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The non-homologous end-joining pathway of *S. cerevisiae* works effectively in G1-phase cells, and religates cognate ends correctly and non-randomly

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Abstract

DNA double-strand breaks (DSBs) are potentially lethal lesions repaired by two major pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). Homologous recombination preferentially reunites cognate broken ends. In contrast, non-homologous end-joining could ligate together any two ends, possibly generating dicentric or acentric fragments, leading to inviability. Here, we characterize the yeast NHEJ pathway in populations of pure G1 phase cells, where there is no possibility of repair using a homolog. We show that in G1 yeast cells, NHEJ is a highly effective repair pathway for gamma-ray induced breaks, even when many breaks are present. Pulsed-field gel analysis showed chromosome karyotypes following NHEJ repair of cells from populations with multiple breaks. The number of reciprocal translocations was surprisingly low, perhaps zero, suggesting that NHEJ preferentially re-ligates the “correct” broken ends instead of randomly-chosen ends. Although we do not know the mechanism, the preferential correct ligation is consistent with the idea that broken ends are continuously held together by protein-protein interactions or by larger scale chromatin structure.

INTRODUCTION

DNA double-strand breaks (DSBs) lead to cell death if left unrepaired. DNA DSBs can be generated by endogenous cellular processes such as DNA replication or free radicals from oxidative metabolism, and also by exogenous factors such as ionizing radiation or genotoxic agents. Yeast have two different kinds of pathways for repairing DSBs: homologous recombination (HR) (which includes homology-dependent sub-pathways such as double-strand break repair via double Holliday junctions, synthesis-dependent strand annealing, break-induced replication, and single-strand annealing) and non-homologous end-joining

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(NHEJ) (Reviewed by (Cejka 2015; Haber 1999; Haber 2000; Jasin and Rothstein 2013; Kraus *et al.* 2001; Krogh and Symington 2004; Lewis and Resnick 2000; Lieber 2010; Mathiasen and Lisby 2014; Mehta and Haber 2014; Pannunzio *et al.* 2014; Reid *et al.* 2015; Symington and Gautier 2011)).

Homologous Recombination is the predominant pathway for repairing DSBs. Genes involved in HR repair include *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*. The *RAD50* series of genes (i.e., “*RAD*” genes with a number of 50 or higher) were originally discovered in screens for yeast mutants sensitive to X-rays (Game and Mortimer 1974; Nakai and Matsumoto 1967). One crucial gene is *RAD51*, which encodes a strand exchange protein that plays a central role in recombination. It has homologs in most species, from *E. coli* (where its homolog is the classic recombination protein RecA) to humans (Game and Mortimer 1974; Shinohara *et al.* 1992). Rad52 interacts with Rad51, and stimulates its binding to single-stranded DNA (Sung 1997). Mutant strains lacking *RAD51*, *RAD52*, or most of the other *RAD50* series genes are highly sensitive to gamma rays, bleomycin, MMS, and other agents that induce double-strand breaks (Game and Mortimer 1974; Jasin and Rothstein 2013; Lewis and Resnick 2000; Mehta and Haber 2014; Symington and Gautier 2011).

Although repair by homologous recombination (HR) is efficient and accurate, it has an Achilles’s heel in that it requires a homologous sequence to template repair. A broken chromosome in a diploid or polyploid cell always has access to a homolog, and so, from this point of view, repair of a DSB via homologous recombination may be possible in a diploid at any cell cycle stage. In haploid cells, a homolog (a sister chromatid) is available after S phase until the time of nuclear division, but is not available in G1 phase. Thus, at some stages of the cell cycle, such as G1 phase, a haploid cell cannot usually repair a double-strand break via homologous recombination, because the broken chromosome has no homolog.

The second kind of pathway for repair of DSBs is non-homologous end-joining (NHEJ) (Chen *et al.* 2001; Frank-Vaillant and Marcand 2001; Hefferin and Tomkinson 2005; Kegel *et al.* 2001; Lewis and Resnick 2000; Valencia *et al.* 2001). In this mode of repair, broken ends are brought together and ligated, possibly with some sequences deleted. This ligation typically benefits from complementary overhangs on the two ends, but these may be very short, often just a few bases (Pannunzio *et al.* 2014) which can be found by chance. In vertebrate cells, NHEJ plays critical role not only in the repair of DSBs but also in V(D)J recombination and class switch recombination (Lieber 2010). Yeast genes important for NHEJ include *YKU70*, *YKU80*, *DNL4/LIG4*, *LIF1*, *NEJ1*, *RAD50*, *MRE11*, and *XRS2* (for review see (Daley *et al.* 2005; Dudasova *et al.* 2004; Lewis and Resnick 2000). Yku70 and Yku80 form a heterodimer that comprises the Ku DNA end-binding protein (Milne *et al.* 1996), which is important for telomere maintenance and recruits other repair proteins. Mre11, Rad50 and Xrs2 form the conserved MRX complex (Tsukamoto *et al.* 2001), which is important for processing broken ends as a precursor to both homologous recombination and also NHEJ. Dnl4/Lig4, Lif1, and Nej1 form a complex specifically needed for the ligation event, where Dnl4 provides the ligase catalytic activity. Nej1 may also play a role in the binding of Yku70/Yku80 to DNA ends (Chen and Tomkinson 2011).

Since repair via non-homologous end-joining can occur even in the absence of a homologous sequence, it is natural to think that G1 phase haploid yeast might depend on NHEJ as their sole pathway for repair of double-strand breaks, and indeed that is the current view in the literature. That is, one would expect that in the absence of the NHEJ repair pathway, G1-phase haploid cells would be extremely sensitive to agents inducing DSBs. However, as discussed below, the evidence for this is surprisingly fragile.

