

# Transmission of persistent ionizing radiation-induced foci through cell division in human primary cells

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### 1 Abstract

2 Unrepaired DNA double-strand breaks (DSBs) induced by ionizing radiation are associated with lethal 3 effects and genomic instability. After the initial breaks and chromatin destabilization, a set of post-4 translational modifications of histones occurs, including phosphorylation of serine 139 of histone H2AX 5 (yH2AX), which leads to the formation of ionizing radiation-induced foci (IRIF). DSB repair results in the 6 disappearance of most IRIF within hours after exposure, although some remain 24 hours after irradiation. 7 Their relation to unrepaired DSBs is generally accepted but still controversial. This study evaluates the 8 frequency and kinetics of persistent IRIF and analyzes their impact on cell proliferation. We observed 9 persistent IRIF up to 7 days postirradiation, and more than 70% of cells exposed to 5 Gy had at least one 10 of these persistent IRIF 24 hours after exposure. Moreover we demonstrated that persistent IRIF did not 11 block cell proliferation definitively. The frequency of IRIF was lower in daughter cells, due to asymmetric 12 distribution of IRIF between some of them. We report a positive association between the presence of IRIF 13 and the likelihood of DNA missegregation. Hence, the structure formed after the passage of a persistent 14 IRI focus across the S and G2 phases may impede the correct segregation of the affected chromosome's 15 sister chromatids. The ensuing abnormal resolution of anaphase might therefore cause the nature of IRIF 16 in daughter-cell nuclei to differ before and after the first cell division. The resulting atypical chromosomal 17 assembly may be lethal or result in a gene dosage imbalance and possibly enhanced genomic instability, 18 in particular in the daughter cells.

19

#### 20 Keywords:

21 Ionizing radiation, γH2AX foci, cell division, chromosome break, DNA double strand break.

#### 1 1. Introduction

2 DNA damage is a key event in cell response to ionizing radiation, one involving genetic and epigenetic 3 modifications that may affect the homeostasis of healthy tissues in exposed individuals. In particular, DNA 4 double-strand breaks (DSBs) that are incorrectly or not repaired are associated with lethal effects and 5 genomic instability [1]. DSBs trigger activation of phosphatidylinositol-3'-OH kinase-related kinases 6 (PIKK), such as ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK); 7 these, in turn, phosphorylate the histones H2AX located around the break, at serine 139 [2-5]. This 8 phosphorylation of H2AX (yH2AX) quickly spreads over several megabases on the adjacent chromatin 9 [5,6] and results in the formation of microscopically visible nuclear foci, known as ionizing radiation-10 induced foci (IRIF). One explanation for this extensive phosphorylation postulates that DSBs cause 11 massive relaxation of chromatin coiling [6]. yH2AX initiates the formation of a platform to attract and retain 12 proteins, such as Nijmegen breakage syndrome 1 (NBS1), mediator of DNA damage checkpoint protein 1 13 (MDC1), breast cancer susceptibility 1 (BRCA1), and p53-binding protein 1 (53BP1); these lead in turn to 14 the recruitment of DNA damage repair proteins [7-10].

15 The number of yH2AX IRIF peaks at 30 minutes after exposure, and most of these IRIF disappear within 16 hours of exposure [5,6]. This disappearance is thought to be related to DSB repair and restoration of 17 chromatin integrity and structure [11,12]. However, some IRIF remain 24 hours after exposure [13], and 18 their association with residual DSBs is generally accepted since DDR proteins are still present within 19 these persistent IRIF [13,14]. Although the nature, role, and consequences of these IRIF are still unclear, 20 some studies suggest that they are involved in the inhibition of G1/S progression [13] but also in radiation-21 induced cellular senescence and death [15,16]. 22 In this work, we evaluated the kinetics of the appearance and disappearance of yH2AX and 53BP1 IRIF. 23 We characterized persistent IRIF up to 7 days after exposure to ionizing radiation and evaluated their 24 impact on the resumption of the cell cycle and the division of normal human primary cells irradiated during

the G0/G1 phase.

26

#### 27 2. Materials and Methods

#### 28 2.1. Cell cultures and irradiation

29 Primary human umbilical vein endothelial cells (HUVECs, C2519A, lot. 0000087758) were isolated by 30 Lonza from human tissue (from 3 females and 1 male) donated after permission was obtained for its use 31 in research applications by informed consent or legal authorization. All cells tested negative for 32 mycoplasma, bacteria, yeast, and fungi. Cell lots and donors were tested and negative for HIV-1, hepatitis 33 B, and hepatitis C. The HUVECs were cultured at 37 °C, with 95% humidity and 5% CO<sub>2</sub> in EGM-2 media 34 optimized for the proliferation of endothelial cells and supplemented with 5% fetal bovine serum, 35 hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, gentamicin, and amphotericin-B (EGM-36 2MV BulletKit, CC-3202, Lonza). We used HUVEC primary cells at low passages and evaluated their

