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Human somatic cells deficient for *RAD52* are impaired for viral integration and compromised for most aspects of homology-directed repair

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Abstract

Homology-directed repair (HDR) maintains genomic integrity by eliminating lesions such as DNA double-strand breaks (DSBs), interstrand crosslinks (ICLs) and stalled replication forks and thus a deficiency in HDR is associated with genomic instability and cancer predisposition. The mechanism of HDR is best understood and most rigorously characterized in yeast. The inactivation of the fungal radiation sensitive 52 (*RAD52*) gene, which has both recombination mediator and single-strand annealing (SSA) activities *in vitro*, leads to severe HDR defects *in vivo*. Confusingly, however, the inactivation of murine and chicken *RAD52* genes resulted in mouse and chicken cells, respectively, that were largely aphenotypic. To clarify this issue, we have generated *RAD52* knockout human cell lines. Human *RAD52*-null cells retain a significant level of SSA activity demonstrating perforce that additional SSA-like activities must exist in human cells. Moreover, we confirmed that the SSA activity associated with *RAD52* is involved in, but not absolutely required for, most HDR subpathways. Specifically, a deficiency in *RAD52* impaired the repair of DNA DSBs and intriguingly decreased the random integration of recombinant adeno-associated virus (rAAV). Finally, an analysis of pan-cancer genome data from The Cancer Genome Atlas (TCGA) revealed an association between aberrant levels of *RAD52* expression and poor overall survival in multiple cancers. *In toto*, our work demonstrates that *RAD52* contributes to the maintenance of genome stability and tumor suppression in human cells.

Keywords

Homology-directed repair (HDR); DNA double-strand breaks (DSBs); Radiation sensitive 52 (*RAD52*); Genome stability; Single-strand annealing (SSA)

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Conflict of interest statement

E.A.H. declares that he is a member of the scientific advisory boards of Horizon Discovery, Ltd. and Intellia Therapeutics, companies that special in applying gene editing technology to basic research and therapeutics.

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1. Introduction

DNA DSBs are the most cytotoxic form of genomic lesions for living cells. They can arise from both endogenous DNA replication errors and exogenous exposure to DNA damaging agents. In yeast, the toxicity of DSBs is profound as even a single unrepaired DSB can lead to cell lethality [1]. To ensure that DSBs are repaired, most eukaryotes have developed at least two major pathways of DSB repair: non-homologous end joining (NHEJ) [2] and HDR that are differentially utilized depending upon the actual DSB lesion, the phase of the cell cycle and the organism in which the lesion occurs [3]. NHEJ is a process that results in the covalent ligation of the two broken ends of a DSB in the most expeditious (if not always the most accurate) way possible in an attempt to restore the physical integrity of the affected chromosome. NHEJ is preferentially utilized during G₁ of the cell cycle [4] and it is thought to be predominately, but certainly not exclusively [5], an error-prone process [2]. In contrast, HDR precisely repairs DSBs using the genetic information from a homology donor, which predominately occurs in the S and to a lesser extent in the G₂/M phases of the cell cycle [6]. HDR is required to repair complex genomic lesions such as ICLs and stalled replication forks. Besides DNA repair and replication, HDR also plays roles in meiotic segregation [7] and telomere maintenance [8]. Furthermore, deficits in HDR, caused by mutations in Breast Cancer Allele 1 or 2 (BRCA1 or BRCA2), predispose patients to breast and ovarian cancers [9–11]. Ironically, tumor cells may then rely on mutagenic NHEJ or other poorly-defined backup repair pathways to maintain genome integrity as these cells show increased sensitivity to chemo- [12, 13] and synthetic-lethality-based therapies [14–16]. These studies highlight the importance of HDR in basic and clinical research [11].

HDR is composed of at least the DSB repair (DSBR) [17], synthesis-dependent strand annealing (SDSA) [18] and SSA [19, 20] subpathways. Which subpathway is engaged for a particular repair event is determined by a large variety of factors including, but not limited to, the cell type in which the DSB occurs, the stage of the cell cycle when the DSB occurs or is repaired, the proximity of repetitive sequences to the DSB, and the expression levels of the relevant DNA repair factors. Regardless, a key protein that contributes to all of these subpathways is the RAD52 protein. The yeast homolog of *RAD52* has a recombination mediator activity that is required to help load the essential strand transfer protein, radiation sensitive 51 (RAD51) onto DSB ends by displacing replication protein A (RPA) [21, 22], as well as the unique activity to anneal RPA-coated ssDNAs together [23, 24]; the latter activity of which is likely required in all forms of HDR [16, 25]. However, unlike the yeast mutant that is extremely sensitive to ionizing radiation (IR^s) and defective for most subpathways of HDR [25], *RAD52*-null DT40 chicken cells [26] and the *RAD52* knockout mouse exhibited no IR sensitivity and only mild defects in HDR [27, 28]. Thus, while much of the knowledge concerning *RAD52* was obtained from studies in yeast, it is unclear what the vertebrate-specific functions of *RAD52* might be [29].

Although one of the key activities of the yeast RAD52 protein is its recombination mediator property that recruits the strand transfer activity of RAD51, purified human RAD52 protein demonstrates little of this activity under physiological concentration [30–32]. Instead, it is hypothesized that the tumor suppressor gene breast cancer allele 2 (BRCA2) has evolved to function as the predominant recombination mediator and that *RAD52* may serve as its

backup in human cells [14, 15, 33, 34]. Consistent with this model, *RAD52* inactivation is synthetically lethal with the loss-of-function of *BRCA2*, breast cancer allele 1 (*BRCA1*) or partner and localizer of *BRCA2* (*PALB2*) [14, 15]. Both *BRCA1* and *PALB2* are thought to help participate in recruiting *BRCA2*, which in turn directly recruits *RAD51*, to the site of a DSB. Thus, in human cells, it appears to take 3 (or more) proteins to do what *RAD52* does by itself in yeast. Whether *RAD52* has functions apart from serving as *BRCA2*'s back-up is unclear, but it seems likely for multiple reasons [16]: 1) *RAD52* interacts with *RAD51* upon phosphatase and tensin homolog (PTEN)-mediated sumoylation [35], 2) *RAD52*'s depletion leads to aberrant *RAD51* foci formation [35, 36] and 3) human *RAD52* seems to have preserved part of its SSA activity [37–39]. *RAD52* may also play a role in replication fork preservation in a *RAD51*-independent pathway [38, 40, 41] and it has very recently been shown to involved in DNA replication restart of stalled or broken DNA replication forks [42–44]. These results indicate several *RAD51*-independent roles for *RAD52* in DSB repair.

