Review

Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up

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ABSTRACT

The occurrence of DNA double-strand breaks (DSBs) induced by ionizing radiation has been extensively studied by biochemical or cell imaging techniques. Cell imaging development relies on technical advances as well as our knowledge of the cell DNA damage response (DDR) process. The DDR involves a complex network of proteins that initiate and coordinate DNA damage signaling and repair activities. As some DDR proteins assemble at DSBs in an established spatio-temporal pattern, visible nuclear foci are produced. In addition, post-translational modifications are important for the signaling and the recruitment of specific partners at damaged chromatin foci. We briefly review here the most widely used methods to study DSBs. We also discuss the development of indirect methods, using reporter expression or intra-nuclear antibodies, to follow the production of DSBs in real time and in living cells.

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Ionizing radiation (IR) produce a wide variety of DNA lesions among them double-strand breaks (DSBs), considered to be the major actor responsible for cell death. If unrepaired or improperly repaired, DSBs contribute to chromosomal aberrations, which may lead to human disorders including cancer [1]. Consequently different approaches have been undertaken to identify the mechanisms involved in the production, signaling and repair of DSBs.

The production of DSBs can be quantified by biochemical techniques such as the pulsed field gel electrophoresis (PFGE). In addition, DSBs production can be followed by cell imaging either globally, with the neutral comet assay, or damage specific, through immunostaining of marker proteins or recruitment of fluorescent proteins to the breaks. To analyze the recruitment of signaling and/or repair proteins, a clear understanding of the DNA damage response (DDR) is needed, supported by the development of cell imaging after IR or, more recently, after microlaser irradiation. We will briefly introduce the panel of biochemical and cell imaging techniques. Their insights into the DSBs repair kinetics, largely obtained by microlaser irradiation and fluorescent protein recruitment, will be presented. Since such techniques based on the over-expression of protein might generate artifacts, indirect approaches have also been developed. We discuss here the potential benefits of using intracellular antibodies, in particular directed against post-translational modifications of DDR proteins, as well as the use of different reporter systems.

Human DNA damage response after ionizing radiation treatment

In mammalian cells, the production of DSBs initiates a global cellular response, including checkpoint signaling and repair (Fig. 1) [2]. The MRN (MRE11/RAD50/NBS1) complex binds to DSBs (Fig. 1B and C) and facilitates the activation of ATM (Ataxia Telan-giectasia Mutated), a key PI3K related kinase in the DDR[3]. At the break site, ATM autophosphorylates, allowing its activation and the subsequent phosphorylation of numerous substrates in the sur-rounding chromatin. Among ATM substrates, H2AX – an H2A his-tone variant called γH2AX when phosphorylated – is considered as one of the earliest markers of the DSB signaling [4]. H2AX phos-phorylation reaction is amplified by the recruitment of MDC1

(Fig. 1C and D), a central player of the DDR through its interaction with γH2AX [5]. The accumulation of DDR proteins at damaged sites leads to the formation of foci, visible after staining under microscopic examination. The signaling amplification by MDC1 participates to the recruitment of multiple DDR members

