Editorial

A Perspective on Chromosomal Double Strand Break Markers in Mammalian Cells

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The DNA damage response (DDR) in mammalian cells is a complex and highly orchestrated signaling process that regulates the recruitment of specific DDR proteins to the DNA damage sites [1,2]. The most lethal type of DNA damage is the double-strand break (DSB), which is generated by ionizing radiation (IR), radiomimetic drugs and also drugs of DNA topoisomerase 2 poison family [3]. DSBs are also endogenously caused during replication of single-strand breaks generated by reactive oxygen species or at stalled replication forks [4,5]. While IR is extensively used in treating various cancers, IR-induced DSBs and other genomic lesions can cause mutations and gross genome rearrangements in normal cells as well, possibly leading to secondary leukemia and other cancers. Fortunately, multiple DSB repair (DSBR) systems, in coordination with cell cycle checkpoint control, have evolved to prevent both the cell lethality and mutations arising from unrepaired genome lesions. Activation of the master regulator ATM (Ataxia Telangiectasia Mutated) initiates the complex choreography of signaling required to interface with the repair pathways to protect cells after DSB induction [6]. In view of the critical role of DSBR in differential survival of healthy vs. tumor cells during radiotherapy, and its role in ageing and neurological diseases [7-9], reliable and sensitive biomarkers of DSBs and DSBR kinetics are necessary to monitor cellular responses. These would not only be useful for pre-clinical studies but also for evaluating the efficacy of radiotherapy in the clinical setting by assessing therapy-induced DNA damage or characterizing the inherent genomic instability of precancerous lesions. Radiation-induced DSBs in DNA mostly result from closely spaced (typically within 10 base pairs), bi-stranded single-strand breaks caused by ionization tracks. In response to DSB induction, the Mre11/Rad50/NBS1 (MRN) complex is initially recruited to damage sites to activate ATM, a serine/threonine kinase [10,11]. Activated ATM phosphorylates the H2AX variant of histone H2A at serine 139 at the DSB sites, which then triggers recruitment/accumulation of other DDR proteins including RNF8, RNF168, 53BP1 and BRCA1, at these damage sites [12]. Protein molecules localized in the vicinity of DSB termini, after conjugation with fluorescent antibodies, appear under a fluorescence microscope as bright dots termed foci. Many DDR proteins could be visualized as discrete foci at DSB sites by immuno-fluorescence (IF). However, it is important to note that not all proteins accumulating at damage sites are detectable as foci because accumulation of at least 100 molecules is needed to visualize a discrete focus [13]. While the mechanism underlying protein focus formation has begun to emerge only recently, the phenomenon is being extensively used.

Commonly used DSBR markers in Mammalian Cells

Phosphorylated H2AX - In principle, any DSBR protein that forms IF focus is a candidate for a DSB biomarker. However, phosphorylated H2AX [also known as gamma (γ)-H2AX] was first identified as a quantitative DSB marker due to its high sensitivity and almost immediate formation (within seconds) after DSB induction [14]. The γ-H2AX foci level is linearly related to the number of DSBs and IR dose in the range of 1.2 mGy and 2 Gy, as analyzed in primary human

linearly related to the number of DSBs and IR dose in the range of 1.2 mGy and 2 Gy, as analyzed in primary human fibroblasts. Detection of DSBs by γ-H2AX foci formation is 100-fold more sensitive than by other available methods at clinically relevant radiation doses (1-10 Gy) [15,16]. The half-maximal number of foci is reached within 1 min and the maximum in 9 to 30min after irradiation [17]. Only few base pairs are involved in the initial DSB formation, commensurate with a small but distinct focus being formed initially at a DSB site. However, over time, the focus spreads to adjacent areas to cover up to 2 Mbp chromosomal DNA, and contains estimated 2000 γ-H2AX molecules. This suggests significant signal amplification, probably involving chromatin modification to facilitate the binding of a large number of DSBR components. Based on DSBR kinetics, 15-30min after irradiation appear to be the appropriate interval for precise and sensitive foci counting to quantitate DSBs [18]. However, spontaneous γ-H2AX foci are detectable in both normal and cancer cells, likely as a result of endogenous DSBs generated by DNA replication stress. The basal level of foci varies with the cell type, but commonly 1-2 foci/cell have been observed in normal tissues (colon, breast, ovary and human primary fibroblasts) while in cancer cell lines the number is larger,1-20 foci per cell, and more variable [19,20]. Although IF-based foci analysis is the most sensitive approach for DSB detection, it should be noted that the overlap of multiple foci due to high DSB density poses a major challenge for accurate manual or software-based quantitation, especially with high radiation doses [18].

