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Double strand break repair functions of histone H2AX

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Abstract

Chromosomal double strand breaks provoke an extensive reaction in neighboring chromatin, characterized by phosphorylation of histone H2AX on serine 139 of its C-terminal tail (to form "γH2AX"). The γH2AX response contributes to the repair of double strand breaks encountered in a variety of different contexts, including those induced by ionizing radiation, physiologically programmed breaks that characterize normal immune cell development and the pathological exposure of DNA ends triggered by telomere dysfunction. γH2AX also participates in the evolutionarily conserved process of sister chromatid recombination, a homologous recombination pathway involved in the suppression of genomic instability during DNA replication and directly implicated in tumor suppression. At a biochemical level, the γH2AX response provides a compelling example of how the "histone code" is adapted to the regulation of double strand break repair. Here, we review progress in research aimed at understanding how γH2AX contributes to double strand break repair in mammalian cells.

1. Introduction

DNA double strand breaks (DSBs) pose a threat to the stability of the genome, since their misrepair may lead to chromosome rearrangements, chromosomal deletions or other potentially damaging mutations. Defects in DSB repair are strongly associated with cancer predisposition, aging, neurodegeneration and immune deficiency [1–7]. DSBs may arise directly through the action of exogenous agents such as ionizing radiation, chemical exposure or from scheduled chromosome breakage induced during development of the adaptive immune system, such as V(D)J recombination and immunoglobulin gene class switch recombination (CSR) [8, 9]. Alternatively, the indirect action of numerous different DNA damaging agents can also lead to DSB formation, especially in replicating cells. The reason for this is that DNA lesions that are relatively benign when encountered by a repair-competent cell during G1 or G2, (i.e., when DNA is duplex) may degenerate into a DSB when encountered in the context of DNA replication [10, 11]. Breaks arising during the S phase may be preferentially repaired by sister chromatid recombination (SCR), a potentially error-free repair pathway of homologous recombination (HR), in which the broken chromosome invades the neighboring, intact sister chromatid and copies the missing information into the broken chromatid [12] (Figure 1). In contrast, a DSB generated in a non-replicating cell is a candidate for repair by non-homologous end joining (NHEJ)—a process by which the two DNA ends are religated without extensive reference to the DNA sequences at the site of breakage [13] (Figure 1). HR and NHEJ each have the potential to

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be error-free but are also potential sources of mutation. In certain contexts, HR can be mutagenic [14–18], while "clean" (i.e., chemically unmodified) DNA ends may be efficiently religated in an error-free manner by canonical NHEJ (C-NHEJ; end-joining mediated by the classical NHEJ factors including Ku, XRCC4 and DNA ligase IV) [13]. In the absence of key C-NHEJ genes, rejoining of DNA ends is surprisingly efficient [19–23]. This "alternative end joining" (A-EJ) mechanism entails greater degrees of DNA end resection than C-NHEJ and characteristically involves limited homology (microhomology) between the two DNA ends at the break point [24, 25]. During A-EJ, a mutagenic pathway, the ligation product is likely stabilized by limited base pairing between the two single stranded (ss)DNA tails (Figure 2).

Successful execution of HR requires the sequential action of a number of distinct enzymes on the DSB [26, 27]. In somatic cells, the major HR pathway, termed "synthesis dependent strand annealing" (SDSA), entails the following steps: processing of the DNA end to a 3′ ssDNA overhang; loading of Rad51 onto the resected ssDNA tail; the Rad51 nucleoprotein filament-mediated homology search (for example, sampling the neighboring sister chromatid); repair synthesis; and finally termination by homologous pairing (annealing) with the second end of the DSB [12] (Figure 2). Each of these steps is potentially prone to error. The stage in this multi-step pathway at which an error occurs can often be deduced by analysis of DSB repair products. Thus, a failure to undertake resection will cause a global reduction in flux through repair pathways that require the initial resection step. These pathways include HR as well an error-prone outcome termed single strand annealing (SSA) [12]. Single strand annealing requires the exposure of tracts of ssDNA at homologous repeats to produce its characteristic repair product, the formation of a homologous deletion (Figures 1 and 2) [12]. SSA does not require Rad51 and does not engage the homology search of a neighboring DNA molecule that defines HR. Inactivation of the protein complex responsible for loading of Rad51 onto ssDNA, in which the *BRCA2* gene product plays a critical role, causes a characteristic reduction in HR and skewing of repair in favor of SSA [28, 29]. DNA ends that have undergone resection but do not engage HR may instead be repaired by "microhomology-mediated end joining" (MMEJ), which is characterized by the presence of short stretches of microhomology at the site of rejoining [30] (Figures 1 and 2). Although there is a close association between A-EJ and MMEJ, the two are not necessarily synonymous [24]. A-EJ is defined genetically (rejoining in the absence of C-NHEJ genes), whereas MMEJ is strictly a descriptive account of the DNA sequence at the site of break rejoining.

The context in which the DSB arises critically influences how it is repaired. For example, DSBs encountered in heterochromatin exhibit different repair properties to those arising in euchromatin [31]. In addition, the cell cycle phase during which the break is generated or encountered will decide whether a sister chromatid is available to support SCR and whether the DNA end is likely to undergo extensive resection. In yeast as well as in vertebrate cells, high levels of cdk activity, such as occur in cycling cells during the S/G2 phases of the cell cycle, target and activate certain mediators of DNA end resection [32–34]. Consistent with this, HR in mammalian cells is largely restricted to the S/G2 phases of the cell cycle [35, 36]. The regulated activity of DNA end resection enzymes provides an additional opportunity for dysfunction. Under certain aberrant conditions, DNA end resection might be activated inappropriately in G0/G1 cells, when an efficient error-free HR/SCR pathway is not available due to the absence of a sister chromatid. Under these circumstances, a DSB that might normally be religated efficiently and with minimal error by C-NHEJ might instead engage a more error-prone rejoining pathway.