During exponential growth, the NHEJ-specific mutants *dnf4*, *lif1*, and *nej1* show little or no sensitivity to classical break-inducing agents such as gamma rays. Not only were these genes not identified in any of the original screens for X-ray sensitive mutants, (Game and Mortimer 1974; Nakai and Matsumoto 1967) they were also not identified as X-ray sensitive in the genome-wide screen of Bennett et al. (Bennett *et al.* 2001) or even in the genome-wide screen of McKinney et al., (McKinney *et al.* 2013) which used an inducible EcoRI to make double-strand breaks. McKinney et al. specifically looked at *yku70*, *dnf4*, and *nej1* mutants, and stated they were not sensitive. Many other studies of exponentially-growing cells also failed to see any significant gamma ray, bleomycin, or MMS sensitivity in mutants specifically and solely defective in NHEJ ((Herrmann *et al.* 1998; Schar *et al.* 1997; Teo and Jackson 1997; Wilson *et al.* 1997); reviewed by (Lewis and Resnick 2000)).

Another kind of experiment has been to combine a mutant defective in NHEJ with a mutant defective in homologous recombination. Results have been mixed. For example, Herrmann et al. (Herrmann *et al.* 1998) (Fig. 3A) and Schar et al. (Schar *et al.* 1997) (Fig. 3C) compared the X-ray sensitivity of a *rad52* mutant to a *dnf4 rad52* double mutant during exponential growth, and saw no noticeable difference between them. On the other hand, Siede et al., Wilson et al., and especially Teo and Jackson (Siede *et al.* 1996; Teo and Jackson 1997; Wilson *et al.* 1997) did observe differences between *rad52* single mutants and *rad52 dnf4* or *rad52 yku70* double mutants. There were striking differences between these latter three papers in the degrees of difference seen, from modest to large.

Since there is an expectation that NHEJ should be most important in G1 phase in haploids, experiments have also been done (with and without mutations in homologous recombination) in haploid cell populations enriched for G1 cells. In this extreme situation, NHEJ mutations sometimes show sensitivity, but not always, and often the increased sensitivity is somewhat modest. Nunes et al. (Nunes *et al.* 2008) grew WT or *yku70* cells to late stationary to enrich for G1 cells, and irradiated with gamma rays. At low doses (50 Gy; ~90% killing for the WT) the *yku70* mutants were about 2.5-fold more sensitive than WT, but at higher doses (100 and 200 Gy) there was no significant difference. Likewise Siede et al. (Siede *et al.* 1996) grew WT or *yku70* cells to late stationary and irradiated with gamma rays, and saw a modestly increased sensitivity of the *yku70* mutants, which was probably statistically significant in some experiments (Fig. 8A) but not others (Fig. 8B, *yku70* vs WT). Karathanasis and Wilson (Karathanasis and Wilson 2002) also compared haploid *rad52* and *rad52 dnf4* cells grown to stationary phase to enrich G1 cells. The *rad52 dnf4* cells were about 4.5-fold more sensitive, but the size of the error bars suggests the difference may not have been statistically significant. *rad52* and *rad52 dnf4* cells were also compared after use of alpha-factor to arrest cells in G1 phase (albeit with limited purity of about 90% unbudded cells (Karathanasis and Wilson 2002)), and in this case no difference was seen.

Finally, experiments have been done with mutants defective in NHEJ using continuous expression of *GAL-HO* to make breaks at *MAT*. These experiments are done in the absence of a second, uncuttable copy of *MAT*, so that homologous recombination cannot be used for repair. In these experiments, unlike the gamma ray experiments cited above, all workers seem to find that a defect in NHEJ gives a high degree of sensitivity (Bonetti *et al.* 2013; Mahaney *et al.* 2014; Shim *et al.* 2005; Yu and Volkert 2013). Breaks made by HO, unlike breaks made by gamma rays, leave a specific structure in the DNA that may be favorable for ligation. Moreover, this *GAL-HO* system makes exactly one break per haploid genome (so there is no issue of which two ends to join), whereas gamma rays can make multiple breaks.

Here, given these complex results, we revisit the question of why mutants lacking the NHEJ pathway show only modest and variable gamma ray sensitivity in previous studies. One concern is that previous methods for enriching for G1-phase cells may not have been sufficient. For instance, if a population is 90% in G1 (Karathanasis and Wilson 2002), but results are assayed after killing to 0.01% survival (Karathanasis and Wilson 2002), then the 10% of cells that were not in G1 is a substantial fraction, and could have a major impact on the final results. We use our previous experience in cell cycle synchronization (Futcher 1999; Nash *et al.* 2001; Spellman *et al.* 1998; Tyers *et al.* 1993; Wang *et al.* 2009) to prepare very pure populations of G1-phase haploid cells. Using these pure populations, we consider three hypotheses for the modest and variable sensitivities seen previously for NHEJ-deficient mutants. First, that repair by NHEJ ligation is simply inefficient. Second, that it is efficient and effective in G1 cells, but that previous studies may not have had sufficiently pure populations of G1 cells. Third, that it is efficient, but randomly rejoins broken ends, such that when there are multiple broken chromosome in the same cell, most of the “repaired” cells are nevertheless inviable because of the generation of dicentric and acentric chromosomes. We find that in populations of G1 cells that are 99% or more pure, NHEJ is highly efficient and effective and important for survival. By using CHEF gels to characterize karyotypes of cells repaired via NHEJ, we show that in the vast majority of cases, cognate ends must have been correctly and non-randomly religated. In fact, we see no clear case of any reciprocal translocation, a product expected for random re-ligation.

