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PRINCIPAL INVESTIGATOR: Aylin Rizki

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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13. ABSTRACT *(Maximum 200 words)*

Telomeres, ends of eukaryotic chromosomes, need to be maintained for long-term cellular proliferation. In most eukaryotes, the reverse transcriptase telomerase is responsible for replicating and maintaining the telomeres. Absence of telomerase activity results in progressive telomere shortening with continued cell division. When telomeres are too short to maintain function, most cells stop dividing. However, a subpopulation of cells can escape death via telomerase-independent pathway(s). Telomerase-independent survivors have been observed in budding yeasts, in fission yeast and in human cells.

In *S. cerevisiae* survivors, two types of telomeric sequences are affected: the terminal $G_{1\text{-}3}T$ repeats and subtelomeric repeats called Y' elements; both sequences are substantially amplified by *RAD52*-dependent recombination. Since the terminal G-rich sequences are not perfectly homologous, this could provide a potential barrier to recombination. We have tested this, by demonstrating that mutations in genes in the mismatch repair pathway, previously shown to increase homeologous recombination frequencies, enhance telomerase-independent survival in a *RAD52*-dependent manner. Since little or no Y' amplification is observed in these survivors compared to just telomerase-minus survivors, this suggests that the absence of mismatch repair genes removes a block to $G_{1\text{-}3}T$ to $G_{1\text{-}3}T$ recombination.

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A. Rizki

PI - Signature Date

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RESEARCH SUMMARY

Introduction

Telomeres are replicated and maintained primarily through telomerase, a reverse transcriptase that adds sequences to chromosome ends and ensures a constant length through extended generations of growth. In *Saccharomyces cerevisiae* mutations in *EST2*, the catalytic component of telomerase, in *TLC1*, the internal RNA template component of telomerase, and in three other genes, *EST1, EST3* and *CDC13* result in progressive loss of viability, called senescence and also in progressive shortening of telomeres (Lendvay et al., 1996). When telomeres get too short to maintain function, the majority of the cells die. However, a small subpopulation of cells is able to survive the catastrophic effects of telomerase-deficiency (Lendvay et al., 1996; Lundblad and Blackburn, 1993).

Telomerase-independent survival in *Saccharomyces cerevisiae* is recombination dependent and involves gross rearrangements and amplification of telomeric and subtelomeric repeat sequences (Lundblad and Blackburn, 1993). The observed telomeric profile of telomerase-independent survivors suggests that either homologous recombination between the 99% identical subtelomeric Y elements or homeologous recombination between the telomeric $G_{1\text{-}3}T$ repeat tracts (or both) may play a role in generating survivors.

We have tested this by demonstrating that mutations in mismatch repair genes, previously shown to increase homeologous recombination rates, enhance telomerase independent survival in a *RAD52*-dependent manner. Furthermore, such enhancement of survival is accompanied by little or no increase in Y' amplification levels, suggesting that the primary mechanism for this survival enhancement is by promoting recombination between telomeric repeats.

Some mismatch repair genes have been shown to function in the single strand annealing (SSA) pathway of direct repeat recombination. Therefore, we tested the effect of mutations in other genes in the SSA pathway. Such mutations had no detectable effect on telomerase-independent survival or on Y' amplification levels in survivors, suggesting that the roles of mismatch repair proteins in the SSA pathway is not necessarily relevant in the analysis of the effect of mismatch repair mutations in telomerase-independent survival.

More general characterization of the effects of telomerase-deficiency showed that *RAD52* was required for maintenance of survivors as well as for their establishment.

Methods

Yeast strains

The *MSH2, MSH3, MSH6, MLH1, PMS1, RAD1, RAD10,* or *genes* were disrupted in diploids heterozygous for *est1-Δ* or *est2-Δ,* using PCR fragments generated from PCR amplification of the KANMX2 cassette. *msh3-Δmsh6-Δest2-Δ* triple heterozygote diploid was prepared by mating *msh6-Δest2-Δ* and *msh3::URA3:hisG:URA3* disrupted haploids. Diagnostic PCR and southerns were used to confirm disruptions.