1 cytogenetic status by M-FISH during passages 2 (P2) and 4 (P4) [17]. No clonal abnormality was 2 observed, and the proportions of males and females obtained by cytogenetic analysis were consistent with 3 the initial cell pools (3 females and 1 male). To obtain cells in G0/G1 phase of the cell cycle at irradiation, 4 we synchronized cells by contact inhibition induced in confluent culture. Subsequently, synchronized cells 5 were seeded on glass in Nunc® Lab-Tek® II chamber slide systems (Thermo Fisher Scientific) for 5 hours 6 before irradiation. The experimental protocol was designed to ensure that the DNA damage took place in 7 cells in G0/G1 (initial synchronization of the cells) but also that nothing other than irradiation could block 8 resumption of the cell cycle. We therefore verified that at 5 hours post-seeding, cells were in G0/G1 and 9 almost all adherent and that the density of seeding would allow future cell growth. An Elekta Synergy 10 Platform (linac accelerator) was used to deliver X-rays with a maximum energy of 4 MeV (4 MVp) at a dose rate of 2.3 Gy-min<sup>-1</sup> and with a delivered dose uncertainty rate of 7%. We delivered two different 11 12 doses: 1 and 5 Gy. Culture media were not renewed after irradiation. To monitor cell proliferation we 13 added 50 µM 5-bromo-2'deoxyuridine (BrdU; B-5002, Sigma-Aldrich) to the cell culture medium just after 14 its exposure to X-rays.

15

#### 16 2.2. Immunofluorescence staining

17 Cells were washed with 1X PBS (14190-094, Life Technologies), fixed with 4% paraformaldehyde solution 18 (199431LT, Affymetrix), and permeabilized with 0.5% Triton X-100 (T8787, Sigma-Aldrich). The following 19 primary antibodies were used in this study: mouse IgG1 monoclonal anti-phospho-histone H2AX (Ser139) 20 antibody (dilution of 1/800; 05-636, clone JBW301, Upstate), rabbit IgG polyclonal anti-53BP1 antibody 21 (dilution of 1/1000; A300-272A, Bethyl Laboratories), and rat IgG2a monoclonal anti-BrdU antibody 22 (dilution of 1/200; ab6326, clone BU1/75 (ICR1), Abcam). Antibodies were diluted in 1X PBS with 2% 23 (w/v) BSA (bovine serum albumin; A9418, Sigma-Aldrich) and incubated with cells for 1 hour at room 24 temperature (RT). After washing, the following secondary antibodies were used: donkey anti-mouse IgG polyclonal antibody conjugated to FITC (1 mg.ml<sup>-1</sup>; dilution of 1/100; AP192F, Chemicon), donkey anti-rat 25 IgG polyclonal conjugated to Alexa Fluor® 594 (2 mg.ml<sup>-1</sup>; dilution of 1/500; A21209, Life Technologies), 26 and goat anti-rabbit IgG polyclonal conjugated to Alexa Fluor® 647 (2 mg.ml<sup>-1</sup>; dilution of 1/1000; A21245, 27 Life Technologies) or chicken anti-goat IgY polyclonal conjugated to Alexa Fluor® 647 (2 mg.ml<sup>-1</sup>, dilution 28 29 of 1/500; A21469, Life Technologies). They were diluted in PBS with 2% BSA and incubated with cells for 1 hour at RT. DNA was stained with DAPI (0.2 µg.ml<sup>-1</sup>; 1050A, Euromedex) and mounted with ProLong® 30 Antifade Reagents (P36930, Life Technologies). For BrdU labeling, we performed a DNA denaturation 31 32 step between fixation and permeabilization by incubation for 30 minutes at RT in 1 M HCL (Prolabo).

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34 2.3. Analysis of binucleated cells

To obtain binucleated cells, cytokinesis was blocked with cytochalasin B (0.35 μg.ml<sup>-1</sup>; c2743, Sigma Aldrich) and added to culture medium 24 hours after exposure to 5-Gy irradiation. *In situ* immunofluorescence staining was performed 24 hours after cytochalasin B treatment began.

4

#### 5 2.4. Image acquisition and analysis

6 Images were acquired and analyzed with the Scan<sup>R</sup> platform (Olympus), as described previously [17]. 7 Briefly, images were acquired on an inverted Olympus IX81 fluorescence microscope with a UPLSAPO 8 100XO oil immersion objective (Olympus) and an NA of 1.4; the microscope was coupled with an Orca R<sup>2</sup> 9 CCD camera (Hamamatsu) and a motorized SCAN IM IX2 stage (Märzhäuser). Image analysis was 10 performed with Scan^R analysis software (Olympus). An edge segmentation algorithm based on Canny's 11 method [18] was used to detect nuclei in the DAPI channel (main object) and yH2AX foci in the FITC 12 channel (sub-object 1). A first selection based on the area and circularity of the nuclei excluded clusters of 13 cells and cellular debris. Cells were selected in the different phases of the cell cycle by assessing the 14 integrated intensity of the DAPI signal (DNA content) combined with the integrated intensity of the yH2AX 15 signal in the entire nucleus, which increased dramatically increased in S phase [17,19].