(Fig. 1D–F), such as RAP80, 53BP1, KAP-1 and BRCA1 [6]. The recruitment of 53BP1 and BRCA1 proteins in ionizing-radiation-induced foci (IRIF), triggered by γH2AX and the MDC1 binding, is also dependent on the participation of the RNF8/RNF168 E3-ubiquitin ligases [7,8]. The overall signaling pathway leads to the
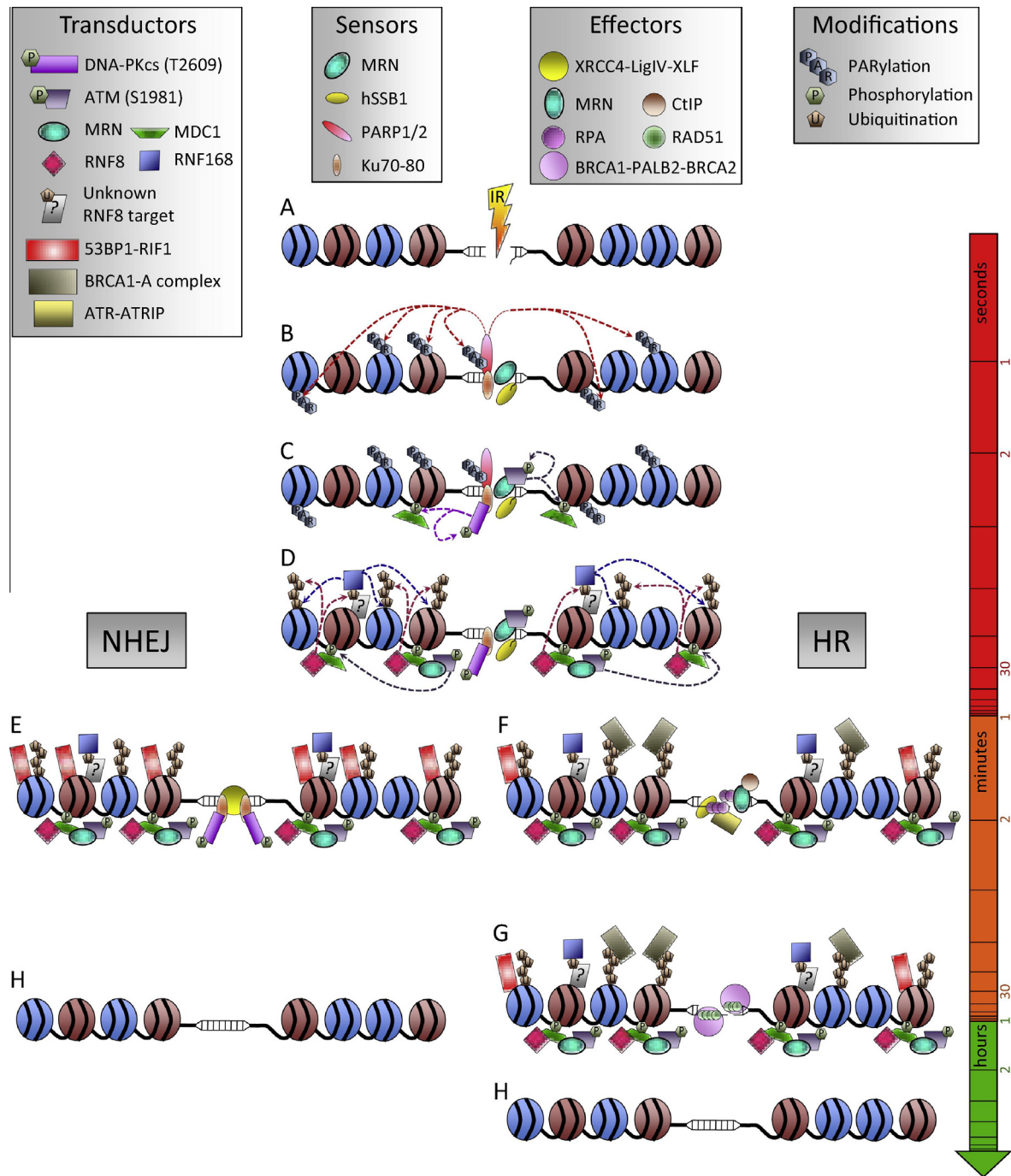

Fig. 1. Kinetics of DDR protein recruitment and modification at IR-induced DSBs. (A) Exposure to ionizing radiations (IR) induces DSBs. (B) Sensor proteins immediately recognize DSB formation. These include PARP1, which induces its own PARylation together with the PARylation of the surrounding chromatin and many DDR actors. MRN and Ku70-80 interact with DSB ends, while hSSB1 binds to the ssDNA regions. (C) H2AX phosphorylation happens in the first seconds after IR. ATM and DNA-PKcs are recruited to DSB through their interaction with MRN and Ku80, respectively. ATM and DNA-PKcs autophosphorylation drive their activation and phosphorylate H2AX (γH2AX), close to the DSB site (H2AX-containing nucleosomes are shown in red) and MDC1 rapidly binds γH2AX. (D) Modification of the chromatin flanking the DSB within the first minute after IR. MDC1 recruits more MRN–ATM complexes, which phosphorylate more distal H2AX. RNF8, recruited through its interaction with MDC1, promotes the ubiquitylation of a yet unidentified non-nucleosomal target. Once ubiquitylated, this protein is recognized by RNF168 that initiates H2A and H2AX ubiquitylation. RNF8/RNF168 then promotes K63 ubiquitin chain formation. (E and F) Factors determining the DSB repair pathway choice take place during the first minutes after IR. (E) NHEJ pathway. 53BP1 and RIF1 are recruited via 53BP1 binding to H4-K20me2, therefore preventing BRCA1 accumulation and inhibiting resection. The XRCC4-LigIV-XLF complex promotes DSB ligation. (F) HR pathway. BRCA1 complex binds to K63 ubiquitin chains at DSB, leading to 53BP1 exclusion. CtIP cooperates with MRN to initiate resection, removing Ku70-80 from DSB ends. ssDNA overhangs, bound by hSSB1 and RPA, recruit ATR-ATRIP. (G) The BRCA1–PALB2–BRCA2 complex recruits RAD51 to DSB around 30 min after IR. (H) Loss of DSB markers after repair completion. The right arrow outlines the timing of events after DSB appearance, with a simplified logarithmic scale.

downstream phosphorylation of CHK2, p53 and CDC25, triggering checkpoint activation and cell cycle arrest, in G1/S and/or G2/M. These checkpoints induce transient cell cycle arrests, allowing sufficient time for DNA repair.

