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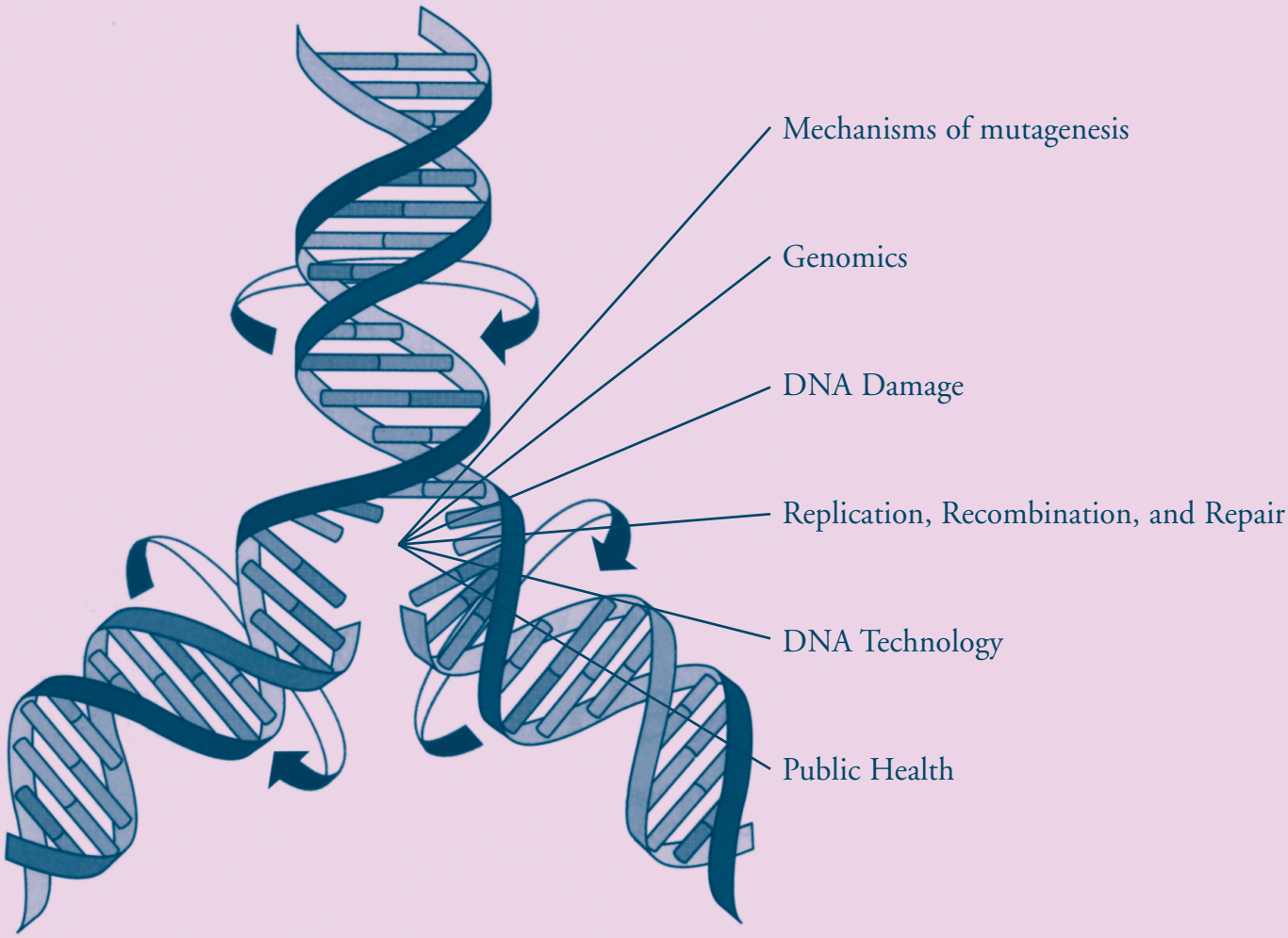

In this issue: Rothkamm et al review the use of DNA damage foci to understand the cellular and individual responses to DNA damaging agents.

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Review

DNA Damage Foci: Meaning and Significance

Kai Rothkamm,[1,2]* Stephen Barnard,[1] Jayne Moquet,[1] Michele Ellender,[1] Zohaib Rana,[1] and Susanne Burdak-Rothkamm[3]

[1]*Public Health England, Centre for Radiation, Chemical and Environmental Hazards, Chilton, United Kingdom*

[2]*Department of Radiotherapy, Laboratory of Radiation Biology and Experimental Radiation Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

[3]*Department of Cellular Pathology, Oxford University Hospitals, Headley Way, Headington, Oxford, United Kingdom*

The discovery of DNA damage response proteins such as γH2AX, ATM, 53BP1, RAD51, and the MRE11/RAD50/NBS1 complex, that accumulate and/or are modified in the vicinity of a chromosomal DNA double-strand break to form microscopically visible, subnuclear foci, has revolutionized the detection of these lesions and has enabled studies of the cellular machinery that contributes to their repair. Double-strand breaks are induced directly by a number of physical and chemical agents, including ionizing radiation and radiomimetic drugs, but can also arise as secondary lesions during replication and DNA repair following exposure to a wide range of genotoxins. Here we aim to review the biological meaning and significance of DNA damage foci, looking specifically at a range of different settings in which such markers of DNA damage and repair are being studied and interpreted. Environ. Mol. Mutagen. 56:491–504, 2015.

Key words: γH2AX; 53BP1; DNA double-strand break; ionizing radiation; genotoxicity

WHAT ARE DNA DAMAGE FOCI?