DSB repair pathway "choice" and the completion of DSB repair occur in the context of chromatin, the basic unit of which is the nucleosome [37]. The idea that chromatin structure

might respond to DNA damage and, in turn, influence subsequent DNA repair was suggested several decades ago from work on nucleotide excision repair of UV-induced DNA lesions [38]. This concept acquired dramatic support from the discovery by Rogakou *et al.* that histone H2AX, a variant form of histone H2A, undergoes phosphorylation on serine 139 of its C terminal tail in response to ionizing radiation (IR) [39, 40]. Serine 139 phosphorylated H2AX (γH2AX) was shown to accumulate in foci in response to IR and careful quantitation suggested that each IR-induced DSB triggers a local γH2AX response, visualized initially as an IR-induced focus [41]. The Bonner lab estimated that IR-induced DSBs elicit a γH2AX response extending up to a megabase of chromatin flanking the break [41]. The H2AX S139 phosphorylation site is a canonical target of the nuclear DNA damage PI3 kinase-like signaling kinases, Atm, Atr and DNA-PKcs [42–47]. Interestingly, H2AX appears to be the primordial form of histone H2A. Although it accounts for <10% of all histone H2A species in mammalian cells, the two histone *H2A* genes in *Saccharomyces cerevisiae* each encode proteins with an H2AX-like C-terminal tail and these H2A species, like H2AX, are also subject to C terminal phosphorylation during the DNA damage response [48, 49].

2. The γH2AX chromatin domain

The original calculations of the Bonner lab have held up well with the development of chromatin immunoprecipitation (ChIP) methods to visualize the γH2AX chromatin domain in mammalian cells. ChIP analysis of *S. cerevisiae* γH2A was reported in a strain in which an HO endonuclease-induced DSB could not be repaired [47]. This provoked a strong γH2AX response, with a marked depletion of γH2AX signal in the ~1 kb proximal to the DSB. Work in mammalian cells studied the extent of the γH2AX domain in primary thymocytes and in immortalized *bcr-abl* expressing pre-B cells induced to undergo V(D)J recombination in G1 in response to abl kinase inhibition [44]. By examining the response in *Artemis* null cells, in which a hairpin intermediate of V(D)J recombination cannot be cleaved (i.e., an “unrepairable DSB”), the authors were able to map the γH2AX domain over several hundred kilobases. Deletion of a major DSB signaling kinase, Atm, reduced the extent of the γH2AX domain, but did not abolish the γH2AX response in close proximity to the DSB. This implicates at least one DSB response kinase other than Atm (likely, DNA-PKcs and/or Atr) in the γH2AX response closer to the DNA end. A second approach entailed the controlled induction of a restriction endonuclease, *Asi*SI, to induce DSBs at discrete chromosomal loci in a human osteosarcoma cancer cell line, U2OS and in a human glioblastoma cell line, T98G [50]. Mapping of the γH2AX domain revealed characteristically locus-specific, asymmetric distributions of γH2AX flanking the defined break sites, with a broadly similar pattern observed in each cell line. Exceptions to the similar distributions between the two cell lines were observed in cases of actively transcribed genes, which correlated with regions of low staining intensity (“holes”) located within a γH2AX domain. One defined molecular modifier of γH2AX distribution is the cohesion complex, which restricts the spread of γH2AX [51]. Cohesin may play a role in isolating actively transcribed genes from the impact of a nearby γH2AX response.

These observations make a number of important points. First, the extent of spread of the γH2AX domain is not defined simply by the “distance” in base pairs from the break site, but appears to conform to a pre-existing locus-specific chromatin architecture that is to a certain extent common to different cell types. Second, the γH2AX signal is dynamic, being suppressed by the presence of actively transcribed genes. At present, it is not clear what defines the outer boundary of an individual γH2AX domain. Work in yeast suggests that the presence of heterochromatin suppresses γH2A locally but is not an absolute block to the spread of the γH2A signal [47]. The significance of the γH2A(X)-free zone near the break, which has also been observed in other ChIP analyses of γH2AX in mammalian cells,

remains unclear [52]. The temporal element is important in the DNA damage response, and the use of “unrepairable” DSBs or recurrent endonuclease-induced breaks at a single site likely produces a “steady state” DSB signal. The regulation and chromatin distribution of γH2AX signals in this steady state may differ from the dynamic, perhaps more transient γH2AX response to a normal, repairable DSB, such as might occur during replication.

