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RAD51AP1-deficiency in vertebrate cells impairs DNA replication

Ann C. Parplys[1], Katja Kratz[2], Michael C. Speed[3], Stanley G. Leung[4], David Schild**, Claudia Wiese*

Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, United States

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ABSTRACT

RAD51-associated protein 1 (RAD51AP1) is critical for homologous recombination (HR) by interacting with and stimulating the activities of the RAD51 and DMC1 recombinases. In human somatic cells, knockdown of RAD51AP1 results in increased sensitivity to DNA damaging agents and to impaired HR, but the formation of DNA damage-induced RAD51 foci is unaffected. Here, we generated a genetic model system, based on chicken DT40 cells, to assess the phenotype of fully inactivated *RAD51AP1* in vertebrate cells. Targeted inactivation of both *RAD51AP1* alleles has no effect on either viability or doubling-time in undamaged cells, but leads to increased levels of cytotoxicity after exposure to cisplatin or to ionizing radiation. Interestingly, ectopic expression of *GgRAD51AP1*, but not of *HsRAD51AP1* is able to fully complement in cell survival assays. Notably, in *RAD51AP1*-deficient DT40 cells the resolution of DNA damage-induced RAD51 foci is greatly slowed down, while their formation is not impaired. We also identify, for the first time, an important role for RAD51AP1 in counteracting both spontaneous and DNA damage-induced replication stress. In human and in chicken cells, RAD51AP1 is required to maintain wild type speed of replication fork progression, and both RAD51AP1-depleted human cells and *RAD51AP1*-deficient DT40 cells respond to replication stress by a slow-down of replication fork elongation rates. However, increased firing of replication origins occurs in *RAD51AP1*-/- DT40 cells, likely to ensure the timely duplication of the entire genome. Taken together, our results may explain why RAD51AP1 commonly is overexpressed in tumor cells and tissues, and we speculate that the disruption of RAD51AP1 function could be a promising approach in targeted tumor therapy.

1. Introduction

Genome stability is dependent on the accurate repair of DNA double-strand breaks (DSBs). DSBs can arise from exogenous DNA damaging agents or, spontaneously, during DNA replication. One major mechanism by which DSBs are repaired is by homologous recombination DNA repair (HR). HR utilizes the sister chromatid as a template, and is considered a comparatively faithful DNA repair pathway that can be subdivided into three major stages: the pre-synaptic stage characterized by strand stabilization, the synaptic stage characterized by strand invasion and branch migration, and the post-synaptic stage which includes the formation of Holliday junctions and their resolution.

RAD51AP1 is a RAD51-associated protein that binds to both the RAD51 and DMC1 recombinases, and is critically important for wild-type levels of homologous recombination in mitotic and meiotic cells [1–3]. Knockdown of RAD51AP1 in human cells leads to increased levels of genomic instability and to decreased levels of homology-directed DNA repair [3,4]. Human cells with silenced *RAD51AP1* are viable, but are sensitized to the cytotoxic effects of interstrand cross-linking agents and of ionizing radiation. However, human cells with depleted RAD51AP1 are able to form RAD51 DNA damage foci with normal kinetics [3,4]. Purified human RAD51AP1 binds avidly to DNA, and prefers D-loop structures over double-stranded DNA, and double-stranded DNA over single-stranded DNA [3,4]. Collectively, these results have shown that in mitotic human

* Corresponding author at: Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, United States.
Tel.: +1 970 491 7618; fax: +1 970 491 0623.
** Corresponding author at: Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, United States.
Tel.: +1 510 486 6013; fax: +1 510 486 6816.
E-mail addresses: dschild@lbl.gov (D. Schild), claudia.wiese@colostate.edu (C. Wiese).
[1] Present address: Campus Science 27, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.
[2] Present address: Laboratory for Cell Biology and Genetics, The Rockefeller University, New York, NY, United States.
[3] Present address: Department of Natural Sciences, Del Mar College, Corpus Christi, TX, United States.
[4] Present address: Lorry I. Lokey Stem Cell Research Building, G2065, Stanford University, CA, United States.

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cells RAD51AP1 functions downstream of the pre-synaptic stage of HR and through its interaction with RAD51.

Here we generated *RAD51AP1*-deficient DT40 cells to assess the phenotypic consequences of the total loss of RAD51AP1 expression. In this model system, we are able to show, for the first time, that vertebrate cells are viable without any expression of RAD51AP1. Confirming our results for human cells with RAD51AP1 knockdown, chicken RAD51AP1 also is not essential for the formation of DNA damage-induced RAD51 foci. However, chicken RAD51AP1 is required for the timely resolution of RAD51 DNA damage foci, data that are difficult to obtain in human cells, because of the transient and likely incomplete depletion of the RAD51AP1 protein in RAD51AP1 knockdown experiments. We find that *RAD51AP1*-deficient DT40 cells are sensitized to the cytotoxic effects of cisplatin and of ionizing radiation, and that ectopic expression of *Gg*RAD51AP1, but not of *Hs*RAD51AP1, fully complements in these cell survival assays. Last, we show that RAD51AP1 is critical for overcoming replication stress in both human and in chicken cells, and we provide a model that may help explain why the human RAD51AP1 protein frequently is expressed at elevated levels in a variety of human cancer cells and tissues.