There are a number of different names in use for DNA damage foci, such as (ionizing) radiation-induced foci (IRIF or RIF) or DNA repair foci. In general they all refer to local accumulations or modifications of DNA damage response proteins that form at the sites of DNA double-strand breaks and can be visualized through microscopic imaging following immunocyto- or -histochemical detection or fluorescent protein tagging (Figs. 1 and 2). The first and most prominent protein for which foci formation at the site of a double strand break was described is the histone variant H2AX which gets phosphorylated at its C-terminal Ser-139 residue by the DNA damage-activated kinases ATM, ATR, and DNA-PK [Stiff et al., 2004] to form γH2AX. As this phosphorylation event is restricted to a chromosomal region surrounding an unrepaired double-strand break but involves hundreds to thousands of histone modifications within this region, it can be detected microscopically as a distinct spot or 'focus' of several hundred nanometres diameter following immunostaining against the phosphorylated form of the histone [Rogakou et al., 1999]. γH2AX then acts as a docking station for other DNA damage signaling factors such as MDC1 and 53BP1 which accumulate to form foci in a histone-modification-dependent manner (recently reviewed in [Panier and Boulton, 2014]).

TECHNICAL ASPECTS OF FOCI DETECTION

DNA damage foci have been studied in a wide range of established cell lines, primary cell cultures, peripheral blood lymphocytes which are of great relevance in patient studies, three-dimensional in vitro tissue models as well as histological sections of human and animal tissues. Foci are most commonly detected by immunofluorescence

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*Correspondence to: Kai Rothkamm, University Medical Center Hamburg-Eppendorf, Laboratory of Radiation Biology and Experimental Radiation Oncology, 20246 Hamburg, Germany. E-mail: k.rothkamm@uke.de

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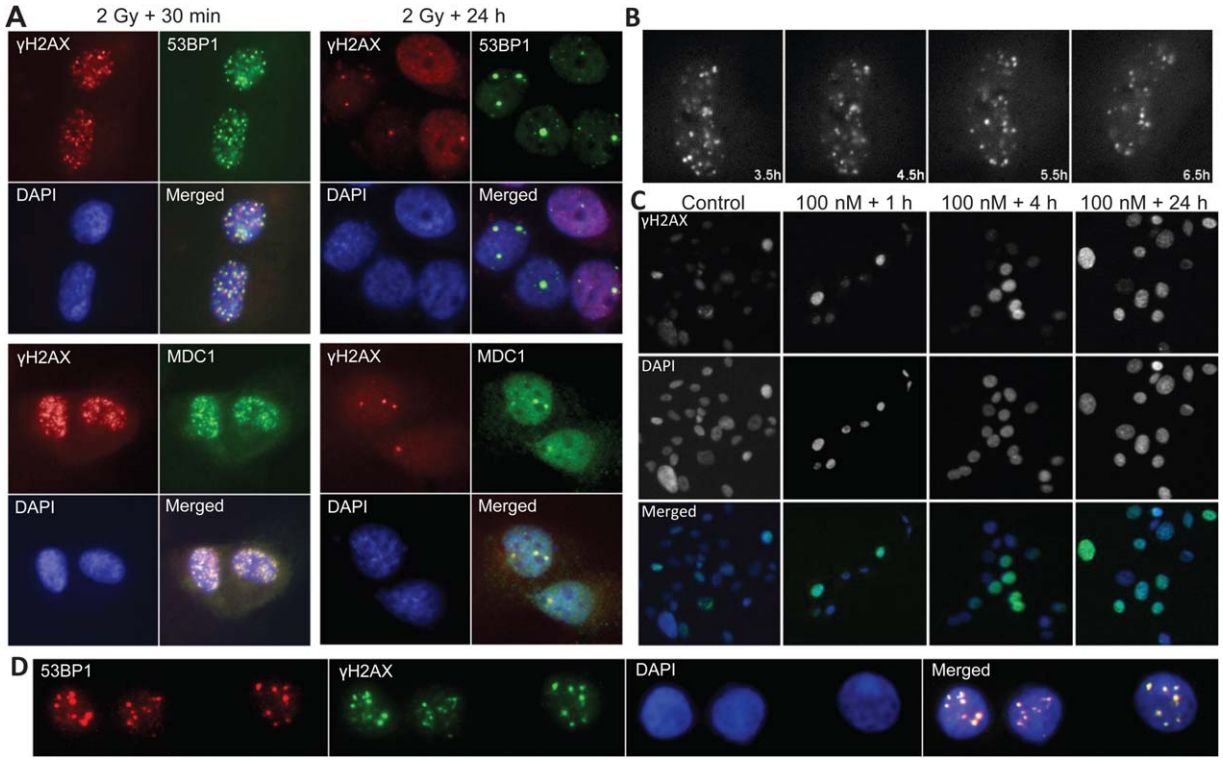

Fig. 1. Examples of DNA damage foci in cultured cells. (A) Colocalizing γH2AX, 53BP1, and MDC1 foci indicative of DSBs in UTSCC14 head and neck squamous cell carcinoma cells 30 min and 24 h following treatment with 2 Gy X-rays. Fluorescence microscopy images were taken following co-immunofluorescence staining and DNA counterstaining with DAPI. Each image is 35 μm wide. (B) Time lapse fluorescence microscopy images of one HeLa cell following transient transfection with a mammalian expression vector encoding a green fluorescent protein-53BP1 fusion protein. Pictures were taken using a live cell imaging microscope at 3.5 to 6.5 h post 2 Gy X-irradiation. Each picture is 21 μm wide. (C) Time-course of γH2AX induction in UTSCC14 cells following treatment with 100 nM gemicitabine, a nucleoside analogue which stalls DNA replication. Pan-nuclear γH2AX staining is observed in an increasing fraction of cells over time, rather than distinct subnuclear foci as in the case of X-rays. Each image is 190 μm wide. (D) Colocalizing γH2AX and 53BP1 foci in peripheral human blood lymphocytes 24 h following 4 Gy X-irradiation ex vivo. Each image is 40 μm wide.

microscopy, but can also be analysed using fluorescent protein fusion constructs, enabling foci formation and loss to be monitored in live cells. Immunohistochemical staining methods using chromogenic substrates have also been successfully employed to detect foci in sections obtained from formalin-fixed paraffin-embedded tissue blocks. Foci quantification can be performed by manual scoring through the eyepieces of a microscope, manual scoring of digital microscope images or automated scoring using commercial or open source image analysis software packages. In all cases it is of crucial importance to define and maintain strict scoring criteria. As scoring is severely influenced by staining quality and imaging characteristics, it is good practice to include positive and negative reference samples which help confirm the validity and reproducibility of the results obtained in a particular experiment. One frequently voiced caveat of manual scoring, lack of objectivity of the scoring procedure, can be easily overcome by coding samples to remove any bias.