16

## 17 3. Results

18 3.1. Characterization of IRIF as a function of time and dose

To investigate the IRIF dynamics induced in G0/G1 phase, we exposed synchronized G0/G1 phase human umbilical vein endothelial cells (HUVECs) to 1 or 5 Gy of 4 MV X-rays and studied IRIF from 10 minutes to 7 days after exposure by monitoring γH2AX foci. Using automated detection of nuclei and foci, we analyzed a mean of 4,000 cells for each condition so that we could screen subpopulations of cells or foci by different characteristics, such as size, shape, or cell-cycle phase, and weight their representativeness within the entire population of exposed cells. All results presented here concern only cells in the G0/G1 phase of the cell cycle.

26 The number of yH2AX IRIF peaked 30 minutes after exposure (Fig. 1A), consistent with previous reports 27 [5,6,20]. By 24 hours after exposure, the frequency of yH2AX IRIF per nucleus decreased by more than 28 90% for both doses. Some IRIF nonetheless remained observable, their numbers dose-dependent; we 29 observed 4.6 times more IRIF for the cells exposed to 5 Gy compared with 1 Gy of irradiation: 4.2 ± 0.5 30 and 0.9 ± 0.2 yH2AX IRIF per nucleus, respectively (Table 1). By 7 days after exposure to 5 Gy, the mean 31 number of yH2AX IRIF per nucleus was still 0.8 ± 0.3, still significantly more than that measured in 32 unirradiated cells (0.4  $\pm$  0.2 per nucleus; Table 1). Co-immunostaining of yH2AX and 53BP1 showed 33 nearly invariable co-location between these proteins from 10 minutes to 7 days after exposure (Fig. 2C). 34 To characterize these yH2AX/53BP1 IRIF over time, we measured the area of about 100,000 to 460,000

early IRIF (before 24 hours postexposure) and 9,600 to 22,000 persistent IRIF (from 24 hours
 postexposure). Their area increased as their number per nucleus decreased (Fig. 1). Most early IRIF were

1 small, whereas persistent IRIF were characterized by their larger size (Fig. 1B), in accordance with 2 previous work [13]. Comparison of the distributions of IRIF areas at 30 minutes and 72 hours confirmed 3 this observation. Fig. 2 depicts the two different ranges of size observed for early (Fig. 2A) and persistent 4 (Fig. 2B) IRIF induced specifically by ionizing radiation (i.e., normalized with respect to the corresponding 5 non-irradiated condition). Hence, early IRIF were characterized by an area ranging from 0.2 to 1.3 µm<sup>2</sup> 6 (Fig. 2A) and persistent IRIF by an area greater than 1.1 µm<sup>2</sup> (Fig. 2B). We therefore use these areas as 7 thresholds to study persistent IRIF in the rest of this work and report in Table 1 the frequencies of these 8 IRIF as a function of time since irradiation and dose. A basal rate of foci with size characteristics similar to 9 the persistent IRIF was observed in non-irradiated cells.

At 24 hours after exposure,  $12.7\% \pm 3.3\%$  of the cells exposed to 1 Gy irradiation contained at least one persistent IRI focus (Fig. 3), that is, about one fifth of the percentage among 5 Gy irradiated cells:  $73.2\% \pm$ 12.8%. These results underline the substantial number of cells affected, especially as only  $4.3\% \pm 0.01\%$ 

- 13 of non-irradiated cells contained at least one large  $\gamma$ H2AX focus.
- 14

#### 15 3.2. Persistent IRIF and cell proliferation

16 We investigated the role of the cell cycle in IRIF behavior. To identify the cells that had completed a full 17 cell cycle, we added BrdU just after irradiation. Fig. 4A and 4B show the cell distribution at different 18 phases of the cell cycle as a function of dose and time. The results for unirradiated cells were consistent 19 with normal HUVEC cell growth in our cell culture conditions. At 24 hours after exposure, the cell cycle 20 had resumed, dose-dependently (Fig. 4A). Consistent with the cell synchronization in the G0/G1 phase at 21 irradiation, the main activated checkpoint was G1/S. The lack of any significant increase in the proportion 22 of cells in G2 at 24 (Fig. 4A) or 48 hours (Fig. 4B) after irradiation suggests that the G2/M checkpoint was 23 not significantly activated in our experimental conditions.

24 The density of the cell monolayer (Fig. 4C) was consistent with cell cycle resumption between 24 and 48 25 hours after irradiation. Interestingly, although BrdU-positive cells in G0/G1 accounted for almost half of the 26 cell population exposed to 5 Gy (Fig. 4B), monolayer density did not increase significantly (Fig. 4C). 27 Phase contrast live-cell imaging of synchronized cells exposed to 5 Gy showed that around 45% of cells 28 that reached M phase (when the adherent cells became transiently spherical) died. Of the 157 cells that 29 entered M phase, 86 produced adherent daughter cells, while the other 71 died during this phase. This 30 finding may explain the apparent stability of the cell monolayer density after irradiation with 5 Gy, despite 31 the presence of dividing cells.