Mammalian cells encompass two major pathways to repair DSBs, the non-homologous end-joining (NHEJ) pathway being the major one. The second one, homologous recombination (HR), is mostly involved in the repair of secondary DSBs that occur later after IR during the S-phase, when the replication fork collapses at unresolved single-stranded DNA (ssDNA) lesions.

The NHEJ reaction proceeds through DSBs recognition, processing of the damaged DNA ends to remove non-ligatable groups and finally ligation (Fig. 1E and H), to restore the strands continuity [9]. The first stage of the reaction is the binding of the Ku70/Ku80 heterodimer to DNA ends. The core NHEJ complex is then recruited to Ku-bound DNA ends [10]. NHEH core components include the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XLF and the XRCC4/DNA Ligase IV (X4LIG4) complex [11]. DNA-PKcs binds to the carboxy-terminus of Ku80 at break ends, providing protection within a synaptic structure, associated with protein kinase activity [12,13]. The autophosphorylation of DNA-PK induces a conformational change, required for the activation of end processing nucleases and the DNA-PKcs subunit dissociation from DNA ends [14,15]. The last stage, requires the XRCC4-Ligase IV complex and XLF which stimulates the ligation reaction [16–18]. Other proteins such as Artemis, PNK, pol λ and μ are involved in the reaction depending on the chemistry of the lesion. Moreover, new proteins involved in NHEJ are still being discovered, as the NF90/NF45 complex known to interact with DNA-PK and participating in DSB repair *via* NHEJ [19], or NONO whose recruitment to the damaged site is PARP-dependent [20].

In contrast to NHEJ, HR uses the sister chromatid sequence, synthesized during the replication, as a template for error-free DSBs repair. The first step of the HR process is a DNA ends resection reaction (Fig. 1F), initiated by the cooperation between CtIP and the MRN-complex [21] and extended by other factors including the BLM helicase and the EXO1 and DNA2 nucleases [22]. Subsequently, the recombination mediators (e.g. BRCA2, PALB2) displace RPA from the ssDNA 3′ tails and stimulate the formation of RAD51 nucleoprotein filament (Fig. 1G, reviewed in[6]). The RAD51 nucleofilament and accessory factors then catalyze homology search and strand invasion, in order to form a D-loop structure that allows the extent of DNA end to restore lost information (Fig. 1H). Several pathways can then occur through (1) D-loop dissociation leading to noncrossover products (NCO) or (2) second-end capture and double Holiday junction formation – such HR intermediates eventually being dissolved by BLM helicase to form NCO or resolved by specific endonucleases to produce either NCO or crossovers [23].

The balance between HR and NHEJ is highly regulated. At least three factors affect the choice between NHEJ and HR (Fig. 2): chemical complexity of the breaks, chromatin conformation, and cell cycle [24–27]. Indeed, although NHEJ is active throughout the cell cycle, HR is restricted to S and G2 where the sister chromatids are available to allow recombination processing [28]. At a molecular level, DDR proteins recruitment and modification regulate DSB repair. Basically, 53BP1-RIF1 complex stimulates NHEJ by inhibiting end resection whereas BRCA1-CtIP antagonizes 53BP1-RIF1 to promote HR [29]. It has also been shown that DNA-PK plays a negative action on HR [27,30]. In conclusion, spatio-temporal control of post-translational modifications along with a coordinated recruitment of signaling and repair proteins at the break ends are critical events in the DDR [2].

Biochemical and cellular quantification of DSBs

Radiosensitivity is governed by the amount of DNA damage resulting from exposure, and also by individual capacity to correctly repair these insults. In this regard, it is important to not only predict the DSB level arising from a given IR dose, but also to consider the DSB repair rate, which might considerably fluctuate between tissues and persons.