MATERIALS AND METHODS

Yeast strains

The strains used in this paper are purchased from Genetic Research. Deletions were confirmed by PCR as described (http://www-sequence.stanford.edu/group/yeast_deletion_project/single_tube_protocol.html). The *rad52 yku70* double deletion was constructed by crossing *MATa rad52* to *MATa yku70*. The *rad51 yku70* double deletion was constructed by crossing *MATa rad51* to *MATa yku70*.

Radiation sensitivity of asynchronous haploid yeast cells

Spotted assay: Cells were inoculated into a 96-well plate with 140 µl medium/well containing 1% yeast extract, 2% peptone and 2% glucose (YPD) supplemented with 50 mg/l adenine, 50 mg/l leucine and 200 µg/ml ampicillin. Cells were cultured at 23°C for one week. By microscopy, >95% of the cells were unbudded (after sonication). Cells were then

treated with 80 krad ionizing radiation at 49 rad/sec. 3.5 µl of five 5-fold serial dilutions were plated on YPD plates. Plates were incubated at 30°C for 48 h.

Quantitative assay: Cells were grown to middle exponential phase at 30°C in medium containing 1% yeast extract, 2% peptone and 2% glucose (YPD) supplemented with 50 mg/l adenine, 50 mg/l leucine and 200 µg/ml ampicillin. The cell suspension was exposed to a ⁶⁰Co source at 8.3 krad/min. Cells were serially diluted with PBS pH 6.5. Relevant dilution fractions were spread on YPD plates in triplicate. Plates were incubated at 30°C and the colonies were counted after 48 hours. The survival rate was calculated for each deletion mutant by comparing to the corresponding untreated cells.

Elutriation and Gamma radiation

Cells were inoculated into medium containing 1% yeast extract, 2% peptone and 2% ethanol as carbon source, supplemented with 50 mg/l adenine and 50 mg/l leucine and 200 mg/l ampicillin. Cells were cultured at room temperature with shaking at 200 rpm for one week. Growth in ethanol accentuates the size difference between mother and daughter cells, allowing collection of very pure populations of unbudded daughters (Futcher 1999). Cells were collected by centrifugation. Elutriation was carried out to purify small G1 phase, unbudded cells using the culture supernatant (Futcher 1999). The percentage of budded cells was < 1%. However, the vast majority of the rare budded cells observed had low phase contrast, suggesting they were not intact. (The most common contaminant of the G1 fraction is broken cells.) Thus, in terms of viable cells, the G1 fractions had purities higher than 99%, possibly greater than 99.9%. Because the contaminating budded cells were rare, and because the difference between an intact cell and a broken cell is not always obvious by phase contrast microscopy, it was difficult to identify any clear cases of contaminating, viable budded cells, so a lower limit on budded cells cannot be given.

Elutriated cell suspension in cold conditioned medium was exposed to a ⁶⁰Co source at 8.3 krad/min. Cells were serially diluted with PBS pH 6.5. Relevant dilution fractions were spread on YPD plates in triplicate. Plates were incubated at 30°C and the colonies were counted after 48 hours. The survival rate was calculated for each deletion mutant by comparing to the corresponding untreated mutant.

Pulsed field gel electrophoresis

23 colonies of each genotype (*yku70*, *rad52* and *rad52 yku70*) and wild type that survived after 40 krad of radiation as described above were chosen randomly from the plates. Cells grown in YPD medium were pelleted and washed with equal volume ice cold 50 mM EDTA (pH 7.5) twice. Pellets were resuspended in 100 µl 50 mM EDTA, and mixed with 40 µl solution I (1 M sorbitol, 0.1 M sodium citrate, 50 mM EDTA, pH 7.5 containing 5% 2-mercaptoethanol (V/V), 1 mg/ml zymolyase) and 160 µl of 1% low melting agarose in 0.125 M EDTA, pH 7.5. The mixture was then cast into plug molds. After standing at 4°C for 30 min, the plugs were transferred into solution II (0.45 M EDTA, 10 mM Tris-HCl, 7.5% 2-mercaptoethanol (V/V), 10 µg/ml DNase-free RNase A, pH 7.5) and incubated at 37°C for 2 hr. Then the buffer was replaced by solution III (0.25 M EDTA, 10 mM Tris-HCl, 1% SDS, 1 mg/ml proteinase K, pH 7.5). The plugs were incubated for 3 days with daily changes of

solution III. The plugs were then washed with cold TEN (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0) to remove SDS and protein.

A Gene Navigator Pulsed Field System (Amersham Biosciences) was used to run the gel. Electrophoresis in 1% agarose (Bio-Rad) was carried out in 0.5X TBE. The running conditions were 12V/cm, at 7°C and the following pulse program: 70 seconds switch time for 17 hours, and then 120 seconds switch time for 5 hours with buffer recirculation. The yeast chromosome marker was purchased from Bio-Rad. The gels were stained in 0.5 µg/ml ethidium bromide for 1h and destained for 2h.