Further details of biosampling, foci staining, detection and quantification have been discussed elsewhere [Olive, 2004; Nakamura et al., 2006; Rothkamm and Horn, 2009; Löbrich et al., 2010; Ivashkevich et al., 2011; Redon et al., 2011; Barnard et al., 2013; Vignard et al., 2013; Pouliliou and Koukourakis, 2014]. Intensity-based approaches such as flow cytometry or Western blotting are also commonly used to study foci-forming DNA damage response proteins (see e.g. [Olive, 2004; Tanaka et al., 2007, 2009; Pope et al., 2011; Rosen et al., 2014]). However, it must be stressed that these assays, which merely measure total abundance of the protein or modification, are typically less sensitive than imaging approaches (e.g. [Horn et al., 2011]) and blind to the intranuclear spatial distribution of the proteins of interest.

DNA damage foci are now widely studied in a range of different research areas, utilizing very diverse organisms, such as yeasts, plants, rodents and humans. The following sections discuss their meaning and significance in different applications.

DNA DOUBLE-STRAND BREAKS

Each human cell has to repair numerous DNA lesions every day as a result of spontaneous decay, replication

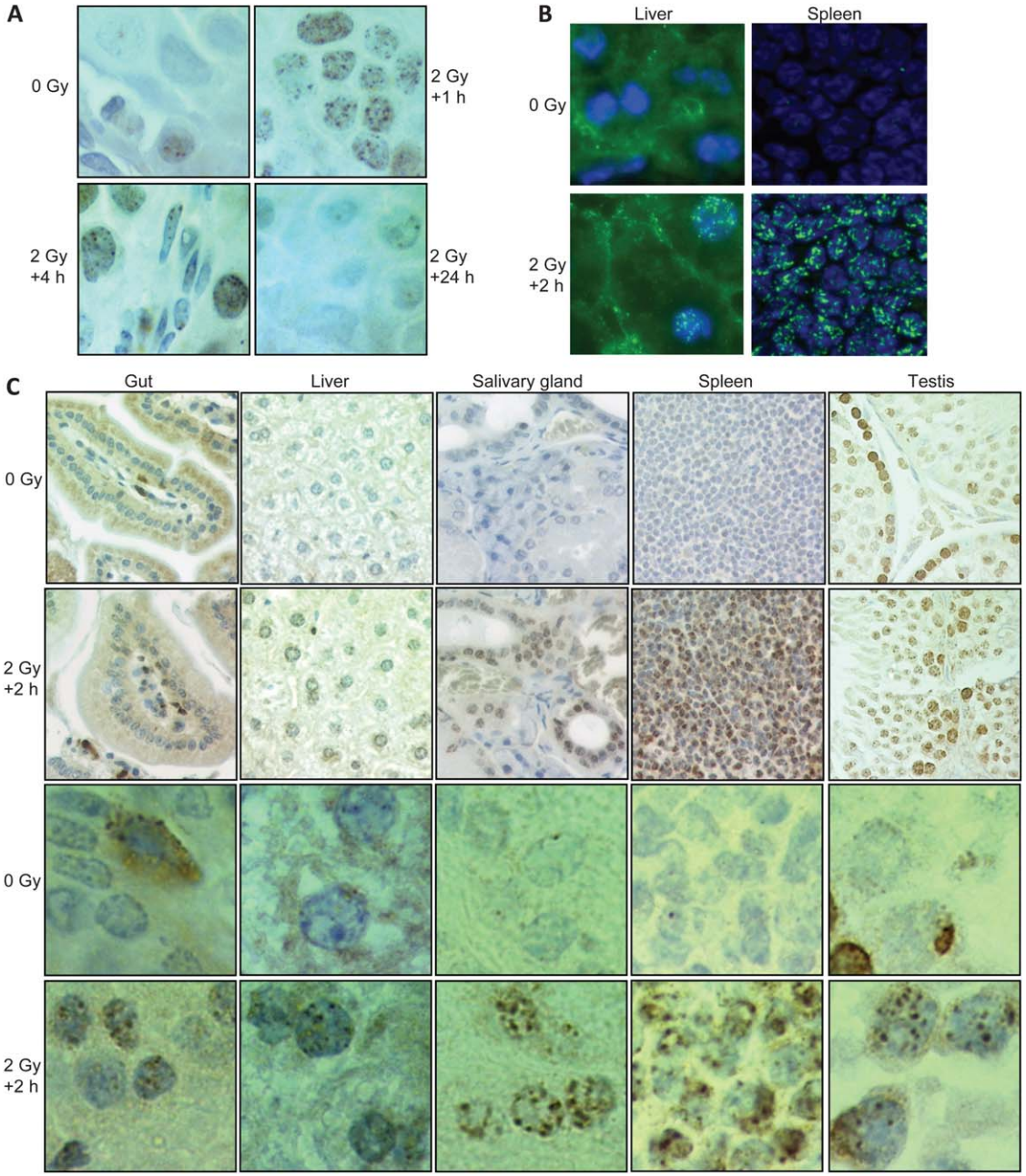

Fig. 2. X-ray-induced γH2AX foci in tissue sections. (A) Chromogenic staining for γH2AX (brown) with hematoxylin counterstaining in 5 μm sections of formalin-fixed paraffin-embedded UTSCC14 xenograft tumours. Each image is 54 μm wide. (B) Immunofluorescence staining for γH2AX (green) and DNA counterstaining with DAPI (blue) in frozen 10 μm sections of murine liver and spleen. Images show maximum projections of z-stacks obtained using a fluorescence microscope with structured illumination attachment. Each image is 63 μm wide. (C) Chromogenic staining for γH2AX (brown) with hematoxylin counterstaining in 5 μm sections of different formalin-fixed paraffin-embedded murine tissues. Images in the upper two rows are each 310 μm wide, in the bottom two rows 42 μm.