Physical approaches to measure DSBs

The first approaches developed to study DNA breakage were based on the physical separation of undamaged from fragmented DNA. In the 70's, neutral sucrose gradient sedimentation was the method used to measure DSBs in irradiated mammalian cells
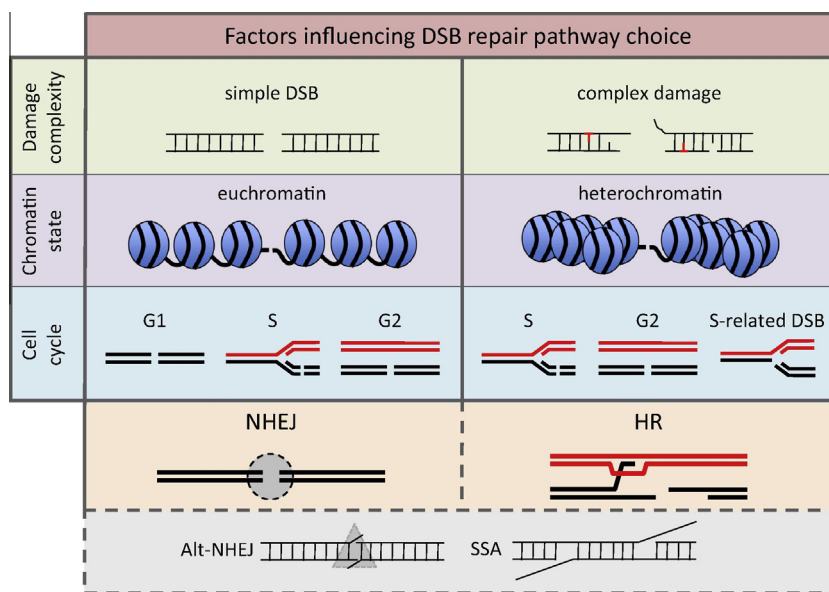
Fig. 2. Factors influencing the repair pathway choice after IR-induced DSBs. Damage complexity – Damage complexity increases with LET of radiation. Simple DSBs or clustered (also named complex) lesions are most probably repaired by NHEJ or HR, respectively. Chromatin state – DSBs produced in euchromatin is mainly repaired by NHEJ, while DSBs in heterochromatin are mainly processed by HR mechanisms. Cell cycle – NHEJ is effective during the whole cell cycle, whereas HR is restricted to S and G2 phases. Indirect DSBs occurring through fork collapse (S-related DSB) have to be repaired by HR in order to restart the fork. When NHEJ- or HR – is defective (dashed lines), the DDR can switch to the second pathway or to alternative mechanisms, namely alternative NHEJ (Alt-NHEJ) or Single Strand Annealing (SSA).

[31]. In the 80's, neutral elution [32] and electrophoretic methods allowed to detect more sensitively DNA breaks and their repair kinetics. Pulse-field gel electrophoresis (PGFE), first developed to separate yeast chromosomes [33], has been deflected to visualize broken DNA in mammalian cells after IR [34]. The comet assay has been described at the same time, allowing the visualization of damaged DNA in individual cells embedded in agarose [35]. Both PFGE and comet assay are still routinely used to measure DSBs, but one has to consider that these methods (i) necessitate some technical expertise, (ii) suffer poor reproducibility between labs and (iii) may reveal other types of lesions than strictly DSBs [36]. More troublesome, the non-random distribution of IR-induced DSBs across the genome [37], the heterogeneity of cellular response between cells (i.e. apoptotic DNA fragmentation, repair processes) and the atypical migration pattern of S-phase related replication fork structures lead to only approximate the DSB levels in living cells by PFGE [38,39]. Inter-laboratory variation is also a well-known limitation of the comet assay [40], albeit new high-throughput approaches are under development to overcome such impediment [41]. In conclusion, although direct detection approaches give information related to cell survival, recent developments of cell imaging led to gain more insights in the DSBs repair mechanisms.

Immunocytological detection of DSBs

Identification and characterization of new DDR components considerably increased in the last twenty years. Proteins involved in these processes function as sensors, signal transductors or at the repair mechanism level. Hence, the vast majority of these actors have to accumulate at damaged sites, a feature which makes them immunocytologically visualizable as docking structures called foci. The MRE11 nuclear relocalization in irradiated human cells leads to a local concentration, at DSBs sites, named IRIFs for ionizing radiation-induced foci [42]. However, proteins have to accumulate enough at IRIFs to be detectable. In the light of this remark, the components of NHEJ, major pathway of irradiation-induced DSB repair in mammals, are very difficult to visualize because (i) they are highly expressed and (ii) only one or two molecules are required per DSB during the repair process [43,44]. Consequently, a cellular fractionation assay has been developed to reveal the chromatin remobilization of NHEJ factors tightly bound to the breaks in contrast to their weak binding to the undamaged chromatin [45]. Thus, this approach accounts for global activation of the NHEJ pathway and simply qualitatively assesses the presence of DSB. On the other hand, many proteins involved in HR accumulate at IRIFs, including RAD51, RAD52, RAD54, BRCA2, PALB2, RPA and CtIP [21,46,47]. As mentioned above, HR is only required in S/G2 at IR-induced DSBs repair, being involved in approximately 15% of total DSBs, mainly corresponding to heterochromatin-associated DSBs [48]. Thus, analysis of HR-related IRIFs only leads to estimate a specific subset of DSBs and is not useful for non-cycling cells.