Modeling Expected Chromosome Abnormalities

To calculate the expected number of translocations (Table 2), we made the following conservative assumptions and simplifications. First, to calculate the fraction of the population with 0 breaks, we assumed that the *rad52 yku70* double mutant had no ability to repair DSBs. However, if this assumption was incorrect, then there would have been more DSBs than we calculated, and hence an even higher expected number of translocations. Thus the assumption is conservative with respect to the eventual conclusions. Second, we assumed a Poisson distribution of breaks. If this assumption was incorrect, and breaks were clustered and not independent, then there would have been more breaks, and hence more expected translocations than we calculated. Again, this is a conservative assumption. Third, we assumed that although the mean number of breaks in the population was 10.8 (for detail explanation see Results section), that all survivors came from cells with 4 or fewer breaks (the minimum break classes needed to account for the overall percentage of survivors). Again, if this assumption were incorrect, then the expected number of translocations would be even higher than we calculated, and so the assumption is conservative.

We neglected the possibility of multiple breaks in a single chromosome and the generation of inversions, but these can have only a small effect on the expected number of translocations. We also ignored lethality due to disruption of essential genes at translocation breakpoints, but this is a relatively minor effect given that only about 10% of the yeast genomic DNA consists of essential genes. Furthermore, to the extent that loss of essential genes at breakpoints does diminish survival, it would require that the survivors actually observed come from even higher break classes (i.e., 5 breaks per cell) which would raise the expected number of translocations. Thus most of our assumptions and simplifications are conservative; changes in these assumptions would generally create an even larger discrepancy between the observed result and the null hypothesis of random rejoining.

RESULTS

Asynchronous NHEJ mutants are not sensitive to gamma radiation

Consistent with most previous work, none of the haploid NHEJ mutants (*yku70*, *yku80*, *nej1*, *dnl4* and *lif1*) displayed any sensitivity to gamma radiation when tested as asynchronous cells (Fig. 1, Fig. 2). In contrast, a *rad52* mutant was highly sensitive (Fig. 1, Fig. 2). The results that the *rad51 yku70* and *rad52 yku70* mutants were no more sensitive than the *rad51* or *rad52* single mutants results are highly consistent with the results of

Herrmann et al. and Schar et al., somewhat different from results of Siede et al. and Wilson et al., and very different from the results of Teo and Jackson (Herrmann *et al.* 1998; Schar *et al.* 1997; Siede *et al.* 1996; Teo and Jackson 1997; Wilson *et al.* 1997); we have no explanation for this variability in the published results.

Interestingly, the stationary phase *rad52* cells of Figure 1 are about 25-fold more sensitive to radiation than WT at 80 krad, while the exponential phase *rad52* cells of Fig. 2 are about 200-fold more sensitive even at 20 krad. Previous studies have shown that stationary phase cells are more resistant to gamma rays than exponentially-growing cells (PETIN and PETIN 1995; NISHIMOTO et al. 2015), even if the cells are *rad52* mutants (PETIN and PETIN 1995). This could be because stationary phase cells induce genes of the “Environmental Stress Response”, and these include many genes for repair of DNA damage and recovery from stress (GASCH et al. 2000).

These strains are haploid; since their resistance depends on *RAD52*, and since repair by homologous recombination requires a homolog, the *RAD52*-dependent resistance suggests that the surviving cells were in G2 or M phase when irradiated, and had two copies of each chromosome available. By this reasoning, we would expect that a pure G1 population of haploid cells would not be able to use homologous recombination for repair. Such G1 haploid cells might be dependent on NHEJ for repair of DSBs.

G1-phase NHEJ mutants are highly sensitive to gamma radiation

We examined haploid cells in G1 phase. Cells of various genotypes were grown to early stationary phase in ethanol medium. This (a) provides a preliminary enrichment for G1 cells, since stationary cells accumulate in G1 phase; and

(b) accentuates the size difference between mother cells and newly abscised buds (buds that form at low growth rates are small). Centrifugal elutriation was used to isolate small, unbudded G1-phase cells. Contaminating budded cells were less than 1% of the population by microscopy, possibly less than 0.1% (Methods and Materials). Cells were treated with gamma radiation, and survival was assayed (Fig. 3).

The G1 cells gave markedly different results from asynchronous cells. Whereas in the asynchronous cells, the *rad51* and *rad52* mutants were very sensitive to radiation (Fig. 2), in the G1 cells, the *rad51* mutant had only a very slight phenotype, while the *rad52* mutant had a modest phenotype. The NHEJ mutants gave complementary results. In the asynchronous cells, the NHEJ mutants had almost no phenotype (Fig. 2), but in the G1 cells, the NHEJ single mutants were very sensitive to radiation: whereas the WT had a survival of 9.75% $\pm$ 1.49% at 20 krad, the *yku70* and *yku80* mutants had survivals of 1.17% $\pm$ 0.10%, or 0.86% $\pm$ 0.08%, respectively, about a 10-fold difference. The *rad52 yku70* and *rad51 yku70* double mutants were only slightly more sensitive than the *yku70* single mutants (0.38% $\pm$ 0.02%, or 0.38% $\pm$ 0.01%, respectively), consistent with the idea Rad51 and Rad52 are largely ineffective in G1 cells, and also consistent with the fact that these populations are highly purified for G1 cells. These results show that the NHEJ pathway does play an important role in repairing DSBs in haploid, G1-phase yeast.

Chromosome Aberrations after Non-Homologous End-Joining: Is End-Joining non-random?