errors, and cellular metabolism. One relatively rare but very potent type of lesion is the DNA double strand break (DSB). DSBs occur when the two complementary strands of the DNA are broken within a distance of a few base pairs. This leads to the dissociation of the two DSB ends, potentially resulting in erroneous repair and recombination with other DNA fragments. DSBs can be induced by exogenous agents such as ionizing radiation, chemicals, anti-cancer drugs and environmental stress or endogenously as a result of reactive oxygen species (ROS) produced during normal cell metabolism or when DNA replication forks collapse. DSB formation may also be

seen in cells treated with topoisomerase inhibitors, upon replication of DNA molecules affected by lesions (such as SSBs), during V(D)J recombination and class switch recombination in lymphocytes, meiotic recombination in germ cells, mating type switching in yeast and also as a consequence of DNA fragmentation in cells undergoing apoptosis.

Overwhelming evidence supports a strong, quantitative correlation between γH2AX foci formation and DNA double-strand break induction following ionizing radiation exposure, based on absolute yields and distributions induced per unit dose [Rothkamm and Löbrich, 2003; Sedelnikova et al., 2003, Barnard et al., 2013], their modulation by microenvironmental factors such as oxygen concentration [Olive and Banath, 2004; Wardman et al., 2007] and by genetic factors such as mutations in relevant DNA repair genes [e.g. Rothkamm and Löbrich, 2003; Rothkamm et al., 2003; Kühne et al., 2004; Riballo et al., 2004]. As radiation-induced γH2AX foci tend to co-localise very reliably with 53BP1 and ATM-pS1981, these other DNA damage response proteins can be used as alternative or, in situations where accuracy is of crucial importance, as additional markers of double-strand breaks through co-immunostaining [Ward et al., 2003; Bekker-Jensen et al., 2006; Rothkamm et al., 2007; Horn and Rothkamm, 2011; Ojima et al., 2011]. Nonetheless, quantitative and spatio-temporal inconsistencies in the relationship between foci and double-strand breaks and heterogeneous foci dynamics within the nucleus have been reported and some of the underlying issues have been explored [Kinner et al., 2008; Costes et al., 2010; Barnard et al., 2013; Chiolo et al., 2013], at least for ionizing radiation. These include the heterogeneous distribution of the H2AX histone in the nucleus [Bewersdorf et al., 2006], the delay between DSB induction and the formation of microscopically visible foci [Rothkamm and Horn, 2009], pan-nuclear H2AX phosphorylation and MDC1 recruitment following localised induction of complex DNA damage [Meyer et al., 2013], expulsion of DNA damage foci from heterochromatin [Jakob et al., 2011], and the possible coalescence of multiple foci in close proximity into one [Neumaier et al., 2012]. The situation is even less clear for "spontaneous" foci and those triggered by other stimuli, whether intrinsic (e.g. replication stress, aging, oxidative damage, DNA metabolism), or extrinsic (e.g. ultraviolet radiation, chemical exposures). Most genotoxins induce foci only as secondary events, when unrepaired DNA lesions cause replication forks to stall and/or collapse in cells passing through S phase following exposure. Consistent with this notion, foci induction has been reported to be delayed, peaking only several hours after exposure to agents such as UV light and alkylating agents [Staszewski et al., 2008; Zhao et al., 2014] and not always showing the distinct pattern of individual spots seen for ionizing radiation-induced DNA damage foci, but instead sometimes a more homogenous, pan-nuclear signal (see e.g. Fig. 1C). It is important to note that the extent of such secondary foci induction may be affected by a number of factors, including the cell cycle distribution at the time of exposure, cell cycle checkpoint control, the efficiency of DNA repair pathways, i.e. nucleotide or base excision repair, that contribute to the removal of lesions prior to S phase entry, and the functionality of relevant DNA damage sensing and signaling pathways that are involved in foci formation. Also it is not absolutely clear whether such secondary foci do in fact always reflect the presence of DSB. For example, ageing haematopoietic stem cells have been reported to harbor replication stress-induced nucleolar γH2AX foci which persist owing to ineffective H2AX dephosphorylation by mislocalized PP4c phosphatase rather than ongoing DNA damage [Flach et al., 2014].

There are continuing discussions over the biological meaning and interpretation of residual DNA damage foci which may persist for many days following high [Ahmed et al., 2012] as well as low dose irradiation [Rothkamm and Löbrich, 2003]. Several different mechanisms have been discussed which may contribute to this effect, including delayed or ineffective γH2AX dephosphorylation, inducible repair that is lacking at low doses but can be stimulated by treatment with hydrogen peroxide [Grudzenski et al., 2010]; the induction of secondary, bystander-type foci which persist for much longer than directly induced foci [Ojima et al., 2011] and accumulation of DNA damage foci in senescent cells [Sedelnikova et al., 2004] where they may be associated with telomeres [Nakamura et al., 2009; Fumagalli et al., 2012; Hewitt et al., 2012]. However, there is still no proof that these persistent foci do indeed reflect unrepaired DSB. The lack of a reliable, alternative assay for the sensitive DSB detection means that this ambiguity is likely to remain unresolved for the foreseeable future, although promising complementary DSB detection methods have been reported recently [Shee et al., 2013].

Despite all these potential flaws and caveats, DNA damage foci, and especially γH2AX, have already been used extensively as markers of DNA damage or repair in human population studies (reviewed in [Valdiglesias et al., 2013]), and this trend will most certainly continue in the coming years.