Other potential DSB biomarkers are proteins binding directly DNA breaks, working as DSB sensors (Fig. 1B). PARP-1 and PARP-2 are among the first molecules recruited to DNA breaks induced by irradiation, and are essential for the binding of another DSB sensor, as the MRN complex [49]. However, as PARPs mobilization and local PARylation events to DSBs – but also SSBs – are very transient, they do not provide good biomarkers. Ku70–Ku80 heterodimer is a specific DSB sensor, but as discussed above, is not easily detectable by classical immunostaining because, among the non-histone proteins, it is one of the most abundant factors in the nucleus. The MRN components are thus the only sensors that may be used to observe DSBs, despite their foci formation being delayed [50]. However, the human ssDNA binding protein 1 (hSSB1), binding ssDNA overhangs of IR-induced DSBs, should be considered as a new DSB sensor as it localizes to DSB with the same kinetics than MRN, and is essential for ATM signaling and MRN recruitment [51,52]. Moreover, due to its role in later HR steps, hSSB1 remains associated longer than MRN to DSBs, and may thus provide a more stable DSB biomarker.

The last group of DDR proteins, mediating damage signaling, provides the most proficient DSBs biomarkers. Upstream the DNA damage signal transduction pathway lie three members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family, recruited to damaged sites *via* interaction with specific sensors [53]. ATM and DNA-PKcs are both involved in DSB signaling (Fig. 1C) [6]. ATM autophosphorylation at serine 1981 drives its activation and localization at IRIFs [54]. However chromatin structure modification independent of DNA breaks induces S1981-ATM phosphorylation. Phosphorylated DNA-PK at threonine 2609 can be detected at IRIFs [44], whereas total DNA-PK cannot [43], probably due to the high content of DNA-PKcs in the nucleus. A third PIKK, ATR (Ataxia Telengiectasia-Rad3 related), can be tracked with its partner ATRIP at IRIFs [55], but specifically stains IRIFs associated with replication fork collapse and HR processing.

DDR kinases phosphorylate hundreds of targets [56], and many of these events orchestrate regulated accumulation of DDR components – or their modified forms – to IRIFs. The most documented example is the phosphorylation at serine 139 of the histone variant H2AX, referred to as γH2AX. DSB-mediated phosphorylation of H2AX spreads up to several Mega bp around damaged sites, and related foci appear within a minute (Fig. 1C and D) [57]. The number of γH2AX IRIFs, 30 min after irradiation, is similar to the number of DSBs estimated by PFGE and γH2AX foci disperse once the breaks are repaired [58]. Therefore γH2AX is currently the DSB biomarker of choice and is broadly exploited in various applications, to monitor DSB formation and repair, including radiosensitivity [59,60], and most commonly in genomic instability studies associated to cancer, aging and inflammatory diseases. Several recent reviews focus on the significance and limitations of measuring γH2AX for such purposes [50,61–64]. However, although the occurrence of one DSB was correlated to the formation of one γH2AX focus, the opposite relation is questionable. Indeed, chromatin modifications can activate ATM in hypoxic cells, independently of the MRN complex, and DNA-PK is also activated under hypoxia [65–67], these kinases being therefore able to phosphorylate H2AX independently of DSBs signaling.

γH2AX concentration at DSBs enables the assembly of downstream DDR proteins. For example, MDC1 interacts directly with γH2AX and forms IRIFs (Fig. 1C and D) [5]. However, until today, antibodies raised against MDC1 are not completely satisfactory for IRIF analyses [50] and MDC1 immunolocalization is rarely achieved. Once accumulated at DSBs, MDC1 serve as a docking platform for numerous effector proteins loading to the damaged site, including IRIF forming E3 ubiquitin ligases RNF8 (Fig. 1D) and RNF168, which locally ubiquitinate H2A and H2AX [7,8]. Besides, visualization of RNF8/RNF168-dependent chromatin ubiquitination at IRIFs can be realized with FK2 antibodies, detecting ubiquitin-conjugates. Chromatin ubiquitination at DSBs promote the recruitment of other ubiquitin-binding proteins including RNF169 and RAD18 [68], and the late-acting antagonizing factors BRCA1 and 53BP1 (Fig. 1E and F), in order to direct DSB repair to HR and NHEJ, respectively. The BRCA1-A complex is mobilized at IRIFs through the UIM domains of RAP80, which binds ubiquitin chains [69]. BRCA1 promotes HR and does not form IRIFs in G1 cells. On the opposite, 53BP1 and its partner RIF1 accumulate at DSBs from G1 to the end of G2 [70,71]. 53BP1 is therefore a second highly useful IRIF marker, that does not accumulate at ssDNA regions formed during replication stress unlike ATR-dependent γH2AX staining that arises in the S-phase [50]. Therefore, recent

studies combine 53BP1 and γH2AX immunofluorescence staining to discriminate DSB-independent γH2AX staining (without 53BP1) from γH2AX foci that really mark DSBs [72,73].

