DNA REPAIR 6 (2007) 280–292

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journal homepage: www.elsevier.com/locate/dnarepair

Collaborative roles of γH2AX and the Rad51 paralog Xrcc3 in homologous recombinational repair

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ARTICLE INFO

Article history:
Received 16 August 2006
Received in revised form
29 September 2006
Accepted 19 October 2006
Published on line 22 November 2006

Keywords:
γH2AX
Rad51 paralog
Homologous recombination
Genome stability
Double-strand break repair

ABSTRACT

One of the earliest events in the signal transduction cascade that initiates a DNA damage checkpoint is the phosphorylation on serine 139 of histone H2AX (γH2AX) at DNA double-strand breaks (DSBs). However, the role of γH2AX in DNA repair is poorly understood. To address this question, we generated chicken DT40 cells carrying a serine to alanine mutation at position 139 of H2AX (*H2AX*$^{-/S139A}$) and examined their DNA repair capacity. *H2AX*$^{-/S139A}$ cells exhibited defective homologous recombinational repair (HR) as manifested by delayed Rad51 focus formation following ionizing radiation (IR) and hypersensitivity to the topoisomerase I inhibitor, camptothecin (CPT), which causes DSBs at replication blockage. Deletion of the Rad51 paralog gene, *XRCC3*, also delays Rad51 focus formation. To test the interaction of Xrcc3 and γH2AX, we disrupted *XRCC3* in *H2AX*$^{-/S139A}$ cells. *XRCC3*$^{-/-}$/*H2AX*$^{-/S139A}$ mutants were not viable, although this synthetic lethality was reversed by inserting a transgene that conditionally expresses wild-type H2AX. Upon repression of the wild-type H2AX transgene, *XRCC3*$^{-/-}$/*H2AX*$^{-/S139A}$ cells failed to form Rad51 foci and exhibited markedly increased levels of chromosomal aberrations after CPT treatment. These results indicate that H2AX and XRCC3 act in separate arms of a branched pathway to facilitate Rad51 assembly.

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doi:10.1016/j.dnarep.2006.10.025

1. Introduction

The DNA double-strand break (DSB) poses a serious challenge to genome stability. Within a few minutes of its formation, a DSB triggers various checkpoint switches and repair processes. One of the earliest responses is the phosphorylation of histone H2AX at serine 139 to form γH2AX. The phosphorylation is carried out by the phosphatidylinositol 3-kinase family kinases, DNA-PK, Atr, and Atm [1–3]. The response is highly amplified with several hundred to several thousand H2AX molecules being phosphorylated in the chromatin around each DSB. A role for γH2AX in a damage checkpoint signaling cascade has been suggested by the findings that γH2AX facilitates the recruitment and retention of a number of damage checkpoint proteins, such as the Mre11/Nbs1/Rad50 (MRN) complex, Brca1, 53BP1, and NFBD1/MDC1 to the DSB sites [4–6]. Mice lacking H2AX are hypersensitive to ionizing radiation (IR), and display a dramatic increase in cancer incidence in a p53-deficient background [7,8].

Genetic studies in yeast demonstrated a causal relationship between defective damage checkpoints and IR hypersensitivity [9]. However, it is an open question whether hypersensitivity to IR in vertebrate cells is due to defective cell cycle arrest. The cause of radio-sensitivity in ataxia telangiectasia (A-T) cells remains controversial, being variously attributed to compromised cell cycle arrest at the G_1/S and G_2/M boundaries, loss of apoptosis, and repair abnormalities [10–17]. Likewise, damage checkpoint dysfunction in cells deficient in Nbs1 does not necessarily account for their IR sensitivity, as reconstitution with a hypomorphic mutant Nbs1 reverses their IR sensitivity, but not their defective cell cycle arrest [18]. A similar hypomorphic mutation has also been identified in the BRCA1 gene [18]. Thus, the defective checkpoint of H2AX deficient mice does not necessarily explain their genome instability and IR sensitivity. Alternatively, γH2AX might directly affect DSB repair, as accumulating evidence suggests that the damage checkpoint pathways can control DSB repair pathways [16,17].

