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Mini-review

Assembly and function of DNA double-strand break repair foci in mammalian cells

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ABSTRACT

DNA double-strand breaks (DSBs) are among the most cytotoxic types of DNA damage, which if left unrepaired can lead to mutations or gross chromosomal aberrations, and promote the onset of diseases associated with genomic instability such as cancer. One of the most discernible hallmarks of the cellular response to DSBs is the accumulation and local concentration of a plethora of DNA damage signaling and repair proteins in the vicinity of the lesion, initiated by ATM-mediated phosphorylation of H2AX (γ-H2AX) and culminating in the generation of distinct nuclear compartments, so-called Ionizing Radiation-Induced Foci (IRIF). The assembly of proteins at the DSB-flanking chromatin occurs in a highly ordered and strictly hierarchical fashion. To a large extent, this is achieved by regulation of protein–protein interactions triggered by a variety of post-translational modifications including phosphorylation, ubiquitylation, SUMOylation, and acetylation. Over the last decade, insight into the identity of proteins residing in IRIF and the molecular underpinnings of their retention at these structures has been vastly expanded. Despite such advances, however, our understanding of the biological relevance of such DNA repair foci still remains limited. In this review, we focus on recent discoveries on the mechanisms that govern the formation of IRIF, and discuss the implications of such findings in light of our understanding of the physiological importance of these structures.

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1. Introduction

DNA damage arises continuously as the result of intracellular metabolism and upon the exposure of cells to a multitude of genotoxic agents [1,2]. If left unrepaired, such insults can be life-threatening for cells and organisms as they alter the content and organization of the genetic material. To overcome this challenge to genomic stability, cells have evolved a global signaling network known as the DNA damage response (DDR) that senses different types of genotoxic stress to mount a coordinated and multi-faceted response, which includes modulation of cell cycle transitions and transcriptional processes, and stimulation of DNA repair [3–5]. Accordingly, the DNA damage response functions as a major cellular defence mechanism against the accumulation of genetic changes associated with diseases such as cancer and neurodegenerative disorders [4,6]. Besides being activated by DNA damaging agents, the DDR constitutes a key surveillance mechanism that monitors the quality of DNA replication. At the molecular level, the DDR is organized into an elaborate network of interacting pathways, the constituents of which can be grouped into three major classes of proteins that act in concert to translate the signal of damaged DNA into the appropriate downstream response. These comprise (1) sensors, proteins that recognize abnormally structured DNA and initiate the signaling response, and (2) transducers, factors that relay and amplify the damage signal to (3) effector proteins in numerous downstream pathways [3,7].

DNA can be damaged in many ways, ranging from relatively innocuous single base or nucleotide modifications and single-strand breaks to highly cytotoxic lesions such as interstrand crosslinks and double-strand breaks (DSBs) [2,8]. The former types of lesions occur spontaneously in vast numbers during the cell cycle, and normally these are swiftly repaired without eliciting full-blown activation of the DDR. Rather, this phenomenon appears to be restricted to conditions of massive replication problems or the presence of DSBs, a particularly destructive type of DNA lesion [2]. DSBs arise from a number of endogenous and exogenous sources, such as ionizing radiation, oxidative stress, and replication of damaged DNA. In addition, intentional DSBs are formed during genetically programmed processes such as meiotic recombination and V(D)J recombination in developing lymphocytes [2]. Persistent or inappropriately repaired DSBs can lead to mutations or more gross chromosomal aberrations such as deletions and chromosome loss or translocations. DSB repair occurs via two principal mechanisms: non-homologous end-joining (NHEJ) [9] and homologous recombination (HR) [10]. Most non-replication-associated DSBs are repaired by NHEJ, the predominant mode of DSB repair in G0/G1 cells, in which the broken DNA ends are simply pieced together in an efficient but error-prone fashion. In contrast, HR repairs DSBs in an error-free fashion, but because this requires an intact sister chromatid as a template, this mode of DSB repair only takes place in S/G2 phase cells.

While most of the DDR components are present at all times in the cell, activation of the DDR is accompanied by a dramatic increase in the availability of these factors. In bacteria such as *Escherichia coli*, this is ensured by the SOS network, a transcriptional program activated by DNA damage that mediates the rapid production of various DNA repair factors [11]. In eukaryotes, the total amount of DNA repair factors is not regulated by DNA damage on a global scale; rather, the local concentration and availability of these proteins is increased. The purpose, however, is the same: to markedly boost the ability of the DDR to faithfully reestablish genomic integrity. The local up-concentration of DDR proteins into so-called IRIF (Ionizing Radiation-Induced Foci) is a highly regulated yet dynamic process, where numerous proteins are recruited to sites of DSBs by a range of intricate mechanisms [12–14] (Fig. 1A). In yeast, multiple DSBs can be mobilized into the same repair focus (referred to as a DNA repair factory). In mammalian cells, on the other hand, DSBs are generally immobile and hence IRIF largely reflects protein accumulation at single DSBs [13]. The molecular mechanisms underpinning the structure and generation of IRIF have been the subject of intense investigation over the last decade, and while it is clear that the formation of these structures is of great importance for a successful DDR, our understanding of their biological function is still surprisingly limited. In this review, we discuss recent advances in our understanding of the mechanisms that govern IRIF formation, and the physiological importance of these structures.

2. DNA damage induces compartmentalization of the nucleus

The complex protein aggregates at sites of DNA damage that we refer to as IRIF contain all the common hallmarks of nuclear domains and bodies, such as PML and Cajal bodies, Polycomb regions, and replication factories [15,16]. Thus, IRIF can be viewed as an affinity platform for a substantial number of proteins, allowing for the local concentration of these factors. The involved proteins generally do not bind stably and constantly to the affinity platform; rather, they are highly dynamic and transiently shuttle in and out of the IRIF compartment, similar to the behaviour of protein components of other nuclear domains [12,17].

