Mutation Research 756 (2013) 30–36

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Species conserved DNA damage response at the inactive human X chromosome

Iris Müller[a,*], Bruno Merk[a], Kay-Obbe Voss[a], Nicole Averbeck[a], Burkhard Jakob[a], Marco Durante[a,b], Gisela Taucher-Scholz[a,c]

[a] GSI Helmholtzzentrum für Schwerionenforschung, Planckstraße 1, 64291 Darmstadt, Germany
[b] Institut für Festkörperphysik, Technische Universität Darmstadt, Hochschulstraße 6-8, 64291 Darmstadt, Germany
[c] Fachbereich Biologie, Technische Universität Darmstadt, Schnittspahnstraße 10, 64287 Darmstadt, Germany

ARTICLE INFO

Article history:
Received 19 April 2013
Accepted 21 April 2013
Available online 28 April 2013

Keywords:
DNA damage response
Particle irradiation
Heterochromatin
Chromocenter
Inactive X chromosome

ABSTRACT

Chromatin modifications are long known as an essential part of the orchestrated response resulting in the repair of radiation-induced DNA double-strand breaks (DSBs). Only recently, however, the influence of the chromatin architecture itself on the DNA damage response has been recognised. Thus for heterochromatic DSBs the sensing and early recruitment of repair factors to the lesion occurs within the heterochromatic compartments, but the damage sites are subsequently relocated from the inside to the outside of the heterochromatin. While previous studies were accomplished at the constitutive heterochromatin of centromeric regions in mouse and flies, here we examine the DSB repair at the facultative heterochromatin of the inactive X chromosome (Xi) in humans. Using heavy ion irradiation we show that at later times after irradiation the DSB damage streaks bend around the Xi verifying that the relocation process is conserved between species and not specialised to repetitive sequences only. In addition, to measure chromatin relaxation at rare positions within the genome, we established live cell microscopy at the GSI microbeam thus allowing the aimed irradiation of small nuclear structures like the Xi. Chromatin decondensation at DSBs within the Xi is clearly visible within minutes as a continuous decrease of the DNA staining over time, comparable to the DNA relaxation revealed at DSBs in mouse chromocenters. Furthermore, despite being conserved between species, slight differences in the underlying regulation of these processes in heterochromatic DSBs are apparent.

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1. Introduction

DNA double-strand breaks (DSBs) represent one of the most dangerous threats to genome integrity in all living organisms. Unrepaired or not properly repaired DSBs can lead to chromosome aberrations, cell death or various forms of cancer. Repair is accomplished mostly by two major repair pathways either by homologous recombination (HR), which allows generally accurate repair, or by nonhomologous end joining (NHEJ), which is often error prone. The choice of the pathway depends on the phase of the cell cycle, the complexity of the breaks and the surrounding chromatin (for review see [1,2]). In both pathways a large number of repair and signalling proteins assemble at the damage sites in a highly ordered spatial and temporal fashion. Detection of the DNA lesion by early repair factors is accomplished as part of an elaborate network of protein interactions and modifications of the surrounding chromatin, including phosphorylation of the histone variant H2AX (γH2AX) which itself serves as a platform for the binding of several other repair factors [3,4]. Also acetylation, methylation, sumoylation and ubiquitination of different kinds and proteins of the surrounding chromatin have been found to influence the repair process (for review see [5–7]). Together with these chromatin modifications, local as well as global chromatin decondensation have been observed [8–10] and are thought to be necessary to enable the access of repair factors to the site of damage at various stages of the repair process (for review see [11]). However, not only has the DNA damage an impact on the surrounding chromatin, also the chromatin structure itself influences the repair mechanism. Chromatin can mainly be separated into euchromatin representing a more open, actively transcribed state of the chromatin and heterochromatin as a highly compacted, transcriptionally inactive state. Compared to euchromatic breaks, heterochromatic DSBs are repaired with slower kinetics [12] in an ATM-dependent manner [13,14]. While the actual recognition of the DNA lesion occurs inside the heterochromatin, with early repair proteins accumulating within this densely compacted chromatin, the DSB sites move out of the heterochromatic domains to a region of more open chromatin to be repaired. This was shown by Chiolo et al. [15] for the highly repetitive DAPI-bright heterochromatin region in *Drosophila* cells and was measured in our group after aimed irradiation of repetitive pericentric heterochromatin, so called chromocenters, in mouse embryonic fibroblast cells [14]. In both cell systems this

* Corresponding author. Tel.: +49 6159711791.
E-mail address: I.Mueller@gsi.de (I. Müller).