As stated above, chromatin ubiquitination drives the recruitment of several proteins to DSBs, but other ubiquitination events have to be considered. Indeed, when DSBs occur in the S-phase, ubiquitination of two Fanconi Anemia proteins, FANCD2 and FANCI, promotes their accumulation to IRIFs [74,75]. On the contrary, RNF8 triggers the ubiquitination-mediated Ku80 removal from damaged sites for an efficient NHEJ repair [76]. Moreover, other protein modifications take place at DSB, like small ubiquitin-like modifier (SUMO) on several DDR proteins [77]. It seems however that SUMOylation events, at DSBs, target protein groups rather than specific substrates, in order to promote physical interactions and stabilize repair complexes [78]. In addition, SUMO E3 ligases PIAS1 and PIAS4 localize to DSBs and are essential for the RNF4/RNF168 ubiquitin ligases and BRCA1 recruitment [79,80], illustrating the tight relationship between SUMO and ubiquitin-mediated signaling pathways.

Live analysis of DSBs

Although immunofluorescence is a sensitive technique, allowing for example the detection of a single γH2AX focus, it requires cell fixation and therefore limits the study of the protein recruitment kinetics as well as the DSBs resolution. Valuable insights on the spatio temporal dynamic of proteins at the IRIFs have been given by time-lapse microscopy studies, following fluorescent-tagged proteins in living cells. Most of these studies have been realized after laser micro-irradiation and the choreography of foci temporal organization has been recently reviewed in great detail [6].

Spatiotemporal dynamics of early DDR proteins on complex DNA lesions have been described [81]. Using ionizing charged particle irradiations, the response of living cells expressing fluorescently tagged proteins was imaged. Information on the mobility and binding rates of the recruited proteins was obtained from Fluorescence Recovery after Photobleaching (FRAP). Interestingly, it appears that NBS1 is recruited faster with increasing lesion density and saturates at very high damage levels. This faster recruitment for increasing lesion complexity is also observed for MDC1, but not for 53BP1 [81,82], and a model has been proposed to explain this different recruitment behavior at low and high Linear Energy Transfer (LET) [81]. However, such irradiators have low accessiblity procedures and, consequently, time-lapse microscopy is often achieved after X- or γ-irradiation. The drawback of these experiments is that early time points of DSB formation cannot be studied because such irradiations take a few minutes. However, later studies (around a minute or few minutes after irradiation) are still feasible and led to visualize, for example, the dose-dependent recruitment of 53BP1 at DSB [83].

Because transitory recruited proteins at the DSB could be missed in such experiments, laser microirradiation has been widely used in laboratories. For example, proteins that function at the early phase of the DSB response are often recruited to the damaged sites. This is the case of the nuclear NONO/SFPQ heterodimer, which displays diverse functions in nucleic acid metabolism and has been shown to enhance DSB repair in vitro [84]. After laser irradiation, SFPQ/NONO is rapidly recruited to DNA damage in a PAR-dependent manner [20]. However, because NONO recruitment is optimal about 2 min after laser irradiation and returns to background levels at 10 min, classic immunofluorescence analysis after IR would not have led to IRIFs observation. For these reasons, transitory recruited proteins are not pertinent biomarkers to follow DSB in living cells. In addition, after laser irradiation, damages are highly concentrated at a cell point (or along a laser line), induc-

ing a bias in the study of the DSB recruited proteins. From these limitations, other tools to study DSB in live conditions have been developed.

New tools to study DSB in live conditions

An original reporter system *in vivo*, based on the luciferase reconstitution after DSB induction, has been recently developed [85]. H2AX and the carboxy-terminal BRCT domains of MDC1 are fused to luciferase N- and C-half parts, respectively (Fig. 3A). The association between γH2AX and the BRCT domains of MDC1, at the DSBs, leads to the luciferase reconstitution and to a quantitative light signal. However, the authors do not describe foci but rather a global signal, associated to two waves of luciferase activation. As the first wave could reflect the signal associated to DSB, the second one could be due to a massive apoptotic signal. This split-luciferase-based method, although necessitating more development to study DSB repair kinetics *in vivo*, is still promising.

Conventional antibodies have been widely used in research, diagnostics and therapeutics. However their large size is a serious disadvantage for their production and folding. To replace and miniaturize classic antibodies, single-chain variable fragments (scFvs) have been developed. ScFv is a fusion of the variable regions of the heavy and light chains of immunoglobulins, connected with a linker peptide (Fig. 3B). ScFvs targeting the DNA-PKcs have been developed, but are shown to modify the IR response in living cells [86]. The radiosensitization by anti DNA-PKcs scFvs has been described [87,88], but no fluorescent scFv were developed, which is consistent with the fact that total DNA-PK cannot form IRIFs [43]. Recently, a phosphospecific scFv has been generated [89]. This demonstrates that phosphospecific scFv can be developed and open the way to a whole world of biomarkers, such as phospho-Thr2609 DNA-PK scFv, in order to detect IRIFs in living cells. A scheme of the resulting system is depicted (Fig. 3B). However, the scFv needs an adequate folding of its two variable domains to be functional, and this proved to be a limiting factor within living cells.