High-resolution analysis of the architecture of IRIF has revealed that the nuclear regions containing damaged DNA consist of two distinct compartments that are spatially separated, have a different structural basis, and contain different subsets of proteins [18] (Fig. 1B). In the first and larger compartment, the chromatin surrounding the DNA lesion serves as an immobile structural platform for the assembly of the repair machinery. The DSB-flanking chromatin areas are sequentially modified by DNA damage-inducible phosphorylation, ubiquitylation, and other post-translational modifications as described below, and these tags serve as binding sites that strongly increase the propensity of DDR factors to interact with chromatin and thus prolong their residence time in repair foci. Such DDR-mediated chromatin modifications have been estimated to extend for as much as up to 1–2 megabases around DSBs, and occur at all stages of the cell cycle [18,19]. Interestingly, these modifications are not uniformly distributed along the affected chromatin fibers and their spreading appears to be highly dynamic [20].

A key step of HR-mediated repair of DSBs in the S and G2 phases of the cell cycle is resection of the broken DNA ends to create long stretches of single stranded DNA (ssDNA) that are rapidly coated by the protective ssDNA-binding RPA protein complex [21]. In this second and comparably much smaller compartment, RPA-coated ssDNA serves as a unique scaffold, onto which protein complexes can form and aggregate. Most prominently, these include factors that are critically involved in the actual DNA repair process such as Rad51, BRCA1, and BRCA2. In addition, signaling factors such as the ATR kinase, its associated proteins ATRIP and TopBP1, and the Rad9-Hus1-Rad1 (9-1-1) complex, all of which play key roles as sensors of DNA lesions and in coordinating DNA repair with cell cycle progression, are recruited to the ssDNA compartment [18]. Some DDR factors, including BRCA1 and the MRE11-NBS1-RAD50 (MRN) complex, perform important functions in both of these DNA damage-induced compartments, and while the nuclear regions they occupy are both non-overlapping and structurally distinct, a significant degree of crosstalk between them is evident. For instance, H2AX, the most proximal component of the chromatin compartment is excluded from the smaller DNA repair compartment, yet it is of key importance for faithful DNA repair [22,23]. Likewise, the duration of the chromatin response impinges directly on the duration of the DNA repair process [24]. Here, we focus on the construction and functions of the chromatin-associated IRIFs that