If a single DSB occurs in a cell, then NHEJ could rejoin the two broken ends, restoring the chromosome. However, if there are two breaks, and so four broken ends, it is less clear what would happen. One possibility is that due to higher-order chromatin structure, cognate broken ends would be held in close proximity to each other until they are ligated together, regenerating the original chromosomes. Alternatively, the chromosomes might completely dissociate at the sites of the breaks, and then NHEJ would rejoin randomly-chosen ends. Assuming that each of four broken ends is eventually joined to some other broken end, there are three possibilities of equal probability. One restores the original chromosomes; a second generates a reciprocal translocation; and a third generates an inviable cell, because it has generated one dicentric and one acentric chromosome. If the number of induced breaks is more than two, similar reasoning can be applied, but the proportion of “correct” repair events goes down, while the proportion of translocation and inviable dicentric and acentric events goes up. Thus, if there are multiple breaks and ends are rejoined at random, then most cells would be inviable, and the rare survivors would be likely to contain one or more reciprocal translocations. We note that this could explain the modest gamma ray sensitivity seen for *nhej* mutants in the literature: If broken ends from multiple breaks are re-ligated randomly, leading to inviable cells, then the presence of the pathway would make only a modest difference to overall survival.

We used the idea that survivors would contain reciprocal translocations and CHEF gels to test the hypothesis that NHEJ joins random ends. We took wild-type, *rad52*, *yku70*, and *rad52 yku70* mutants, obtained pure G1-phase haploid cells by elutriation, and treated with 20 krad or 40 krad of gamma radiation to induce a large number of DSBs. The proportion survival was assayed (Table 1).

Assuming there is no repair in the *rad52 yku70* double mutant, then the surviving fraction, 0.000022 at 40 krad, is an estimate of the fraction of cells that had 0 lethal hits (i.e., the cells that happened to escape damage). This interpretation is strengthened by the fact that none of the surviving double mutants showed any chromosome abnormalities after pulsed field gel electrophoresis (see below, Fig. 4), consistent with the idea that these cells were never damaged. Using the Poisson distribution,

$$P(n) = (e^{-\lambda} \lambda^n) / n!$$

where n is the number of hits, $P(n)$ is the probability of having exactly n hits, and λ is the mean number of hits, we can calculate that $P(0) = 0.00002$ when the mean number of hits, λ , is 10.8. Making the assumption that a lethal hit from gamma radiation is a DSB, this implies that the population of yeast cells treated with 40 krad of gamma radiation had an average of 10.8 DSBs. Consistent with this, the surviving fraction of *rad52 yku70* cells from 20 krad was 0.0029, implying an average of 5.8 DSBs from the Poisson distribution (i.e., half the dose appears to have caused half the damage, as expected).

We also ran the DNA on CHEF gels immediately after irradiation, and from the loss of intensity of the larger chromosomes made a second, independent estimate of the number of breaks. In this assay, we estimated 3.3 breaks per cell at 20 krad, and 4.4 breaks per cell at 40 krad. We feel these are compatible with the genetic estimates, especially given that the physical estimates are highly sensitive to issues such as background subtraction, linearity of signal, etc.

As described above, if these broken ends are repaired by random religation, then for each end, there are three possible outcomes: correct ligation to its cognate end (allows viability), incorrect ligation to generate a monocentric translocation chromosome (allows viability), or incorrect ligation to generate a dicentric or acentric chromosome (does not allow viability). In Table 2, we calculate the relative proportions of these different events for 0, 1, 2, 3, and 4 DSBs per cell, assuming random re-joining of ends, assuming that all ends are rejoined, and ignoring the possibility of multiple breaks on a single chromosome and inversions. To make this calculation, we assume that a G1 *rad52 yku70* double mutant has no ability to repair a DSB. From Table 1, we see that at a dose of 40 krad, a G1 phase *rad52 yku70* culture yields survivors at the rate of 0.00002. According to our assumption, these survivors represent the cells that had zero double-strand breaks. Then, using the Poisson distribution, we can calculate the fraction of cells with exactly 1, 2, 3, or 4 *etc.* double-strand breaks in the same irradiated population. If we then assume that these ends are ligated back together at random, and enumerate all possibilities, it yields the rest of the results in Table 2. Although there is some uncertainty in the original assumptions, our final conclusions are not very sensitive to changes in these assumptions. Furthermore the assumptions are conservative with respect to the eventual conclusion. (See Methods and Materials for further discussion of the assumptions).

Survival of the *rad52* cells (i.e., cells where the only mode of repair was NHEJ) was 0.0044 at 40 krad, while survival of wild-type cells was 0.014 (about 700-fold greater than the double mutant). As can be seen from Table 2, assuming 10.8 initial double-strand breaks, wild-type and *rad52* survivors must have included cells that originally had at least four double-strand breaks, because even if 100% of cells with 0, 1, 2, and 3 lethal hits survive, this fraction is smaller than 0.0044. Thus, from the cumulative reciprocal translocation column CRT(n) of Table 2, random rejoining of ends predicts that at least 79% of the wild-type and *rad52* survivors would bear one or more reciprocal translocations. Furthermore, from the cumulative events per survivor column CES(n), the average number of events (aberrant chromosomes) per survivor should be 2.22.

To test these predictions of the random rejoining model, we chose 23 colonies of each genotype that survived after 40 krad of radiation. The chromosomes of these survivors were analyzed by pulsed field gel electrophoresis (PFGE). Examples of these gels are shown in Fig 4.

One DNA preparation failed, leaving 91 survivors analyzed. Although at least 12 of the 91 surviving colonies had an abnormal karyotype, this is a much lower frequency of abnormal karyotypes than predicted by the random rejoining model.

We saw four kinds of abnormalities. First, some colonies were apparently missing entire chromosomes (wild-type #4 and #8, and *rad52* #10). Wild-type #4 seemed to be missing chromosome 5 and chromosome 10; wild-type #8 seemed to be missing chromosome 7 and chromosome 15. Since all chromosomes are needed for viability, we presume that these chromosomes are present in some altered form that we cannot see on these gels. One possibility is that they have circularized, because of breaks and re-joining near the telomeres. Circular chromosomes would be trapped in the wells of these gels, and so would not be visualized.