2.1 γH2AX nucleates a specialized, multistep chromatin structure at sites of chromosome breakage

The biochemical and functional consequences of H2AX S139 phosphorylation near mammalian chromosomal DSBs has been the subject of intense research for more than a decade. The biochemical picture that has emerged is of a specialized chromatin domain that may affect chromatin compaction and that recruits a specific set of DNA damage response factors to chromatin flanking the DSB [53]. A large nuclear protein, MDC1, binds directly to γH2AX via a tandem C-terminal BRCT repeat of MDC1 [54–58]. MDC1 is a multi-domain scaffolding protein that appears to orchestrate most, if not all γH2AX functions. MDC1 recruits the heterotrimeric Mre11/Nbs1/Rad50 (MRN) complex by direct interaction of casein kinase-phosphorylated MDC1 target sites with the FHA domain of Nbs1 [59]. The MRN complex has critical roles in early responses to the DSB, contributing to both HR and NHEJ; Mre11 endonuclease activity has been implicated in DNA end resection [60–63]. MRN bound to DNA ends also recruits and activates the Atm signaling kinase [64, 65], and this Atm association is retained when MRN interacts with MDC1 within the γH2AX chromatin domain at a distance from the break site [66]. This suggests that MDC1 amplifies the Atm response to the DSB. Indeed, the deliberate tethering of MDC1 to a chromosomal array in the absence of a DSB triggers a γH2AX response—presumably mediated in part by this Atm-dependent amplification mechanism [67]. This is supported by the finding, in pre-B cells undergoing arrested V(D)J recombination, that the γH2AX signal intensity is reduced in *MDC1* null cells, although the extent of spread of the γH2AX (measured by ChIP) appears not to be reduced by deletion of *MDC1* in the way that it is by loss of *Atm* [44]. The γH2AX domain is a dynamic chromatin structure. Work in *Drosophila melanogaster* has provided evidence of histone replacement of the H2AX homolog, H2Av [68]. In mammalian cells, histone H2AZ has been detected on chromatin at DSB sites, suggesting that some histone replacement occurs within γH2AX chromatin [69].

A number of proteins implicated in HR or NHEJ, in addition to the MRN complex, assemble on γH2AX/MDC1 chromatin [70]. These include BRCA1, the product of the hereditary breast/ovarian cancer predisposition gene, together with its heterodimeric partner BARD1, and the DNA damage response protein 53BP1 [71]. The formation of extensive IR-induced nuclear foci (a cytological reflection of the chromatin response) by these proteins is dependent upon both *H2AX* and *MDC1*, suggesting that BRCA1 and 53BP1 serve functions in chromatin downstream of the initial γH2AX/MDC1 response. The mechanism of 53BP1 recruitment requires its interaction with a constitutive chromatin mark, histone H4 dimethylated on lysine 20 (H4K20me2) [72, 73]. This is mediated by direct physical interaction between the tandem Tudor repeat of 53BP1 and the H4K20me2 mark. Affinity measurements *in vitro* suggested that 53BP1 can equally well bind H4K20me1 and this is supported by *in vivo* analysis [74, 75]. This also presented a paradox: if the H4K20me2 mark is ubiquitous in chromatin, how is the specific recruitment of 53BP1 to γH2AX/MDC1 chromatin achieved?

2.2 Ubiquitin-dependent elements of the γH2AX chromatin response

A major breakthrough in understanding 53BP1 and BRCA1/BARD1 recruitment to γH2AX/MDC1 chromatin came from the discovery that a set of E3 ubiquitin ligases, RNF8 and RNF168, are required for efficient recruitment of these proteins to γH2AX/MDC1

chromatin [76–80]. RNF8 directly binds MDC1 and ubiquitylates chromatin components, including histone H2A species in chromatin at the break site by generating K63-linked ubiquitin chains. The RNF8-associated E3 ubiquitin ligase, RNF168 (the loss of which has been identified as a cause of the human RIDDLE syndrome of radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties [81]), is recruited by RNF8 to γH2AX/MDC1 chromatin but acts biochemically upstream of RNF8 by monoubiquitylating histone H2A on K13/K15 residues—a motif distinct from the known polycomb RING1B/Bmi1 complex target site on histone H2A or H2AX K118/119 [82]. Monoubiquitylation of H2A or H2AX K13/K15 by RNF168 and its E2, UbcH5, acts as a priming mark for the action of RNF8 and its E2, Ubc13, in the formation of major K63-linked polyubiquitin species on histone H2A/H2AX [83]. H2AX is formally dispensable for 53BP1 recruitment provided the appropriate H2A ubiquitin marks can be established on chromatin, as is the case for spontaneous 53BP1 focus formation on incompletely replicated DNA structures transmitted through mitosis to the subsequent G1 cell cycle phase [84–86]. Thus, the MDC1/RNF8/RNF168 response generates a specific set of ubiquitylated chromatin marks that lay a foundation for specific recognition of DNA damage response complexes by ubiquitin-binding domains.

The mechanism by which these ubiquitin marks recruit 53BP1 remained unclear for some time and a number of additional factors influence 53BP1 recruitment [87], some possibly in a cell type-specific fashion [75, 88]. Mapping of the domains of 53BP1 that are required for its recruitment to γH2AX/MDC1 chromatin has implicated regions close to the tandem Tudor repeat in dimerization; indeed, homodimerization is required for efficient recruitment of 53BP1 to γH2AX/MDC1 chromatin [89]. This is consistent with the finding that the binding affinity of H4K20me2 for the tandem Tudor repeat is in the micromolar range [72]. Recently, it was shown that 53BP1 recognizes mono-nucleosomes containing H4K20me2 and H2A ubiquitylated on K15 (H2AK15Ub) [90]. A region C-terminal of the 53BP1 tandem Tudor repeat, termed the ubiquitylation-dependent recruitment (UDR) motif, interacts with H2AK15Ub and this dual interaction of dimerized 53BP1 with H4K20me2 and H2AK15Ub is required for its stable interaction with nucleosomes. Thus, 53BP1 is secured on chromatin by a low affinity, high avidity mechanism entailing multiple binding sites with regulated histone marks, which include RNF168-mediated H2AK15Ub. This conforms to the canonical mechanism for regulating chromatin-associated proteins through alterations in the “histone code”, whereby combinatorial binding *via* individually low affinity interactions with histone marks allows for complex regulation of chromatin association and for the rapid accumulation or release of the chromatin-associated protein in response to the appropriate cue [91].