At 24 hours after exposure, most of the cells with persistent γH2AX/53BP1 IRIF were halted in G0/G1, that is, were still BrdU-negative (Fig. 5A). Surprisingly, the situation was reversed at 48 hours: as most of the cells with persistent γH2AX/53BP1 IRIF were G0/G1 BrdU-positive (16.7%±1.3% and 42.3%±3.6% for 1 and 5 Gy, respectively, see Fig. 5A and B). These results indicate that the presence of persistent IRIF does not permanently prevent cells from progressing through the cell cycle. It is interesting to note that BrdU incorporation significantly increased the frequency of large foci measured in unirradiated cells after cell division; at 48 hours, their frequency of cells with large foci was close to that measured for cells exposed to a 1-Gy dose (Fig 5A). To prevent this from interfering with subsequent measurements, we focused on cells exposed to the 5 Gy dose.

5

#### 6 3.3. Transmission of persistent IRIF to daughter cells

7 To investigate the transmission of persistent IRIF through cell division, we determined its frequency as a 8 function of BrdU labeling and time since exposure. The frequency of persistent IRIF decreased 9 significantly between 24 and 48 hours after exposure, apparently simultaneously with the resumption of cell cycle progression (Figs. 4, 5, and 6A). Interestingly, the frequency of persistent IRIF remained stable 10 11 over time in each subpopulation of G0/G1 BrdU-negative cells and G0/G1 BrdU-positive cells (Fig. 6A). 12 This finding suggests that cell division, rather than a repair process occurring in G0/G1 after exposure, 13 was primarily responsible for reducing the frequency of persistent IRIF. These IRIF therefore do not 14 permanently block cell proliferation, which is indeed likely to influence the number of IRIF in the daughter 15 cells. To examine this point in more detail, we analyzed the distribution of G0/G1 BrdU-negative and 16 BrdU-positive cells according to their number of persistent IRIF (Fig. 6B and C). The shape of the 17 distribution in G0/G1 BrdU-negative cells, like the frequency, remained the same between 24 and 18 48 hours (gray bars in Fig. 6B and C). The proportionately equal decrease for all classes indicates that the 19 number of persistent IRIF per cell did not influence ability to progress through the cell cycle. In addition, 20 the distribution observed for G0/G1 BrdU-positive (compared with G0/G1 BrdU-negative) cells clearly 21 shifted to the classes with fewer IRIF. This result indicates a class change between the mother cell and its 22 daughter cells; daughter cells had fewer persistent IRIF than their mothers. To verify this hypothesis, we 23 blocked cytokinesis with cytochalasin B 48 hours after exposure to 5 Gy. Around half of the binucleated 24 cells had an asymmetric number of persistent IRIF in the daughter nuclei (Fig. 7). Our data thus suggest 25 that the decrease in the frequency of persistent IRIF over time may be linked not to a repair process but 26 rather to a substantially asymmetric distribution of IRIF between daughter cells. Furthermore, this 27 asymmetry suggests that IRIF segregation during anaphase may be abnormal.

To confirm this hypothesis, we analyzed the missegregation events in G0/G1 BrdU positive cells observed at 48 hours after exposure (without cytochalasin B treatment). Among the entire population of G0/G1 BrdU-positive cells, the number of micronuclei and/or nucleoplasmic bridges in mono- and binucleated cells clearly increased and did so dose-dependently (Table 2). More than 85% of these missegregation events were associated with the presence of a  $\gamma$ H2AX signal, however; this finding suggests that the structure formed after the passage of a persistent IRI focus across the S and G2 phases may impede the correct segregation of the affected chromosome's sister chromatids (Fig. 8 and Table 2).