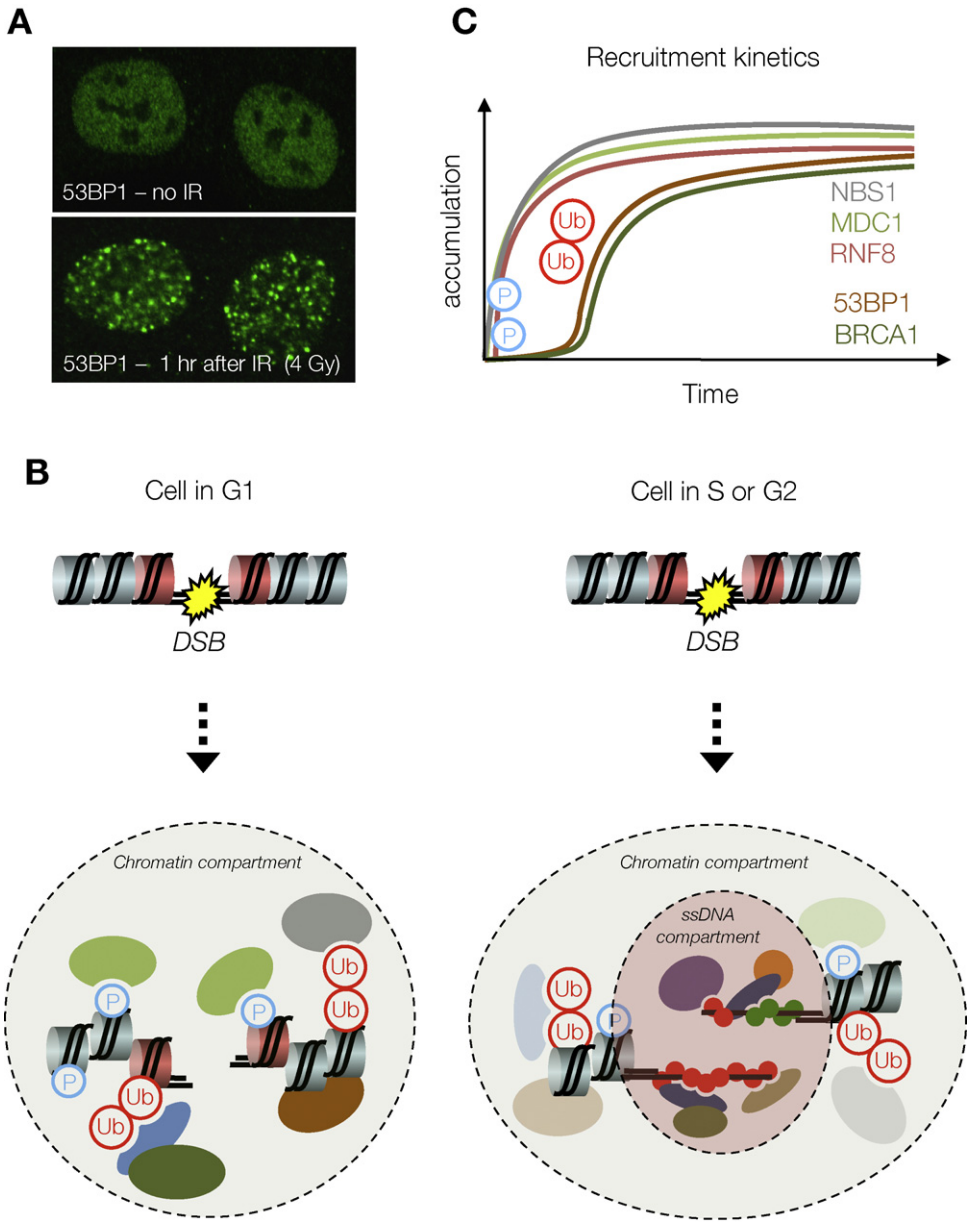

Fig. 1. Spatio-temporal properties of the DNA damage response. (A) A large number of DDR regulators accumulate at nuclear sites of DSBs, forming so-called Ionizing Radiation-Induced Foci (IRIF). A striking example is that of 53BP1, which is homogenously distributed in the nucleus of unperturbed cells but undergoes a dramatic relocalization to the damaged regions upon exposure to DSB-inducing agents such as IR. (B) Two structurally distinct nuclear compartments are formed around DSBs. One such compartment is formed in the chromatin regions distal to the break, irrespective of cell cycle stage (left). In the S- and G2 phases of the cell cycle, a subset of DSBs are processed for repair by homologous recombination. This involves resection of the breaks and generation of long stretches of single-stranded DNA, to which a number of repair and DDR signaling factors bind. The resulting DSB-associated compartment is distinct from the chromatin compartment, both in terms of its structure and protein composition (right). (C) Recruitment of DDR proteins to the chromatin compartment of IRIF occurs in two distinct kinetic waves. The early wave is governed by phosphorylation of the DSB-surrounding chromatin, which provides an affinity platform for MDC1 and its binding partners, such as NBS1 and RNF8. A subsequent wave of protein accumulation requires DSB-induced histone ubiquitylation, restructuring chromatin to a state permissive for the retention of downstream factors such as 53BP1 and BRCA1. IR: Ionizing Radiation, P: Phosphate, Ub: Ubiquitin, ssDNA: single-stranded DNA.

are formed distal to DSBs, while the orchestration of DNA repair pathways in the ssDNA compartment is described in other reviews in this issue.

3. Order and dynamics of protein recruitment to DNA repair foci

3.1. γ-H2AX: the most proximal marker of IRIF formation

The assembly of proteins at the DSB-flanking chromatin occurs in a highly ordered, strictly hierarchical, and rapid fashion (Figs. 1C and 2). Key to the formation of IRIF is the DNA damage-induced phosphorylation of H2AX, a histone H2A variant that comprises 10–15% of total cellular H2A in higher eukaryotes, on S139 (to form γ-H2AX) [23,25]. While several of the PI3K-like kinases, including ATM, ATR, and DNA-PK, seem capable of performing this function [23,26], ATM has emerged as the master kinase for both the direct phosphorylation of H2AX and many ensuing phosphorylation events required for the proper formation of higher order protein complexes at sites of DNA damage. The mechanism by which ATM becomes activated in response to DNA damage has been the subject of a long-standing debate. One dominating view is that ATM can somehow sense the stochastic chromatin changes that are associated with DNA breakage, which

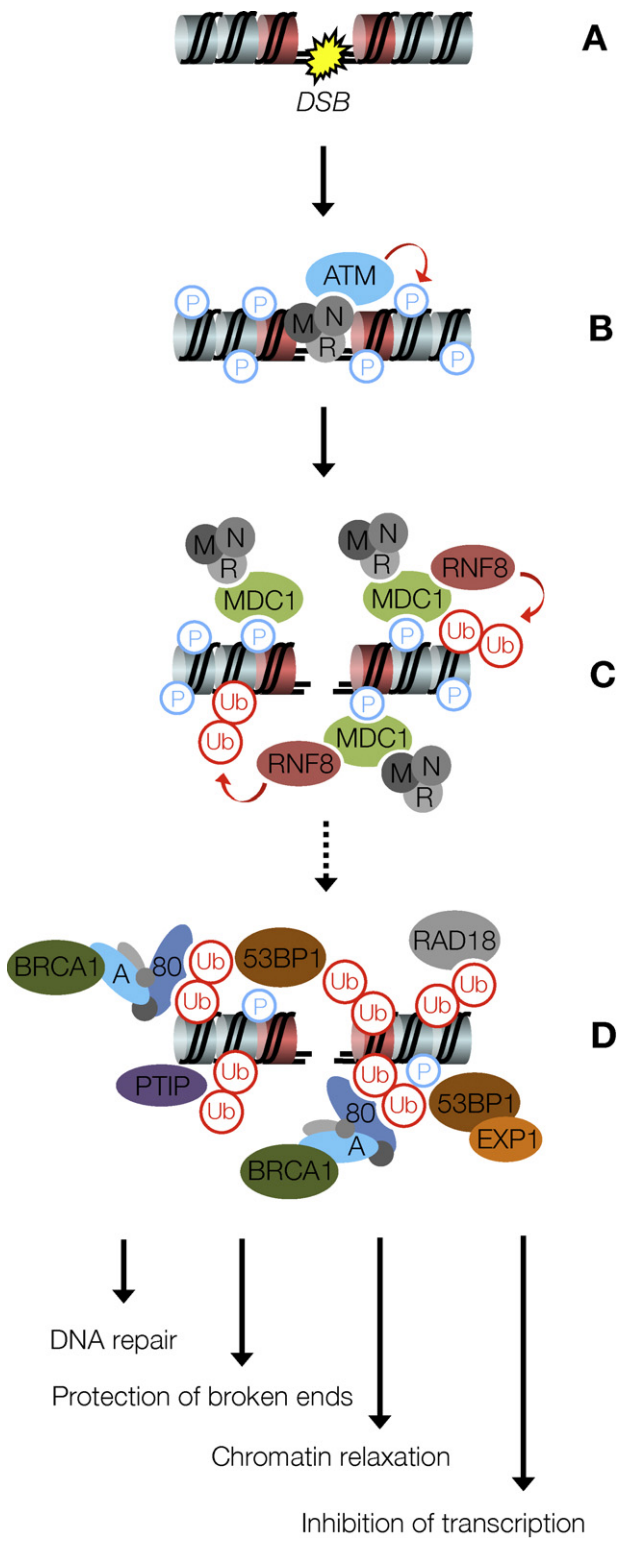

Fig. 2. Hierarchical assembly of IRIF. Genomic DSBs (A) are sensed by the MRN (MRE11-RAD50-NBS1) complex, which recruits the ATM kinase to the vicinity of the lesions. The resulting ATM-mediated phosphorylation of the histone variant H2AX (B) allows for accumulation of the MDC1 protein, along with its binding partners (C). These include the MRN complex and RNF8, a ubiquitin ligase which initiates histone poly-ubiquitylation at sites of DNA damage. This chromatin modification allows for a second wave of protein accumulation, including factors such as 53BP1, the BRCA1 A complex, RAD18 and PTIP (D). By controlling various DNA and chromatin transactions and stimulating DNA repair, the formation of IRIFs is critically important for the maintenance of genomic stability. P: phosphate, M: MRE11, N: NBS1, R: RAD50, Ub: Ubiquitin, A: Abraxas (ABRA1), 80: Rap80, EXP1: EXPAND1.