To gain further insights into the function of human *RAD52*, we have created a *RAD52* knockout human HCT116 colorectal carcinoma [45, 46] somatic cell line. The *RAD52*-deficient cells exhibited mild growth defects and sensitivity to mitomycin C (MMC) and hydroxyurea (HU). By constructing a novel HDR reporter, we demonstrated that *RAD52* is involved in, but not absolutely required for, all forms of HDR, and that its deficiency leads to decreased DSB repair, SDSA and SSA activity. Surprisingly, we also observed a dramatic decrease in rAAV random integrations in the *RAD52*-knockout cells. Collectively, these findings indicate that *RAD52* plays a role in the HDR repair of complex genomic lesions and the integration of viral genomes, and they suggest the existence of an alternative SSA protein/pathway in human cells. Finally, bioinformatics analysis of published cancer genome data revealed that aberrant levels of *RAD52* in tumors is associated with poor patient survival.

2. Materials and methods

2.1. Cell lines and plasmids

The parental HCT116 cell line was obtained from ATCC. The *RAD54B*^{-/-}, *MUS81*^{-/-} and *XRCC3*^{-/-} HCT116 cell lines were obtained from Dr. Kiyoshi Miyagawa [47–49]. The *LIG4*^{-/-} [50], *LIG1*^{flax/-} (Kan *et al.*, unpublished) and *FANCD2*^{-/-} (Thompson *et al.*, in preparation) HCT116 cell lines were created by rAAV-mediated gene targeting. The *RAD52* and *PIGA* gene targeting vectors were assembled as described [51, 52]. The *RAD52* cDNA expression vector was obtained from GeneCopoeia (EX-Q0572-M68). The DR-GFP, SA-GFP and EJ2-GFP reporter plasmids were obtained from Drs. Maria Jasin and Jeremy Stark [53–55]. The RIG reporter plasmid was derived from a rearrangement of the MMR-IR3 plasmid, which was obtained from Dr. Chengtao Her [56]. All the stop codons were removed and re-introduced into the donor and recipient copies of RFP-IRES-GFP, which was then re-assembled into the MMR-IR3 backbone. The PX458 plasmid for Cas9 and sgRNA expression was obtained from Addgene. The complete sequences of the *RAD52* and *PIGA* targeting vectors, the RIG reporter and the *PIGA* sgRNA can be found in Supplemental Sequences.

2.2. Cell culture and viruses

The HCT116 cell line and its derivatives were cultured in McCoy's 5A medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin in a humidified 37 °C incubator with 5% CO₂. rAAV-mediated gene targeting was performed to inactivate the *RAD52* locus as described [51]. PCR screening was carried out using primers illustrated in Fig. 1 and Fig. S1, and the exact primer sequences can be found in Supplemental Sequences. Lentiviral packaging and infection were performed using the RetroMax™ System according to the manufacturer's (EMGENEX) protocol.

2.3. Growth rate analysis

Cells were seeded in a 6-well-plate at a density of 4×10^3 cells per well and counted at designated time points using the Countess™ Automated Cell Counter (Invitrogen).

2.4. Clonogenic assays

Cells were seeded in a 6-well-plate at a density of either 1×10^2 or 3×10^2 cells per well. Only the peripheral 4 wells of a 6-well-plate were used to provide exactly the same ventilation and moisture conditions. After seeding, cells were cultured for 24 hr and irradiated with the designated dose of X-ray radiation or the culture medium was supplemented with the indicated concentration of etoposide, MMC and HU. The cultures were continued for 14 days in the drug-containing medium. Colonies were fixed in methanol and stained with 0.4% crystal violet and counted manually. All colony formation efficiency under IR or drug treatment was first normalized to the untreated controls, and then to the parental HCT116 cell line.

2.5. PIGA targeting and rAAV random integration assays

Cells were seeded in a 6-well-plate at a density of 10^6 cell per well, cultured for 24 hr and infected with 100 μ L of the designated *PIGA* targeting virus. For CRISPR/Cas9-mediated plasmid targeting, the cells were transfected with 1.25 μ g of PX458 containing the proper sgRNA and 1.25 μ g of plasmid-based *PIGA* targeting vectors using Lipofectamine LTX (Invitrogen). The cultures were continued for 2 days before the cells were dissociated with TrypLE™ (Invitrogen), counted, and transferred into 10 cm plates at a density of 2×10^6 cells per plate. The cells were then selected under 1 mg/mL G418 for 14 days. All G418-resistant colonies were dissociated and fixed with 4% formaldehyde for 15 min. The fixed cells were then resuspended in phosphate buffered saline (PBS) at a low density and stained with 5×10^{-9} M fluorescent Alexa aerolysin (FLAER) 488 (Pinewood Scientific Services) for 15 min. Eventually, the percentage of FLAER-negative cells was quantitated by flow cytometry (FACSCanto II, BD Biosciences).

For the rAAV random integration assay, the infected cells were plated into 10 cm plates with limiting dilutions in the presence or absence of 1 mg/mL G418. The random integration efficiency was calculated as the number of colonies formed from the same amount of infected cells in the G418-containing plates normalized to the no-G418 plates.

2.6. DNA repair assays

Cells were seeded in a 6-well-plate at a density of 5×10^5 cells per well and subsequently cultured for 24 hr. For the DR-GFP, SA-GFP and EJ2-GFP assays, the cells were transfected with 0.5 μ g mCherry, 1.0 μ g of an *I-SceI* or *ISceI*-Trex expression vector and 1.0 μ g of the corresponding reporter plasmid using 10 μ L of Lipofectamine LTX. For the RIG assay, the cells were transfected with 1.25 μ g of the RIG plasmid and 1.25 μ g of the *I-SceI* expression plasmid. The EGFP- and RFP/mCherry-expressing cells were quantitated using flow cytometry 48 hr post transfection. For the pDVG94 assay, cells were cultured in the presence or absence of 1 μ M of the DNA-PK_{CS} inhibitor, NU7441, and 24 hr later, transfected with 2.5 μ g of pre-linearized [using EcoRV and AfeI restriction enzyme digestions] pDVG94 plasmid. The repaired plasmids were recovered using Qiagen mini-prep columns, and the repair junctions were amplified using flanking primers. The PCR products were digested with BstXI and resolved on a 6% polyacrylamide gel. The repair efficiency was first normalized to transfection controls and then to the parental or cDNA complementation cell lines. For the RIG assay, the DSB activity was internally normalized to SDSA; and for pDVG94, the alternative NHEJ (A-NHEJ) activity was internally normalized to classical NHEJ (C-NHEJ) initially.