The disappearance kinetics of the γ-H2AX foci, presumably linked to H2AX dephosphorylation, closely follows that of DSBR, irrespective of the repair pathway employed by the cell. However, the mechanism of de-phosphorylation is still controversial. Few studies reported the persistence of a small number of γ-H2AX foci even after apparent completion of DSB repair, particularly after formation of a high level of DSBs due to time required for resetting of chromatin after repair[16]. Nevertheless, the loss of γ-H2AX correlates well with DSBR at low-to-moderate levels of genome damage (<150 DSBs/genome) in repair proficient cells [21]. A unique advantage of using γ-H2AX foci as a DSB biomarker is that these foci are formed in all cell cycle phases. However, under certain circumstances, γ-H2AX foci may not exclusively reflect DSBs because disregulated DNA metabolism may also cause ATR-mediated H2AX phosphorylation in growing cells, which form DSB-independent background foci, in addition to heat mediated H2AX foci, that do not involve DSBs [22,23].

Phosphorylated ATM - ATM, which is activated via auto-phosphorylation at serine 1981, is recruited to DSB sites in the very early stage of DSBR and was proposed as an alternative DSB marker in growing cells [24]. Phospho-ATM foci formation follows a linear relationship with IR dose over the 10 mGy to 1 Gy range, and the number of foci is accurately correlated with the number of DSBs. However, one limitation of phospho-ATM as a DSB marker is that the senescent cells display phospho-ATM signal in the absence of DSBs [25,26].

Other DSBR pathway-specific proteins as DSB markers - In addition to phosphorylated H2AX and ATM, certain unmodified DSBR proteins also form foci at DSB sites, such as p53 binding protein 1 (53BP1), MDC1, RAD50 and BRCA1, and are often used as DSB markers [27,28]. 53BP1 co-localizes with γ-H2AX at DSB sites and the number of foci reaches a maximum at 15-30 min after IR treatment, followed by steady decrease to the background level after 10-16h. Foci disappearance matches the kinetics of DSBR occurring via non-homologous end joining (NHEJ); however, 53BP1 migrates or is released from the DSB sites in the case of other two modes of DSBR, namely, homologous recombination (HR) and alternative end joining (Alt-EJ) [13,29]. Furthermore, unlike phosphorylated H2AX/ATM, 53BP1 recruitment is specific to DSBs, except when DSBs occur during mitosis [30,31]. Other DSBR proteins including MDC1, RAD50, and BRCA1 also accumulate at DSB sites in sufficient numbers to form foci and were utilized as DSB markers in several studies [32]. However, their universal utilization is limited by their lack of correlation with DSBR kinetics in all cell types and all modes of DSB repair.

As already mentioned, not all DSBR proteins are capable of forming foci. For example, several key DSBR proteins like DNA-PK, Ku70/80, Smc1 and Smc3, although, recruited to DSBs, usually by direct binding to the damaged termini, do not form foci after irradiation [33]. Similarly, Chk2 (phosphorylated by ATM at threonine 68) and Chk1, critical DSB response elements in mammalian cells for damage-dependent cell cycle arrest, do not form foci at individual DSB sites after IR [34]. In fact, phospho-(Thr68)-Chk2 rapidly appears over the entire nucleus in laser microirradiated cells. Similarly, DNA-PK/Ku70 and Smc1 can be detected at laser microirradiated tracks after micro-laser irradiation at a substantial dose [33,34].