Second, some surviving colonies had additional chromosome bands at novel positions (*rad52* #7 and #21, *yku70* #1, #9), but did not lack any bands. We do not know the origin of these extra chromosomes. The extra band in *rad52* #21 seems to be running as a smear, which might indicate that it is constantly losing sequences, possibly at an uncapped or imperfectly capped end. An extra band could arise from the centric fragment of an unrepaired break (Kaye *et al.* 2004; Lobachev *et al.* 2004), which was subsequently stabilized by telomere addition (Kramer and Haber 1993).

Third, some surviving colonies have shifted chromosomes (*rad52*, #14). Again, we do not know the origin of this aberration. Finally, four colonies had complex events where some chromosome bands were missing, while new bands appeared (wild-type #6, #13, and *rad52* #13 and #19). These events might include reciprocal translocations.

It is difficult to interpret these aberrations fully. Circular chromosomes and chromosomes smaller than 200 kb would not be visible on our gels; resolution is only moderate, and so some aberrant chromosomes may be co-migrating with normal chromosomes. In principle, co-migration could be detected by assaying the intensity of each band. Some of the survivors do appear to have unusual band intensities, but we have seen the same effects with untreated cells, and so are reluctant to conclude that these signal a chromosome abnormality. Some of the aberrations could have been placed in a different category; for instance, *rad52* #10 we have categorized as having a missing chromosome 10, but one could instead argue that chromosome 10 has been shifted up to co-migrate with chromosome 14. Finally, the four cases we have classified as possible translocations (WT #6, #13, and *rad52* #13 and #19) are all complex events, they do not contain chromosomes of the sizes predicted for a simple reciprocal translocation, and the classification as a possible translocation could be incorrect. Table 3 summarizes the chromosomal aberrations seen.

Nevertheless several points are clear. First, for the 45 wild-type or *rad52* survivors (i.e., all the survivors capable of NHEJ), the total number of observed translocations (4 or less) and other abnormalities (6) is surprisingly low, considering that the average survivor must have had a little more than 3 DSBs. The random rejoining model predicts 36 (0.79×45) survivors with reciprocal translocations, but only 10 survivors with abnormalities were seen. Based on a chi squared analysis, the difference between observed abnormalities (4 possible translocation, 6 other abnormalities, 10 abnormal karyotypes total) and expected abnormalities (36 translocations) is highly significant (sum of $\chi^2 = 90$, $p < 0.0001$). We take this as evidence that ends are not rejoined randomly, but that instead, some mechanism assists the ligation of “correct” ends. Note that this is a very conservative calculation,

because it assumes that all abnormalities are equivalent to reciprocal translocations (i.e., the greatest possible repair error), and it assumes that all survivors were derived from cells with four or fewer DSBs whereas in reality some survivors probably suffered five or more DSBs. Because the assumptions and calculation are conservative, the conclusion that rejoining is not random is robust.

Second, the random rejoining model predicts that in the 45 WT or *rad52* survivors there should be a total of 100 abnormal chromosomes (45×2.22 , from the CES(n) column of Table 2). If we count the putative missing chromosomes and the putative translocations as 2 events per cell, and the shifted chromosomes and additional chromosomes as 1 event per cell, then the total number of abnormal chromosomes seen or inferred is 17. 100 is significantly different from 17 ($p < 0.0001$). Again, this analysis rejects the random rejoining model.

Third, the random rejoining model predicts that many survivors will have three or four translocation chromosomes, but we saw no such events.

Fourth, none of the *rad52 yku70* double mutants showed any abnormalities. This supports the idea that these double mutants have no ability (i.e., no third unknown pathway) for repairing DSBs, and that the rare surviving colonies are derived from rare cells that received no double-strand breaks.

DISCUSSION

Non-homologous end-joining is evolutionarily conserved among different prokaryotic as well eukaryotic species and is the predominant double-strand break repair pathway in mammalian cells. It is also very clear that it operates in yeast cells. Surprisingly, yeast mutants lacking non-homologous end-joining have only modest sensitivity to ionizing radiation. Here, we demonstrate that when very pure populations of G1 phase haploid yeast are isolated, a situation when no homolog is available for recombinational repair, then indeed such cells do depend on NHEJ for survival after induction of DSBs. In this situation, the NHEJ pathway is more important for survival than recombinational pathways, as expected. These results are in contrast to those obtained by Karathanasis and Wilson (Karathanasis and Wilson 2002), who found that *dnf4* mutants have little sensitivity to ionizing radiation even in G1 phase. However, the cells studied after alpha-factor synchronization were described as about 10% budded (Karathanasis and Wilson 2002), which is still a large impurity relative to four logs of killing (Karathanasis and Wilson 2002). We believe that by elutriation, we are able to achieve a purer G1 population than studied previously, and this 99%+ pure G1 population allows us to detect G1-specific repair.