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http://dx.doi.org/10.1016/j.mrgentox.2013.04.006

DSB relocation was found to be associated with local DNA decondensation at the damage sites, measured either as an increase of volume of the whole DAPI-bright heterochromatin compartment in *Drosophila* or directly as a local depletion of DNA staining over time at sites of ion impact within chromocenters. A movement of DNA damage sites has also been reported for the repetitive sequences of rDNA clusters, which are highly expressed gene regions in *Saccharomyces cerevisiae* where the lesion relocates from inside the nucleolus to extranucleolar sites [16]. Relocation of DSBs might be protecting repetitive sequences from repair-related rearrangements within heterochromatin to lower the risk of mismatched repair [15,17]. Here we investigated the chromatin decondensation and relocation of ion-induced DSB at a different, nonrepetitive heterochromatic location by irradiating the inactive X chromosome (Xi) in female human fibroblast cells. We show that relocation is not limited to repetitive sequences but is a general feature of the repair process within heterochromatic regions. Using human cell lines we also demonstrate that the phenomenon of decondensation and relocation within heterochromatin is conserved between flies, mice and humans.

2. Materials and methods

2.1. Cell culture and transfection

NIH3T3 cells (ATCC) were cultured in DMEM medium (Biochrome) containing 10% FCS. IMR90 and Wi38 cells (both ATCC) were grown in EMEM medium (Lonza) with 10% FCS and 1% glutamine (Biochrome). For experiments at the microbeam, cells were seeded on a polypropylene foil with Cell-Tak (BD Biosciences) as described before [18]. For immunofluorescence studies all cells were seeded on coverslips. Transfection of NIH3T3 with XRCC1-RFP was performed using Hiperfect (Qiagen) according to the manufacturer's protocol.

2.2. Irradiation and immunofluorescence staining

Ion irradiation was performed with low energies at between 4.7 and 7.7 MeV/u on target at the UNILAC accelerator (GSI Helmholtzzentrum für Schwerionenforschung, Darmstadt) as described in [19]. Irradiation at the microprobe was performed with low energy ions of 4.8 MeV/u producing short range delta electrons with limited radial extensions of the ion track below 0.72 μm. Cells were fixed at the indicated timepoints in 2% formaldehyde as described earlier [20]. For immunostaining the following primary antibodies were used: anti-γH2AX (Ser139) (mouse monoclonal, clone JBW301) from Millipore (1:500), anti γH2AX (Ser139) (mouse monoclonal, Alexa Fluor 488 conjugated) from Biozol (1:75), anti-53BP1 (rabbit polyclonal) from Calbiochem (1:500) and anti-RPA/p34 AB-1 (mouse monoclonal, clone 9H8) from Thermo Scientific (1:300). Goat anti-mouse F(ab)2 Alexa 488 or 568 and goat anti-rabbit IgG Alexa 488 or 568 (all Invitrogen) as well as ATTO 647 goat anti rabbit IgG (Sigma) served as secondary antibodies. For the staining with three different antibodies, cells were first stained with anti-53BP1 and anti-RPA and the corresponding secondary antibodies. In a second step the cells were fixed for 7 min in 2% formaldehyde, washed three times in PBS and finally stained with the anti γH2AX (Alexa Fluor 488 conjugated) antibody. For DNA counterstaining 3 μM DAPI was used.

