processing of DSBs) take place very early after DNA damage. Opposite conclusions about the mobility of DSBs (suggesting highly mobile and completely immobile DSBs, respectively) have been proposed [3, 5, 6, and others], most probably for two reasons: (1) observation of changes at different PI times, and (2) use of different kinds of radiation to induce DSBs. Petrini and Stracker [31] postulated that late DSB foci (observed hours PI) analyzed in most studies represent sites of unsuccessful repair rather than normally repaired breaks. Further, high-LET particles [6] or microlasers [3], most frequently used for DSB generation, produce clustered DNA breaks [10]; such concentrated fragmentation of chromatin may lead to lesions that are difficult to repair and that may behave differently from the single DSBs usually generated in cells under physiological conditions. Indeed, high mobility of DSBs and their clustering was described after exposure of cells to α-particles [6]. On the other hand, irradiation with ultra-soft RTG rays resulted in immobile lesions [5]. To minimize the problems described above, we used γ-radiation to generate isolated DSB-breaks instead of tracks or foci of clustered DSBs, and monitored changes in higher-order chromatin structure from minutes up to several days PI. Our results “unify” the “breakage-first” [5] and “position-first” [6] theories, that explain the probable genesis of chromatin exchange aberrations: chromatin exchange may arise either between two “stable” DSBs when they are formed sufficiently close to one another, or between two or more DSBs that become clustered by chance.

Another important question associated with exchange aberrations concerns the sensitivity of specific chromatin domains to DNA damage. In accordance with previous studies [32 and others], we found, on average, 38 DSBs per Gy per nucleus with 2–5 min PI. The nuclear distribution of DSB was non-homogenous; most were induced in low-density chromatin (usually containing active genes, [19, 33, 34]). These observations support the view that most DNA lesions induced by γ-radiation are produced by indirect effects of the products of water radiolysis that are more abundant in low-density chromatin. DNA of dense chromatin may also be better shielded from free radicals by its compact structure and bound proteins [35].

Actively repaired DSBs (γH2AX + NBSp foci) specifically colocalize with dimetH3K9 and HP1β, despite being located almost exclusively in low-density euchromatin, as shown by TOPRO-3 staining. Although apparently contradictory, these results probably provide an explanation of how the cell solves the conflicting requirements for chromatin structure in DSB repair. Low-density chromatin in which DSBs occur (due to decondensation of damaged chromatin or looping out of heterochromatic DSBs into euchromatin holes) would permit interactions with repair proteins. Nevertheless, γH2AX foci, representing unrepaired DSBs, did not colocalize directly with acetylH4K12, characteristic of active and open chromatin, but instead were adjacent, indicating exclusion of damaged loci from transcribed domains. Temporarily increased colocalization of γH2AX with acetylH4K12 (and acetylH4K5 + Tip60 histone acetylase, [2]) observed up to 20-30 min PI is in accordance with published results [36, 37] and is probably associated with chromatin decondensation in early stages of repair. DSBs also colocalize with HP1γ and especially HP1β as well as with dimetH3K9. Since dimetH3K9 represents the histone code for binding HP1 proteins [38], responsible for heterochromatinization and gene silencing. It seems that a specific part of the damaged domains, despite being undetectable by TOPRO-3 staining due to its small size, has a condensed, inactive conformation. HP1 proteins may therefore stabilize free DNA ends in the surrounding medium and/or temporarily protect free DNA ends against fusion, as already described for telomeres [22, 39]. Alternatively, due to the progressively increasing frequency of colocalization of dimetH3K9 and HP1β during the PI period, HP1 proteins may be involved in transcriptional silencing [40, 41] of breaks that are difficult to repair or are only repaired slowly, or in re-condensation of decondensed chromatin domains after repair. Reduced colocalization of HP1α compared with β and γ probably...
corresponds to different “playgrounds” of these isoforms [42] but more experiments are required to clarify this question.

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