2. Materials and methods

2.1. Cell lines and reagents

Chicken DT40 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% chicken serum, 10 μM beta-mercaptoethanol, 1% antibiotics and 1% L-glutamine and kept at 37 °C in a humidified incubator containing 5% CO_2. Selection of individual clones derived from DT40 cells was performed in standard growth medium containing 1.7 mg/ml G418 and 0.5 μg/ml *puromycin* for gene targeting constructs, and 2 mg/ml *hygromycin* for lentiviral transduction to express the *HsRAD51AP1* or *GgRad51ap1* ORFs. U2OS cells were grown in Dulbecco's modified Eagle's Medium with 10% fetal calf serum at 37 °C in a humidified incubator containing 10% CO_2. Forward transfections with siRNA were carried out using Lipofectamine RNAiMAX reagent (Invitrogen), essentially as recommended by the manufacturer. Down-regulation of RAD51AP1 was mediated by RAD51AP1-directed siRNAs with the target sense sequences 5′-CCTCATATCTCTAATTGCA-3′ (here: AP1_1) and 5′-TGAACAATCTCCGGAAAGA-3′ (here: AP1_2) and as previously described [3–5]. For negative control siRNA, the following target sequence was used: 5′-GATTCGAACGTGTCACGTC-3′, as previously described [6].

2.2. Growth-rate measurement and cell survival assays

For measuring the cell-doubling times of DT40 wild type and mutant cells, exponentially growing cells were cultured for 5 days and were counted twice a day using a Coulter Counter (Beckman Coulter). Cells were diluted appropriately in pre-heated medium to maintain cell density between 0.2 and 1.4×10^6 cells/ml and cell viability >95%. Each growth rate determined is the average of 3 independent cultures. Cells were treated with the indicated concentrations of cisplatin. Percent survival represents the number of colonies in the presence of the drug compared to the number of colonies in the absence of the drug.

2.3. Plasmids and lentiviral DNA

For the ectopic expression of the FLAG-tagged human and chicken *RAD51AP1* ORFs, lentiviral vectors were constructed based on the Gateway System (Invitrogen), but modified by Dr. E. Campeau [7]. FLAG-*HsRAD51AP1* was generated by sub-cloning a *Sal*I to *Xho*I fragment containing the DNA sequence for the FLAG peptide 5′ to and in frame with the human *RAD51AP1* ORF amplified from pOK24 [8] into pENTR1A (Invitrogen). To obtain pLenti CMV DEST #2 [7] encoding human FLAG-RAD51AP1, pLenti CMV DEST #2 was used and a standard LR recombination reaction was carried out, as described [7]. Similarly, FLAG-*GgRAD51AP1* was generated by sub-cloning a *Sal*I to *Eco*RI fragment containing the DNA sequence for the FLAG peptide 5′ to and in frame with the chicken *RAD51AP1* ORF amplified from chicken EST cDNA clone 3GAL_54E24 into pENTR1A (Invitrogen), and the same strategy, as described above, was used to obtain pLenti CMV DEST #2 encoding chicken FLAG-RAD51AP1. Lentiviruses were produced in 293FT cells as described [7], aliquoted and stored at −80 °C. DT40 cells were transduced in the presence of 6 μg/ml polybrene in 6-well tissue culture plates at about 500,000 cells/ml growth medium without antibiotics. Two days after transduction, cells were spun out to replace the cell culture medium with fresh medium containing *hygromycin*. Cell cultures were expanded and single clones isolated that showed similar levels of expression for both *Hs*RAD51AP1 and *Gg*RAD51AP1, as determined by Western blot analysis.

2.4. Gene disruption constructs for generating the RAD51AP1 knockout in DT40 cells

For cloning of the chicken *RAD51AP1* deletion construct, a *Bam*HI-adapted forward and a *Bam*HI-adapted reverse primers were used to amplify the *puromycin* and *neomycin* genes with *β-actin* promoters from p*Lox*Puro and p*Lox*Neo vectors (kindly obtained by Jean-Marie Buerstedde), respectively. To generate the 3.2 kb 5′ homology flank of the *RAD51AP1* deletion construct a *Kpn*I-adapted forward and a *Sal*I-adapted reverse primer based on NCBI reference sequence: XM_417234.3 were used for amplification using DT40 genomic DNA as a template. To generate the 4.0 kb 3′ homology flank a *Not*I-adapted forward and a *Sac*I-adapted reverse primer were used for DNA amplification. The homology arms were assembled into pBlueScript SK^+ and the *neomycin* or *puromycin* resistance cassettes were cloned in as *Bam*HI-digested fragments.

2.5. RT-PCR

Total RNA was isolated from DT40 wild type cells and derivatives using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. For the generation of cDNA, a standard reverse transcription protocol was carried out using ImProm-II Reverse Transcriptase and oligo(dT)15primer (Promega). The following primers were used: forward_1 (F1; in exon 2) 5′-GGCGCGGATGGTGAGGAG-3′; reverse_3 (R3; in exon 7) 5′-GGAGTCTTCTTTTCTTTTCTAGTCTGCC; reverse_4 (R4; in exon 8) 5′-GGACATCATCTTTTGTGTCTGC-3′. β-Actin primers were used as control, as described previously [9].

2.6. Southern blotting and hybridization

Six μg of genomic DNA from DT40 wild type cells and derivatives was digested with *Pvu*II, ethanol precipitated, re-suspended in TE buffer, and separated on a 0.8% agarose gel in 1 × TAE, at 35 V overnight. The DNA was transferred to a HybondXL membrane (Amersham) using a vacuum blotter, with 10 min hydrolysis in 0.125 N HCl and 1 h denaturation in denaturation buffer (0.5 M NaOH, 1.5 M NaCl). To neutralize, the membrane was rinsed in 2 × SSC for 30 min and stored wet until further use. Hybridizations were carried out at 60 °C in 5 × SSC, 5 × Denhardt's and 0.5% SDS with 100 μg/ml sheared salmon sperm DNA. As a hybridization probe, a ^{32}P-labeled 1.5 kb fragment with 100% homology to the 3′-end of the 5′-flank of the gene targeting vector was generated