2.7. RAD52 expression levels in tumors and overall survival of patients

TCGA gene expression scores from Hiseq RNA-seq V2 pipeline (Level_3__RSEM_genes_normalized) and clinical data (Merge_Clinical.Level_1) were obtained from gdac.broadinstitute.org on November 2015 (date stamp 2015110100). All data processing was done using Bedtools, Python and R (survival package). Genes that had a RSEM expression of 0 in all the samples within each cancer type were discarded. RNA-seq data of the samples that were matched normals (the 14–15th string in the TCGA barcode in range 01 to 09) were separated from the tumor samples. Genes that had a RSEM of 0 in more than 50% of the samples were discarded. The limma package (voom) was used to normalize the data and data was z-score transformed. For each cancer type analyzed, patients with *RAD52* mRNA levels in the top 20th percentile were designated as *RAD52*-high samples and patients with *RAD52* mRNA levels in the bottom 20th percentile were designated as *RAD52*-low samples. To compute the overall survival of patients with high or low *RAD52*, we parsed the vital_status, days_to_death or days_to_last_followup columns from the clinical data. We used the survfit function to compute the Kaplan-Meier estimator and used the survdiff function to perform the log-rank test to determine if the difference between the survival function of low-*RAD52* and high-*RAD52* samples were statistically significant.

3. Results

3.1. Disruption of the RAD52 genetic locus via high-efficiency rAAV targeting

To determine the loss-of-function phenotype of *RAD52* in human cells, we generated a *RAD52*-knockout human cell line [57] in a HCT116 background using rAAV gene targeting technology [51]. To disrupt the *RAD52* locus by HDR, an unusual exon-trapping strategy was employed and compared to the traditional promoter-driven selection strategy [58] in the absence of meganucleases (Fig. 1A and B). In exon-trapping, an initiator methionine (ATG)-

less neomycin resistance gene (*Neo^R*) was targeted to exon 3 of *RAD52* in-frame, generating (following successful gene targeting) an N-terminal *RAD52-Neo^R* fusion gene driven by the endogenous promoter. When the drug selection cassette was removed by Cre recombination, the remaining LoxP scar generated a frame-shift mutation to disrupt the function of the gene (Fig. 1A). Unlike the promoter-driven [58] (Fig. 1B) and similar promoter-trapping strategies [59], in exon-trapping the *Neo^R* cassette can be expressed only upon in-frame fusion into the transcribed exons. Thus, this strategy dramatically enriched for the correctly-targeted events out of the vast majority of random integrants. In the first round of targeting, as expected, the *Neo^R* cassette was precisely targeted to the *RAD52* locus in 28 out of 48 (58.3%) of the G418-resistant clones in exon-trapping (Fig. S1A and B), as compared to 2 out of 144 (1.4%) clones in the promoter-driven strategy (Fig. S1C and D). Interestingly, exon-trapping can be employed both in the absence and presence of meganucleases [60], highlighting its potential application in precise genome engineering and correction-based gene therapy [61].

Subsequently, two independently-targeted clones, #31 and #78, derived from the exon-trapping strategy, were Cre treated and utilized for a second round of targeting using the same strategy (Fig. S1E). 29 out of 44 G418-resistant clones (65.9%) were precisely targeted (Fig. S1F), and (as anticipated) 15 of them (51.7%) were targeted to the second allele (Fig. S1G). The homozygous null cell lines #31 and #78 were Cre treated and used for all further studies (hereafter *RAD52*^{-/-} cells). It is important to note that the ability to readily obtain biallelically-targeted clones clearly demonstrated the non-essentiality of *RAD52* in human cells [62–64], a finding confirmed by the recent description of a *RAD52*-null human U-2 OS cell line [43]. The bi-allelic disruption of the genetic locus and complete depletion of *RAD52* proteins was confirmed by PCR and western blotting, respectively (Fig. 1C and D). In a complementation experiment, a cytomegaloviral (CMV)-driven full-length human *RAD52* cDNA was delivered by lentivirus. Notably, the expression level of *RAD52* in the complemented clones was about 10-fold higher than the parental cell line (Fig. 1D).

3.2. *RAD52*-deficient cells exhibit a mild growth defect and HU sensitivity

The *RAD52*^{-/-} cells exhibited a modest, albeit statistically significant, growth defect (Fig. 2A). The *RAD52*-null cells doubled every 22 to 24 h, as compared to 19 h for the parental HCT116 cell line. The growth defect was much milder compared to a haploinsufficient *LIG1*^{fllox/-} cell line, which was also generated by rAAV gene targeting (Kan *et al.*, unpublished).

The HDR pathways maintain genome stability and provide high-fidelity repair of deleterious lesions such as DSBs and ICLs [11]. They are also involved in the preservation of stalled replication forks and telomere maintenance [65]. To determine if the growth defect of *RAD52*^{-/-} cells was caused by compromised genome stability, we compared their sensitivity to DNA damaging agents to a panel of DNA repair-deficient cell lines (Fig. 2B to E). A deficiency in any of the tested HDR genes — including mutagen sensitive 81 (*MUS81*), X-ray cross complementing 3 (*XRCC3*) and *RAD54B* [47–49] — as well as *RAD52*^{-/-} did not sensitize the cells to IR. As a positive control, cells devoid of *LIG4* [50], an essential component of the C-NHEJ pathway, showed an expected and profound IR sensitivity (Fig.

