Analyzing the Role of *RIF1* in DNA Double-Strand Break Repair

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BRIEF: This work uncovers that the function of *RIF1* in DNA Double-Strand Break repair is likely mediated through the Single-Strand Annealing form of Homology-Directed Repair

ABSTRACT. DNA double-strand breaks (DSBs) are highly deleterious to cells, leading in some cases to mutations and genome rearrangements. However, these breaks can be repaired by multiple protein-dependent pathways. Among these, homology-directed repair (HDR) encompasses a subset of pathways that require extensive homology and depend on the protein Rad52. The protein *Rif1* inhibits HDR in mammalian cells [1], but its role during DSB repair in yeast, a common model organism for studying eukaryotic cellular processes, is unknown. We show that deletion of *RIF1* increases cell survival following an induced DSB. Epistasis analyses demonstrate that this increased rate of repair occurs only in the presence of *RAD52,* suggesting that *RIF1* may inhibit HDR in yeast. Deletion of *RIF1* does not alter cell survival in response to UV damage, indicating that Rif1p may not inhibit all forms of HDR, but may specifically reduce the occurrence of mutagenic single-strand annealing events occurring at an induced DSB. Because Rif1p is shown to inhibit HDR and therefore share a similar function in both yeast and humans, studies of Rif1p mechanism in yeast are likely applicable to that of human cells, which is key to understanding genomic repair in humans.

INTRODUCTION

DNA is a molecule that provides a blueprint for protein production and thus encodes the genetic instructions for functioning of all biological processes. Therefore a change in an organism's DNA, or mutation, can result in changes to all aspects of its life. Many human disorders such as cancer, neurodegeneration, and immunodeficiency are caused by faults in the cellular response to DNA damage [1]. Thus, the study of DNA repair is crucial to understanding these diseases. Particularly, a DNA double-strand break (DSB) is a form of damage that occurs when both strands of the double helix are severed. DSBs occur as a consequence of radiation or erroneous DNA replication and are lethal to the cell if unrepaired [2]. Consequently, there are several mechanically distinct, protein-dependent pathways capable of repairing a DSB [2]. Mistakes that occur during DSB repair can lead to changes in the DNA including loss of DNA sequence (deletions), addition of DNA sequence (insertions), and interchanges between parts of different chromosomes (translocations). These types of mutations are frequently observed in cancer cells and result in the changes in gene function/expression that drive tumor cell growth [1]. Therefore, the ability of the cell to choose the appropriate repair pathway is critical.

One mechanism of DSB repair is non-homologous end joining (NHEJ; Figure 1A), which is essentially a cut-and-paste form of DNA repair that reseals the break without using the sequence as a guide. This pathway utilizes the Ku70 and Ku80 proteins, but also requires the ligase protein Dnl4 [3]. Although NHEJ can result in restoration of the original sequence, it can also result in insertions or deletions at the site of the break.

A second type of DSB repair is homology-directed repair (HDR), and is characterized by the need for a second highly similar DNA sequence in the cell to act as a guide for repair. Gene conversion and SSA are both examples of this mode of repair. SSA uses short regions (30 bases or more) of highly similar (homologous) sequences on the same chromosome and results in loss of all DNA sequences between the homologous repeats. All forms of HDR require function of the protein Rad52.

The final type of repair is microhomology-mediated end joining (MMEJ), a highly mutagenic process mechanistically similar to SSA that results in variable size deletions. Unlike SSA, MMEJ requires only 5-25 bases of microhomology at the breakpoint and is independent of Rad52 (Figure 1A). Both MMEJ and SSA are dependent on the Rad1 protein [3].

Rif1 (*Rap1* Interacting Factor 1) is a regulatory protein found in the cells of humans and other mammals that is recruited to a DSB where it inhibits HDR and promotes NHEJ. However, the mechanism of this effect is not well understood [5]. A homolog of *RIF1* is found in other eukaryotes including *Saccharomyces cerevisiae* (Baker's yeast). *S. cerevisiae* is a useful model organism for the study of human cellular processes due to its similar genetic makeup, the full publication of its entire genome, and the ease of genetic manipulation. Although other functions have been ascribed to *RIF1* in yeast, the lack of a reported function of *RIF1* in yeast DNA repair has been surprising given the clear sequence conservation of the *RIF1* gene from yeast to humans [4, 5].