Growth Analysis

Serial streakout analysis: Growth phenotype was assessed by streaking out successively for up to 4X streakout from freshly dissected spores; successive streakouts were then reassembled on the same plate, growth properties were scored blind, using an arbitrary scale of 4 (like wildtype) to 0 (no growth). Because of the variability of the senescence phenotype, multiple (15-32) spore colonies of each genotype were characterized in this manner. Whether growth of one mutant strain was better than another was determined by comparing the percent streakouts of each genotype showing the same growth characteristic at each successive streakout.

Semi quantitative dilution analysis vs. serial streakout analysis: Serial streakout analysis was done as described above. At the point where successive streakouts were reassembled, cells from the colonies used for reassembly were resuspended in water, counted by a hemacytometer and equivalent numbers of cells were placed in microtiter dishes. 10 fold dilutions were done in dishes and cells were stamped onto rich media plates. Dilutions and streakouts were grown for 2-3 days at 30°C.

Competition experiments: Cells from spore colonies of the desired genotypes (wildtype, *est2-Δ, msh2-Δ, est2-Δmsh2-Δ*) were grown to log phase in rich media. Cells from log cultures of genotypes to be compared were counted by hemacytometer and 10^4 cells/ml of two genotypes were resuspended in 10 ml rich media. At 24, 55, 91, and 127 hours, cells from each culture were counted by hemacytometer, dilutions were plated for viability and genotype determinations, and $2X10^4$ cells/ml were used to inoculate 10 ml rich media. Percentage of each genotype in a culture was determined by replica plating onto -ura or G418 containing plates since *EST2* is disrupted by URA3 and *MSH2* is disrupted by KANMX2.

DNA preparations, southerns, Y' quantitation

Yeast genomic DNA preparations and southerns to quantitate Y' element amplification were performed as previously described (Lundblad and Blackburn, 1993). Phosphorimager quantitation was done to quantitate the long and short Y' length bands compared to an internal control band.

Results

Mismatch repair gene mutations enhance telomerase-independent survival

In *Saccharomyces cerevisiae,* the mismatch repair protein Msh2 binds mismatches either in complex with Msh3 or Msh6 (Strand et al., 1995; Johnson et al., 1996; Alani, 1996; Marsischky et al., 1996). Pms1 and Mlh1 are required to bind to the Msh/DNA complex in order to initiate mismatch repair (Prolla et al., 1994). Mutations in *MSH2*, *MLH1* and *PMS1* have been shown to increase homeologous recombination frequencies (Bailis and Rothstein, 1990; Datta et al., 1996; Chen and Jinks-Robertson, 1998; Kramer et al., 1989; Alani et al., 1994). Msh3 and Msh6 have somewhat redundant functions, such that Msh6 prefers to bind single base mismatches whereas Msh3 binds loop mismatches (Marsischky et al., 1996).

To test the effect of mismatch repair mutations on telomerase-independent survival, I compared the growth phenotypes of serial streakouts from multiple independent isolates of telomerase-minus cells and telomerase-minus cells that were also *msh2-Δ, mlh1-Δ, pms1-Δ, msh3-Δ, msh6-Δ or msh3-Δmsh6-Δ*. At a time point in senescence where the single telomerase-deficient cells displayed minimal colony forming ability, telomerase-deficient cells that were also mutant for *MSH2, PMS1, MLH1* or *MSH3MSH6* showed a greatly enhanced colony forming ability (Figures 1-4). Cells mutated in *MSH3* or *MSH6* showed little or no survival enhancement, as expected from the redundancy in the roles of these genes (Figure 4).

In an effort to establish a more quantitative basis for growth comparisons, semi quantitative dilution analysis and competition experiments were done for a subset of *est2-Δ* vs. *est2-Δmsh2-Δ*

samples. These experiments support the validity of the more qualitative streakout senescence assays: Qualitative growth assessment by serial streakouts paralleled the more quantitative growth assessment by 10-fold dilution series that started with equivalent numbers of cells (data not shown). In five independent competition experiments, a single *est2-Δ* mutant was competed out by an *est-Δmsh2-Δ* mutant, where *msh2-Δ* by itself had no growth advantage over wildtype under these conditions (data not shown).

