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Regulation of DNA repair in the absence of classical non-homologous end joining

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ABSTRACT

Classical non-homologous end-joining (cNHEJ) is the main pathway for the repair of DNA double strand breaks (DSBs) in mammalian cells. In the absence of c-NHEJ, an alternative end-joining (A-EJ) mechanism resolves DSBs. To date, no A-EJ specific factor has been identified. Instead, this mechanism appears to co-opt proteins involved in more than one DNA repair pathway. These include components of base-excision repair (PARP1/XRCC1/LIG3), interstrand cross-link repair (BRCA1/FANCD2), and DSB response/DNA end-resection (MRE11A/RAD50/RBBP8). To clarify the contribution of these factors to A-EJ, here we examined their expression and recruitment to DSBs in correlation with surrogates of cNHEJ (53BP1) and homologous recombination (RAD51) in cells deficient for the cNHEJ end-ligation component XRCC4. This revealed XRCC4-deficient cells exhibited marked increases in the stability of A-EJ transcripts that result in correspondingly elevated levels of associated proteins, in comparison to WT cells. RAD51 was also increased while 53BP1 was unaffected. Treatment with radiomimetic DSB-inducing drug doxorubicin did not influence these activities. However, FANCD2, BRCA1 and XRCC1 foci, prominently associated with 53BP1 foci and hence DSBs resolved by cNHEJ, were only detected in doxorubicin-treated XRCC4-deficient cells. Strikingly, treatment of XRCC4-deficient cells with the PARP-specific inhibitor Niraparib enhanced A-EJ, and substantially induced 53BP1 transcripts and the numbers of A-EJ-associated 53BP1 DNA damage foci. RAD51 was severely inhibited, and upstream cNHEJ (KU70/KU80/DNA-PKCs/ARTEMIS) transcripts were substantially induced. These latter results were recapitulated in BRCA1-deficient cells, which contrastingly did not affect 53BP1 or PARP1 status irrespective of doxorubicin or Niraparib treatment. Hence A-EJ is regulated transcriptionally, reduced by a higher turnover rate in cNHEJ-proficient cells and sustained but fine-tuned by PARP1 in XRCC4-deficient cells to promote DNA repair and survival. Upstream cNHEJ components are similarly transcriptionally down-modulated by PARP1 and BRCA1 in a manner inversely correlated with HR and mechanistically distinct from A-EJ respectively in cNHEJ-deficient and cNHEJ-proficient settings.

1. Introduction

Genomic integrity is maintained by the coordinated actions of multiple network processes that control DNA damage response (DDR) and repair. Homologous recombination (HR) and classical non-homologous end-joining (cNHEJ) are the two major DNA double strand break (DSB) repair pathways in mammalian cells. HR accurately repairs DSBs via a homologous DNA sequence, and is relegated to S and G2 because end-resection is required to activate HR. cNHEJ directly ligates DSBs lacking homology (direct joins) or containing several base-pair homologies, forming microhomology (MH) joins and is active throughout the cell cycle [1]. cNHEJ exclusively resolves RAG endonuclease-induced DSBs generated during lymphocyte-specific V(D)J recombination, and is the major pathway that resolves radiation-induced and IgH class switch recombination (CSR) associated DSBs [2]. The cNHEJ machinery is composed of seven core proteins. KU70 and KU80, which form “KU” DNA binding end-recognition complex, are the first proteins that bind to DSBs. KU:DNA serves as a node for the recruitment the DNA-PKCs:ARTEMIS nuclease and XRCC4:LIG4:XLF end-ligation complex [3]. Studies in cells deficient for KU70, KU80, XRCC4 or LIG4 revealed a robust alternative end-joining (A-EJ) mechanism [2]. While capable of direct joining, A-EJ almost exclusively resolves CSR DSBs with longer

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MH regions in the absence of XRCC4 or LIG4, and generates substantial levels of direct joins in the absence of KU or KU plus XRCC4 or LIG4 [4,5]. These findings led to suggestions of a distinct A-EJ mechanism that uses KU to facilitate MH joining [5]. Alternatively, KU might hinder A-EJ's access to regions lacking MH. cNHEJ is reduced and MH joining of proximal CSR DSBs is increased in B cells deficient in the DSB response factor 53BP1 [6,7], findings that led to the suggestion that 53BP1 bridges distal cNHEJ substrates and suppresses MH joining mediated by A-EJ. Ablation of 53BP1 in BRCA1-deficient cells was also shown to restore HR [8]. These and other studies revealed that 53BP1 antagonizes HR during G_1 phase of the cell cycle by protecting DNA ends from BRCA1 facilitated RBBP8/CtIP-mediated end-resection [8,9]; by promoting DSB response by transducing ATM cell cycle checkpoint signals, and facilitating the recruitment of the MRE11-RAD50-NBS1 (MRN) complex with γH2AX at DSBs [10]. BRCA1 in turn facilitates HR by antagonizing 53BP1-directed cNHEJ during S/G_2 [11]. The amplitude of BRCA1 activity in HR is down-modulated by PARP1 by the addition of poly-ADP-ribose (PAR) polymers on BRCA1, which recruits RAP80 to BRCA1 in a stable complex with PARP1, and reduces BRCA1's avidity to DNA [12]. MH joining is increased in BRCA1/2-deficient tumors, an outcome associated with elevated FANCD2 and recruitment of Polθ to damaged sites in which FANCD2 loss enhanced tumor cell death, in which the latter indicated a synthetic lethal relationship caused by loss of both factors [13]. Whereas elsewhere, compensatory roles for BRCA1 and FANCD2 in the repair of UV induced interstrand cross-links (ICL) were described [14]. Depletion of MRE11 reduces MH usage in cNHEJ-proficient cells and suppresses end-resection in XRCC4-deficient cells [15]. These and other studies suggested MRE11 facilitates the initial end-resection step required for HR and the MH joining that occur in S/G_2 when HR and cNHEJ, and potentially A-EJ, are active [16]. While the components of A-EJ and its regulation remain poorly understood, factors that impact MH joining including PARP1, BRCA1, FANCD2, which have functions in more than one DNA repair pathway and orchestrate DNA repair pathway choice, serve as potential candidates. In the absence of LIG4, A-EJ is expected to use one of the other cellular DNA Ligases, either LIG1 or LIG3. However, the distribution of MH joins used during CSR of B cells disrupted for LIG4 with LIG1 or LIG3 are the same as those only lacking LIG4, which led to the suggestion these two DNA ligases have redundant functions in A-EJ [17]. LIG3 is reportedly stably assembled with the base excision repair (BER) factor XRCC1 by PARP1 at sites of oxidative DNA damage. Robust A-EJ also still occurs in B cells lacking XRCC4 and XRCC1 or depleted for LIG3, demonstrating XRCC1 and LIG3 are not requisite components of A-EJ. To characterize A-EJ, here we examined if putative A-EJ factors are activated and recruited to 53BP1 at DSBs in cells deficient for XRCC4. Our findings in this report expand our understanding of the DNA repair factors recruited with 53BP1 and H2AX in the absence of XRCC4 and the role of PARP1 in their regulation. We also identified novel roles for PARP1 and BRCA1 in the regulation of upstream cNHEJ components (KU70, KU80, DNA-PKCs and ARTEMIS) in manners that appear to be inversely correlated with HR and mechanistically distinct from A-EJ.