The mechanism of BRCA1/BARD1 recruitment to γH2AX/MDC1 chromatin has also been unequivocally linked to a physical interaction with ubiquitylated chromatin components, mediated by an interaction of BRCA1/BARD1 with the ubiquitin-binding protein Rap80 [92–94]. The BRCA1-Rap80 complex, which entails direct binding of the BRCA1 tandem BRCT repeat to Abraxas/CCDC98, contains a number of ubiquitin modifying enzymes, including the deubiquitinating enzyme BRCC36 [95] and the BRCA1/BARD1 dimer, itself an E3 ubiquitin ligase [96]. This suggests a likely role for the BRCA1-Rap80 complex in ubiquitin editing on γH2AX/MDC1 chromatin [97–99]. The Rap80-bridged interaction of BRCA1 with ubiquitylated chromatin components appears to be significant in breast cancer risk, since a missense allele of Abraxas that mislocalizes the protein has been implicated in hereditary breast cancer risk [100].

2.3 Competition between BRCA1 and 53BP1 on γH2AX/MDC1 chromatin

BRCA1/BARD1 contributes to early steps of HR by promoting DNA end resection—a process that involves its interaction with CtIP, the mammalian homolog of the *S. cerevisiae*

Sae2 endonuclease [101]. A remarkable functional antagonism exists between BRCA1 and 53BP1, whereby depletion of 53BP1 restores viability to *Brca1* null cells in vitro [102]. Deletion of *53BP1* in the mouse germ line reverses some of the HR defects in *Brca1* exon 11-deleted hypomorphic mice [103, 104]. This functional antagonism, which is discussed in more detail below, is reflected in mutual antagonism between BRCA1 and 53BP1 in the γH2AX/MDC1 chromatin response. Although these functional antagonists are co-recruited to γH2AX/MDC1 chromatin, responding to overlapping histone ubiquitylation marks, recent work using three-dimensional structured illumination microscopy suggests that BRCA1 and 53BP1 occupy mutually exclusive domains within a single γH2AX/MDC1 chromatin domain [105]. The N-terminal tail of histone H4 is subject to acetylation by the Tip60 histone acetyl transferase complex, and H4K16 acetylation inhibits binding of H4K20me2 to the 53BP1 tandem Tudor repeat [106]. Tip60 may therefore help to disrupt 53BP1 chromatin association so as to allow BRCA1/BARD1 and other resection enzymes access to chromatin flanking the DSB.

3. The “concentration of repair factors” hypothesis: the chromatin domain *vs*. the DNA domain

Chromatin structure plays a major role in the regulation of chromosomal processes such as transcription and DNA replication. In keeping with the multifunctional characteristics of chromatin, an Atm-dependent process, acting in *cis* with respect to a chromosomal DSB, has been implicated in localized repression of transcription during the acute response to a DSB [107]. H2AX also has DNA damage signaling functions [66, 84]. Indeed, the number of post-translational modifications of chromatin and enzymatic activities associated with γH2AX/MDC1 chromatin is diverse pointing to additional functions of this domain; these have been the subject of recent review [108]. However, the fact that the specialized γH2AX/MDC1 chromatin domain recruits known DNA repair proteins to chromatin flanking the DSB led to the proposal that γH2AX/MDC1 supports DSB repair by concentrating repair factors near the break site. In this section we will review to what extent this hypothesis has survived the test of time.

3.1 Homologous recombination functions of H2AX

Mice lacking any one of *Brca1*, *Mre11*, *Rad50* or *Nbs1* show early embryonic lethality [109, 110], with spontaneous chromosome structural abnormalities due to an underlying severe HR defect. In contrast, *H2AX* null mice are viable, exhibiting male sterility and a modest impairment in class switch recombination (CSR) at the *IgH* locus in developing B cells [111]. Thus, the genetic data suggest that BRCA1 and the MRN complex execute their major HR functions independently of *H2AX*. Consistent with this, minor fractions of BRCA1, 53BP1 and the MRN complex are detectable at DSB sites in *H2AX* null cells [112]. Thus, the response to a chromosomal DSB could be considered to entail two (or more) compartments: a DNA domain, in which major DSB repair enzymes act to negotiate effective DSB repair, and the γH2AX/MDC1 chromatin domain, in which some of the same DSB repair factors appear, separated from the break by some hundreds of kilobases of chromatin (Figure 3).

The mild phenotype of *H2AX* null mice prompted a search for DSB repair functions that are specific to H2AX. Examination of DSB-induced HR in *S. cerevisiae* and in mammalian cells revealed a ~4-fold defect in HR/SCR in *H2A(X)* null cells [113, 114]. The *H2AX* null HR defect was reversed by expression of wild type H2AX but not by H2AX S139 mutants, which cannot form γH2AX species. Similar observations were made in chicken DT40 lymphoblastoid cells [115]. Collectively, these observations suggest that the HR/SCR function of H2A(X) is evolutionarily conserved. Consistent with a role in HR/SCR, many