2B). This result indicates that the bulk of IR-induced DSBs are processed by the C-NHEJ pathway in human somatic cells, and also likely explains why the *RAD52* knockout chicken cells [26] and the *RAD52*-null mouse are not IR sensitive [27]. Consistent with previous reports [48, 49], the *XRCC3*^{-/-} and *RAD54B*^{-/-} cells, but not *RAD52*^{-/-} nor *MUS81*^{-/-} cells were slightly sensitive to prolonged etoposide (a radiomimetic) treatment, albeit only at the highest dose tested (Fig. 2C). The discrepancy between the IR and etoposide treatments is likely explained by the fact that HDR is only required to repair certain types of lesions during the S phase [11, 65]. The one-time IR treatment kills a small portion of cells in S phase, which is undetectable by our colony formation assay, whereas the prolonged (multiple days) etoposide treatment takes effect whenever the cells enter S phase. Interestingly, *RAD52* and *Mus81* were not essential for this HDR-mediated DSB repair, indicating the existence of alternative recombination mediators [14, 66] and Holliday junction resolvases/dissolvases [67; {Wyatt, 2017 #90}] in human cells.

To examine if *RAD52* plays a role in the ICL repair and replication fork recovery, we also tested the sensitivity of the isogenic cell lines to mitomycin C (MMC) and HU (Fig. 2D and E). All the HDR- and C-NHEJ- compromised cell lines were slightly more sensitive to MMC than the parental counterpart (Fig. 2D), suggesting a role for both HDR and C-NHEJ in ICL repair [68–70]. As a positive control, a Fanconi anaemia, complementation group D2-null (*FANCD2*^{-/-}) HCT116 cell line generated by rAAV targeting (Thompson, E., in preparation) exhibited, as expected, extreme MMC sensitivity. Surprisingly, the *RAD52*^{-/-} and *XRCC3*^{-/-} cells, but not any of the other HDR-deficient cells [49] were sensitive to HU (Fig. 2E), indicating a potential role for *RAD52* and *XRCC3* in repairing stalled replication forks via a *RAD51*-independent HDR pathway (Fig. 2F), consistent with recent reports [42–44].

3.3. *RAD52*-deficient cells exhibit decreased HDR activities

To pinpoint the specific defective DNA repair pathways leading to the compromised genome stability, we tested the HDR activity in the *RAD52*^{-/-} cells using plasmid reporters (Fig. 3). The DR-GFP reporter [53] (Fig. 3A) contains two inactive green fluorescent protein (GFP) genes along with a recognition site for the *I-SceI* meganuclease. Expression of *I-SceI* can cleave one of the inactive GFP genes and if HDR occurs it can restore GFP expression resulting in easily-quantitated green fluorescing cells. This reporter measures the gross non-crossover products generated by the DSBR and SDSA subpathways [71]. Both *RAD52*^{-/-} clones showed decreased DR-GFP repair activity compared to the parental cell line and to their respective cDNA complemented derivatives (Fig. 3B), indicating a role for *RAD52* in the DSBR and/or SDSA pathways. As a positive control and as expected, a cell line null for the HDR gene *RAD54B* [50], was even more defective in the repair of the DR-GFP plasmid. In a complementary fashion, the SA-GFP reporter [54] measures the SSA repair of long direct repeats (Fig. 3C). In this reporter, there are also two inactive GFP genes, one of which contains a recognition site for *I-SceI*. Unlike the DR-GFP reporter, however, the two genes contain only limited homology (the long direct repeats) such that the only way to reconstitute a functional GFP gene is via SSA (Fig. 3C). The *RAD52*^{-/-} cell lines were also significantly reduced for this activity in a fashion that was more comparable to that observed for the *RAD54B*-null cells (Fig. 3D). Interestingly, but tangential to the focus of this work,

this appears to be the first demonstration that human *RAD54B* also plays a role in SSA, which might not have been predicted from the yeast studies [72]. Finally, we also measured the alternative-NHEJ (A-NHEJ) activity and A-NHEJ/C-NHEJ ratio using the EJ2-GFP [55] and pDVG94 [73] reporters (Fig. 3E, G and Fig. S2). In the EJ2-GFP reporter a single GFP gene has been rendered non-functional by the introduction of a translational stop codon flanked by small direct repeats of 8 bp (*i.e.*, microhomology) and an *I-SceI* recognition site. Upon expression of *I-SceI*, GFP expression can be restored if the repair event utilizes the small direct repeats (*i.e.*, A-NHEJ). The pDVG94 reporter has been designed such that cleavage with *Eco47III* and *EcoRV* results in a blunt-ended linear substrate with 6 bp direct repeats at both ends. C-NHEJ results in the retention of some of both repeats whereas A-NHEJ generates a single repeats, which can subsequently be diagnostically cleaved by the *BstXI* restriction enzyme [74]. Thus, the EJ-GFP reporter measures total A-NHEJ activity in a cell whereas the pDVG94 reporter is more a measure of the relative A-NHEJ to C-NHEJ activity. The *RAD52*^{-/-} cells showed a mild but consistent decrease in absolute and relative A-NHEJ activity (Fig. 3F, H), respectively, which was not observed in the *RAD54B*-null cell lines and suggested that the SSA activity of *RAD52* (Fig. 3D) may contribute to the annealing of microhomology in A-NHEJ. Finally, for all the mutants tested, the simultaneous inhibition of the catalytic subunit of the DNA-dependent protein kinase complex subunit (*DNA-PKcs*) — and therefore C-NHEJ — with the small molecule inhibitor NU7441 increased the absolute amounts of A-NHEJ observed, but not significantly more so in the mutants than in the controls and parental population (Fig. 3H and Fig. S2). Thus, the deficit in HDR caused by the absence of *RAD52*, was not sufficient to render the cells synthetically sick/lethal with the loss of C-NHEJ. Lastly, for all these assays, the *RAD52*-null clone #31 was always slightly more deficient than the *RAD52*-null clone #78 (Fig. 3). We posit that this was due simply to the clone-to-clone variability inherent in human somatic cell culture. Importantly, for both clones, the deficits observed were completely rescued by the re-expression of the *RAD52* cDNA and are thus *RAD52*-dependent and not due to differences/variability in the genetic backgrounds of the two clones.