in turn causes ATM auto-phosphorylation on S1981, resulting in dissociation of latent ATM dimers into active monomers [27]. However, this model has been challenged by the finding that mice solely expressing an ATM S1987A mutant (corresponding to S1981A in human ATM) were proficient for ATM-dependent responses at the cellular and organismal level [28]. Recently, auto-phosphorylation of ATM was shown to mediate its binding to MDC1, allowing for a sustained retention of the kinase in IRIF [29]. These findings may help to reconcile the conflicting views of the importance of ATM auto-phosphorylation. On the other hand, the MRN complex has been shown to be critically important for ATM activation [30,31], and accordingly this complex emerges as the most proximal sensor of DSBs, possibly mediated through its intrinsic DNA binding capability [32]. Exactly how ATM activity feeds on DNA lesions remains enigmatic, and it is likely to involve components that are yet to be discovered or which we do not currently appreciate in full. One such candidate factor is the Tip60 acetyl transferase, which modifies chromatin at sites of DNA damage [33,34], as well as ATM itself [35], and hence may impact on DNA damage-induced ATM activation.

Like other core histones, the C-terminal tail of H2AX contains several amino acid residues that are subject to various post-translational modifications. While DNA damage-induced S139 phosphorylation remains the best studied of these, other modifications may also play important roles in regulating the formation and dynamics of IRIF, or the propensity of different nuclear regions to support the generation of these structures. Thus, similar to epigenetic regulation, the DNA damage response could be subject to regulation through a histone code that is considerably more complex than mere phosphorylation of S139. Recently, this concept was supported by the dissection of the relationship between phosphorylation of the very C-terminal Tyrosine residue of H2AX (Y142) and IRIF dynamics. In essence, Y142 was shown to undergo dephosphorylation by the EYA phosphatase following induction of DSBs, and ablation of this activity markedly diminished the levels of γ-H2AX induced by IR [36]. The atypical Tyrosine kinase WSTF, the catalytic domain of which bears no obvious homology to known kinase folds, was found to be responsible for Y142 phosphorylation [37], and it has been suggested that the different phosphorylation marks in the C-terminus of H2AX could thus determine the potential of DNA damage sites to attract factors that are involved in DNA repair and cell fate decisions (such as apoptosis), respectively. However, it is still unclear how the opposing activities towards Y142 phosphorylation is regulated in response to DNA damage, and exactly how the S139 and Y142 modifications impact on each other [38].

3.2. *MDC1: the master organizer of protein assembly at IRIF*

The key function of γ-H2AX is to provide a high-affinity binding site for the MDC1 protein, which in turn orchestrates the recruitment of essentially all of the downstream IRIF-associated factors damaged to chromatin. The C-terminal tandem BRCT repeats of MDC1 represent the only well-characterized binding partner for γ-H2AX, and in addition to serving as a key recruitment platform, this interaction protects γ-H2AX from de-phosphorylation and determines the dynamic extension of IRIF [39]. Interestingly, lower eukaryotes such as yeast do not contain obvious MDC1 orthologues. Instead, other conserved IRIF-forming factors (such as the 53BP1 orthologues Rad9 (*Saccharomyces cerevisiae*) and Cut5 (*S. pombe*), and the PTIP homologue Brc1 in *S. pombe*) can directly bind phosphorylated H2A [40–42], which is exclusively present at sites of DNA damage [43,44].

One of the most prominent binding partners of MDC1 is the MRN complex, which is robustly recruited to MDC1-decorated chromatin by virtue of this interaction [45]. The interaction between MDC1 and the MRN component NBS1 is constitutive and not

affected by DNA damage. It primarily occurs via binding of the FHA domain of NBS1 to a series of conserved clusters of CK2 phosphorylation sites in the N-terminal part of MDC1 [46–49]. These clusters contain doubly phosphorylated SDT motifs, in which phosphorylation of both the Serine and Threonine residues are required for binding to NBS1. This points to a unique and unusual feature of the NBS1 FHA domain in recognizing doubly phosphorylated peptides. Recently, it was demonstrated that the two BRCT domains downstream of the FHA domain in NBS1 also participate in the phosphorylation-dependent binding to MDC1 [50,51], and that NBS1 interacts with CtIP by an analogous mechanism to promote resection of DSBs [51,52].

It has been demonstrated that DSB factors accumulate in the chromatin compartment surrounding DNA lesions according to a pre-determined time schedule [45,53]. Thus, γ-H2AX formation and recruitment of MDC1 and its associated binding partner, the MRN complex, are the most proximal events following DSB induction. The recruitment kinetics of these factors to foci following exposure to DSB-inducing agents is exceedingly rapid, reaching maximal accumulation within a few minutes. After a short but significant lag of approx. 1–2 min, this is then followed by a second wave of protein accumulation at sites of DNA damage [54] (Fig. 1C). The group of proteins arriving with such delayed kinetics includes the DNA repair factors 53BP1 and BRCA1, and until recently, the signals required to facilitate the retention of these factors at the DSB-associated chromatin remained elusive. A major breakthrough in resolving this important issue came with the discovery by several groups that the ubiquitin ligase RNF8 plays an instrumental role in promoting the maturation of DSB-associated chromatin [54–57]. Through its direct interaction with MDC1, RNF8 is recruited to DSB sites along with the other factors in the initial wave of protein accumulation at IRIF [54]. Here, RNF8 initiates a complex and tightly regulated ubiquitylation cascade of histones H2A and H2AX at the DSB-flanking chromatin, which causes chromatin restructuring (through incompletely understood mechanisms) associated with the generation of binding sites for protein complexes that accumulate downstream of these early factors (Fig. 3) [54,55]. Like NBS1, RNF8 interacts with MDC1 through its FHA domain, which recognizes distinct phosphorylation sites in the N-terminal half of MDC1. As opposed to the MDC1-NBS1 interaction, however, the MDC1-RNF8 complex is only formed in response to genotoxic insults. Thus, the three highly conserved phosphorylation sites on MDC1 that create binding sites for RNF8, all fall within a conserved T-Q-X-F motif, a consensus sequence for phosphorylation by ATM and related kinases closely matching the phosphorylation-dependent binding site preference for the FHA domain of RNF8 [54,55].