The survival enhancement effect of mismatch repair gene mutations on telomerase-independent survival is RAD52-dependent

A telomerase-deficient strain that is *rad52-Δ* can not be propagated past about 60 generations of growth; therefore it is not possible to get any survivors in the absence of Rad52. I compared the growth (by multiple streakout analysis) of *est2-Δrad52-Δ* with strains that were also deleted in *MSH2, MLH1, PMS1, MSH2 or MSH6* and found that it is not possible to get survival past about 60 generations in the absence of Rad52 even when mismatch repair genes are mutated (Figure 5). This suggests that the enhancement of telomerase-independent growth by mismatch repair gene mutations is *RAD52*-dependent and not a bypass effect.

Mismatch repair gene mutations have little or no enhancement effect on Y' amplification in survivors

If the recombination events that are increased in frequency in the absence of mismatch repair are due to Y' - Y' recombination events, then I would definitely expect the telomerase-independent growth enhancement to be accompanied by a large increase in Y' amplification levels as well. If the recombination events that are primarily increased in frequency are $G_{1\text{-}3}T$ - $G_{1\text{-}3}T$ recombination events, then I expected to see either little or no increase in Y' amplification levels to accompany the telomerase-independent survival enhancement effect of mismatch repair mutations.

At a time point in senescence where telomerase-independent survival is clearly enhanced by the absence of mismatch repair genes *MSH2, MLH1, PMS1*, or *MSH3MSH6*, there was either no increase (Figure 6A, Figure 7) or at most a two-fold increase in Y' amplification over the Y' level of a single telomerase-deficient strain (Figure 6B, 6C)

Mutations in genes of a pathway intersecting with the mismatch repair pathway, the single strand annealing pathway, have no detectable effects on telomerase-independent survival

The *RAD1, RAD10, and EXO1* genes are required for the single strand annealing pathway of direct repeat recombination, where the end product is loss of sequences between tandem repeats (Ivanov and Haber, 1995; Ivanov et al., 1996). Deletions of *MSH2* and *MSH3* result in substantial decreases in SSA products where homeologous sequences are forced to recombine in a certain orientation (Paques and Haber, 1997; Saparbaev et al., 1996; Sugawara et al., 1997). In addition, *EXO1* was found to have a two-hybrid interaction with *MSH2* (Fiorentini et al., 1997; Tishkoff et al., 1997). In order to determine whether or not the roles of *MSH2* and *MSH3* in SSA would need to be taken into consideration, I also tested the effects of mutations in *RAD1*, *RAD10* and *EXO1* on telomerase-independent survival by comparing multiple serial streakouts of single telomerase-minus and telomerase minus mutants that also had a mutation in one of these SSA genes (Figure 8). *RAD1, RAD10* or *EXO1* mutations have no detectable effect on telomerase-independent survival, suggesting that the role of *MSH2* or *MSH3* in SSA is not relevant in the survivor pathway.

Other

RAD52 is required for maintenance of survivors, as well as for establishment

RAD52 is required for the initial amplification of telomeric and subtelomeric sequences that results in the establishment of survivors (Lundblad and Blackburn, 1993). If these amplified sequences can be inherited stably in the absence of *RAD52,* then this gene will not be required for the maintenance of survivors. To test this, an *est1-Δrad52-Δ* strain carrying the *RAD52* gene on a *URA3* marked plasmid was used to generate 10 stably growing survivors by successive streakouts selecting for the *RAD52*-containing plasmid. For each survivor, the frequency of 5-FOA-resistance (cells that lost the plasmid) and total cell viability were determined and used to calculate the frequency of plasmid loss. Table 1 shows the frequency of pRAD52 loss of *est1-Δrad52-Δ*pRAD52 survivors after about 250 generations of growth. Frequency of loss of pRAD52 if these survivors is about 300 fold lower than that of wildtype/pRAD52 on average implying that *RAD52* is required for the maintenance of survivors, as well as for their establishment.

Conclusions and Future Work

The absence of substantial Y' amplification increases in mismatch repair mutant survivors suggest a Y'-amplification independent component to the survival enhancement effect. To pursue this more directly, we are developing a telomeric recombination assay to measure frequencies of $G_{1\text{-}3}T$ - $G_{1\text{-}3}T$ recombination in *S. cerevisiae*. This assay will also help us answer other interesting questions such as whether or not a telomerase-deficient strains go through a "crisis" like stage where a burst of telomeric recombination helps a subpopulation of cells survive. Approaching this question from a different perspective, I am also in the process of testing whether or not telomerase-deficient strains exhibit an epigenetic suppression of mismatch repair proficiency at any point in senescence, possibly leading to enhanced telomeric recombination frequencies to allow survival.