Recent work in our laboratory has uncovered a role for yeast *Rif1* in DSB repair. Unpublished data indicate that Rif1p inhibits the ability of yeast cells to survive a DSB produced by persistent endonucleolytic cleavage at a single site *in vivo*. Only cells that repair the DSB and, consequentially, mutate/delete the endonuclease cleavage site are able to survive, suggesting that *Rif1* may normally inhibit one or more of the error-prone repair pathways described above. Genetic epistasis analyses suggest that *Rif1* primarily inhibits SSA during the endonuclease cleavage assay, a function that may be required to increase the fidelity of DNA repair in response to a DSB. Epistasis refers to the masking of a phenotype by the mutation of a certain gene. Epistasis analysis is a method of identifying a gene responsible for the function of a cellular pathway by comparing phenotypes upon mutating genes responsible for those phenotypes. Preliminary results suggest that the function of *Rif1* may be specific to the SSA pathway of HDR. If *Rif1* is shown to inhibit aspects of HDR in both yeast and human cells, then studies of the mechanism through which *Rif1* affects repair pathway choice in yeast are likely to be applicable to understanding the analogous pathways in human cells, an issue of critical importance to the understanding of the maintenance of genomic integrity and prevention of potentially cancer-causing or other harmful chromosome rearrangements in human cells.

MATERIALS AND METHODS

HO Endonuclease Induction

Yeast strain JRL017 has a genotype designed in a manner advantageous to studying DSB repair [6]. YKF905 *rad52Δ::TRP1*, YKF905 *rif1Δ::LEU2*, and YKF905 *rad52Δ::TRP1rif1Δ::LEU2* were created by KLF using standard one-step gene replacement [7].

Single colonies of YKF905 or related strains were inoculated in synthetic media lacking uracil and containing 2% raffinose (-ura+raff). Raffinose is a non-repressing sugar that allows induction of the HO endonuclease gene in the added presence of galactose. After overnight growth at 30˚C, cultures were chosen based on optimum optical density (OD_{600}) of 0.3-0.9. Cells were plated on solid rich media containing glucose (yeast extract, peptone, 2% glucose; YPD) to monitor total cell number on rich media containing galactose (yeast extract, peptone, 2% galactose; YPgal) which induces the HO endonuclease that creates DSBs at the HO cleavage site. The undiluted cultures were plated on each of 4-6 YPgal plates and incubated at 30˚C for three days, while a 1000x dilution of the

original culture was plated on each of two YPD plates and incubated at 30˚C for two days. The frequency of survival after the HO-induced DSB was determined by dividing the average number of colonies that survived on each YPgal plate by the average number of colonies growing on each YPD plate x 1000. Colonies that survived on galactose were patched onto media lacking uracil to monitor retention of the URA3 marker located distal to the HO cleavage site.

Ultraviolet (UV) Sensitivity Assay

To test for the ability of mutated strains to repair DSBs induced by UV radiation, single colonies of YKF905 and derivative strains were inoculated into YPD and grown to an OD of ~0.6. A ten-fold dilution series in YPD was prepared up to a 10,000x dilution. Each dilution was plated on YPD and varying amounts of UV radiation was induced according to the dilution so that cells with a greater dilution received lower doses of UV. UV was delivered in specific doses to open petri plates using a UV Stratalinker (Stratagene) after ensuring that no visible liquid remained on top of the agar surface. Within each trial, each dilution/dosage was done twice. After incubating the plates for three days, the number of colonies growing on each plate was counted and the average number of colonies on the duplicate plates was determined. Percent survival was calculated by dividing the number of cells that grew on each dilution/dosage of UV by the number of cells that grew with no UV radiation after correction for the dilution factor. This assay was repeated three times. Trial data were averaged to form a survival curve.

Statistical Analysis

A student's T-test was utilized to compare the rates of survival between WT/*rif1Δ*, *rad1Δ/rad1Δrif1Δ*, *rad52Δ/rad52Δrif1Δ*, *lif1Δ/lif1Δrif1Δ*, and *ku80Δ/ku80Δrif1Δ* strains. P values less than 0.05 were considered statistically significant.