3.3. Mechanisms of BRCA1 and 53BP1 accumulation in IRIF

Similar to how γ-H2AX attracts MDC1 and other proteins belonging to the first wave of protein accumulation at IRIF, ubiquitylated histones at sites of DNA damage mark the spot for the important downstream factors 53BP1 and BRCA1. How recruitment of the latter protein feeds on ubiquitylation of the DSB-flanking chromatin has become clear from the isolation of the so-called BRCA1 A complex that bridges the interaction between BRCA1 and ubiquitylated histones [58]. A central component of this complex is the RAP80 protein [59–62], which contains two Ubiquitin Interaction Motifs (UIMs) that were shown to directly bind ubiquitylated histones at sites of DNA damage [63]. RAP80 forms part of a relatively tight complex with the proteins Abraxas, BRCC36, BRE, and NBA1 (also called MERIT40) [58]. Within this complex, the coiled-coil domain protein Abraxas seems to form the structural core, onto which the other factors can assemble [64]. Abraxas also provides the point of contact for BRCA1 itself, and the binding between BRCA1 and Abraxas involves the tandem BRCT domains of BRCA1 and a phosphorylation site in the extreme C-terminus of Abraxas [60,65]. The BRCA1-Abraxas association appears to be rather transient compared to the interactions between the other components. Thus, the availability of several of the core components of the BRCA1 A complex strongly impact on the stability of the other components, and in contrast to BRCA1, which exhibits low levels during G1, the core Abraxas complex remains stably expressed throughout the cell cycle [64,66]. Hence, besides bridging the interaction between BRCA1 and ubiquitylated histones, this complex is likely to play additional roles at sites of DNA damage.

As opposed to the clear mechanistic insight into the requirements for BRCA1 accrual, it remains an enigma how histone ubiquitylation promotes 53BP1 accumulation to sites of DNA damage. Like BRCA1, 53BP1 also possesses a set of tandem BRCT domains in its C-terminus, but these are not required to target the protein to IRIF [67]. So far, efforts aimed at isolating binding partners of 53BP1 have not led to the identification of factors that could perform a similar function for 53BP1 as the BRCA1 A complex does for BRCA1. It is possible that such factors could exist for 53BP1 as well, but have simply escaped detection so far. Alternatively, 53BP1 could possess ubiquitin-binding activity itself, which would obviate a requirement for such associated proteins. A well-studied feature of 53BP1 is the presence of an extended Tudor domain upstream of its BRCT domains. This domain has been shown to be capable of binding methylated Lysine residues on core histones, an activity that is absolutely required for targeting the protein to IRIF [68]. Though initially isolated as a domain that binds methylated K79 on histone H3 [68], subsequent studies in human, mouse, and fission yeast have pinpointed methylated K20 on histone H4 as a more likely physiological binding partner of the 53BP1 Tudor domain [69–71]. The domain pocket preferentially accommodates the di-methylated form of H4K20 while showing low affinity for the mono-methylated form, as well as for methylated H3K79 [71]. Despite its clear importance for 53BP1 biology, the precise function of the Tudor domain in targeting 53BP1 to repair foci remains elusive. None of the candidate histone marks for 53BP1 binding are induced by DNA damage [68,72], or appears differentially exposed at sites of DNA damage, so the binding to such marks cannot by itself explain the increased affinity of 53BP1 for DSB-modified chromatin. It is possible, however, that local histone ubiquitylation is accompanied by a restructuring of chromatin, which somehow renders the binding sites for the Tudor domain of 53BP1 more accessible [72], resulting either in an increased density of binding sites, or allowing for a prolonged interaction. Further studies of the biology of 53BP1 will be crucially required to answer these outstanding questions.

4. Ubiquitin-dependent assembly of DNA repair factors at IRIF

4.1. Sequential H2A poly-ubiquitylation by RNF8 and RNF168

The discovery that histone ubiquitylation plays a key role in promoting the retention of DDR factors at sites of DNA damage was spurred by the identification of RNF8 as a novel and critically important DDR protein. Since then, an overwhelming amount of new discoveries have highlighted the regulatory complexity and biological importance of this seemingly simple ubiquitylation reaction. Following the identification of RNF8 as a central regulator of the DSB response, two independent genome-wide siRNA screens identified another E3 ubiquitin ligase, RNF168, as being critically important for 53BP1 and BRCA1 foci formation [73,74]. RNF168, like RNF8, is also a component of IRIF, and harbors in addition to its RING domain two Motifs Interacting with Ubiquitin (MIUs) [75]. These domains target RNF168 to sites of DNA damage through recognition of ubiquitylated forms of H2A and H2AX produced by