The DR-GFP reporter does not distinguish between DSBR and SDSA activity; however we were keen to establish if a *RAD52* deficiency affected a particular subpathway or both. Thus, we constructed a red fluorescent protein-internal ribosome entry site-GFP (RFP-IRES-GFP; RIG) reporter to determine the ratio of DSBR to SDSA (Fig. 4A). The RIG reporter was derived from a rearrangement of the MMR-IR3 plasmid [56]. Briefly, a cytomegaloviral (CMV)-driven RFP-IRES-GFP expression cassette was disrupted by an *I-SceI* restriction enzyme site and two stop codons in the open reading frames for the fluorescent proteins on both sides of the IRES cassette. The distance between the *I-SceI* site and the stop codons was 924 bp for RFP and 179 bp for GFP. A truncated homology donor containing part of the undisrupted RFP-IRES-GFP sequence is supplied in the same plasmid in the reverse direction. After *I-SceI* cleavage, HDR can be initiated by the 3' ends and products generated via either the DSBR (when both 3'-ends invade the template) or SDSA (when there is invasion of only one 3'-end) pathways (Fig. 4B), resulting in 4 types of repair products (Fig. 4A, B and Fig. S3). Type II products correct the stop codons on both sides of the *I-SceI*, which is characteristic of the DSBR pathway [60]; type I products can be generated by both

DSBR and SDSA, although predominantly by SDSA as it has much higher activity in short-tract gene conversion [3, 18]; in contrast, SDSA has dramatically diminished activity in producing type III products by correcting the stop codon far away from the DSB [75] (Fig. S3 and Kan *et al.*, manuscript submitted), thus these products — which were, in any case, rare (type III products were at least 6X less abundant than type II products, Fig. S3) — were not taken into the calculation. An additional product can be formed (type IV), but this repair event does not result in the restoration of either the RFP or GFP sequences and is thus inconsequential. Thus, the ratio of DSBR to SDSA can be measured as the percentage of GFP⁺:RFP⁺ (type II) over GFP⁺:RFP⁻ (type I) cells. Importantly, the inactivation of *RAD52* did not change the *ratio* of DSBR to SDSA, in contrast to the disruption of canonical HDR genes such as *RAD54B* and *MUS81*, which greatly reduced the frequency of DSBR (Fig. 4C). These results showed that in contrast to *RAD54B* and *MUS81* the inactivation of *RAD52* equally impairs the DSBR and SDSA pathways, probably because its SSA activity is involved in both pathways.

3.4. RAD52-deficiency dramatically reduces rAAV random integrations

rAAV-mediated gene targeting using a central drug selection marker is mediated by the two-ends, ends-out DSBR subpathway [60] (Fig. 5A). To determine if *RAD52* inactivation had any effect on rAAV targeting, we determined the rAAV targeting efficiency in the *RAD52*-null cell lines at the phosphatidylinositol glycan anchor biosynthesis, class A gene (*PIGA*) locus. Using a similar exon-trapping strategy as was used to inactivate *RAD52* itself, a stop codon and an IRES-driven *Neo^R* cassette was targeted into the 6th exon of the *PIGA* locus (Fig. 5B). The stop codon was used to terminate the translation of *PIGA*, whereas the IRES hijacked its transcription to express the *Neo^R* cassette upon correct targeting. *PIGA*, which conveniently resides on the X chromosome (and is therefore hemizygous in the male HCT116 cell line) serves as a screening marker because it is required for the biosynthesis of glycosylphosphatidylinositol (GPI) anchors. The inactivation of *PIGA* by a single round of gene targeting leads to the loss of GPI anchors in male cell lines. The absence of GPI anchors, in turn, can be quantitated using a specific stain (an Alexa-488 conjugated proaerolysin variant called FLAER) that normally binds to the anchors [52]. To demonstrate the quantitative nature of FLAER staining, we mixed *PIGA*-targeted HCT116 cells with the parental counterparts at designated ratios, and measured the percentage of GPI-negative cells as indicated by (the lack of) FLAER staining. The percentage of FLAER-negative cells precisely reflected the percentage of *PIGA*-targeted cells (Fig. 5C). Thus, the relative targeting efficiency (the ratio of correct targeting to random integrations) can be calculated as the ratio of FLAER-negative to FLAER-positive cells after G418 selection [52]. Using this system, *RAD54B*^{-/-} cells showed a dramatic decrease in rAAV-mediated gene targeting efficiency compared to the parental ones, as was expected from earlier studies using more conventional plasmid targeting methodologies [47]. In contrast and unexpectedly, the rAAV-mediated targeting efficiency was enhanced by 4- to 7-fold in the *RAD52*^{-/-} cell lines compared to their corresponding cDNA complemented derivatives (Fig. 5D). To confirm this surprising result, we also determined the targeting efficiency of three other rAAV vectors targeted to different regions of the *PIGA* locus in parental and *RAD52*^{-/-} cells. For each rAAV vector, the relative targeting efficiency was consistently increased by ~3-fold in the *RAD52*-deficient background (Fig. S4).

Since rAAV targeting is mediated by the DSB repair pathway [60], enhanced relative targeting efficiency can be caused either by increased DSB repair activity or decreased rAAV random integrations. To gain more insight into this unexpected result, we also measured the efficiency of rAAV random integration in the *RAD52*-deficient background. Surprisingly, but in accordance with increased relative gene targeting, rAAV random integrations were dramatically reduced in the *RAD52*^{-/-} cell lines (Fig. 5E). Thus, the increase in relative gene targeting efficiency was not as much caused by increased DSB repair activity, as by decreased random integrations. As a positive control and as expected, the relative rate of random integrations of rAAV vectors in a *RAD54B*-null background was elevated. To demonstrate that this was an rAAV-specific phenotype, we also determined the frequency of CRISPR/Cas9-induced HDR using plasmid-based donors (Fig. 5B). The targeting efficiency was greatly reduced in the *RAD54B*-null background but did not increase in the *RAD52*-deficient background (Fig. 5F), which is consistent with our conclusion that the enhanced rAAV targeting efficiency in the *RAD52*-null was due to dramatically decreased random integrations.

3.5. Aberrant expression of RAD52 is associated with poor overall survival of certain cancer patients

The data described above demonstrated that in human somatic cells, there are deficiencies in DSB repair associated with *RAD52* loss-of-function. In contrast, when *RAD52* was disrupted in the mouse, the resulting animals were essentially aphenotypic [27, 28]. Thus, to extend our studies, we were interested in ascertaining whether there were any differences in organismal phenotypes related to human *RAD52* expression. To this end, we used RNA-seq and clinical data from TCGA to determine if cancer patients with either very high or very low levels of *RAD52* have different overall survival. We analyzed data from 8 different cancer types (Fig. 6). For most of the cancer types, no significant difference in survival was evident based upon *RAD52* expression. However, we found a statistically significant reduction in the overall survival of patients with low, relative to high, *RAD52* expression levels in patients with bladder urothelial carcinoma (BLCA) (log rank $p = 0.020$) and head and neck squamous carcinoma (HNSC) (log rank $p = 0.027$) (Fig. 6). Intriguingly, patients with kidney renal clear cell carcinoma (KIRC) revealed an opposite trend with lower survival associated with higher *RAD52* expression, however the data were slightly shy of statistical significance (log rank $p = 0.065$) (see Material and methods). *In toto*, these data provocatively suggest that, in humans, both the complete absence (on a cellular level) and the aberrant expression (on a tissue level) of *RAD52* are likely associated with significant pathologies.