DNA DOUBLE-STRAND BREAK REPAIR

Because of their high potential impact on genome stability and cell survival, several complex pathways have evolved for repairing DSB. Accordingly, DSB repair deficiency has been associated with chromosomal breaks and translocations resulting in cell death, cell transformation and tumorigenesis, developmental defects, neurodegeneration,

immunodeficiency, radiosensitivity, sterility, and cancer disposition [Polo and Jackson, 2011]. The two main mechanisms for repairing DSBs are homologous recombination (HR) and nonhomologous end-joining (NHEJ) [Chapman et al., 2012; Goodarzi and Jeggo, 2013; Davis et al., 2014] which complement each other. HR involves a complex machinery to resect DSB ends to produce long single-stranded DNA overhangs, search for sequence homologies, and exchange strands with an intact sister chromatid that serves as a template for repair. On the other hand, NHEJ involves three steps which result in the ligation of two DNA ends in close proximity: (a) recognition of break ends and their binding by the Ku subunit of the DNA-dependent protein kinase (DNA-PK), (b) removal of nonligatable termini, and (c) joining of the ends by DNA ligase IV, supported by the scaffold proteins XLF and XRCC4. These pathways are evolutionary conserved in eukaryotes, but their significance differs between species and changes during the cell cycle. For example, HR is favored in simple eukaryotes, such as yeast and is generally more active during or after DNA replication. On the other hand, NHEJ is the dominant pathway in mammals and is active throughout the cell cycle, whereas HR is active only in the S- and G2-phases [Rothkamm et al., 2003; Kakarougkas and Jeggo, 2014] when a sister chromatid is available as a template for recombination and DSB repair is assisted by the cohesin complex [Nasmyth and Haering, 2009; Bauerschmidt et al., 2010].

Over the past decade, a number of alternative DSB repair mechanisms were identified. These include alternative end joining pathways (or backup NHEJ, in contrast to conventional DNA-PK-dependent NHEJ) which operate independently of the core-end joining factors such as DNA-PK, XRCC4, and DNA ligase IV. They are suspected to be more error-prone than DNA-PK-dependent NHEJ [Mladenov and Iliakis, 2011; Schipler and Iliakis, 2013]. PARP-1, DNA ligases 1 and 3 as well as XRCC1 have been found to contribute to backup end joining. Furthermore, single-strand annealing and break-induced replication may contribute to DSB repair, especially in association with replication. Both of these processes have long been characterised in yeast but have recently also been observed in mammalian cells [Constantino et al., 2014; Kuhar et al., 2014] and may contribute to copy number variation following irradiation [Gribble et al., 2013; Arlt et al., 2014]. These alternative DSB repair processes are thought to gain special importance in tumour cells, in which the canonical DNA damage signaling and repair pathways are often disturbed.

The temporal and spatial dynamics of proteins accumulating to form foci in response to DNA damage, and their resolution during repair, have been studied in great detail and have improved considerably our understanding of the genetic, epigenetic, and biochemical basis of mammalian DNA double-strand break repair pathways and their regulation [Ciccia and Elledge, 2010; Lukas et al., 2011; Scully and Xie, 2013; Smeenk and van Attikum, 2013; Goodarzi and Jeggo, 2013; Kakarougkas and Jeggo, 2014; Daley and Sung, 2014; Gupta et al., 2014; Panier and Boulton, 2014]. Apart from the cell cycle position, discussed above, DSB repair pathway choice and the composition of DNA damage foci may also be influenced by the nature of DSB ends as well as by the localisation of the DSB within the nucleus and its chromatin context.

The chemical nature of DSB ends can have a major impact on their processing [Aparicio et al., 2014], with NHEJ being inhibited by modified/damaged DNA ends, but also by those with extended single-stranded DNA overhangs. A range of specialised enzymes act to process DNA ends to restore them to a ligatable state and at the same time determine the choice of repair pathway to be utilized. These include polynucleotide kinase 3' phosphatase (PNKP) [Jilani et al., 1999], Ku itself which can excise nucleotide damage in the vicinity of DNA ends [Roberts et al., 2010], the neurogenerative disease protein aprataxin which removes adenylate groups [Ahel et al., 2006] and the phoshodiesterases TDP1 and 2 which remove DNA topoisimerase adducts [Cortes Ledesma et al., 2009]. In addition, damaged DNA ends can be trimmed by endonucleases such as Artemis or Metnase [Mohapatra et al., 2013], the Aprataxin and PNK-like factor (APLF) [Kanno et al., 2007], the RecQ helicases WRN and BLM in cooperation with the helicase/endonuclease DNA2 [Sturzenegger et al., 2014] and the MRE11/RAD50/NBS1 (MRN)/CtIP complex [Neale et al., 2005], to make break ends ligatable. Especially the MRN/CtIP complex has been implicated in DSB sensing, initiating the resection of the 5'-DNA strand to produce 3'-single-strand DNA overhangs and facilitate the search for sequence homology required for homology-dependent repair. The distinct endo- and exonuclease activities of MRE11 generate 3' overhangs [Shibata et al., 2014] once NBS1 has recruited the endonuclease CtIP. The activities of MRE11, RAD50, CtIP, and the nucleases EXO1 and DNA2—which perform the bulk of end-resection required for HR—are all regulated by the DNA damage kinases ATM and ATR [Symington and Gautier, 2011; Jasin and Rothstein, 2013]. Not surprisingly, DSBs that are accompanied by additional lesions in close vicinity of the break ends, so-called complex DSBs which are frequently induced by densely ionizing radiations such as alpha particles, are much more likely to be processed by end-resection than "simple" DSBs [Yajima et al., 2013; Averbeck et al., 2014]. Radiation quality therefore influences repair pathway choice.