DNA damaging agents provoke predominantly S-phase γH2AX responses in cycling cells. Interestingly, several groups reported alterations in (but not the abolition of) Rad51 focus formation in *H2AX* null cells [115, 116]. In addition to the observed HR defect, *H2AX* null mouse ES cells reveal a skewing of DSB repair in favor of single strand annealing [114]. This pattern (reduced HR, skewing in favor of SSA) is reminiscent of the DSB repair defect observed in cells lacking Rad51 or its loading enzyme, BRCA2. However, a number of distinct mechanisms could account for the characteristic imbalance between HR and SSA seen in *H2AX* null cells. Although γH2AX might conceivably act upon the Rad51 filament, a number of points favor an indirect mechanism. First, γH2A chromatin in *S. cerevisiae* colocalizes with cohesin complexes, suggesting a possible mechanism of action of H2AX in DSB repair that operates in parallel to enzymes acting at the DSB [113, 117]. Second, inactivation of mammalian complexes that mediate Rad51 filament formation or stability (BRCA2, the Rad51 paralogs) causes strong skewing of HR in favor of long gene conversion tracts, whereas this skewing is not seen in *H2AX* mutants [63, 118–121].

Further genetic analysis of H2AX revealed a tight connection between the HR function of H2AX and its ability to bind MDC1 [122, 123]. *MDC1* null cells also reveal an HR defect and domain analysis suggest that the RNF8-binding region of MDC1 is dispensable for its HR function; in contrast, the Forkhead-associated (FHA) and PST repeat domains are required for this function. Thus, the RNF8/RNF168-dependent recruitment of BRCA1 or 53BP1 does not appear to participate in the core HR function of H2AX [122]. This apparently paradoxical result is in fact consistent with the idea of an evolutionarily conserved HR/SCR function to H2AX, since the *S. cerevisiae* genome does not contain a *BRCA1* homolog. This result is also consistent with recent data on the role of the Rap80/BRCA1 complex in HR (discussed below). One clue as to how γH2AX/MDC1 might enact its HR function is suggested by the finding that the FHA domain of MDC1 can bind Rad51, suggesting a possible role for MDC1 as an accessory mediator of Rad51 loading [124]. However, this model does not explain the contribution of the MDC1 PST repeats to HR. The MDC1 FHA domain has a major role in DNA damage-induced homodimerization, which is required for efficient activation of MDC1 [125, 126]. To what extent MDC1 dimerization affects its ability to interact with Rad51 is currently unclear.

A study of the relationship between *H2AX* and *Atm* in HR revealed further insight into the HR function of H2AX [127]. As noted above, Atm is responsible for the formation of an extensive γH2AX domain; in its absence the spread of γH2AX signal is significantly reduced, the remaining phosphorylation of H2AX S139 likely being mediated by DNA-PKcs and/or Atr in close proximity to the DSB. Surprisingly, *Atm* deletion has no effect on DSB-induced HR, at least in euchromatin, and *H2AX*-dependent HR is strictly *Atm*-independent in this context [127]. Thus, it appears that the limited formation of γH2AX chromatin that occurs in *Atm* null cells is fully sufficient to support γH2AX/MDC1-mediated HR—at least, in the repair of a “clean” enzyme-induced euchromatic DSB.

In summary, analysis of the HR function of H2AX/MDC1 appears to provide little evidence in support of the “concentration of repair factors” hypothesis, as originally proposed. The core HR functions of *BRCA1*, *Mre11, Rad50* and *Nbs1*—each of which is required for organismal viability in mammals—are independent of *H2AX*. The domains of MDC1 that mediate its HR function do not include the RNF8 binding domain and therefore the HR function of *H2AX/MDC1* is genetically separable from its ability to recruit BRCA1 to chromatin. This conclusion is underscored by the specific contribution of the BRCA1-Rap80 complex (i.e., the major γH2AX/MDC1-associated, RNF8-dependent fraction of BRCA1) to HR. Inhibition of Rap80 has a stimulatory effect on HR in some cell types, likely reflecting perturbed control of DNA end resection [128, 129]. Thus, the BRCA1 fraction that is associated with RNF8/RNF168/Rap80 appears to have HR regulatory functions quite

different from the "core" HR functions of BRCA1, which act at the DSB independently of *H2AX* to promote DNA end resection and to mediate recruitment of BRCA2/Rad51 to the resected DSB (Figure 1). However, the full set of DSB response functions associated with the Rap80-BRCA1 complex is not yet defined and there may be crosstalk between Rap80-BRCA1 and other "core" HR functions of BRCA1 in normal physiology.

3.2 H2AX in non-homologous end joining

Class switch recombination (CSR) is a specialized example of programmed DNA breakage and rejoining that occurs in developing B cells as they undergo conversion of immunoglobulin heavy chain (IgH) isotypes from IgM to the mature isotypes IgG, IgA or IgE. This process entails localized chromosome breakage and rejoining of highly repetitive switch (S) regions in proximity to the constant region alternative exons Cμ, Cγ, Cα and Cε. Cytokine activity targets the Activation-Induced (DNA-cytosine) deaminase (AID) to Sμ and one of Sγ, Sα or Sε [8, 9]. The action of AID, assisted by uracil DNA glycosylase and the apyrimidic/apurinic-endonuclease, results in break formation localized to the targeted S regions, followed by rejoining of the two distantly located broken switch regions [130, 131]. This places the variable region of the Ig heavy chain in proximity to the new constant region Cγ, Cα or Cε and results in IgH isotype switching. AID-initiated breakage can occur at multiple sites within Sμ, and rejoining of these intra-switch breaks can result in internal Sμ deletions. CSR is accompanied by an extensive γH2AX response, focused on switch regions undergoing AID-induced breakage. Analysis of *H2AX* null mice revealed a modest reduction in CSR but no alteration in somatic hypermutation, a second AID-mediated process that targets the hypervariable region of the Ig gene for affinity maturation [111, 132]. *H2AX* null cells similarly reveal no reduction in internal Sμ deletions, consistent with the idea that the induction of DSBs in Sμ is not perturbed by deletion of *H2AX*.