4. Discussion

4.1. Human RAD52 is non-essential

The inactivation of *RAD52* in *S. cerevisiae* and *S. pombe* results in yeast strains that, while viable, were decidedly not healthy and were slow to propagate [25]. In contrast, the disruption of *RAD52* in either the DT40 chicken cell system or the mouse resulted in cells and animals, respectively, that showed no growth defects and were essentially aphenotypic with only slight deficits in either gene targeting [26] and/or HDR [27, 28]. Our ability to generate *RAD52*-null human cells clearly demonstrates that for human somatic cells *RAD52*

is similarly not an essential gene. Our data confirm and extend a very recent report that *RAD52* is also non-essential in the human osteosarcoma U-2 OS cell line [43]. With that said, however, the phenotypes of human *RAD52*-null cells phenocopied neither the yeast nor the chicken/murine systems, but instead seemed to display unique phenotypes. Thus, human *RAD52*-null cells showed a significant reduction in their proliferation rate (Fig. 2A) that was neither as severe as the comparable yeast strain, but which was more significant than either of their chicken or murine counterparts. Like the chicken and murine systems, however, — and in stark contrast to yeast — the *RAD52*-null cells were not IR sensitive, confirming that in vertebrates *RAD52* is not relevant for the repair of this type of DNA damage. Similarly, both budding and fission strains of *RAD52*-null yeast are severely defective for gene targeting [25, 65], whereas the chicken and mouse are only ~2-fold reduced [26, 27]. In contrast, the human *RAD52*-null cells showed no *relative* reduction in gene editing whatsoever (Fig. 5F). Thus, while the human *RAD52*-null cells more closely resemble their vertebrate counterparts rather than their fungal ones, they seem to present with their own unique spectrum of phenotypes.

To our knowledge, a *RAD52*-null patient has never been described. This fact would normally suggest that such loss-of-function individuals are either not viable or so aphenotypic that they have not been identified. The reduced proliferation rates of human *RAD52*-null cells withstanding, our data clearly favor the latter prediction. Thus, many patients — who were later discovered to have germline mutations in DNA repair genes — were first identified due to an adverse reaction to clinical chemo- or radiotherapy [2]. Our data clearly demonstrate that on a cellular level, *RAD52*-null patients would be very unlikely to be as radiosensitive as say a LIGIV-defective patient (Fig. 2B) or as sensitive to cross-linking agents as a Fanconi anemia (Fig. 2D) patient and thus might go undetected.

4.2. Human *RAD52* functions as an SSA protein in all HDR processes

Yeast *RAD52* possesses the dual activity of a recombination mediator and a SSA protein and it is the central factor for HDR in yeast. Importantly, the recombination mediator activity does not fully account for the extreme HDR deficits in a *RAD52*-null background, because *RAD52* mutants exhibit more severe recombination defects than their *RAD51*-null counterparts [25, 65]. On the other hand, the SSA activity is likely required for all forms of HDR in yeast, including second-strand capture during DSBR, and the strand annealing that occurs during SDSA and SSA [24, 25, 65]. Unlike yeast, the *RAD52* knockout mouse [27, 28] and human cells (Fig. 2B, 3B, D) demonstrated no IR sensitivity and only mild HDR defects, indicating that both activities of mammalian *RAD52* protein may be lost, partially replaced by, and/or redundant with other proteins. Indeed purified human *RAD52* exhibited little recombination mediator activity *in vitro* [30–32], in contrast to *BRCA2* [33]. These studies eventually led to the conclusion that *BRCA2* has evolved as the major recombination mediator in mammalian cells, whereas *RAD52* only serves as a backup in the case of a *BRCA* deficiency [14, 15, 33, 34] (Fig. 7A).

While evolution may have diminished *RAD52*'s role as a recombination mediator, our study demonstrates that the SSA activity of human *RAD52* cannot be fully compensated for by other proteins. *RAD52*, but not *BRCA2*, possesses the activity of annealing complementary

ssDNA and DNA:RNA duplexes [33, 37, 39, 76]. In addition, RAD52 arrives late in IR-induced DSB repair, suggesting a *RAD51*-independent role of *RAD52* in DSB repair [38]. Also, *RAD52*-deficient human cells have a universal reduction (at least to some degree) in all of the HDR pathways (Fig. 3B, D, 4C). These results collectively demonstrate that the SSA activity of human *RAD52* is involved in all major HDR processes (Fig. 7A–C). Interestingly, the HDR defects in *RAD52*-deficient human cells are not as robust as their yeast counterparts [25, 65], indicating the existence of other SSA proteins in human cells. To complicate this story, *RAD59* which can serve as a backup SSA protein in yeast has no known human paralog [25, 65]. Thus, the identification of other SSA proteins will be important for the understanding of human HDR. Indeed, very recently a gene, *MGM101*, with structural and functional similarities to *RAD52* has been described [66]. In the future, it will be important to determine the synthetic interactions between *RAD52* and *MGM101*.