RESULTS

A system for the study of double-strand break repair

The yeast strain YKF905 was used to monitor DSB repair. As diagrammed in Figure 1B, this strain contains a galactose-inducible HO endonuclease gene. Production of the HO endonuclease protein in response to plating of cells on media containing galactose results in a single DSB on chromosome V at an HO endonuclease recognition site. If the break is repaired correctly, the HO endonuclease recleaves the chromosome. Therefore, cells remain in a cycle of cleavage and repair until a mutation occurs that heals the chromosome. The *URA3* gene is integrated about 10 kb distal to the HO cleavage site. Therefore, growth on media lacking uracil can be used to select for those cells that retained the distal portion of chromosome V.

RIF1 inhibits repair of an induced DSB in a RAD1- and RAD52-dependent manner

The lab had observed that deletion of *RIF1* from YKF905 increases the fraction of cells that survive growth on galactose (i.e. survive cleavage with HO endonuclease) while retaining the *URA3* gene (Figure 2A; p value <0.001). The presence of *URA3* indicates that these cells incurred a mutation within the HO cleavage site while undergoing DSB repair. This result suggests that deletion of *RIF1* either increases the frequency and/or decreases the fidelity with which the HO endonuclease-induced DSB is repaired.

To determine which mechanism of DSB repair is normally regulated by *RIF1*, a genetic epistasis analysis was conducted. This analysis individually eliminates each type of repair mechanism and examines the consequence of additionally eliminating *RIF1*. If the repair that is unregulated upon *RIF1* deletion cannot occur, there should no longer be an increase in the production of *URA3* colonies that survive on galactose upon deletion of *RIF1*. To conduct this, deletion of *RIF1* was combined with individual deletions of several genes known to affect DSB repair pathways. When *LIF1* or *YKU80* were deleted to eliminate NHEJ, the result was a decrease in overall survival in comparison to the wild-type strain (Figure 2A). However, the deletion of *RIF1* in addition to either *LIF1* or *YKU80* led to increased survival compared to the deletion of *LIF1* and *YKU80* alone (Figure 2B; p values of 0.003 and 0.001 for deletion of *YKU80* and *LIF1*,

Figure 1A

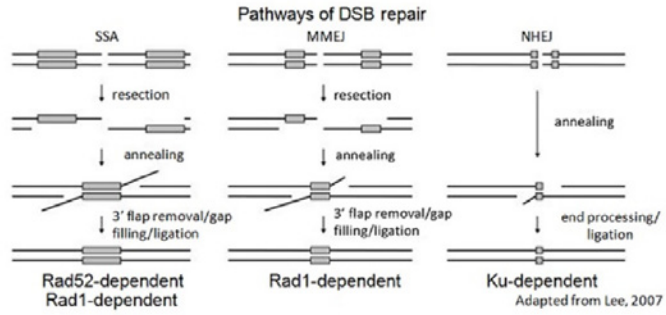

Figure 1B

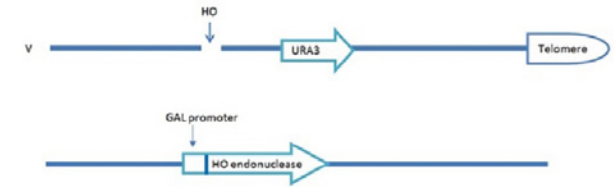

Figure 1A: Diagram of pathways of DSB repair, including NHEJ, MMEJ, and one form of HDR (SSA). Note that each pathway is dependent on specific proteins, and that SSA requires function of both Rad52 and Rad1.

Figure 1B: Schematic of JRL017; on Chromosome V, the URA3 gene is inserted about 10 kb distal (toward the telomere) from the HO cleavage site. The gene that encodes the HO endonuclease is expressed from a galactose-inducible promoter.

respectively). Note that y-axes in Figures 2A and 2B are different to account for the decrease in survival upon deletion of *LIF1* or *YKU80* in comparison to the wild-type. The increase in survival upon deletion of *RIF1* in strains unable to utilize NHEJ for DSB repair demonstrates that the major repressive effect of *RIF1* is not mediated through NHEJ.

The effect of eliminating SSA and MMEJ through the deletion of *RAD1* was then investigated. In contrast to the result in strains lacking *YKU80* and *LIF1*, deletion of *RIF1* did not increase the frequency with which galactose-resistant, *URA3* colonies were generated in the absence of *RAD1* (Figure 2A; p value 0.640). This lack of change suggests that *RIF1* may inhibit either MMEJ or SSA, both of which require Rad1.