We are also interested in investigating whether or not mismatch repair defects in other organisms enhance survival in the absence of telomerase. I am in the process of cloning the *MSH2* gene of *Kluvyeromyces lactis. K. lactis* is a budding yeast that also has a *RAD52*-dependent pathway of telomerase-independent survival pathway (McEachern and Blackburn, 1996). Contrary to *S. cerevisiae*, *K. lactis* has perfectly homologous telomeres but there is very little information available about its subtelomeric region sequence elements. It will be interesting to see if possibly through an effect on somewhat heterogeneous subtelomeric regions, a mismatch repair mutation in *K. lactis* can enhance telomerase-independent survival.

Mismatch repair mutations in the human homologues of *MSH2, MLH1, PMS1* and *PMS2* genes have been shown to cause Human Hereditary Non-Polyposis Colon Cancer (HNPCC), as well as some sporadic tumors (Jiricny, 1994). If the enhancement effect of mismatch repair mutations on telomerase-independent survival that we have demonstrated in *S. cerevisiae,* proves to be applicable to other organisms, it will be intriguing to investigate whether cells from HNPCC patients have a diminished requirement to activate telomerase activity for continued proliferation leading to cell immortalization.

Other

Poster presentation at the Yeast Genetics Meeting of the Genetics Society of America, at the University of Maryland, College Park. July 1998.

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Figure legends

Figure 1. *Mutations in MSH2, PMS1, and MLH1 enhance telomerase-independent survival.*
Growth of haploid *est1-Δ* or *est2-Δ* strains in the absence (upper panel) or presence of *msh2-Δ* or *mlh1-Δ* or *pms1-Δ* mutations (lower panel) assayed by successive streakout analysis, starting with cells from spore colonies from freshly dissected diploids.

Figure 2. *Survival enhancement by msh2-Δ.*
21 *est1-Δ* and 23 *est1-Δmsh2-Δ* spore colonies were used to produce serial streakout assemblies. Each senescence time point (1X to 4X) of each streakout assembly was scored in a blind manner. Numbers from 0 (no growth) to 4 (healthy growth) were assigned to each streakout taking into consideration the colony density and size, as well as heterogeneity of colony size. The graphs shown here represent the percentage of streakouts in each genotype group that exhibited a certain growth phenotype. The top panel represents the senescence progression of *est1-Δ*, the middle panel is of *est1-Δmsh2-Δ* and the bottom panel is a comparison of the senescence progression of the single vs. double mutants.

Figure 3. Survival enhancement by *pms1-Δ* and by *mlh1-Δ.*
Senescence progression of 15 *est2-Δ* and 22 *est2-Δpms1-Δ* streakout assemblies are compared in the top panel. Senescence progression of 21 samples of *est2-Δ* and 26 samples of *est2-Δmlh1-Δ* streakouts assemblies are compared in the bottom panel. (See figure 2 legend for details)

Figure 4. *Mutations in MSH6 or MSH3 have little to no effect on survival without telomerase. Mutating both genes increases survival substantially.*

Growth comparisons of *est2-Δmsh3-Δ*, *est2-Δmsh6-Δ*, or*est2-Δmsh3-Δmsh6-Δ* mutations with a single *est2-Δ* at a time point in senescence (3X) when the single *est2-Δ* mutant exhibits minimal growth.

Figure 5. *Telomerase-independent survival enhancement effect of mutations in mismatch repair genes is RAD52-dependent.*

Growth comparisons of telomerase-deficient (*est1-Δ* or *est2-Δ*), recombination deficient (*rad52-Δ*) in the presence or absence of mismatch repair mutations *msh2-Δ* (left panel), *mlh1-Δ* (right panel), *pms1-Δ*, *msh3-Δ* or *msh6-Δ* (data not shown) by serial streakouts. Multiple independent samples of each genotype were analyzed and found to produce a similar result.

Figure 6. *There is little or no increase in the extent of Y' amplification in msh2-Δ, mlh1-Δ, and pms1 survivors.*

Comparison of Y' levels normalized to an internal control band. Yeast genomic DNA was digested with XhoI, an enzyme that cuts once within Y' elements. Southerns of such DNA were probed with a poly (dG/dT) probe that recognizes telomeric sequences, including those flanking the Y' elements and a constant internal band. The Y' and control band signal intensities were determined by phosphorimager analysis. Cells for analyis were taken off of 1X, 2X or 3X streakout colonies and grown for DNA preparation. 10-20 samples of each genotype were analyzed and the mean and SD plotted.