4.3. The SSA activity of RAD52 is likely involved in stalled replication fork recovery

RAD52-deficient human cells were sensitive to the replication inhibitor HU at high concentrations (Fig. 2E). Similar HU sensitivity was observed in cells deficient for *XRCC3*, but not for canonical DSB repair genes such as *RAD54B* and *Mus81*. This result indicates a role of *RAD52* and *XRCC3* in stalled replication fork recovery through a *RAD51*-independent SSA pathway. Stalled replication forks can be repaired via translesion synthesis, fork cleavage and fork regression mechanisms [77, 78]. The fork regression pathway bypasses the genomic lesion via the formation and reversal of a “chicken foot” structure, and both of these steps require SSA activity. Indeed yeast *RAD52* can repair stalled replication forks via a SSA-dependent faulty template-switch mechanism [76]. Consistent with a role in stalled replication fork dynamics, human *RAD52* forms nuclear foci in response to HU treatment [38]. It also repairs stalled replication forks and rescues the resulting genomic stress in a *CHK1*-deficient background [40]. Most recently *RAD52* has been shown to be involved in DNA replication restart of stalled or broken DNA replication forks in human cells [42–44]. These results indicate that there are likely several *RAD51*-independent roles for *RAD52* in DSB repair and that the SSA activity of *RAD52* may be required for stalled replication fork recovery via the fork regression mechanism. This conclusion seems superficially at odds with the admittedly mild HU-sensitivity of the *RAD52*-null cells. We hypothesize, however, that the *RAD52*-deficient cells only exhibited a mild HU sensitivity because the bulk of stalled replication forks may be processed via translesion synthesis, via initiation replication from dormant replication origins [79] or via break-induced replication [42–44].

To our knowledge, we are the first to report that *XRCC3*- and *LIG4*-deficient cells also exhibit increased HU sensitivity, to a level similar to that of *RAD52*-deficient cells (Fig. 2E). *XRCC3* physically associates with *RAD52* and RPA in chicken DT40 cells [48]. Although no SSA activity has been documented *in vitro*, *XRCC3*-deficient human cells demonstrated reduced SDSA and SSA activity in our hands (Fig. S5). Interestingly, the inactivation of *RAD52* is synthetically lethal with *XRCC3* in DT40 cells [80]. Given the mildness of the *RAD52* and *XRCC3* knockout phenotypes, the synthetic lethality possibly indicates that these genes may complement each other in the same stalled replication fork repair pathway via their SSA activity. Alternatively, and we think more likely, *RAD52* and *XRCC3* may complement each other in repairing certain forms of genomic lesions that cannot be resolved

by the other pathway. In addition, the increased HU sensitivity in *LIG4*-deficient cells suggests C-NHEJ may play a role in repairing stalled replication forks, possibly in the fork cleavage pathway. It will be interesting to dissect the roles of these proteins in a translesion synthesis-deficient background.

4.4. The inactivation of RAD52 alters the mode of rAAV integration

Unexpectedly, we observed that *RAD52* inactivation altered the mode of rAAV integration in favor of HDR (Fig. 5D, Fig. S4). Perhaps even more surprising was that this alteration was due to a sharp decrease in random integrations instead of an increase in the DSBR activity that is required for rAAV targeting (Fig. 5E). *RAD52* binds to the DNA ends of rAAV, the human immunodeficiency virus (HIV-1) and the herpes simplex virus (HSV-1) and the competitive binding of *RAD52* with the C-NHEJ factor, Ku, may determine the fate of these viral genomes [57, 81, 82]. Interestingly, *RAD52* seems to exert a different effect on HIV-1 compared to rAAV and HSV-1. *RAD52* decreases the infection, integration and potentially circularization of HIV-1 [82], whereas it promotes the circularization and random integration of rAAV and HSV-1 [57, 81] (Fig. 5E). Part of the difference may be explained by the fact that the intermolecular ligation of these viruses is mediated by different pathways: the circularization of HIV-1 requires NHEJ [82], whereas rAAV and HSV-1 seem to be dependent on HDR [57, 83]. Notably, the role of *RAD52* in reducing HIV-1 transduction depends solely on its DNA binding activity (and not its recombination mediator activity), suggesting an HDR-independent role of *RAD52* in competitively binding DNA ends and dictating the fate of viral genomes [83]. Although the exact mechanism of *RAD52* in controlling the mode of rAAV integration is unclear, our work suggests that *RAD52* potentially could be targeted as a molecular switch to improve HDR-mediated rAAV genome editing and gene therapy [61]. Lastly, it is also important to note that *RAD52*'s effect appeared to be viral-specific as it appeared to play no role in CRISPR/Cas9-mediated HDR using plasmid donors (Fig. 5F).

4.5. Aberrant expression of RAD52 mRNA is associated with poor overall survival of patients in multiple cancers

To extend the biological significance of these data we sought to correlate *RAD52* expression in cancer patients to survival outcome. Unexpectedly, in the case of bladder and head and neck cancers low mRNA levels of *RAD52* correlated significantly with a poor overall survival (Fig. 6A, D). Interestingly, a naturally-occurring variant of *RAD52* (the "TT" genotype) was recently shown to be indicative of longer survival in colorectal patient cohorts [84]. Whether this allele correlates with reduced or increased *RAD52* expression is, however, currently not known. In contrast, although not quite statistically significant, a trend towards poorer survival was observed in kidney renal clear cell carcinoma patients that overexpressed *RAD52* (Fig. 6E). Moreover, although it was not evident in our data set (Fig. 6G) the overexpression of *RAD52* has been shown to be associated with lung squamous cell carcinomas genesis and progression [85]. Lastly, the relative overexpression of *RAD52* was also shown to lead to earlier mortality in a mouse model genetically engineered to be predisposed to colorectal carcinomas [43].

In toto, these results are consistent with the role of *RAD52* in DSBR and given our findings that human *RAD52* is involved in all three HDR subpathways (DSBR, SDSA and SSA) we speculate that non-physiological levels (either too high or too low) of *RAD52* promote dysregulated HDR, which possibly up-regulates error-prone backup repair pathways. Needless to say, further experiments, which are beyond the scope of this manuscript, will be required to determine if the observed correlation between aberrant levels of *RAD52* and poor survival is caused by *RAD52*'s role in tumor suppression or via other mechanisms. Why aberrant levels of *RAD52* limit survival in only certain cancers is also currently unclear, but it seems likely that it may be correlated with the expression levels of other repair factors that in turn regulate the penetrance of the *RAD52* deficiency/overexpression.

In conclusion, we have generated a *RAD52*-null human cell line model, which should serve as a useful tool to uncover the mechanism via which *RAD52* contributes to HDR. This cell line model may also help us understand how *RAD52* contributes to tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Construction of human somatic cell lines deficient for RAD52
- Demonstration that RAD52-null cells are reduced, but not completely deficient, in activity for almost all HDR sub-pathways
- Description of a novel HDR reporter (the RIG reporter) that permits the relative use of DSB-R versus SDSA HDR sub-pathways
- Demonstration that RAD52-null cells are decreased in the random integration of recombinant adeno-associated virus
- Demonstration that aberrant expression of RAD52 correlates with poor overall survival for several cancer types

Fig. 1.