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#### 1 4. Discussion

2 Many studies have described the correlation of transient IRIF with DSBs [5,19,21,22]. ATM 3 phosphorylation of histone H2AX on serine 139 near the break, its amplification over megabases around 4 the break and the resulting observation of nuclear foci have been well described in mammalian cells 5 [6,7,19]. Moreover, this histone modification leads to relaxation of the chromatin architecture, which in turn 6 allows the DNA repair machinery to access the spatially confined region surrounding the break [23-25]. 7 The disappearance of IRIF is generally associated with DSB repair [22] and restoration of the initial 8 chromatin structure [24]. Our data show that, depending on the dose, 5 to 10% of the total IRIF observed 9 at 30 minutes after exposure remain 24 hours after irradiation in 10 to 70% of cells. Relatively few studies 10 have examined the nature and cellular impact of these persistent IRIF. Consistently with the progressive 11 increase of the size of 53BP1 and ATM IRIF reported by Yamauchi et al [13], we observed that the area of 12 yH2AX/53BP1 IRIF increased over time as the frequency of yH2AX/53BP1 IRIF decreased (Fig. 1). At 72 13 hours after exposure, persistent vH2AX IRIF were predominantly large, with areas 2 to 6 times bigger than 14 those measured at 30 minutes (Fig. 2). Some studies suggest that chromatin movement may cause 15 adjacent IRIF to merge, thus contributing to both the reduction of their frequency and their increase in size 16 [26–29]. These observations, however, involved asynchronous cells, in which DSB repair by homologous 17 recombination (HR) can occur when DNA damage is produced during S or G2. In our study, we ensured 18 that DNA damage occurred mainly to cells in G0/G1, thus preventing the use of HR and limiting putative 19 chromatin movements [30]. In this case, persistent yH2AX IRIF may grow larger over time through the 20 amplification of yH2AX signals, probably due to persistent ATM presence at their site [13,15,31,32]. The 21 cause of the persistence and amplification of the phosphorylation nonetheless remains unclear. No repair 22 activity appears to take place at the site of these persistent IRIF [33], although several DNA damage 23 signaling proteins, such as 53BP1 (Fig. 2C), MRE11, and NBS1, co-locate there [32-34]. In addition, and 24 consistent with other studies [32-34], we observed that promyelocytic leukemia nuclear bodies were 25 almost invariably associated with these persistent IRIF (data not shown).

26 Consistently with the results of Yamauchi et al [13], a majority of cells with persistent yH2AX/53BP1 IRIF 27 were halted in G1 until 24 hours after exposure (Fig. 5A). However, analyzing later post-irradiation time 28 points, we observed that this inhibition was not permanent in our model of normal primary endothelial cells 29 (Fig. 4B and 5A). The persistent IRIF analyzed in our study were compatible with cell-cycle progression 30 and were transmitted to daughter cells: HUVECs irradiated in the G0/G1 phase that had persistent IRIF 24 31 hours after exposure — and not just a few of them — were able to complete a whole cell cycle. Moreover, 32 the ability of cells to progress through a complete cell cycle did not appear to depend on their number of 33 persistent IRIF: between 24 and 48 hours; the frequency of BrdU-negative cells decreased at the same 34 rate, regardless of their number of persistent IRIF (gray bars in Fig. 6B and C). 35 These observations raise questions about the real nature of these IRIF. Normal cells, such as primary

Huve observations raise questions about the real nature of these RRP. Normal cells, such as primary
 Huve cs, have active cell-cycle checkpoints able to block the cycle's progression until the resolution of
 DSBs [35–37]. Thus, if persistent IRIF correspond to a somehow stabilized unrejoined DSB, our

1 observations would seem to indicate that this mechanism failed to detect this structure. Several studies 2 suggest that the G1/S phase checkpoint can be bypassed, despite unrepaired DSBs, particularly if cells 3 are in the late G1 phase during irradiation and have passed a point where they can no longer initiate a 4 global shutdown of the S phase [38]. This hypothesis seems unlikely in our case, as it would mean that 5 this restriction point would have been passed 24 hours before the cells entered the S phase. Moreover, 6 DSBs induced in the G0/G1 phase can also persist after DNA replication, as shown by G2 premature 7 chromosome condensation assays combined with FISH using pantelomeric PNA probes [39]. In this case, 8 the observation of incomplete chromosome elements (chromosome fragments without a telomere signal at 9 each end) demonstrated the presence at G2 phase of unrejoined chromosome breaks from DSBs induced 10 in the G0/G1 phase. Another study reported the presence in G2 phase of chromosome breaks (without 11 discriminating complete from incomplete chromosome elements) and vH2AX IRIF in fibroblasts irradiated 12 in the G0/G1 phase [40]. On the other hand, the G2/M checkpoint appears to induce a drastic reduction in 13 the rate of incomplete chromosome elements in M-phase cells [39], although the rate of incomplete 14 chromosome elements per cell in M phase remains dose-dependent. The selection that appears to occur 15 during the G2/M transition may be consistent with the difference in the frequency of persistent IRIF 16 observed in our experiments between G0/G1 BrdU-negative cells and G0/G1 BrdU-positive cells (Fig. 6A). 17 However, the absence of any significant arrest in G2 in our model suggests that this mechanism may not 18 apply here (Fig. 4A).

Thus if these incomplete chromosome elements are able to reach cytokinesis, they should be transmitted to daughter cells, as were the persistent IRIF observed in our study. Analyzing metaphase cells, Martin *et al.* reported that persistent IRIF do not seem to co-locate with either telomere-free chromosome ends or misrepaired breaks [41]. It is nonetheless important to note that histone H2AX phosphorylation takes place on serine 139 along the chromosome arms from metaphase to telophase, independently of any DNA damage induced [42–44]. Thus, it appears difficult, or even impossible, to distinguish clearly between the H2AX phosphorylation that occurs normally during M phase and that induced by DDR.