In theory, NHEJ should be most important to yeast in haploids in G1 phase. It should be less important in diploids, because diploids always have a homologous chromosome available for recombinational repair. NHEJ should also be less important in the G2 and M phases of the cell cycle, because homologous chromosomes are available at these times even in haploids. There is evidence that both these ideas are true. NHEJ appears to be down-regulated in diploid cells because of transcriptional down-regulation of *NEJ1* and *LIF1* (Chen *et al.* 2001;

Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Valencia *et al.* 2001). Furthermore, there seems to be reciprocal regulation of NHEJ and HR repair through the cell cycle, such that NHEJ is most active in G1 phase, while recombinational repair is most active in G2 and M phases (Aylon *et al.* 2004; Cejka 2015; Ferreira and Cooper 2004; Karathanasis and Wilson 2002; Lieber 2010; Mathiasen and Lisby 2014; Symington and Gautier 2011). This regulation is not just at the level of the presence or absence of a homolog; the cyclin dependent kinase complexes controlling the cell cycle appear to play a critical role as well (Aylon *et al.* 2004; Cejka 2015; Ferreira and Cooper 2004; Ferretti *et al.* 2013; Karathanasis and Wilson 2002; Langerak and Russell 2011; Lieber 2010; Mathiasen and Lisby 2014; Symington and Gautier 2011).

Which Ends are Joined?

After a double-strand break has occurred, the two broken ends may separate from each other. If so, this poses a problem for later repair: the ends must somehow be brought together, and, if there are several pairs of broken ends, there is the further issue of whether the correct pairs of ends are brought together. Alternatively, it may be that protein-protein interactions, or larger scale chromatin structures, keep pairs of broken ends together despite the lack of continuity of the DNA. Several studies have shown that a single pair of HO-induced cognate broken ends can be held together (Kaye *et al.* 2004; Lisby and Rothstein 2004b; Lobachev *et al.* 2004). Since these studies examined the location of ends several hours after the induction of HO, it is not entirely clear whether the connection between the broken ends is maintained continuously from the time of the initial break, or whether the ends are brought together after an initial period of separation, but in any case these studies do establish that there is some mechanism that can hold ends together even while the double helix is broken. This could promote directed ligation of the correct ends, rather than ligation of random ends.

Yu and Gabriel (Yu and Gabriel 2004) examined populations where two breaks were made using the HO endonuclease. Like us, they found that only a small fraction of survivors contained reciprocal translocations, consistent with the idea that there is directed ligation of the correct ends. However, there are numerous differences in assay and approach that make it difficult to compare our study and the study of Yu and Gabriel. For instance, in the study of Yu and Gabriel, a HO-induced break that is repaired without change or loss of nucleotides (either by perfect ligation to the cognate end, or to a non-cognate end, generating a translocation) will once again be a substrate for cleavage by HO, leading to futile cycles of cleavage and repair. The ultimate survivors assayed are all cells where repair has (eventually) been imperfect, and has destroyed the HO sites.

Haber and Leung (Haber and Leung 1996) also studied the fates of two simultaneously broken chromosomes. In contrast to our results and to the results of Yu and Gabriel, Haber and Leung found that reciprocal translocations were about equal in frequency to correct repair, consistent with the random ligation of ends. However, a possibly critical difference in this study was that each of two chromosomes was cut by HO in two places, at opposite ends of a 2 kb fragment carrying the *LEU2* gene (i.e., 4 breaks total, in two chromosomes). The survivors studied were *leu2⁻*; i.e., had lost the 2 kb fragment originally flanked by HO sites. Thus in this study a 2 kb gap was created rather than a break, and this gap may have resulted

in random, rather than directed, joining of ends. Interestingly, Rothkamm et al. (Rothkamm et al. 2003) found that in mammalian cells, the correct ends tended to be rejoined when radiation doses were low, and DSBs were well separated, but incorrect ends tended to be rejoined when radiation doses were very high, and DSBs were close together.

Broken ends and relevant repair proteins are recruited to a small number of nuclear foci (Lisby et al. 2003a; Lisby et al. 2004; Lisby et al. 2003b; Lisby and Rothstein 2004a; Lisby et al. 2001). One break (two broken ends) induces one focus, but even a very large number of breaks (~80) induces only a few foci (~2) (Lisby et al. 2003b). These foci have been called “factories” for DNA repair, and it is thought that each focus may contain many pairs of broken ends, and the proteins needed to put them back together.

Given that a single focus can contain many pairs of broken ends, it is unclear whether non-homologous end-joining would bring together cognate ends, or whether it would simply re-ligate ends randomly. If cognate ends are held together continuously from the time of the initial break, then obviously this would facilitate re-ligation of the correct ends. On the other hand, if ends separate after the initial break, and then are all brought back together in the repair focus, it is unclear which ends would be rejoined.

Here, we have generated a G1-phase population with an average of more than 10 double-strand breaks per cell, where repair is exclusively or almost exclusively by NHEJ, and where the average survivor must have sustained over 3 DSBs. In a model where random ends are rejoined, we expect at least 36 of 45 (79%) of these survivors to contain reciprocal translocations, and we expect to see a total of 100 abnormal chromosomes in 45 survivors. In fact, only 10 of 45 survivors showed any chromosomal abnormality, and at most 4 of these (and perhaps zero) were reciprocal translocations. Only about 17 abnormal chromosomes were seen. These results are inconsistent with models where random ends are re-joined, and support models that postulate a continuous connection between cognate broken ends.

Although end-joining seems to be non-random, it is not perfect, since we did see some chromosome abnormalities. At present however it is difficult to say very much about the nature of the imperfections in NHEJ repair of X-ray damage, in part because we do not understand the nature of the chromosome abnormalities we observed. None of the abnormalities unambiguously corresponded to a reciprocal translocation, which is the simplest and most obvious abnormality that one might expect. Possibly NHEJ is extremely efficient with a simple break of the kind that could lead to a simple translocation; the abnormalities seen in Fig. 4 may derive from very severe and unusual damage. It will be interesting to see from future studies exactly what kinds of events are occurring in these survivors.