Inactivation of *RAD52* in human somatic cells.

(A) Schematic of rAAV-mediated inactivation of *RAD52* via the exon-trapping selection strategies. Schematic elements: wide boxes, exons; narrow boxes, drug selection cassettes; thin black lines, genomic DNA; thick grey arrows, mRNA transcripts; triangles, LoxP sites; hairpins, rAAV inverted terminal repeats; black arrowheads, primers. (B) PCR confirmation of the *RAD52* heterozygous and homozygous knockout cell lines using primers P1 and P2. The 762 bp band corresponds to the wild type exon and the 832 bp band corresponds to the targeted exon. “+” refers to the wildtype and “-” to the correctly targeted alleles, respectively. (C) Western blot analysis confirmation of the *RAD52*-null and complemented cell lines. β -actin was utilized as a loading control.

Fig. 2.

Sensitivity of *RAD52*-deficient cells to DNA damaging agents.

(A) Growth curves of *RAD52*-deficient cells. The *LIG1*^{flox/-} HCT116 cell line was used as a negative control. (B–E) Sensitivity of *RAD52*-deficient cells to IR (B), etoposide (C), MMC (D) and HU (E). All data are averaged from 3 biological replicates and showed as the mean $\pm$ SEM. All values were first normalized to the untreated controls, and then to the parental HCT116 cell line. Statistical analysis (t-Test) was performed at representative data points and the results shown. The *LIG4*^{-/-} and *FANCD2*^{-/-} HCT116 cell lines served as positive controls.

Fig. 3.

The HDR and A-NHEJ activity of *RAD52*-deficient cells.

(A, C, E and G) Schematics of the DR-GFP (A), SA-GFP (C), EJ2-GFP (E) and pDVG94 (G) reporters. Schematic elements: intact and fragmented boxes, intact and truncated reporter elements; black boxes, *I-SceI* sites; green boxes, functional GFP expression; black arrows, promoters; orange, microhomology; red crosses, stop codons. (B, D, F and H) Normalized repair activity of DR-GFP (B), SA-GFP (D), EJ2-GFP (F) and pDVG94 (H) reporters. To inhibit C-NHEJ activity, cells were pre-treated with 1 μ M of the DNA-PK_{cs} inhibitor NU7441 for the pDVG94 (H) assay. All data are averaged from 3 biological replicates and shown as the mean $\pm$ SEM except for the pDVG94 assay. For these data all values were first normalized to the respective internal controls, and then to the parental or cDNA complementation cell lines. Statistical analysis (t-Test) was performed between the corresponding groups and the results shown. The raw, absolute wild-type levels of activity for all the assays shown were between 5% and 10%.

Fig. 4.

A novel RIG reporter to measure the ratio of DSB to SDSA activity.

(A) Schematics of the RIG reporter and its HDR repair products. All schematic elements are coded as Fig. 2, except that red indicates functional RFP expression. (B) Schematic illustration of the DSB and SDSA repair of the RIG reporter and the resulting products. Red, homology recipients; blue, homology donors; arrows, newly synthesized DNA. (C) Normalized DSB/SDSA activity (defined as the ratio of Type II/Type I recombination products) in *RAD52*-deficient cells. All data are averaged from 3 biological replicates and shown as the mean $\pm$ SEM. All values were normalized to parental or cDNA complementation cell lines. Statistical analysis (t-Test) was performed between the corresponding groups and the results shown. The raw, absolute wild-type levels of GFP⁺RFP⁺/GFP⁺ control cells for these assays were between 0.1% and 0.15%.

Fig. 5.

The efficiency of rAAV targeting and random integration in *RAD52*-deficient cells. (A) rAAV targeting is mediated by the DSBR pathway. Schematic elements: black, viral sequences; grey, genomic DNA; hairpins, inverted terminal repeats; lightning bolts and PacmenTM, endo- and exo-nucleases; arrows, directions of Holliday junction resolution. (B) The *PIGA* targeting vector and the intronic CRISPR/Cas9 cutting site. All schematic elements are described in Fig. 1. (C) Quantification of the FLAER staining. In all instances correct targeting events corresponded to a *PIGA*-null/FLAER-null phenotype whereas random integration of the targeting vector corresponded to a *PIGA*⁺/FLAER⁺ phenotype. (D–F) Normalized efficiency of rAAV targeting at the *PIGA* locus (D), rAAV random integration (E) and CRISPR/Cas9-mediated plasmid targeting (F) in *RAD52*-deficient cells. All data are averaged from 3 biological replicates and shown as the mean $\pm$ SEM. All values were first normalized to the respective internal controls, and then to the parental or cDNA complementation cell lines. Statistical analysis (t-Test) was performed between the corresponding groups and the results shown. The raw, absolute wild-type levels of correct *PIGA* targeting in the control cells were between 1 to 5 events per 1×10^4 cells.

Fig. 6.

The correlation of *RAD52* expression with cancer survival.

The TCGA was mined for survival and *RAD52* mRNA expression levels as detailed in the *Materials and methods*. For each cancer type analyzed, patients with *RAD52* mRNA levels in the top 20th percentile were designated as *RAD52*-high samples and patients with *RAD52* mRNA levels in the bottom 20th percentile were designated as *RAD52*-low samples. Cancer types are abbreviated as follows: (A) bladder urothelial carcinoma, (BLCA); (B) breast cancer, (BRCA); (C) colon adenocarcinoma, (COAD); (D) head and neck squamous carcinoma, (HNSC); (E) kidney renal clear cell carcinoma, (KIRC); (F) lung adenocarcinoma, (LUAD); (G) lung squamous cell carcinoma, (LUSC); (H) uterine corpus endometrial carcinoma, (UCEC).

Fig. 7.

The role of human *RAD52* in the HDR pathways.

(A–C) In human cells, HDR is mainly composed of the DSBR (A), SDSA (B) and the SSA (C) subpathways. Although the recombination mediator function of *RAD52* in recruiting *RAD51* to ssDNA is largely replaced by *BRCA2* in the DSBR pathway, it still plays a role in the second-strand capture step of DSBR, and the strand annealing steps of the SDSA and SSA pathway through its activity of annealing RPA-coated complementary ssDNA ends.