The Variable domain of Heavy chain antibody (VHH domain), produced by a particular animal species, is sufficient for antigen interaction (see left part of Fig. 3C, reviewed in [90]). The VHH domain can be cloned and expressed as monomeric domain of 14–15 kDa and is ten times smaller compare to a classic antibody. The VHH properties led to diverse and original applications, in particular as intrabodies (also called single-domain antibodies (sdAbs) or nanobodies) associated to fluorescent tag [91]. Thus, they can be used in living cells to track a specific target and are, in addition, easily producible in *Escherichia coli* [92]. The study of replication forks, in living cells and in real-time, has been achieved recently, through as a stable cell line expressing the anti-PCNA sdAb fused to the GFP (Fig. 3C), with or without a replicative stress [93]. Regarding sdAb, it has been reported that linking two sdAbs to form a diabody may increase its avidity [94], allowing it to stay longer on the target. Another way to improve sdAb affinity, hence signal specificity, would be to fuse them to self-associating peptides and generate multimeric antibodies [95].

Post-translational modifications (ubiquitination, SUMOylation, acetylation, methylation, PARylation and phosphorylation) are playing major functions in IRIFs [2], and new tools to allow live studies – through post-translational modifications – have been developed. Indeed, time-lapse studies using fluorescent-tagged proteins are not giving any indication on their post-translational modification status. The new techniques are essentially based on the immunological recognition of specific post-translational modifications, and the new systems are mainly targeting the widely used γH2AX marker. So, labelledanti-γH2AX antibodies, associated to the HIV transcription activator (Tat) as an entry peptide and to a Nuclear Localization Sequence to address the construct to the
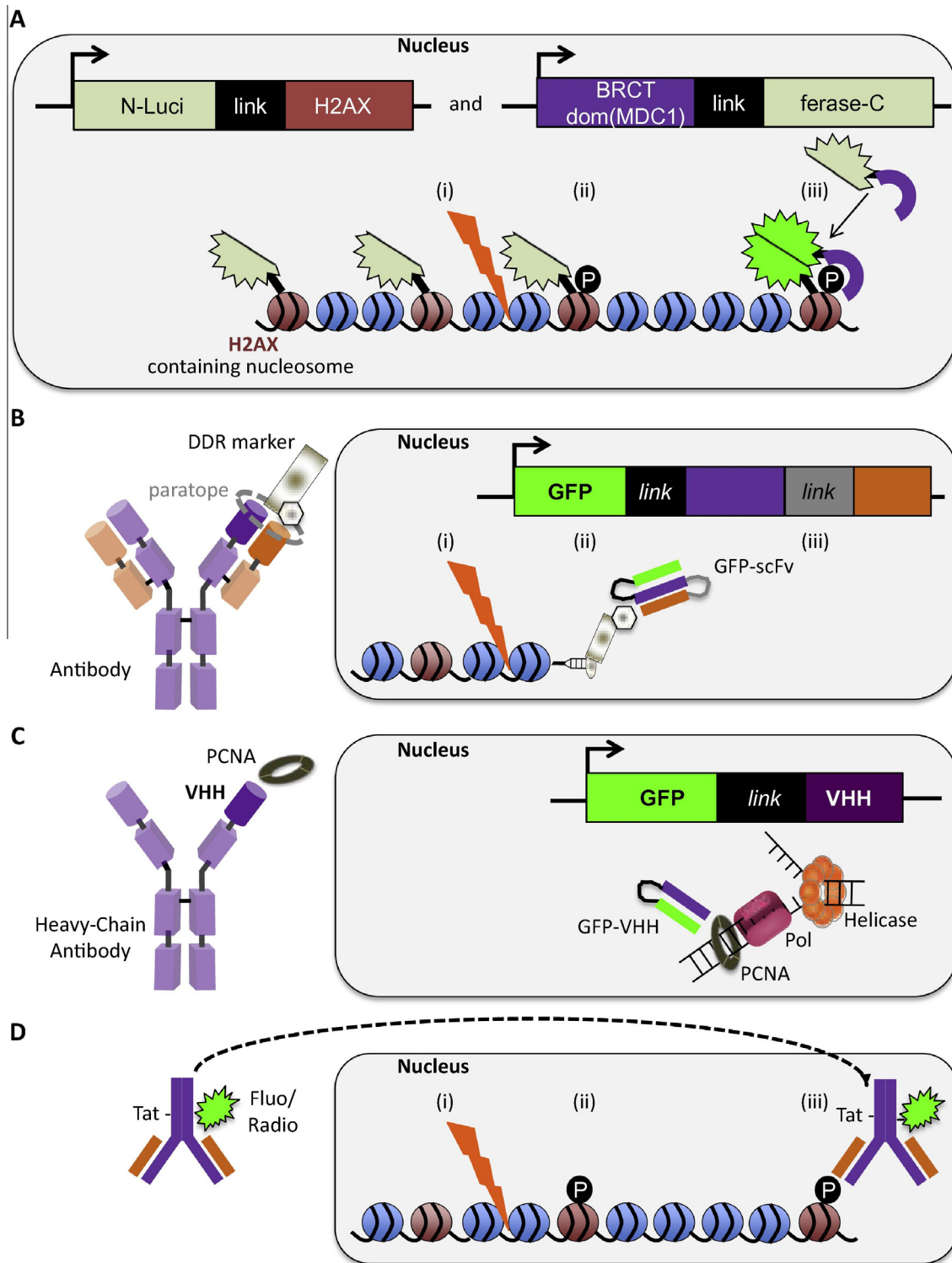
Fig. 3. Following Double-Strand Breaks *in vivo*. (A) H2AX/MDC1 Luciferase reporter system. The H2AX and BRCT-MDC1 sequences are cloned in fusion with half of the luciferase (N-luci and ferase-C, respectively). These constructs are transduced in cells and, after irradiation (i), H2AX phosphorylation around the DSB (ii) leads to the reconstitution of a functional luciferase (iii). (B) Genetically encoded fluorescent scFV. In classic antibodies, the paratope is formed of the heavy and light chain variable domains. A construct, associating an scFv recognizing a DDR marker (or an appropriate post-translational modification) and the GFP protein, is proposed. In a stable cell line, after irradiation and DSB induction (i and ii), the recruitment of the GFP-scFv could be followed (iii), allowing the real-time DSB analysis in living cells. (C) Genetically encoded fluorescent sdAb. Diagram of a heavy-chain antibody displaying the VHH domain and the PCNA antigen interaction. The construct made of the anti-PCNA VHH domain, fused to the GFP, recognizes PCNA and allows the replication analysis in living cells and in real-time. (D) Fluo/radio-labeled anti-γH2AX-Tat antibodies. Representation of the labeled anti-γH2AX antibodies, coupled to a Tat peptide allowing their cell entry and nucleus localization. Tat-antibodies are coupled to a radio- or fluorescent marker for their analysis.