26 Nevertheless, the asymmetric distribution of persistent IRIF between daughter cells (Fig. 7) suggests that 27 it may be difficult for this structure to segregate correctly during anaphase. The normal distribution of 28 chromosomes between daughter cells is driven by the presence of a centromere that allows sister 29 chromatids to segregate equally in each cell [45,46]. Thus, the structure formed after the passage of a 30 persistent IRI focus across the S and G2 phases may impede the correct segregation of the affected 31 chromosome's sister chromatids. The rate of missegregation associated with the presence of a vH2AX 32 signal strongly supports this hypothesis (Table 2 and Fig. 8). All these observations suggest that the 33 nature of the IRIF must differ at least slightly before and after the first cell division following exposure. If 34 DNA damage is induced in G0/G1 phase but the cell nonetheless reaches metaphase, the process of 35 chromosome segregation during anaphase may encounter numerous problems, including the conversion 36 of a remaining DSB into chromosome breaks (CRB) (Fig. 9). This transition into CRBs of DSBs induced in 37 the G1 phase and reaching metaphase has been described in yeast where two types of associations may 1 occur: one between the two sides of the DNA break (intrachromosomal association) and the other 2 between sister chromosomal fragments from either side of the DNA break (interchromosomal association) 3 [47]. These two associations lead to a kind of "tug-of-war" between the two spindle poles during the 4 anaphase, which almost always results in co-segregation of the acentric chromosome fragments [47]. 5 According to this model, the persistent IRIF observed after cellular division in our study might correspond 6 to several kinds of atypical chromosomal structures composed of segregated chromatids with unrejoined 7 DSBs either stabilized or converted into CRBs. These chromosome elements may be included in the cell 8 nucleus or may form micronuclei (Fig. 9).

9 In conclusion, we observed that cells containing persistent IRIF do not block the cell cycle permanently 10 and that these IRIF can be transmitted to daughter cells. Moreover the asymmetric distribution of persistent IRIF between daughter cells indicates that cell division likely affects the structure signaled by 11 12 persistent IRIF and may lead to the formation of an atypical chromosomal structure in daughter cells. This 13 atypical chromosomal assembly may be lethal or result in gene dosage imbalance and possibly enhanced 14 genomic instability in daughter cells. The consequences of this phenomenon should be further analyzed, 15 especially as more than 50% of the progeny of cells exposed to 5 Gy of irradiation (10% for 1 Gy) may 16 carry this abnormal structure.

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- 19 Conflict of interest
- 20 None
- 21
- 22

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## 23 Figure Legends

24 Fig. 1. Numbers and areas of yH2AX foci in G0/G1 primary HUVECs as a function of time after irradiation 25 by 1 Gy (light gray boxes), 5 Gy (dark gray boxes), and in non-irradiated cells (white boxes). Box-and-26 whisker plots of (A) the number of vH2AX foci per nucleus, and (B) their corresponding areas. Bold black 27 bars of boxplots correspond to medians. The lower and upper borders of the box correspond to the first 28 and third quartiles, respectively, and the upper and lower whiskers to 1.5 times the interquartile distance. 29 The numbers of yH2AX foci per nuclei and their respective areas were evaluated with image analysis 30 software on around 3,000 cells for each post-irradiation time, corresponding to one representative 31 experiment. The number of yH2AX IRIF peaked 30 minutes post-exposure. The area of yH2AX IRIF 32 increased as the number per nucleus decreased. Most early vH2AX IRIF (before 24 hours after exposure) 33 were small, but persistent IRIF were characterized by a larger size.

34

1 Fig. 2. Distribution of IRIF area in G0/G1 cells after X-ray exposure at 1 Gy. Frequencies of yH2AX foci 2 area at (A) 30 minutes and (B) 72 hours after irradiation were normalized relative to the corresponding 3 non-irradiated condition to define the different ranges of size observed for early and persistent IRIF. 4 Because spontaneous foci are also present in irradiated cells, to focus only on the size of IRIF (foci 5 induced by irradiation), we normalized them, that is, calculated a ratio (irradiated versus control) of the 6 frequency of each class of the size distribution. The results shown are obtained by pooling the data of 5 7 experiments and correspond to the analysis of the distribution of size of about 460,000 IRIF at 30 minutes 8 and 21,000 IRIF at 72 hours after irradiation. Early IRIF were characterized by an area ranging from 0.2 to 9 1.3 µm<sup>2</sup> and persistent IRIF by an area greater than 1.1 µm<sup>2</sup>. (C) Representative pictures of the co-10 labeling by immunofluorescence of yH2AX (FITC) and 53BP1 (Alexa Fluor® 594) corresponding to early 11 (upper panel, 30 min) and persistent (lower panel, 72 h) IRIF in HUVECs. DNA was stained with DAPI. 12 We observed co-location of yH2AX and 53BP1 IRIF for both early and persistent IRIF. Scale bar 13 corresponds to 10 µm.