The mechanism of H2AX action in CSR was suggested by the finding that *H2AX* null mice reveal increased frequencies of unrepaired breaks at *IgH* and associated chromosomal translocations involving *IgH* [133]. This suggested that H2AX (and also Atm, MDC1 and 53BP1) contributes to the timely rejoining of AID-induced DSBs at *IgH*. Whether this specialized case of NHEJ reflects a more general function for H2AX in NHEJ is not yet clear. In this regard, the modest reduction of CSR efficiency and grossly normal V(D)J recombination in *H2AX* null mice argues against a "core" role for H2AX in NHEJ. Consistent with this, the simple rejoining of two proximate chromosomal DSBs induced by a restriction endonuclease is not disrupted by *H2AX* deletion [63]. The mechanisms by which H2AX supports NHEJ during CSR are currently unclear. In yeast, an association between γH2A and cohesion has been noted, suggesting a possible role for γH2AX in "tethering" DNA ends during DSB repair [113]. Limited support for this model is suggested by live cell imaging of the stability of DNA ends following DSB induction in mammalian cells [134]; depletion of H2AX modestly disrupted DNA end stability over time.

Although V(D)J recombination is grossly normal in *H2AX* null mice, the elevated frequency of IgH J_H-translocations in *H2AX/Trp53*-deficient mice hints at possible abnormalities in the execution of V(D)J recombination [135, 136]. Compelling evidence of a role for H2AX in V(D)J recombination came from two studies. First, aberrant processing of hairpin intermediates of V(D)J recombination induced was noted in B-lymphocytes of mice lacking both Artemis and H2AX [137]. Artemis is the major endonuclease responsible for cleaving hairpin intermediates of V(D)J recombination. In *Artemis* null cells, the hairpin is maintained intact, but in *Artemis/H2AX* null cells, hairpin opening occurs, accompanied by extensive CtIP-mediated resection of the opened DNA end, which is rejoined with an increased frequency of MMEJ breakpoints. Second, combined deletion of *H2AX* and the C-NHEJ factor *XLF* causes defective V(D)J recombination, which is associated with marked degradation of unjoined V(D)J ends [138]. These observations implicate H2AX in V(D)J

recombination fidelity—a function which is normally masked by functional redundancy in wild type cells.

Telomere integrity is maintained by an interaction of nucleoprotein complexes on telomeric DNA, a central role being played by the shelterin complex [139]. Disruption of shelterin components can provoke a DNA damage response, due to exposure of DNA structures at the end of the disrupted telomere. Inhibition of the shelterin component TRF2 releases telomere ends and promotes interchromosomal fusion of telomere ends—a process that is mediated by C-NHEJ [140, 141]. Consistent with a role for H2AX/MDC1 in NHEJ, depletion of MDC1 disrupts the fusion of dysfunctional telomeres caused by loss of TRF2 [142].

3.3 Relationships between 53BP1 and H2AX in DSB repair: 53BP1 goes freelance

The observation that 53BP1 recruitment to DSB-induced nuclear foci is controlled by H2AX/MDC1 and by the RNF8/RNF168 ubiquitin response suggested that γH2AX/MDC1 might control 53BP1 DSB response functions. However, work in recent years has argued against this simple model. As noted above, spontaneous 53BP1 focus formation in G1 cells is not abolished by deletion of *H2AX* [84]. In contrast to *H2AX* null mice, *53BP1* null mice reveal a profound defect in CSR—indeed, more severe than that of *RNF8* null or *RNF168* null mice [143, 144]—suggesting that the contribution of 53BP1 to this end-joining process is at least in part independent of *H2AX* [145, 146]. Second, analysis of H2AX- and MDC1-dependent HR/SCR led to the finding that 53BP1 inhibition stimulates HR, suggesting that 53BP1 normally antagonizes HR in this setting. This function is independent of *H2AX* but is abolished in *XRCC4* null cells, suggesting a primary role for 53BP1 in C-NHEJ [122]. A primary function for 53BP1 in NHEJ was further supported by the finding that the fusion of dysfunctional telomeres is virtually abolished by removal of 53BP1—a pattern strongly reminiscent of the impact of 53BP1 loss on CSR [147].

Some phenotypes of *BRCA1* hypomorphic mutant mice are suppressed by co-deletion of *53BP1*, and 53BP1 suppression restores viability to *Brca1* null embryonic stem cells, as well as resistance to poly(ADP-ribose) polymerase inhibitors and other DNA damaging agents [102, 103]. 53BP1 inhibition did not restore viability to *BRCA2* mutant cells [102]. Crucially, deletion of *H2AX* or *Atm* does not rescue the growth of *BRCA1* mutant cells, indicating that 53BP1 is acting independently of the prime mediators of the γH2AX/MDC1 chromatin response in its adversarial relationship with BRCA1. The likely point in DSB repair at which 53BP1 and BRCA1 enact this antagonism is in the regulation of DNA end resection, BRCA1 playing a positive role in this process and 53BP1 suppressing resection [104]. 53BP1 makes phosphorylation-dependent interactions with Rif1, which recent work has shown to be an important mediator of 53BP1's anti-resection/NHEJ functions [148–151].