To distinguish possible effects of *RIF1* on SSA or MMEJ, the effect of deleting *RAD52* (required exclusively for SSA) was examined by conducting three independent assays in *rad52Δ* and *rad52Δrif1Δ* strains and calculating the rate at which URA3 cells survived growth on galactose. Deletion of *RIF1* and *RAD52* did not increase the frequency with which galactose-resistant, *URA3* colonies were generated. Indeed, a statistically significant decrease in survival was observed (p value 0.007; Figure 2A). The observation that increased survival upon HO cleavage in the absence of *RIF1* depends upon both *RAD1* and *RAD52* is consistent with the hypothesis that *RIF1* normally inhibits the SSA pathway of DSB repair.

Ultraviolent (UV) Sensitivity Assay

The assay used in the previous section does not measure effects of *RIF1* deletion on the rates of most types of HDR. Although the epistatic relationship described above is consistent with *RIF1* inhibiting SSA, it is possible that *RIF1* has a more general role in the inhibition of HDR. Previous studies showed that deletion of *RAD52* impairs survival of cells after treatment with ultraviolet (UV) light [8], indicating that HDR is important for cellular response to UV-induced DNA damage. Therefore, if *RIF1* inhibits general HDR and not only SSA, there should be increased resistance to treatment with UV light (i.e. improved repair) upon deletion of *RIF1*. To assess this possibility, the wild-type strain and strains lacking either *RAD52* alone, *RIF1* alone, or both genes were treated with UV light. As expected, deleting *RAD52* increased sensitivity to UV

treatment (At 10 J/m² p value=0.054, at 25 J/m² p value=0.059), confirming that HDR contributes to cell survival under these conditions [8]. However, deletion of *RIF1* did not increase rate of survival (Except for an anomaly at 10 J/m² where p value=0.361; however at 25 J/m², p value=0.914). Likewise, the *rad52Δ* and *rad52Δrif1Δ* strains did not exhibit a significant difference in the survival rate upon treatment with UV (Figure 4, at 10 J/m² p value=0.051, at 25 J/m² p value=0.656).

Figure 2A

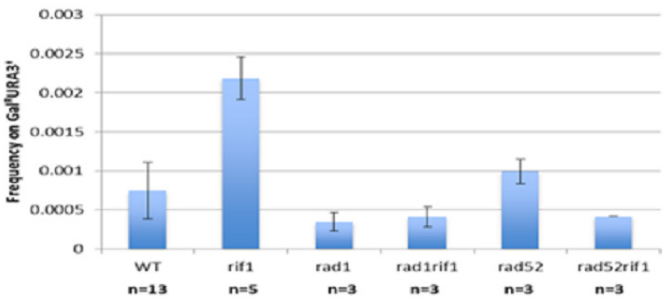

Figure 2B

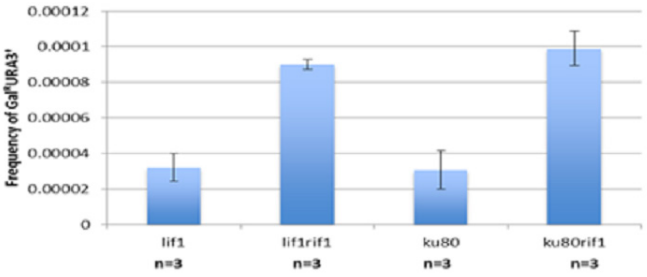

Figure 2: Survival rates of the indicated mutant strains in the presence and absence of *RIF1*. N values indicate the number of times that the experiment was repeated. Error bars represent standard deviation. Student's T-test performed in pairs indicated that the difference between survival rates of WT/*rif1Δ* (p-value <0.001), *rad52Δ/rad52Δrif1Δ* (p-value 0.007- note that difference is due to a decrease rather than an increase), *lif1/Δlif1Δrif1Δ* (p-value 0.001), and *ku80Δ/ku80Δrif1Δ* (p-value 0.003) is statistically significant. As expected, comparison of *rad1Δ/rad1Δrif1Δ* did not demonstrate a significant difference (p-value 0.640). Data for *rad52Δ* strains are produced by YEY. Other data produced by KLF.