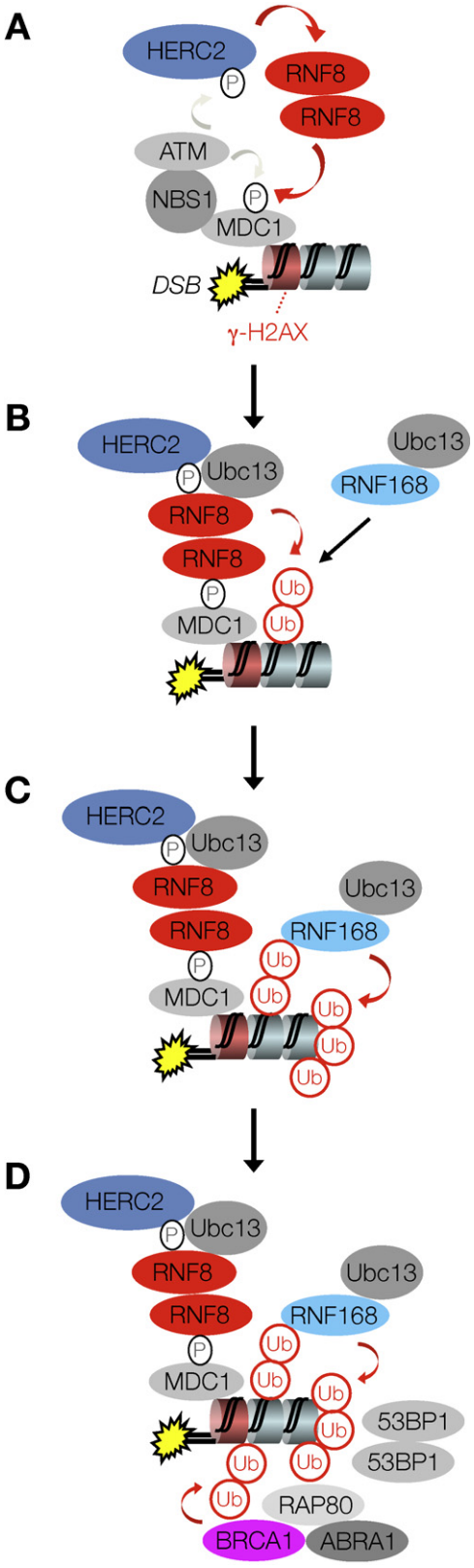

Fig. 3. Ubiquitin-dependent assembly of genome caretakers at damaged chromatin. (A) In response to DSBs, ATM-mediated phosphorylation of γ-H2AX-bound MDC1 on conserved T-Q-X-F motifs generates binding sites for the FHA domain of RNF8 to promote its recruitment to IRIF. Simultaneously, ATM phosphorylates HERC2 on T4827, which also stimulates an interaction with the FHA domain of RNF8. Together with an intrinsic ability of RNF8 to form dimers or oligomers, these ATM-mediated phosphorylations enable the formation of a ternary MDC1-RNF8-HERC2 complex at sites of DNA damage. (B) HERC2 stabilizes the interaction between RNF8 and Ubc13 to promote initial, RNF8/Ubc13-mediated K63-linked poly-ubiquitylation of H2A-type histones. This in turn serves as a binding site for the MIU domains of RNF168, allowing it to accumulate at the damaged chromatin. (C and D) RNF168 augments H2A poly-ubiquitylation at sites of DNA damage in a Ubc13-dependent manner to an extent permissive of recruitment of DNA repair factors such as the BRCA1 A complex and 53BP1. Accumulation of the BRCA1 A complex at IRIF is directly mediated by the ability of the UIM domains of RAP80, an integral component of the complex, to bind K63-linked poly-ubiquitin chains on H2A/X. The ubiquitin-dependent mechanism underlying 53BP1 retention at the DSB-flanking chromatin is currently unknown. Ub: Ubiquitin.

RNF8 (Fig. 3B) [73,74]. By means of its E3 ligase activity, RNF168 subsequently amplifies ubiquitylation of H2A-type histones to levels sufficient to promote the ubiquitylation-dependent recruitment of downstream factors such as 53BP1 and BRCA1 (Fig. 3C and D) [73,74]. Through its ability to both promote and associate with H2A ubiquitylation, RNF168 may effectively self-reinforce and expand histone ubiquitylation at the DSB-flanking chromatin. Such two-step model involving the sequential actions of RNF8 and RNF168 is consistent with the finding that RNF168 arrives slightly, but significantly, later than RNF8 at sites of DNA damage in a manner fully dependent on functional RNF8 [73].

Histone H2A is potentially the most abundantly ubiquitylated protein in the cell, raising the question of how ubiquitylated H2A in IRIF differs qualitatively from the bulk of ubiquitylated H2A present thoughout the nucleus. Available evidence suggests that in response to DNA damage, RNF8 and RNF168 generate K63-linked, non-proteolytic ubiquitin chains on histones [55,73,74], which are distinct from the dominant mono-ubiquitylated form of H2A. Consistent with this idea, Ubc13, the only E2 ubiquitin-conjugating enzyme known to exclusively catalyze the formation of K63-linked ubiquitin chains, is required for RNF8 and RNF168 function in the DDR [55,74,76]. Both of these E3 ligases interact with Ubc13 and display in vitro ubiquitin ligase with Ubc13-Mms2 [54,74]. In further support of this notion, structural analysis of RAP80 has recently demonstrated that the length of the linker region separating the tandem UIM motifs that promote its relocalization to sites of DNA damage enables its UIMs to recognize K63- but not K48-linked ubiquitin chains [77,78]. These and related findings strongly argue for a role of Ubc13-catalyzed, K63-linked ubiquitin chains on core histones (and perhaps other chromatin-associated proteins) in marking the site for downstream factors to accumulate in IRIF.

4.2. HERC2: a novel ubiquitin ligase required for DSB-associated histone ubiquitylation

Recently, yet another E3 ubiquitin ligase was found to be required for the cellular ability to promote non-proteolytic histone polyubiquitylation at sites of DNA damage. In response to DSBs, RNF8 forms a complex with HERC2, a giant protein of almost 5000 amino acids and an E3 ligase by virtue of a C-terminal HECT domain, and this interaction promotes HERC2 relocalization to IRIF [79]. The mechanistic basis for the RNF8-HERC2 interaction involves DNA damage-dependent phosphorylation of HERC2 on T4827 in its extreme C-terminus by ATM and related kinases, which provides a strong binding site for the FHA domain of RNF8 (Fig. 3A) [79]. An intrinsic ability of RNF8 to di- or oligomerize enables it to simultaneously interact with MDC1 and HERC2, allowing the formation of a ternary MDC1-RNF8-HERC2 complex at sites of DNA damage (Fig. 3B) [79]. The sheer size of the HERC2 protein has so far precluded attempts at addressing whether the E3 ligase activity of HERC2 is required for H2A poly-ubiquitylation and DDR signaling. It is clear, however, that HERC2 is needed for RNF8 to promote Ubc13-dependent poly-ubiquitylation of H2A-type histones. Like many other ubiquitin ligases, RNF8 is capable of interacting with several E2 enzymes [80,81], and HERC2 was shown to mediate a

preferential interaction of RNF8 with Ubc13 [79]. Hence, HERC2 seems to define a novel type of auxiliary factor capable of providing specificity to E2-E3 interactions, ensuring the formation of K63-linked ubiquitin chains at sites of DNA damage. Future, more targeted approaches to manipulate HERC2, such as knock-in mouse models, will be required to address the functions of the various domains in HERC2 and its exact role in the DDR.