There are two major DSB repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), which appear to contribute differentially to DSB repair, depending on the origin of the DSBs. "Accidental" DSBs, such as those induced by IR are preferentially repaired by NHEJ in mammalian cells. On the other hand, DSBs resulting from blocked replication are repaired primarily by HR [19,20]. Accordingly, HR is essential for genome stability in cycling cells, and plays a critical role in cellular tolerance to the DNA topoisomerase I inhibitor, CPT, which causes replication blocks that ultimate lead to DSBs [21]. HR is a multi-step process involving a number of repair proteins. During its early steps, the MRN complex and other unknown nucleases resect the DNA at DSB sites to generate 3′ single strand (ss) overhangs, which associate with Rad51, a RecA homolog [22]. Although Rad51, Rad52, and Rad54 similarly contribute to HR in budding yeast, Rad51 plays a considerably more important role than Rad52 or Rad54 in vertebrate cells. Accordingly, vertebrates cells posses a number of Rad51 cofactors that control activity of Rad51, including the five Rad51 paralogs, Brca1, and Brca2. The five Rad51 paralogs, which include XRCC3, appear to act as a functional unit to promote Rad51 assembly at DSB sites [23–25]. The resulting Rad51–ssDNA filaments invade other intact homologous sequences to form a D-loop. Finally, DNA synthesis from the invading strand results in gene conversion [26] (reviewed in Ref. [27]). In the budding yeast, histone H2A at serine 129 is quickly phosphorylated upon DSB formation. However, the role of γH2A in DSB repair remains to be elucidated, because an alanine substitution at serine 129 has no effect on HR or NHEJ in yeast.

The DT40 cell line provides a unique opportunity for dissecting the mechanism of HR, because its highly efficient targeted integration makes it possible to construct a wide range of HR mutants [28,29]. In attempt to identify a role for γH2AX in HR-mediated DSB repair, we constructed a chicken DT40 cell line, in which one H2AX allele carried a mutation changing serine 139 to alanine, the other allele being completely deleted ($H2AX^{-/S139A}$). $H2AX^{-/S139A}$ cells grew with nearly normal kinetics and exhibited a modest defect in HR, as previously reported [30]. However, deletion of the *XRCC3* gene in $H2AX^{-/S139A}$ cells resulted in loss of Rad51 foci formation at IR-induced DSBs, extensive chromosomal breaks, and subsequent cells death, as observed in Rad51 depleted cells [31] Our data unmask a critical role for γH2AX in preventing chromosomal breaks by partially substituting for a Rad51 paralog. Our findings suggest a novel function for histone modification acting with Rad51 cofactors in facilitating homologous recombination by facilitating Rad51 polymerization at DSBs.

2. Materials and methods

2.1. Plasmid construction

A 9 kb genomic fragment containing the chicken H2AX gene was isolated from a DT40 genomic library [32]. pBSK containing the 9 kb fragment was recircularized at *Eco*RI sites to remove a 3 kb fragment containing the single H2AX exon and 3′ sequence (pH2AX5′). A PCR-amplified 1.3 kb 3′ arm was inserted into the pH2AX5′ containing 6 kb 5′ arm (pH2AX5′3′). To construct the H2AX knockout vector, a bsr resistant cassette was inserted into unique *Bam*HI site between 5′ and 3′ arms of pH2AX5′3′. To construct the H2AX knock in constructs, a hisD resistant cassette was inserted into a unique *Bam*HI site, followed by inserting PCR-amplified genomic *Eco*RI–*Xho*I fragments containing wild-type H2AX or H2AXS139A, generated by site-directed mutagenesis, at *Eco*RI (in the genome) and *Xho*I site (in the hisD cassette). To construct the H2AX expression vector, chicken H2AX cDNA was inserted into an expression vector driven by tet-repressible promoter followed by IRES-luciferase (tet-H2AX-IRES-luciferase) [33].

2.2. Generation of H2AX mutants

DT40 cells were sequentially transfected with the H2AX-bsr knockout vector, and subsequently the wild-type H2AX-hisD, H2AXS139A-hisD or H2AXnull-hisD knockin vector to obtain $H2AX^{-/wild}$, $H2AX^{-/S139A}$ or $H2AX^{-/-}$ cells, respectively. For the generation of $H2AX^{-/S139A}/XRCC3^{-/-}$ mutant, $H2AX^{-/S139A}$ cells were transfected with the XRCC3-hyg