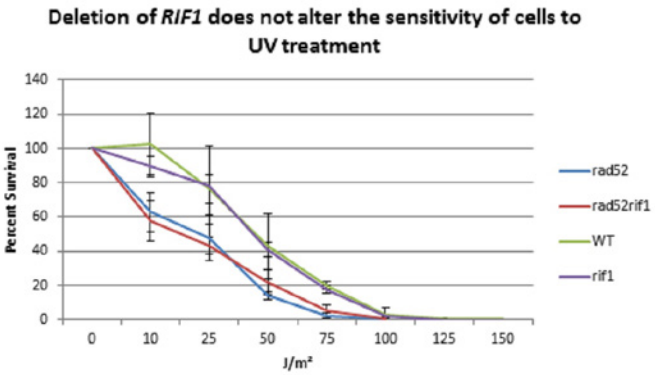

Figure 3: UV survival data for *rad52Δ*, *rad52Δrif1Δ*, *rif1Δ* and WT; x-axis shows increasing dosages of UV radiation; there does not appear to be a difference in the repair ability of *rad52Δ* and *rad52Δrif1Δ*; and WT and *rif1Δ* strains. Data produced by YEY.

DISCUSSION

Epistasis analysis indicated that Rif1p does not repress NHEJ, since deletion of *RIF1* increased cell survival even in the complete absence of NHEJ (Figure 2B). In contrast, the absence of an effect on cell survival upon deleting *RIF1* in a *RAD1*-mutant background suggested that *RIF1* functions to inhibit a Rad1-dependent pathway: MMEJ or SSA. It was then shown that the function of *RIF1* in response to a DSB depends on both *RAD1* and *RAD52*, highly suggestive that *RIF1* functions to inhibit SSA, the only DSB repair pathway known to require both proteins for function (Figure 2A) [3].

To address whether *RIF1* may repress additional forms of HDR, we utilized a UV sensitivity assay. When repairing DSBs that occur as a result of UV exposure, it is most likely that cells will utilize gene conversion for repair. The importance of HDR for the cellular response to UV damage is indicated by the reduced ability of cells lacking *RAD52* to survive UV treatment (Figure 3) [8]. Deletion of *RIF1* did not have any effect on cell survival in response to UV damage, suggesting that *RIF1* is unlikely to negatively regulate the types of HDR being used for repair under these circumstances (Figure 3). Together, these results suggest that *RIF1* specifically inhibits SSA.

The conclusion that Rif1p functions to inhibit SSA is further supported by additional unpublished data from the laboratory. Induction of HO endonuclease cleavage in the absence of NHEJ function (in a *YKU80* mutant strain) results in large deletions that appear to result from both MMEJ and SSA, the former requiring small amounts of homology and the latter occurring between regions of 20-35 bp of homology. When *RIF1* is deleted in a strain lacking *NHEJ*, the fraction of the deletions occurring in areas with extensive microhomology (>20 bp) is indicative of SSA increases. This result is consistent with the epistasis results presented here and supports the hypothesis that *RIF1* normally inhibits SSA.

In summary, this work indicates that the role of Rif1p in yeast is likely in the SSA form of HDR, expanding current knowledge of the role of this protein. This finding demonstrates that by inhibiting HDR, yeast *RIF1* does indeed act in a similar manner as mammalian *RIF1*. This assay should be useful to future studies of DSB repair in both yeast and human cells that may have great relevance in the study of human genomic processes.

Future Directions

It is noteworthy that induction of UV radiation generates several different forms of direct DNA damage, including production of thymine dimers. DSBs are also often generated by UV but are likely not the most prominent form of damage that occurs upon irradiation. If DSBs are not being induced/repaired to a large degree, then this assay may not be sensitive to additional roles of *RIF1* during HDR.

Therefore, in the future, *RIF1* should be studied specifically within the context of gene conversion. This study will utilize yeast strain JRL480, which has been designed to study gene conversion. Survival rates on galactose have been reported to be ~70% for this strain [6]. If *RIF1* normally inhibits gene conversion, this survival rate may increase upon deletion of *RIF1*. The *RIF1* gene will be knocked out and the cells monitored for the ability to repair DSBs through gene conversion.

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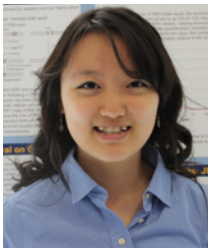

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