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Highlights

- Repair of double-strand breaks in G1 yeast depends on non-homologous end-joining.
- Non-homologous end-joining is an effective repair pathway even for multiple breaks.
- Non-homologous end-joining preferentially or exclusively rejoins cognate ends.

Fig. 1. Sensitivity of asynchronous haploid cells to gamma radiation (spot assay). Cells were grown for one week in YPD medium. Cells were irradiated (80 krads) and serially diluted (left to right) by 5-fold, then a 3.5 μ l sample of each dilution was plated on YPD. Plates were incubated at 30°C for 48 h.

Fig. 2.

Sensitivity of rapidly growing haploids to gamma radiation. Cells grown in YPD medium to mid-log stage were irradiated at 5, 10 and 20 krad, and serially diluted. Relevant diluted fractions were spread on YPD plates in triplicate. The plates were incubated at 30°C for 48 h. Survival was calculated for each strain after normalizing to a 0 dose plate of the same strain. Error bars show one standard deviation. Error bars are small (many colonies were counted) and typically not visible.

Fig. 3.

Sensitivity of G1-phase NHEJ mutants. G1-phase cells (at least 99% pure G1) were obtained by elutriation, then irradiated and serially diluted. Relevant diluted fractions were spread on YPD plates, in triplicate. The plates were incubated at 30°C for 48 h. Survival was calculated for each strain after normalizing to a 0 dose plate of the same strain. Error bars (often overlapping with data point symbols, and often not visible) show one standard deviation. The *rad51 yku70* and the *rad52 yku70* curves were not statistically different from each other; the *yku70* and *yku80* curves had a marginal statistical difference from each other; all other pair-wise comparisons were different with a high statistical significance ($p < 0.01$).

Fig. 4.

Karyotype analysis of chromosome aberrations after DSB repair. Haploid G1-phase cells were isolated by elutriation as before, and irradiated with 40 krad. Random survivors were chosen from YPD plates, and analyzed by pulsed-field gel electrophoresis. Chromosome abnormalities are indicated by arrows. The size of marker and the corresponding chromosome number (from top to bottom, in kb) are 2,200 (XII), 1,600 (IV), 1,125 (XV and VII), 1,020 (XVI), 945 (XIII), 825 (II), 785 (XIV), 750 (X), 680 (XI), 610 (V), 565 (VIII), 365 (III), 285 (VI) and 225 (I). Note that chromosome XIII in the marker strain is smaller than in the experimental strain.

Table 1

Survival after 20 or 40 krad.

	20 krad	40 krad
WT	0.12	0.0140
<i>rad52</i>	0.054	0.0044
<i>yku70</i>	0.0074	0.0030
<i>rad52 yku70</i>	0.0029	0.000022

Table 2

Proportion of expected translocations after NHEJ DSB Repair at 40 krad

Breaks	Fraction	PV	FV	Cum. FV	FT	Cum. FT	E/S	Cum. E/S
0	0.00002	1	0.00002	0.00002	0	0	0	0
1	0.0002	1	0.00020	0.00022	0	0	0	0
2	0.001	0.67	0.00067	0.00089	0.5	0.38	1	0.75
3	0.003	0.40	0.00120	0.00209	0.83	0.64	2	1.47
4	0.009	0.23	0.00200	0.00409	0.96	0.79	3	2.22

Breaks is the initial (before repair) number of double-strand breaks in a given cell. Here, we are equating "lethal hits" with "breaks".

Fraction is the fraction of cells at 40 krad with the indicated number of breaks (i.e., 0.00002 of the population received 0 breaks). The fraction with a given number of breaks is calculated from a Poisson distribution, and from the fact that the fraction of a *rad52 yku70* population that survives 40 krad of radiation is 0.00002 (Table 1).

PV is Probability that a cell is with a given number of breaks is Viable, assuming (i) all breaks are re-sealed by NHEJ; (ii) the pairs of ends resealed are chosen randomly; and (iii) cells die if repair generates one dicentric or one acentric. For instance, at 4 breaks, the probability that a repaired cell is viable is only 0.23, because the other 0.77 have dicentric or acentric chromosomes.

FV is the Fraction of cells with the indicated number of breaks that are Viable; $FV = \text{Fraction} \times PV$. For instance, the fraction of cells in the total population that had 4 hits and are viable is $0.009 \times 0.23 = 0.002$.

Cumulative FV is the cumulative fraction of cells that are viable, summed over all break classes smaller than or equal to the current class. For instance, Cum. FV at 4 breaks is $0.00002 + 0.00002 + 0.00067 + 0.00120 + 0.00200 = 0.00409$.

FT is the fraction of cells with the indicated number of breaks bearing at least one reciprocal translocation (two translocated chromosomes). The higher break classes typically have multiple reciprocal translocations.

Cumulative FT is the Cumulative fraction of cells bearing at least one reciprocal translocation.

E/S is predicted Events per Survivor. An event is an abnormal chromosome in principal detectable by pulsed-field gel electrophoresis. A reciprocal translocation causes two events, since two chromosomes are altered.

Cum. E/S is Cumulative Events per Survivor; it is events per survivor averaged over all preceding break classes.

Table 3

Kind and Frequency of chromosome abnormalities.

	Wild-type	<i>rad52</i>	<i>yku70</i>	<i>rad52 yku70</i>
Normal	18	17	21	23
Missing Chrom	2 (#4, #8)	1 (#18)	0	0
Extra Chrom.	0	2 (#6, #15)	2 (#1, #9)	0
Shifted Chrom.	0	1 (#22)	0	0
Translocations	2 ? (#6, #13)	2? (#4, #21)	0	0