Other methods of quantitating DSBs - Complementary techniques used to measure DSBs include flow cytometry and Western blotting, which qualitatively (or semi-quantitatively at best) measure DSBs and their repair by monitoring covalent modification of DSBR proteins (e.g., γ-H2AX and phospho-ATM and also phospho-Chk2). In addition to the protein-based approaches, single cell electrophoresis to analyze damage-induced DNA fragments, named the comet assay, was developed some three decades ago and has been extensively utilized for the detection and quantitation of DNA single-strand and double-strand breaks. This assay method is based on the principle that fragmented DNA migrates out of the cell according to size during in situ electrophoresis, while intact nuclear DNA is too large to have detectable mobility. After staining with the dye acridine orange, the nucleus appears as a comet head with the smaller fragments migrating out of the cell appearing as a 'tail' under the microscope. The amount and size of the DNA fragments in the tail, measured as the 'tail moment', is proportional to the level of strand breaks [35]. A reasonably sensitive and rapid way to detect DNA damage, as

was first introduced in 1984 [36]. The original comet assay was designed to measure single-strand breaks in the genome because electrophoresis was performed under alkaline conditions. The modified comet assay is able to detect cellular DSBs by carrying out electrophoresis at neutral pH. However, the sensitivity of the comet assay for detecting DSBs is significantly lower than that of γ-H2AX foci analysis [37-39].

Although not particularly sensitive, pulse field gradient electrophoresis (PFGE) and size-based DNA fractionation by sedimentation in an ultracentrifuge have also been used to monitor DNA fragmentation [40,41]. However, like comet analysis, these are not appropriate for quantitating small numbers of DSBs.

Perspective and future directions

A sensitive biomarker for chromosomal DSBs is a major prerequisite for analyzing genome damage and repair, which are important in both the laboratory and clinical settings. Some half dozen DSB markers and techniques have been developed for detection and quantification of the genome damage; the selection of the optimum marker largely depends on the objective of the research/diagnostic procedure, the cell types as well as the agents/drugs used to induce the DSBs. Although, γ-H2AX foci formation is not an exclusive indicator of DSBs, it is still the best marker based on its cell phase-independent formation, tight correlation with repair kinetics and repair pathway independence. Future studies should focus on defining distinct DSBR sub-pathways and identifying specific, quantitative biomarkers for analyzing each mode of DSB repair in cancer versus normal cell types. This may require a greater understanding of and range of DSB markers but could help identify and validate unique DSBR targets for cell/tissue type-specific cancer therapy.

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Table 1. DSBR proteins that form immuno-fluorescence foci, which are commonly used as biomarker and their unique features.

DSB foci-forming marker	Description/limitation
Gamma-H2AX	• Forms foci rapidly, disappearance closely follows DSBR kinetics in a cell cycle, pathway-independent fashion. • May not exclusively represent DSBs under some circumstances.
pATM 1981	• Forms foci rapidly and follows the DSBR kinetics in a pathway-independent fashion. • Forms DSB-independent foci in senescent cells.
53BP1	• Forms DSB-responsive foci in all cell phases except in mitosis. • Matches repair kinetics for NHEJ, may migrate or release from DSB for HR/Alt-EJ. • A small fraction of foci may persist after DSBR.
BRCA1	• DSB-responsive foci for HR in S/G2 cells.
RAD-51	• DSB-responsive foci for HR in S/G2 cells. • Role in Alt-EJ unclear.

Figure 1. A schematic model of repair and damage response proteins recruited at chromosomal DSB sites in mammalian cells. The proteins that are commonly used as immune-fluorescence-based foci biomarkers are highlighted (*).
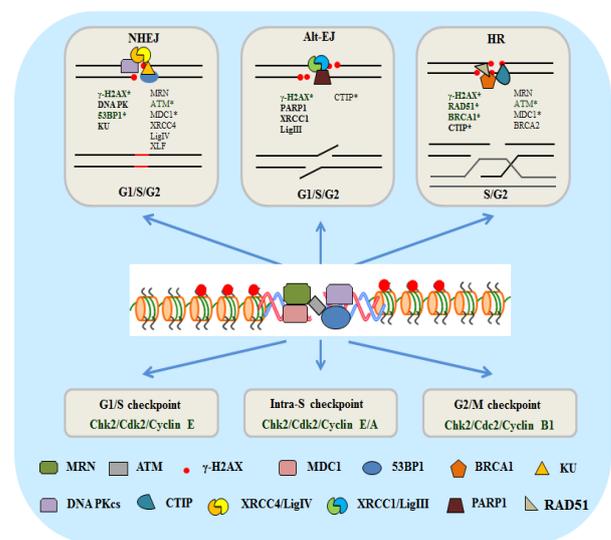
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