The position of a DSB within the nucleus also affects how it is repaired. DSBs located at the nuclear membrane, but not at nuclear pores or in the centre of the nucleus, were shown not to activate the canonical DNA damage response and to be repaired by alternative end-

joining [Lemaître et al., 2014]. Recent electron microscopic studies have provided a more detailed picture of the spatial arrangements of repair proteins within foci structures [Lorat et al., 2012]. DNA damage signaling and foci dynamics differ significantly for DSBs located in different chromatin environments such as hetero- and euchromatin [Goodarzi et al., 2010; Chiolo et al., 2013] and are also affected by the transcriptional status [Aymard et al., 2014]. Furthermore, there is some evidence to suggest that radiation-induced DSB, and thus foci, may move and merge with each other in repair domains if they are located within 1 to 2 μm [Neumaier et al., 2012]. This notion implies a saturation of foci induction with increasing dose, resulting in an underestimation of true DSB yields at high doses. It also provides a mechanistic framework for the formation of chromosomal rearrangements caused by DSB misrejoining [Vadhavkar et al., 2014].

Interestingly, evidence from physical assays for gross DSB rejoining, such as pulsed-field gel electrophoresis, suggests that some of the key proteins used in DNA damage foci assays, i.e. γH2AX, 53BP1, and ATM, are expendable for the bulk repair of most DSBs induced by ionizing radiation, with only a small fraction of repair events appearing to require these factors [Kühne et al., 2004; Riballo et al., 2004]. This surprising finding illustrates the high level of redundancy and wide range of back-up options available to the cell. On the other hand, the severe cellular radiosensitivity as well as the significant developmental and health consequences that are encountered at an organism level in the absence of one of these proteins demonstrate the crucial importance of a tightly regulated hierarchy of DNA damage response functions which is required to minimise the risk of adverse outcomes from erroneous DSB repair. A study using nonphosphorylatable H2AX derivatives has shown that, whilst a wide range of genotoxic agents induce extensive H2AX phosphorylation, this response is important for cell survival mainly for agents that directly induce DSBs [Revet et al., 2011]. Therefore, the induction of γH2AX may serve as a surrogate marker of DNA damage in general but may not always be associated with DSBs. Functionally, however, γH2AX seems to contribute much more critically to the response to DSBs than to other DNA lesions, and specifically to a certain subset of DSBs that also require ATM, MRE11, NBS1, 53BP1, and Artemis for their repair [Riballo et al., 2004].

The points above and the fact that H2AX phosphorylation is dispensible for initial DSB sensing [Celeste et al., 2003], DNA damage signaling, and bulk DSB repair [Yuan et al., 2010] should be kept in mind when quantifying and interpreting DNA damage foci as markers of DSBs. This is especially pertinent in the case of residual foci which, in some studies, have been reported to persist for many days (see section on DNA double-strand breaks). It should always be kept in mind that gamma-H2AX foci represent dynamic events of continued phosphorylation by the DNA damage kinases ATM, DNA-PK, and ATR and dephosphorylation by a range of phosphatases. Just because foci persist this does not necessarily mean that the underlying DSBs are not repaired, just that the foci have not yet been dephosphorylated.

RAD51, on the other hand, is the central player in HR and thus of crucial importance for conventional recombination processes and in fact essential, as RAD51 null cells accumulate DSBs during replication and die [Tsuzuki et al., 1996; Sonoda et al., 1998]. The function of RAD51 in HR-dependent DSB repair has been reviewed in [Krejci et al., 2012; Jasin and Rothstein, 2013]. Briefly, HR requires the extensive resection of DNA ends to generate 3' single strand overhangs (see above) which then form a nucleoprotein filament with the mammalian RecA homolog RAD51, supported by mediator proteins, mainly BRCA2, which promote RAD51 loading by displacing tightly bound replication protein A (RPA) from single-stranded DNA. RAD51 filament formation blocks alternative pathways including single-strand annealing and alternative end-joining in favor of more faithful HR. The RAD51 filament invades an intact template duplex with homologous sequence, such as the sister chromatid in S and G2 phase cells, and the invading 3' end is used as a primer for DNA synthesis. The newly synthesized strand is then displaced by DNA helicases, anneals back to its original complementary strand, and then serves itself as a template for fill-in synthesis on the other strand, followed by ligation. In situations where both DNA overhangs of the DSB undergo strand invasion, the resulting double Holliday junctions can be resolved in a number of different ways which may or may not lead to cross-over events [Jasin and Rothstein, 2013].

It is tempting to equate Rad51 foci formation with homologous recombinational repair functionality. Whilst such a connection has been confirmed in many cases, replication inhibition experiments have demonstrated that a deficiency in RAD51 focus formation, such as that observed in rad51d null mutants, is not necessarily associated with increased cellular sensitivity to agents that block replication [Urbin et al., 2012]. Conversely, Rad51 foci formation and cell death in the absence of DNA damage has been reported in Rad51-overexpressing cells following treatment with the RAD51-stimulatory compound RS-1 [Mason et al., 2014].

In summary, a highly complex picture emerges, in which the choice of DSB repair pathway is made separately for each DSB, depending on a combination of criteria and regulated by a functional network of protein phosphorylation and ubiquitilation in a chromatin context. It is important to emphasise that the significance and functional relevance of DNA damage foci in DSB repair is not always clear, especially in the case of agents that

induce primarily non-DSB lesions [Revet et al., 2011; Cleaver, 2011]. More work is certainly needed to improve our understanding of these issues. Whilst foci assays offer glimpses into the underlying dynamics, decoding the nature of the individual repair event and its consequences for the fate of the cell remains a major challenge.