14

**Fig. 3.** The proportion of G0/G1 cells with at least one persistent IRI focus as a function of time since exposure. The analyses were performed on cells exposed to irradiation of 1 Gy (light gray triangles) and 5 Gy (dark gray squares) and on unirradiated cells (white diamonds). The mean percentages of cells with at least one persistent  $\gamma$ H2AX focus ± standard error (s.e.) were calculated from at least 5 experiments. For each condition, the average number of analyzed cells ranged from about 1,300 to 11,000. The decrease in the percentage of cells with persistent IRIF was more pronounced for those irradiated by 5 Gy, compared with 1 Gy, about 6% per 24 hours for 5 Gy and only 1% per 24 hours for 1 Gy.

22

23 Fig.4. Persistent IRIF and cell proliferation. (A-B) To study the evolution of cell division after irradiation, we 24 added BrdU just after exposure. Cells in the different phases of the cell cycle were selected by 25 assessment of the integrated intensity of the DAPI signal (DNA content) combined with the integrated 26 intensity of vH2AX signal in the entire nucleus (S phase). Percentages of cells in each phase of the cell 27 cycle were measured at 24 hours (A) and 48 hours after irradiation (B) on unirradiated cells (white bars) 28 and on cells exposed to 1 Gy (light gray bars) and 5 Gy (dark gray bars). The mean percentages ± s.e. 29 were calculated from the results of 5 experiments, with an average of about 3,000 cells analyzed per 30 condition. (C) The density of the cell monolayer was evaluated at different time points after exposure for 31 unirradiated cells (white diamonds) and for cells exposed to 1 Gy (light gray triangles) and 5 Gy (dark gray 32 squares). The cell monolayer density was measured by counting of the number of nuclei per cm<sup>2</sup> observed 33 at different time points after exposure. These measurements were performed on around 5,000 images per 34 condition. The percentages were calculated for each condition with respect to the density measured 10 35 min after exposure. The percentages and error bars correspond to the calculation of means and standard 36 errors based on 4 experiments.

37

1 Fig. 5. Persistent IRIF do not permanently block cell proliferation. To recognize cells that had completed a 2 full cell cycle since irradiation, we added BrdU just after exposure and considered only G0/G1 cells. (A) 3 Histograms of the proportion of G0/G1 cells as a function of BrdU incorporation (BrdU+ in light grav and 4 BrdU- in white) and presence (dotted) or not (empty) of persistent IRIF. The percentages were calculated 5 for unirradiated cells, respectively 5, 24 and 48 hours after the addition of BrdU, and for irradiated cells, 6 respectively, 24 and 48 hours after exposure to 1 and 5 Gy and the addition of BrdU. The mean 7 percentages were calculated from the results of 3 experiments, with an average of about 3,000 cells 8 analyzed per condition. (B) Representative pictures of the co-labeling by immunofluorescence of yH2AX, 9 BrdU, and 53BP1 performed 48 hours after 5-Gy irradiation, using primary antibodies against yH2AX, 10 BrdU, and 53BP1, detected respectively by secondary antibodies conjugated to FITC, Alexa Fluor® 594, 11 and Alexa Fluor® 647. DNA was stained with DAPI. Persistent yH2AX/53BP1 IRIF were observed in 12 G0/G1 BrdU-positive cells. Scale bar corresponds to 10 µm.

13

14 Fig. 6. Average numbers and distribution of persistent IRIF per cell in G0/G1 BrdU-positive and BrdU-15 negative cells. We evaluated the number of persistent yH2AX IRIF in G0/G1 BrdU-positive versus G0/G1 16 BrdU-negative cells in the G0/G1 phase. (A) Average number of persistent IRIF per cell analyzed 24 hours 17 (dark gray bars) and 48 hours (light gray bars) after exposure to 5 Gy of X-rays. The frequency of 18 persistent IRIF remained stable over time in each subpopulation of G0/G1 BrdU-negative cells and G0/G1 19 BrdU-positive cells. The reduced frequency of persistent IRIF appears to be associated mainly with cell 20 division rather than time since exposure. The mean number  $\pm$  s.e. was calculated from the results of 3 21 experiments; for each condition the average number of analyzed cells ranged from about 3,000 to 14,000. Student's test: \*\*  $\alpha$  < 0.025 and \*\*\*  $\alpha$  < 0.01. (B-C) Percentages of cells with each number of persistent 22 23 IRIF were calculated in G0/G1 BrdU-positive (black bars) and G0/G1 BrdU-negative (gray bars) cells at 24 (B) 24 hours and (C) 48 hours after exposure to 5 Gy irradiation. The shape of the distribution observed in 25 G0/G1 BrdU-negative cells (gray bars) remained the same at (B) 24 hours and (C) 48 hours. The 26 proportionately equal decrease for all classes indicated that all had the same ability to go through the cell 27 cycle regardless of the number of persistent IRIF per cell. The distribution observed for G0/G1 BrdU-28 positive cells shifted to lower classes (i.e., cells with fewer IRIF) compared to G0/G1 BrdU-negative cells. This result indicates a class change between the mother and daughter cells; the daughter cells have fewer 29 30 persistent IRIF than the mother. Percentages were calculated after pooling the data from 3 independent 31 experiments corresponding to analysis of 3,000 to 14,000 cells, depending on the cell subpopulation 32  $(BrdU^{+} \text{ or } BrdU^{-})$  and time since exposure.