4.3. Complexity of DNA damage-induced histone ubiquitylation

With three ubiquitin ligases required to perform one reaction, the process of DNA damage-induced histone poly-ubiquitylation displays a striking degree of complexity (Fig. 3). The interplay between RNF8, RNF168, and HERC2 is still not completely resolved, and a number of important questions remain to be addressed. First, although it seems clear that H2A undergoes oligo- or poly-ubiquitylation in response to IR [54,73], it is not known whether there could be one E3 for mono-ubiquitylation and another for chain elongation, similar to how PCNA ubiquitylation is orchestrated [82]. Such a distribution of labour between RNF8 and RNF168 seems unlikely, however, since RNF8 is critically required to target RNF168 to sites of DNA damage [73,74]. If RNF8 simply catalyzed mono-ubiquitylation of H2A and H2AX, there would be no binding site for RNF168 that could uniquely discriminate emerging IRIF from other nuclear sites rich in mono-ubiquitylated H2A, such as Polycomb bodies. Moreover, RNF168 has been shown to specifically bind poly-ubiquitylated H2A in an RNF8-dependent manner [73]. Alternatively, HERC2 could catalyze DSB-associated H2A mono-ubiquitylation, which could then be extended to a K63-linked chain by RNF8. This scenario also does not seem likely in view of what we currently know about the function of HERC2, but cannot be formally excluded until the potential role of the HECT domain of HERC2 has been elucidated. Ubc13 is known to have a strong preference for extending existing mono-ubiquitylations rather than catalyzing the formation of these. Thus, DDR-associated H2A ubiquitylation is likely to build on pre-existing mono-ubiquitylated H2A, or alternatively RNF8 could carry out this function with one of its other cognate E2s. The identity of the Lysine residues in H2A-type histones modified by RNF8 and RNF168 is not known, and such information will be required to fully answer these remaining questions about the precise mechanistics of the ubiquitylation reaction. Whether the function of RNF168 in the DDR is merely to boost RNF8-mediated ubiquitylations or whether RNF8 and RNF168 ubiquitylate additional, and perhaps distinct, DDR factors is another important question awaiting to be addressed.

4.4. Regulation of IRIF dynamics by de-ubiquitylating enzymes

Because of the functional importance and regulatory complexity of the pathway governing DSB-induced histone ubiquitylation, it is likely to be subject to regulation by protein de-ubiquitylation, which could be an important means for the cell to control the duration and magnitude of the response, allowing for a fine-tuned regulation of this important facet of the DDR. Indeed, a handful of de-ubiquitylating enzymes (DUBs) have been shown to be capable of reversing H2A ubiquitylation [83–89]. Among these, however, only USP3 has so far been shown to be able to counteract ubiquitin-dependent IRIF formation, at least when over-expressed [73]. Whether this activity has a physiological regulatory relevance in the DDR has not been determined. BRCC36, another DUB and an integral component of the BRCA1 A complex, also appears to play a role in reversing RNF8-dependent ubiquitylation events in the DSB response, although the exact nature of such involvement is not yet known [90]. Given the widespread use of ubiquitylation-dependent regulatory mechanisms in the DDR and the existence of 80–100 catalytically active DUBs in mammalian genomes [91], it seems likely that many aspects of protein recruitment to sites of DNA damage will turn out to be controlled on the level of de-ubiquitylation.

5. SUMOylation-mediated regulation of IRIF formation

Recent evidence has implicated post-translational modification of DDR proteins by the ubiquitin-like modifier protein SUMO as a signaling mechanism which, analogous to and in parallel with protein ubiquitylation, plays an important role in the execution of the chromatin response that governs IRIF formation. Thus, the SUMO E3 ligases PIAS1 and PIAS4 were shown to be required for recruitment of BRCA1 and 53BP1 to IRIF, respectively, and both SUMO1 and SUMO2/3 accumulate in IRIF [92,93]. However, the exact points of intervention of SUMOylation in the DSB-induced chromatin response are still somewhat elusive. Both BRCA1 and 53BP1 were shown to be direct targets of SUMOylation, modifications that were suggested to be required for their recruitment to sites of DNA damage, and, in the case of BRCA1, to boost its E3 ligase activity [92,93]. However, experimental evidence also suggests an important function of protein SUMOylation upstream of the recruitment of 53BP1 and BRCA1 to IRIF. To this end, a robust decrease in DDR-associated ubiquitylation was observed in PIAS4-depleted cells, which fail to recruit RNF168 but not RNF8 to IRIF [92]. This suggests that histone ubiquitylation may itself be regulated through SUMOylation of as yet unknown upstream DDR factors. One attractive scenario is that RNF8, RNF168, and/or HERC2 are targeted by SUMOylation, which could regulate their E3 ligase activities and/or their association with other DDR components. If this is indeed the case, it would imply the existence of yet another regulatory layer imposed on the pathway, further adding to its complexity. Pinpointing the exact relationship between DSB-associated SUMOylation and histone ubiquitylation, and how this impacts on the formation of IRIF will be an important task for future research. As we develop an understanding of how these modifications orchestrate the activation of the DDR, it will be equally important to address the potential involvement of de-SUMOylation activities counteracting these modifications [94], and which, like DUBs, may play a role in regulating the dynamics, magnitude, and duration of the DDR.

6. IRIF and the maintenance of genomic integrity

6.1. What are the biological functions of IRIF?

Despite our growing insight into the constituents of IRIF and the molecular mechanisms that govern their formation, our understanding of the physiological relevance of these structures still remains limited. The accumulation of DDR factors in DSB-containing chromatin regions may help to shelter the broken DNA ends from decay, and prevent illegitimate repair processes, such as those that lead to chromosomal translocations [95,96]. Another simplified explanation is that higher local concentrations of DDR proteins stimulate their activities towards the damaged regions of the genome [13], but this would hardly suffice to describe the collective functions of IRIF. The strong local concentration of DDR signaling factors in IRIF has also been suggested to provide an efficient means of amplifying the DNA damage signal. In support of this idea, local chromatin immobilization of DDR signaling components in both yeast and mammalian cells was shown to be sufficient to trigger robust DDR activation in a lesion-independent fashion [97,98].