4. Summary and future prospects: the resection connection

The data discussed above suggest that the action of γH2AX in DSB repair conforms only partly to a mechanism based upon the concentration of repair factors. Those DSB repair factors that are recruited to γH2AX/MDC1 nuclear foci (MRN, BRCA1 and 53BP1) all demonstrate critical DSB repair functions that are quantitatively more significant than those of H2AX and that persist in *H2AX* null cells. Nonetheless, it is provocative that the functional antagonism between BRCA1 and 53BP1 (enacted at the DNA end without the need for H2AX) is "re-enacted" on γH2AX/MDC1 chromatin in the mutually exclusive distributions of these proteins within a single DNA damage response focus [105, 106].

Each system used to analyze mammalian DSB repair has potential limitations and one wonders how many connections we are missing because of the incomplete nature of assay

systems. For example, the use of endonucleases such as I-SceI to induce DSBs in euchromatin, although an excellent and highly quantitative system, fails to synchronize the timing of DSB induction with common physiological triggers to chromosome breakage, such as replication fork arrest and presents less of a challenge than a DSB formed in heterochromatin. Similarly, the DNA ends generated by I-SceI-induced DSBs are not chemically modified in the way that DSBs induced by ionizing radiation or radiomimetic chemicals are. In this regard, it is interesting to note that the major DSB repair factors that have been reported to accumulate on γH2AX/MDC1 chromatin—MRN, BRCA1 and 53BP1—all have roles in regulating DNA end resection. This raises the possibility that the mature γH2AX/MDC1 chromatin domain serves primarily as a "toolkit" for regulating DNA end resection—a concentration of repair factors that might not be required to fix a clean euchromatic DSB, but whose utility might be revealed in the repair of more challenging, chemically modified or spatially separated DSBs.

H2AX clearly plays important roles in regulating the function and fidelity of the two major pathways of DSB repair, HR and NHEJ. The further molecular and genetic dissection of these *H2AX*-dependent DSB repair functions is important, since they represent some of the clearest examples of the impact of the chromatin response on the fundamental process of DNA break repair and provide compelling illustrations of how the "histone code" is adapted to this process. Throughout the analysis of H2AX and its associated proteins, the connection to human disease is evident. Thus, *H2AX* deletion in mice promotes genomic instability and collaborates with other oncogenic mutations to promote cancer. Indeed, H2AX haploinsufficiency may be a driver of human tumorigenesis [152], as it is in mice [135, 136].

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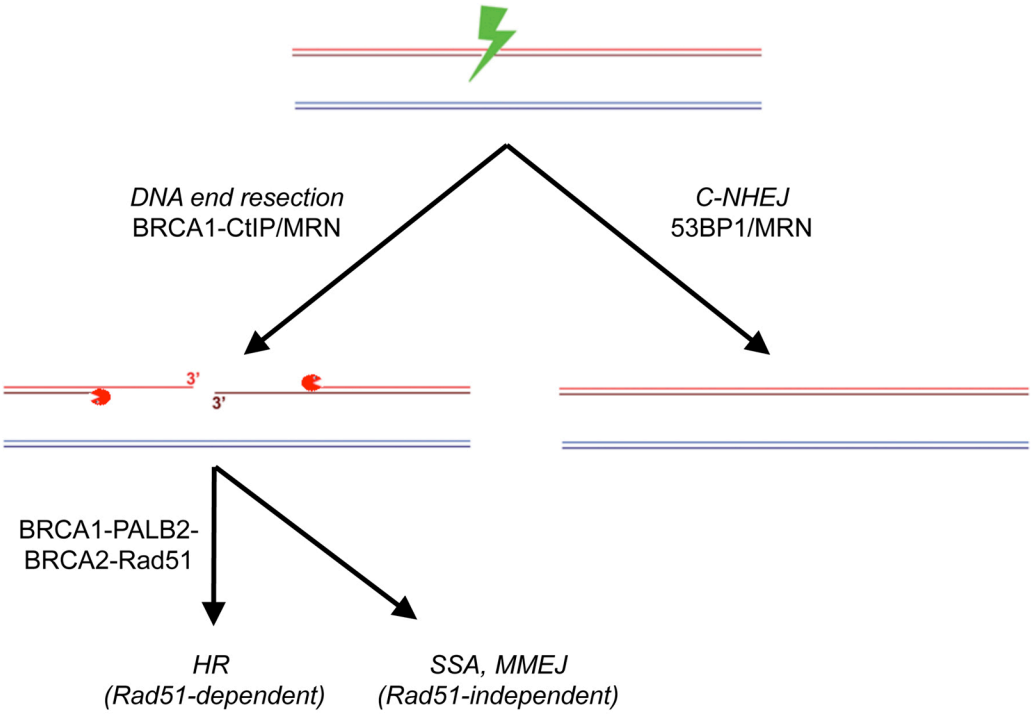

Figure 1. Hierarchy of DSB repair pathways

The engagement of DNA end resection plays a critical role in specifying DSB repair pathway selection. Unresected DSBs are candidates for canonical non-homologous end joining (C-NHEJ). The resected DSB can be repaired in an error-free manner by homologous recombination (HR). If this process fails, mutagenic repair *via* single strand annealing (SSA) or microhomology-mediated end joining (MMEJ) may be engaged. The *H2AX*-independent roles of BRCA1, 53BP1 and the Mre11/Rad50/Nbs1 (MRN) complex are depicted.