nucleus, have been developed [96]. Radio- or fluorescently labeled anti-γH2AX enters living cells, and associate to DSB (Fig. 3D). Used in living cells or organisms, it leads to the visualization of DSBs.

However, this system is based on immunoconjugation, uses a huge amount of antibodies and limits foci analysis to the time of entry of the immunoconjugate within the cells.

Conclusions and perspectives

Until recently, it was impossible to study DSBs production and repair at low irradiation dose (in the range of 1 Gy) due to the very limited sensitivity of the DSB detection methods. Our understanding on the spatio temporal recruitment of repair proteins at low irradiation doses is based on the studies of IRIFs. In these foci, hundreds of recruited proteins have been used as surrogate markers for DSBs. It has been described earlier that pertinent DSB markers should be stably recruited at the break. In order to follow the repair kinetics in real time, live imaging of cells expressing fluorescently tagged proteins, recruited at the breaks, has been developed. This technique allows the determination of the protein mobility and assembly at the damaged site. Cell imaging takes advantage of innovative derivatives such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP) or fluorescence correlation spectroscopy (FCS), allowing the detection of the proteins mobility and interactions *in vivo*. Importantly, these require a stable fluorescent signal in time and led to the development of new fluorescent tools. On the other hand, it is clear that specific DDR markers need to be developed in order to study DSB real time and in living cells. Areas of improvement are multiple, but key steps may be to (i) target post-translational modifications due to DDR signaling pathway activation and (ii) increase the signal specificity. In order to achieve this latter point, it may be necessary to decrease the signal background due to fluorescently tagged protein overexpression.

Indeed, although live cell imaging presents many advantages over immunocytochemistry with fixed cells, this technique encounters some limitations. Among them, fluorescently tagged proteins may produce artifacts and signal background due to the high level of expression. In fact, tagged protein may have a different sub-cellular localization or perturb cell metabolism and/or DNA repair complex. Thus, the knock-in replacement of the endogenous protein may address this question [97], although partly. An elegant alternative may be the use of an indirect approach, with split-luciferase (or split-GFP) or through intracellular antibodies. In addition, intracellular antibodies could be engineered, by molecular evolution in order to modify their affinity or by combining different antibodies. However, such approaches may lead to the inhibition of the repair process. Another potential artifact is linked to the use of microlaser irradiation, which leads to complex damage in a huge nucleus volume. Furthermore, computational analysis of time-lapse imaging data is still challenging and requires procedures of cell segmentation, foci identification and single-particle tracking. At last, in connection with the rapid evolution of the instrumentation and the interaction between biologists and mathematicians in computer science, one should obtain very quickly new insights on the recruitment of proteins at DSBs, and their movement during the repair process.

Acknowledgement

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