An alternative, and by no means mutually exclusive, scenario that has been proposed is that accumulation of a large number of DDR factors to sites of DNA damage provides the cells with a "toolbox" containing all available enzymatic activities relevant for DNA repair and metabolism. This could be especially important in nuclear regions where DNA repair is difficult to achieve, such as heterochromatic regions (reviewed elsewhere in this issue). Thus,

many of the factors associated with IRIF might not per se play an important role in repairing the lesions, yet cells retain the possibility of utilizing all the relevant activities if necessary. In addition, IRIF may be a platform for several opposing activities, and the balance and competition of these could ultimately determine the choice of repair pathway and thus the outcome of the DDR. As an elegant example supporting such a concept, two key constituents of IRIF, 53BP1 and BRCA1, were recently shown to promote different repair pathways, and the proper balance between these activities are crucial for maintaining genomic stability. Thus, whereas knockout of *BRCA1* in mice confers embryonic lethality, a phenotype that can be attributed to a severe defect in DSB repair by HR [99], simultaneous ablation of *53BP1* rescues both the lethality and to a large extent the repair defect of *BRCA1* knockout [100]. In BRCA1-deficient cells, 53BP1 strongly promotes DSB repair via the NHEJ pathway. When this pathway is used illegitimately on broken ends that cannot be rejoined with a partner, such as those that arise from the collapse of stalled replication forks or from the processing of interstrand crosslinks, detrimental lesions such as chromosome fusions and formation of radial chromosomes ensue [100]. This competitive relationship between 53BP1, BRCA1, and the two major pathways for repairing DSBs highlights the IRIF as a platform for integration of DDR activities.

The coordination of DNA repair activities with other processes of DNA metabolism, such as transcription and replication is a key problem to be dealt with by the DDR. Thus, transcription through damaged regions may significantly worsen the extent of DNA damage. Accumulation of DDR factors in IRIF serves to prevent such illegitimate engagement of damaged genes with the transcriptional machinery. Similar to other well-studied processes that mediate transcriptional repression, ubiquitylation of H2A in IRIF seems to constitute an epigenetic mark that repels transcription factors [101]. The establishment of this mark is dependent on the concerted activities of the ATM kinase and the RNF8 and RNF168 ubiquitin ligases, yet the precise nature of the histone modification(s) that negatively regulates transcription at sites of DNA damage may be distinct from those attracting downstream DDR factors such as 53BP1 and BRCA1, and await further elucidation.

6.2. New constituents of IRIF

One major function of IRIF formation appears to be to orchestrate subtle changes in chromatin structure that are compatible with the execution of a proper DNA damage response. The recent identification of new molecular constituents of IRIF has provided additional evidence for this, and 53BP1 could potentially be a platform for assembling such activities in the chromatin regions surrounding DNA lesions. Thus, 53BP1 was recently shown to recruit a novel DDR factor, EXPAND1, to IRIF. This is accompanied by local chromatin relaxation through an as yet unknown mechanism, potentially facilitating the accessibility of DNA repair factors to the lesions [102].

Another factor that interacts avidly with the DSB-surrounding chromatin is the PTIP protein [103]. Despite it is a strong interactor of 53BP1, its recruitment to sites of DNA damage appears to occur independently of 53BP1, but depends on the same upstream signaling, such as γ-H2AX formation and RNF8/RNF168-mediated histone ubiquitylation [104]. Like 53BP1, PTIP and its established interaction partners do not contain obvious ubiquitin binding motifs, and thus it is unclear how PTIP recruitment feeds on histone ubiquitylation. PTIP co-recruits to sites of DNA damage with a partner protein, PA1, and this complex interacts with the MLL histone methylases [104,105]. Though it is not clear if such interaction is relevant to the functions of PTIP in the DDR, it points to a potential role for PTIP, in concert with 53BP1, as a local assembly platform for chromatin modulating activities.

The ubiquitin ligase Rad18 has also been shown to accumulate in IRIF, by virtue of an interaction between its ubiquitin-binding Zinc Finger domain and RNF8/RNF168-deposited ubiquitin [106]. The precise function of Rad18 in the response to DSBs is not clear, but the protein has been suggested to mediate assembly of recombination activities via Rad51C [106]. Regardless of the precise functions of Rad18 recruitment to IRIF, this brings the current tally of ubiquitin ligases present in IRIF to 5 (RNF8, RNF168, HERC2, BRCA1, and Rad18), highlighting IRIF as a nuclear hotspot for ubiquitylation. Though they may well have other critical targets, RNF8, RNF168 and HERC2 seem to be mainly involved in the ubiquitylation of H2A and H2AX. The substrates of BRCA1 and Rad18 in the response to DSBs, however, remain largely obscure, and the identification of such targets will be important to further our understanding of the regulatory importance of DDR-associated ubiquitylation processes.

7. IRIF and human disease

Highlighting its central importance for the cellular response to DSBs, several links between IRIF formation and the biology of human disease have been found. As an example, the activity of the DDR and viral proteins that inhibit the response is known to be an evolutionary battlefield in virus–host interactions [107]. For example, several strains of adenoviruses can inactivate the MRN complex either through its degradation or sequestration into inactive inclusion bodies [108,109]. Such measures are required to prevent detrimental processing of the viral genome in the cell, in turn facilitating its integration into the host genome. Herpes virus has evolved a different but no less remarkable strategy for circumventing genome surveillance mechanisms in the host cell. This virus encodes a ubiquitin ligase, ICP0, which triggers the degradation of RNF8 and RNF168 [110]. Thus, the suppression of DDR-associated histone ubiquitylation plays a key role in the life cycle of herpes virus, and other vira are likely to employ similar sophisticated strategies to harness the host DDR.

As a more prominent example of the importance of IRIFs for human health, mutational inactivation of several of the proteins that accumulate in IRIF are the underlying causes of genomic instability syndromes [6]. Intriguingly, mutations in components whose sole function may be to assemble IRIF, confer equally severe phenotypes. Thus, homozygous inactivation of the gene encoding RNF168 was shown to be the underlying genetic defect in a patient with the RIDDLE syndrome, characterized by marked radiosensitivity as well as immunological and neurological defects [74,111]. So far, this represents the only known case where abrogation of DDR-associated histone ubiquitylation is responsible for a genomic instability disorder. Though the identification of the RIDDLE patient has been instrumental in establishing this link, it will be important to determine whether RNF168 inactivation merely represents a single rare case or is the basis of a real syndrome. Mutation of other factors governing DNA damage induced histone ubiquitylation may also underlie other cases of genomic instability disorders for which the molecular basis is unknown, and it will be important to identify and characterize such cases in the future.

Conflict of interest

The authors declare no conflict of interest.

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