33

**Fig. 7.** Distribution of persistent IRIF between daughter cells. At 48 hours after exposure to 5 Gy, we blocked cytokinesis by adding cytochalasin B to cell culture medium; 24 hours later, we immunostained the cells with an antibody against γH2AX and stained the DNA with DAPI. Representative pictures of the immunofluorescence labeling of γH2AX of binucleated cells with a difference of 0 (left panel), 1 (middle panel), and 2 (right panel) persistent IRIF between the two daughter nuclei. Scale bar corresponds to 10
µm. The percentage of binucleated cell with a difference of 0, 1, or 2 persistent IRIF between the two
daughter nuclei was estimated from 3 independent experiments (around 100 binucleated cells for each).
Around half of the binucleated cells had an asymmetric number of persistent IRIF in the daughter nuclei.

5

Fig. 8. Persistent IRIF induce missegregation phenotypes in BrdU-positive cells. Representative pictures
of BrdU-positive cells with different kinds of missegregation observed 48 hours after irradiation, with
primary antibodies against γH2AX and BrdU (not shown), detected respectively by secondary antibodies
conjugated to FITC and Alexa Fluor® 594. DNA was stained with DAPI. (A) binucleated cells including a
micronucleus and a nucleoplasmic bridge or a broken nucleoplasmic bridge with a γH2AX signal. (B)
G0/G1 cells including a micronucleus with a γH2AX signal. (C) G0/G1 cells including a broken
nucleoplasmic bridge with a γH2AX signal. Scale bar corresponds to 10 µm.

13

14 Fig. 9. Model of inheritance of one persistent IRIF based on the hypothesis of an unrepaired DSB. The 15 two broken ends of a DSB are maintained close by non-homologous end-joining repair proteins 16 (intrachromosomal association; purple circles). After DNA replication, association between sister 17 chromosomal fragments from either side of the DNA break can occur (interchromosomal association; 18 orange circles) [47]. The structure formed after the passage of a persistent IRI focus across the S and G2 19 phases may impede the correct segregation of affected chromosome's sister chromatids. Consequently, 20 the nature of IRIF in the nucleus of daughter cells might differ before and after the first cell division. Thus, 21 the IRIF observed in daughter cells might correspond to several atypical chromosomal structures resulting 22 from abnormal anaphase resolution.







Fig. 3



Post-irradiation time (hours)



Post-irradiation time (hours)

### Fig. 5 A



BrdU- with persistent IRIF

BrdU- without persistent IRIF

BrdU+ with persistent IRIF

BrdU+ without persistent IRIF

В





Number of persistent IRIF

## Fig. 7



in both nuclei in both nuclei 2 persistent IRIF in both nuclei

# Fig. 8





Μ

G0/G1

G2

G0/G1

	Radiation dose	Time after exposure				
		24h	48h	72h	96h	168h
	0Gy	0.27	0.22	0.26	0.25	0.39
Frequency of γH2AX	1Gy	0.86**	0.48***	0.45	0.52	0.59
	5Gy	4.22***	2.23***	1.67***	1.33**	0.84*
	0Gy	0.05	0.05	0.05	0.05	0.05
Frequency of large vH2AX foci per cell <sup>a</sup>	1Gy	0.23*	0.19**	0.16**	0.13**	0.10
	5Gy	2.02**	1.34***	1.19***	0.97***	0.53**

Table 1. Frequency of  $\gamma$ H2AX foci in G0/G1 cells exposed to X-rays

<sup>a</sup> Values represent means calculated on five experiments (about 1 300 to 11 000 cells by condition)

Value significantly different of unirradiated cells using Student T-test with:

\*  $\alpha < 0.05$ , or \*\*  $\alpha < 0.01$ , or \*\*\*  $\alpha < 0.001$ 

Radiation dose	All cells <sup>a</sup>	Cells with $\gamma$ H2AX signal <sup>a, b</sup>	Cells without $\gamma$ H2AX signal <sup>a</sup>
0Gy	9.1% ± 1.0%	59.6% ± 17.8%	40.4% ± 17.8%
1Gy	23.4% ± 3.6%	85.7% ± 0.8%	14.3% ± 0.8%
5Gy	78.0% ± 4.5%	86.5% ± 1.4%	13.5% ± 1.4%

Table 2. Frequency of cells with micronuclei and/or nucleoplasmic bridges in G0/G1 BrdUpositive cells

<sup>a</sup> Values represent means and s.e. calculated on three experiments (around 700 cells by condition)

<sup>b</sup> Corresponding to yH2AX signal observed in nuclei, micronuclei or nucleoplasmic bridges