Figure 7. *Y' amplification effects of msh3-Δ and/or msh6-Δ in survivors*

Comparison of Y' levels for 10-20 samples of each genotype from cells of 1X or 2X streakout colonies, as described in figure 6.

Figure 8. *Mutations in RAD1, RAD10 and EXO1 have no survival enhancement effect.*

Growth comparison of multiple streakout assemblies of each genotype, scored and plotted as described for figure 2.

Figure 1: Mutations in *MSH2*, *PMS1*, and *MLH1* enhance telomerase-independent survival

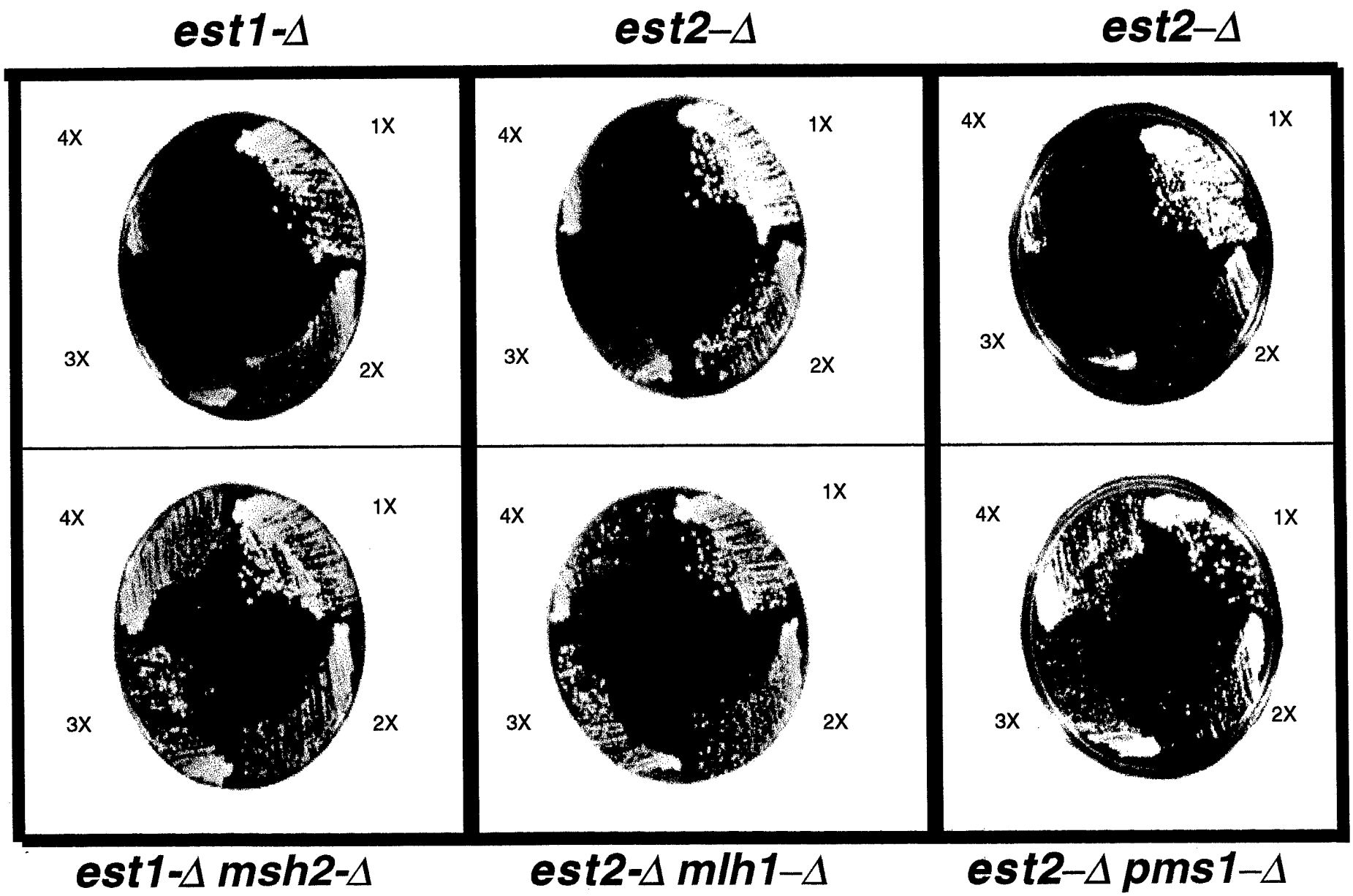


Figure 2: Survival enhancement by *msh2-Δ*