RADIATION EXPOSURE ASSESSMENT

DNA damage foci, and especially γH2AX foci in peripheral white blood cells, are promising biomarkers in biological dosimetry where radiation exposures need to be estimated retrospectively [Rothkamm and Horn, 2009; Redon et al., 2010b, Roch-Lefevre et al., 2010; Horn and Rothkamm, 2011; Horn et al., 2011]. This is largely due to the strong and reproducible dose response of radiation-induced foci which enables foci yields to be converted to dose using a calibration curve. Whilst it must be remembered that the signal decays quickly as foci are lost as a consequence of DSB repair, thus requiring time-specific calibration data, the assay is still useful for biodosimetry for up to several days post exposure [Redon et al., 2010b; Horn et al., 2011; Moroni et al., 2013]. Interestingly, the lack of foci loss at very low doses of X-rays, reported for primary human fibroblast cultures and murine tissues [Rothkamm and Löbrich, 2003; Grudzenski et al., 2010; Ojima et al., 2011], has not been observed in peripheral blood lymphocytes which instead show a rapid decline down to pre-exposure foci levels (unpublished data). Due to the highly dynamic nature of the signal, foci-based dose estimations become very difficult when the time of exposure is unknown. This issue can potentially be addressed by multiplexing with a complementary endpoint with different kinetics, such as apoptosis induction [Horn et al., 2013]. Under conditions where baseline levels of DNA damage foci are very low and exposures are planned, enabling well-defined, short postexposure times, the assay can detect doses in the milligray range. An ideal application is therefore the assessment of patient exposures and the individual DNA damage response during diagnostic and interventional radiological procedures [Löbrich et al., 2005; Rothkamm et al., 2007; Beels et al., 2009; Grudzenski et al., 2009; Kuefner et al., 2009; Kuefner et al., 2010; Beels et al., 2012] as well as during internal [Lassmann et al., 2010; Doai et al., 2013] and fractionated external beam radiotherapy [Sak et al., 2007; Fleckenstein et al., 2011; Bakkenist et al., 2013; El-Saghire et al., 2014; Woolf et al., 2014]. The growing interest in combined treatment modalities and personalised therapies will create an ever increasing demand for reliable markers of individual exposure and effect, which DNA damage foci assays will help to address.

Apart from their ability to detect low dose exposures, the DNA damage foci assays also provide information about the homogeneity of the exposure. Whereas uniform, total body exposures to sparsely ionizing radiation induce DSBs randomly in all cells of the body, localised exposures such as those associated with a partial body CT scan, radiotherapy prescribed to target a localised treatment volume or in fact most accidental radiation exposures cause severe DNA damage only in a fraction of cells. This information can be extracted from foci distributions scored in blood samples using simple mathematical models [Rothkamm et al., 2007; Horn et al., 2011] and can be used to estimate how much of the body was spared from the exposure, a vital piece of information when managing patients following a severe radiation accident [IAEA, 2011]. The applicability of DNA damage foci assays to rapid biodosimetry in large scale radiation accident scenarios has recently been tested in a number of international laboratory intercomparison exercises [Rothkamm et al., 2013a,b; Ainsbury et al., 2014; Barnard et al., in press], which have highlighted large variability in assay performance between participating laboratories. High throughput sample processing and analysis methods have been reported which are aimed at adapting the assay to support rapid radiological triage [Turner et al., 2011; Rothkamm et al., 2012a; Moquet et al., 2014]. Additional effort on standardisation and regular performance testing will be required to fully establish DNA damage foci assays as routine biodosimetric tools.

GENOTOXICITY TESTING

In addition to its well-established role in radiobiological research and radiation biodosimetry, γH2AX is increasingly employed as a biomarker for DSB in environmental, occupational and clinical toxicology [Watters et al., 2009; Khoury et al., 2013; Geric et al., 2014; Nikolova et al., 2014]. As an example, γH2AX foci have been used as an indicator of DSB induced by cigarette smoke following in vitro exposure of human epithelial cells [Albino et al., 2004; Toyooka and Ibuki, 2009] and in peripheral blood mononuclear cells of active smokers [Ishida et al., 2014]. The latter finding suggests that cigarette smoke may induce DSB not only indirectly as secondary events caused by replication stalling and collapse at the site of single-stranded DNA lesions, but also directly in a replication-independent manner. Studies of cell cycle-dependence [Rothkamm et al., 2003; Beucher et al., 2009; Bauerschmidt et al., 2010], the use of pathway-specific foci assays (e.g. Rad51 analysis to test for the involvement of homologous recombination, see below) and analysis of foci induction in different genetic backgrounds such as mutants defective in different DNA repair pathways can provide important mechanistic insights in genotoxicity testing and are therefore prime candidates for inclusion in the battery of follow-up tests for substances that tested positive in the

Ames/*E. coli* bacterial mutagenicity assay [Aardema, 2013]. Multiplexing with other established genotoxicity markers such as the micronucleus test has also been considered [Bryce et al., 2014]. Moreover, the applicability of foci assays not only to cell cultures but also to tissue sections following in vivo exposure [Qvarnstrom et al., 2004; Somaiah et al., 2012; Rothkamm et al., 2012b] opens up exciting opportunities to produce 'genotoxicity maps' across the different organs and cell types of the body. However, it is important to keep in mind that, whilst DSBs tend to be closely associated with DNA damage foci in most situations, there are cases where one may be present without the other.

Obviously, the same concepts and possibilities apply to the use of foci assays as pharmacodynamic tools for characterising anti-cancer therapies [Redon et al., 2010a], combinations of therapeutics [Sak et al., 2009] or DNA damage response modifiers, whether used alone or in combination with DNA-damaging treatments [e.g. Lim et al., 2014; Srivastava et al., 2014; Burdak-Rothkamm et al., 2015b]. In cells undergoing apoptosis H2AX phosphorylation occurs in an intranuclear shell. This response, which microscopic analysis can easily distinguish from foci formation, may serve as an additional pharmacodynamic biomarker for anticancer therapies [Solier and Pommier, 2014].

INDIVIDUAL RESPONSE/SENSITIVITY/SUSCEPTIBILITY

It has long been known that DNA damage foci assays can flag up severe defects in DSB repair in syndromic patients, such as those with ligase IV syndrome, Ataxia telangiectasia, Nijmegen breakage syndrome, radiosensitive severe combined immunodeficiency, etc. More recently, DSB repair measured using DNA damage foci in ex vivo- or in vivo-irradiated peripheral blood lymphocytes has also been proposed as a predictive marker of individual risk of oral mucositis in head and neck cancer radiotherapy patients [Fleckenstein et al., 2011; Goutham et al., 2012; Li et al., 2013]. Similarly γH2AX foci levels have been associated with the risk of acute [Djuzenova et al., 2013; Mumbrekar et al., 2014] and late normal tissue reactions in breast cancer radiotherapy patients [Chua et al., 2011; Henriquez-Hernandez et al., 2011; Chua et al., 2014], for late toxicity in prostate cancer patients [van Oorschot et al., 2014] and for acute radiotherapy toxicities in paediatric cancer patients [Rübe et al., 2010]. However, γH2AX foci results had no predictive power for late normal toxicity in gynaecological cancer radiotherapy [Werbrouck et al., 2010] or in prostate brachytherapy [Olive et al., 2008], and no genetic influence was observed on individual γH2AX signaling/DSB repair capacity in a nonclinical study involving 198 twins [Garm et al., 2013].