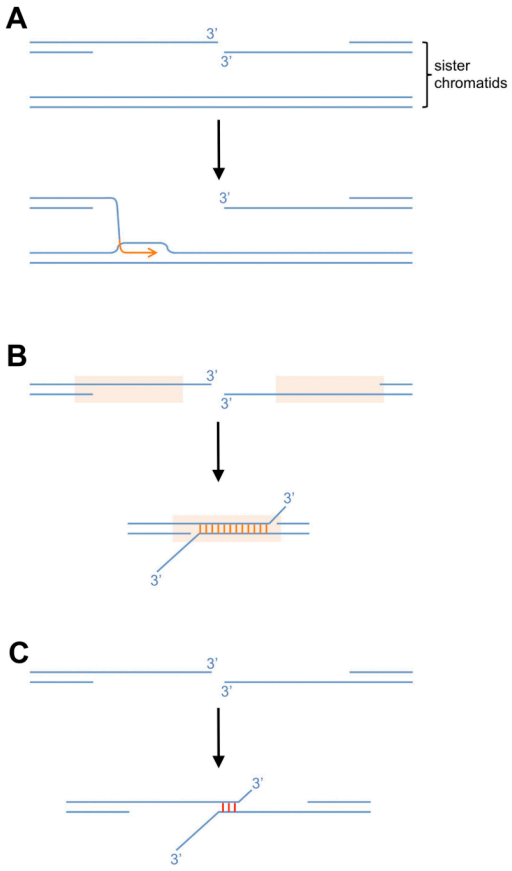

Figure 2. Distinct repair pathways operate on resected DNA ends. A. Homologous recombination/synthesis-dependent strand annealing (SDSA)

Rad51-mediated homologous invasion of the neighboring sister chromatid has potential for error-free repair. **B. Single strand annealing (SSA).** If two regions of homology (orange boxes) are in close proximity to the DSB, the resected ends may anneal (homologous base pairs marked orange), generating a homologous deletion at the site of breakage. SSA is *Rad51*-independent. **C. Microhomology-mediated end joining (MMEJ).** The two resected ends may be stabilized by limited base-pairing (microhomology, base pairs marked red) between the two exposed ssDNA tails of the resected DSB. MMEJ is *Rad51*-independent.

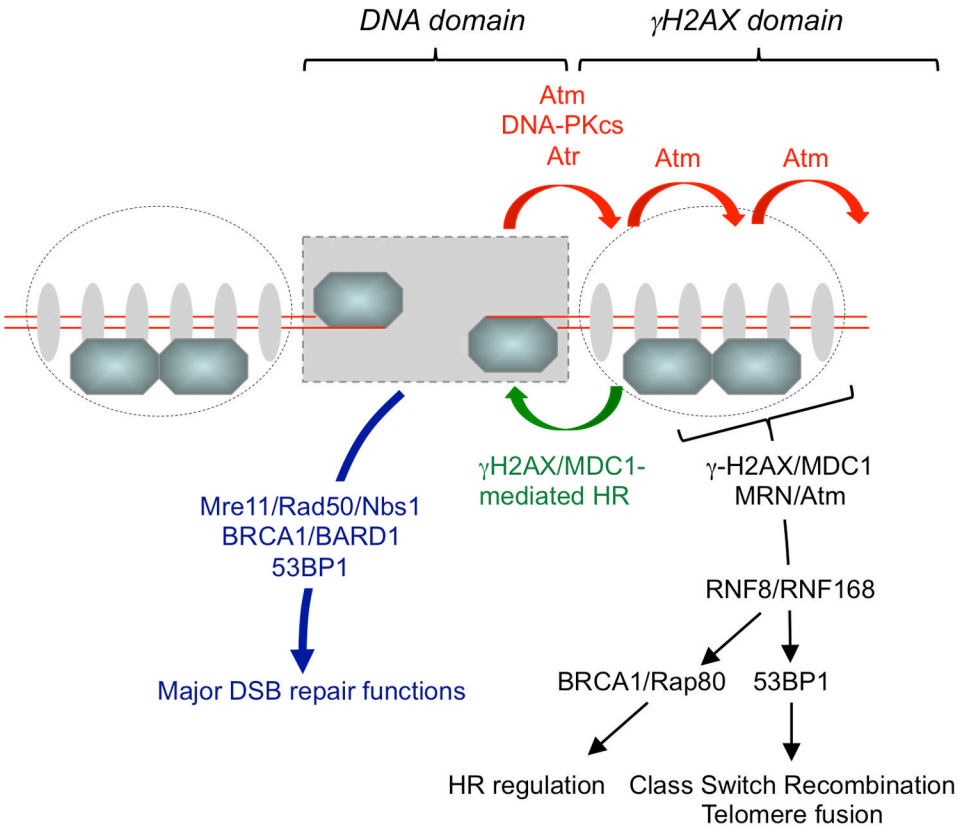

Figure 3. Double strand break repair functions within the γH2AX chromatin domain

Atm kinase activity propagates the γH2AX signal over hundreds of kilobases of chromatin. In its absence, a more localized γH2AX response is mediated by the related DNA damage response signaling kinases DNA-PKcs and Atr. This *Atm*-independent γH2AX response can support the function of H2AX in HR/sister chromatid recombination. The Mre11/Rad50/Nbs1 (MRN) complex, BRCA1 and 53BP1 execute DSB repair functions independently of *H2AX* within the "DNA domain" (see also Figure 1), but their recruitment to extensive chromatin domains flanking the DSB is controlled by γH2AX/MDC1. Non-HR DSB repair functions of γH2AX/MDC1 chromatin include long range rejoining (class switch recombination and fusion of dysfunctional telomeres) and the regulation of DNA end resection.