2.3. Microscopy and image analysis

For fixed cells, image stacks were acquired using the Leica TCS SPE confocal or a Nikon spinning disc microscope. Irradiations within chromocenters or Xi were verified in 3D. For beamline microscopy, cells were preincubated with 0.1 μM Hoechst 33342 and the culture medium was changed to EMEM media without Phenolred (Lonza) containing 1% glutamine and 20 mM Hepes pH7 (both Biochrom). Irradiation and image acquisition was done as described in [21]. The measured fluorescence intensities for each time point were normalised to the overall cellular fluorescence to correct for bleaching. Correction for cell (StackReg by Philippe Thevenaz) movement and analysis of DNA staining intensities was performed using ImageJ.

3. Results

3.1. Targeted induction of chromatin decondensation within chromocenters in mouse cells

Chromatin decondensation at ion-induced damage sites within chromocenters of living mouse embryonic fibroblast was previously reported using the heavy ion beam at the GSI linear accelerator [14]. These experiments had the difficulty that, when using the broad beam, the ion hits are randomly distributed and therefore the observation of irradiated chromocenters is a rather rare event. We were now interested to investigate the response of DSBs within other heterochromatic compartments, preferably in human cells. The inactive X chromosome represents such a large accumulation of heterochromatin also found in human female fibroblast cells, which is visible following DNA staining as a single bright spot mostly at the outer rim of the nucleus ([22] and Figs. 2–4). Because only one big heterochromatic Xi structure is present per cell the chance of hitting it at the broad beam is even lower compared to mouse nuclei containing several chromocenters. Furthermore, it turns out to be nearly impossible to observe such irradiated single heterochromatic structures in living cells where only a limited number of cells can be viewed at a time. To overcome this problem we used the microbeam at GSI for a targeted irradiation of subnuclear compartments in a 1–2 μm range with exactly one single ion [18] and with great accuracy, namely a mean systematic targeting error of only 670 nm [21]. In a first approach we wanted to establish the decondensation measurement on heterochromatic DSBs generated in mouse chromocenters, where based on previous findings DNA relaxation was expected to occur. For that purpose, we used NIH3T3 mouse cells stained with Hoechst 33342 and positioned single particle hits within chromocenters using the *in situ* microscope (see Fig. 1A). Directly after irradiation a time sequence was taken with one image every 10 s for at least 5 min. The position of the DSBs within a chromocenter was verified by the formation of XRCC1 foci, a protein known to accumulate fast after irradiation at DNA lesions with similar kinetics in euchromatin and heterochromatic damage sites [14]. At the XRCC1 foci formed within chromocenters the decondensation of the heterochromatin can be seen directly by eye as a reduction over time in the fluorescence of the Hoechst staining, appearing as a dark focus within the chromocenter (see Fig. 1A). Analysis of a single irradiated chromocenter shows that decondensation can be visualised within single line measurements of the DNA fluorescence intensities as a continuous decrease over time resulting in the formation of a dip (see Fig. 1B). These live cell observations confirm that the dip is unequivocally a marker for decondensation which can be used also for studies in fixed cells (compare Fig. 2A). The comparison of several of the irradiated versus not irradiated chromocenters shows that while the DNA intensities within the unirradiated chromocenters remain stable over time, all the irradiated ones show a continuous decrease (Fig. 1C). These results demonstrate a fast but not instantaneous chromatin decondensation within mouse chromocenters early after ion irradiation, thus confirming previous observations at the broad beam.

3.2. Chromatin decondensation at the inactive X chromosome

Having established the quantitative evaluation of chromatin decondensation in heterochromatin compartments after targeted ion irradiation at the microprobe, we set off to examine the decondensation within the Xi in living cells. The Xi can be detected in interphase nuclei as a bright compacted area using simple DNA staining (Fig. 2A). Additional immunostaining revealed histone H3 lysine 27 trimethylation (Fig. 2A) and histone H3 lysine 9 trimethylation (not shown), confirming the visualisation of this facultative heterochromatin [23,24].

First irradiation experiments of female fibroblast cells at the broad beam coupled with immunofluorescence in fixed cells showed a spot of decreased DNA fluorescence intensity at the site of ion impact already 10–15 min after irradiation (Fig. 2B), suggesting chromatin decondensation within the Xi. This spot of reduced DNA staining was observed in about 90% (28 out of 31) of the hit Xi from

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