2. Materials and methods

2.1. Primary cell culture

Embryonic day 13.5 (E13.5) wild-type (WT) and XRCC4$^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated and cultured as described [18]. BRCA1$^{-/-}$ MEFs [8] were obtained from the laboratory of Andre Nussensweig. All mice were maintained in an AALAC and IACUC approved BL1 animal facility in the Animal Research Facility at the Beth Israel Deaconess Medical Center.

2.2. Genotyping PCR assays

PCR confirming deletion of *XRCC4* floxed allele in genomic DNA extracted from MEFs were performed using primer pairs shown in Table S1.

2.3. Quantitative RT-PCR based analysis of mRNA expression

Quantitative RT-PCR analyses using XRCC4$^{-/-}$, BRCA1$^{-/-}$ and WT MEFs were performed [19] using primer pairs shown in Table S1.

2.4. siRNA-mediated knockdown of XRCC4

siRNA sequences used to target the human *XRCC4* gene were either in a SMARTPool containing four different target sequences:

A, UGACCGAGAUCCAGUCUAU; B, GAACCCAGUAUAACUCAUU; C, CAGCUGAUGUAUACACGUU and D, CCUCUUUGAUGAGAUUUAA (100–500 nM; Dharmacon, USA). Silencer Select Negative Control siRNA (Ambion, UK), which does not target any known sequence in the human genome, was used in control experiments. The specific and non-targeting siRNA sequences were transfected into 293T cells using DharmaFECT 2 reagent as previously described [20].

2.5. Immunofluorescence and microscopy

Immunofluorescence (IF) staining using antibodies against γH2AX (Millipore, 05-636; 1:400), 53BP1 (Novus Biologicals, NB100-904; 1:200), BRCA1 (SantaCruz, sc-642; 1:50), FANCD2 (abcam, ab108928; 1:50), XRCC1 (Abcam, ab47920; 1:50), or RAD51 (SantaCruz, sc-8349; 1:50) were performed as described [19]. Double-labelling using different primary antibodies from same species were performed as described [21]. Images were captured using oil immersion 63× objectives Zeiss Axio Imager A1/Axio Cam MRC and Axiovision LE software (Carl Zeiss, Oberkochen, Germany), with co-localization of foci verified by confocal imaging using a Zeiss LSM 510 Meta Upright Confocal Microscope.

2.6. Immunoblotting analysis

Immunoblotting using antibodies against XRCC4 (SantaCruz, sc-8285; 1:200), BRCA1 (SantaCruz, sc-642; 1:200) or β-actin (Cell Signalling; 4970, 1:3000) was used for detection followed by the incubation with peroxidase-labelled anti-mouse (Cell Signaling; 7076, 1:2000) or anti-rabbit (BioRad; 1662408, 1:2000) IgG secondary antibody. Image J (National Institutes of Health, Bethesda, MD) was used to quantify bands and compare to the loading control.

2.7. Cell cycle analysis

Cell cycle distribution after DOX treatment was measured by propidium iodide staining, as described previously [22].

3. Results and discussion

3.1. A-EJ repair genes are transcriptionally induced in NHEJ-deficient setting

Recently, we reported the mRNA levels of putative A-EJ factors including *BRCA1*, *PARP1*, *FANCD2*, *RBBP8, XRCC1, RAD50, and MRE11A* [23] are elevated in cells (neural stem cells and MEFs) deficient in XRCC4 and p53 [19]. To assess if these activities are altered in response to DNA damage in the presence or absence of cNHEJ, we examined their expression relative to 53BP1 and RAD51, respectively surrogates of cNHEJ and HR, in cNHEJ-deficient (XRCC4$^{-/-}$) compared to cNHEJ-proficient (WT) E13.5 derived MEFs (Fig. S1A) after treatment with the chemotherapeutic DNA cleavage-inducing drug,