More general uses of the DNA damage foci-based functional assays in translational cancer research [Ivashkevich et al., 2012] and in the clinic are being increasingly explored [Redon et al., 2012]. The specific utility of γH2AX as a prognostic biomarker in lung cancer has recently been proposed [He et al., 2013; Matthaios et al., 2013; Chatzimichail et al., 2014]. DNA damage foci assays have been used to study the relationship between DNA repair and radiotherapy fraction size sensitivity [Somaiah et al., 2012, 2013]. RAD51 foci-based functional assays are being developed to profile HR repair pathway activity in tissue biopsies and enable the selection of patients with HR-deficient tumours for specific treatments such as PARP inhibitors [Mukhopadhyay et al., 2010; Shah et al., 2014; Naipal et al., 2014].

NONTARGETED FOCI

A radiation-induced bystander effect (RIBE) was first described as a radiation-induced DNA damage response in cells adjacent to directly targeted cells, manifesting as increased yields of micronuclei, sister chromatid exchanges, apoptosis, mutations, genomic instability and neoplastic transformation [Nagasawa and Little, 1992; Azzam et al., 1996; Watson et al., 2000; Zhou et al., 2000; Bowler et al., 2006]. DNA damage in bystander cells is thought to be initiated by elevated reactive oxygen species (ROS) production [Tartier et al., 2007; Chen et al., 2008]. Subsequent intra- and intercellular signaling events between targeted and nontargeted cells, including the release or activation of nitric oxide [Shao et al., 2003], TGF-β [Shao et al., 2008], cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-kappa B), and mitogen-activated protein kinase (MAPK) [Zhou et al., 2008], result in sustained ROS generation in distant cells, causing genotoxic stress. The initial ROS-induced DNA damage in bystander cells, when present in S-phase, causes replication fork stalling and leads to secondary production of DNA double-strand breaks (DSBs) which are thought to underlie the formation of subnuclear foci of γH2AX [Sokolov et al., 2005; Burdak-Rothkamm et al., 2007; Han et al., 2007] and 53BP1 [Tartier et al., 2007; Burdak-Rothkamm et al., 2008] in bystander cells. These bystander foci occur predominantly in S-phase cells and activate ATM in an ATR-dependent manner [Burdak-Rothkamm et al., 2007, 2008]. The observation of bystander BRCA1 and FANCD2 foci suggests an activation of the Fanconi anaemia (FA)/BRCA DNA damage response pathway [Burdak-Rothkamm et al., 2015a], a key pathway in the resolution of stalled replication [Venkitaraman, 2004], the functional impairment of which has been linked to genomic instability [Howlett et al., 2005].

The RIBE can potentially be interpreted as part of a general genotoxic stress response [Spitz et al., 2004]. In this context, the accumulation of γH2AX foci was observed in other settings which are characterized by the

presence of persistent genotoxic stress: γH2AX foci induction in distant proliferating tissue was reported as manifestation of a systemic tumour-induced bystander effect caused by the presence of a malignant tumour [Redon et al., 2010c; Martin et al., 2011]. In aging mice and in senescent cell cultures, the accumulation of cryptogenic γH2AX foci was observed which are thought to represent unrepairable DSBs caused by persistent genotoxic stress [Sedelnikova et al., 2004]. In pre-cancerous tissues it was shown that increased numbers of DNA double-strand breaks demonstrated by 53BP1 foci accumulation were associated with DNA replication stress [Gorgoulis et al., 2005]. Furthermore, it has been established more recently that nontargeted foci as well as (drug) targeted foci may be generated by transcriptional activity [Sordet et al., 2009; Dickey et al., 2012; Alagoz et al., 2013], with transcriptional R loops likely to play a key role in genomic instability [Gan et al., 2011; Aguilera and Garcia-Muse, 2012; Skourti-Stathaki and Proudfoot, 2014]. DNA damage that arises in nondividing tissues may lead to human pathologies such as neurological disorders or heart failure. Detection of repair foci in these tissues may serve as a tool to better understand the origin of such pathologies.

In conclusion, DNA damage foci have been used to monitor and quantify bystander effects and nontargeted responses in a variety of settings. In particular γH2AX foci are now widely used as a biomarker for nontargeted and systemic genotoxic responses.

CONCLUSIONS

The discovery of DNA damage foci has opened a new dimension to the field of DNA damage signaling. Analysis of the spatiotemporal dynamics of DSB induction and repair in situ or even in live cells has now become a standard method in many different fields. Whilst there is huge scope for DNA damage foci assays to be exploited further as functional biomarkers in cancer therapy trials, radiation dose assessment, and genotoxicity testing, it will require stringent assay standardization and strict quality control measures to ensure good reproducibility and consistency, especially when used in multicenter settings. Beyond the technical issues, there are still a number of fundamental gaps in our understanding of the meaning and significance of DNA damage foci, especially in situations where foci form as secondary events as a consequence of the cellular response to non-DSB damage. However, it is exactly in these areas of general genotoxicology and cellular stress responses that these sensitive functional markers have the potential to provide important insights into the molecular choreography that links DNA damage, repair, chromatin dynamics, replication, and cell cycle control with wider tissue-level responses.

AUTHOR CONTRIBUTIONS

All authors contributed to (i) conception and design of the review, (ii) screening, analysis, and interpretation of the literature, and (iii) drafting the article or revising it critically for important intellectual content. All authors approved the final article.

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