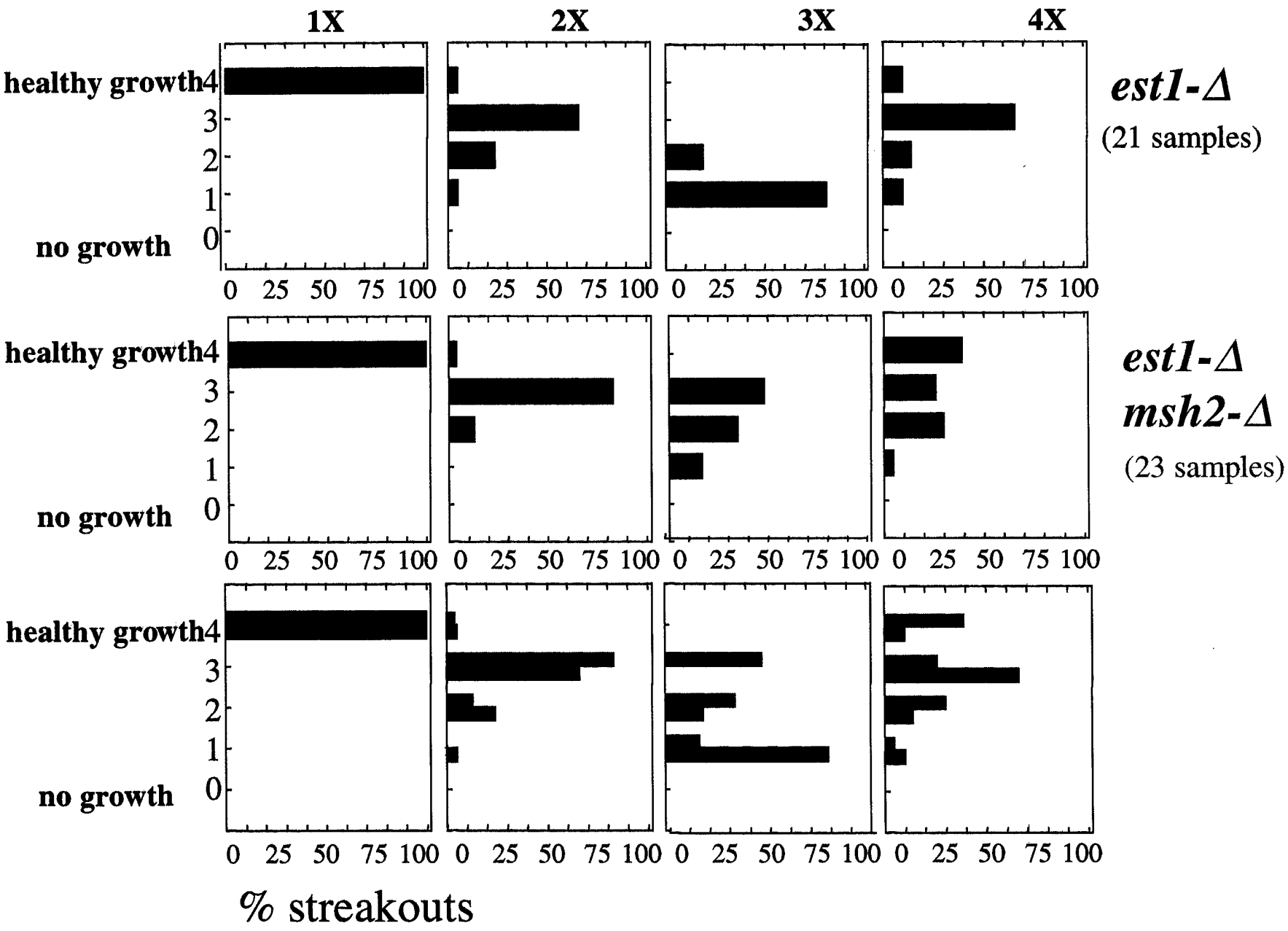


Figure 3: Survival enhancement by *pms1*-Δ and by *mlh1*-Δ

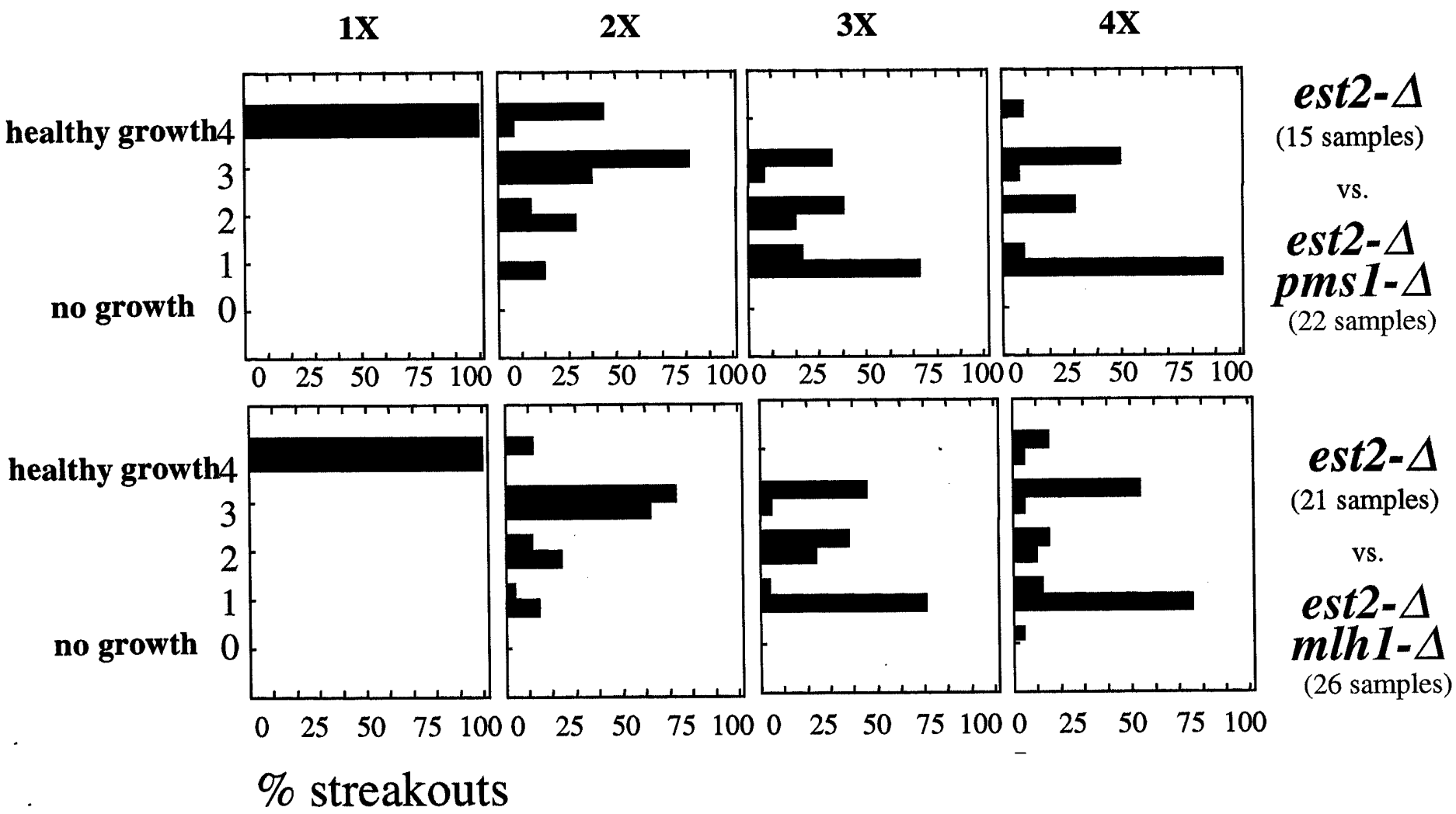


Figure 4.
Mutations in *MSH6 or MSH3* have little to no effect on survival without telomerase. Mutating *both* genes increases survival substantially.

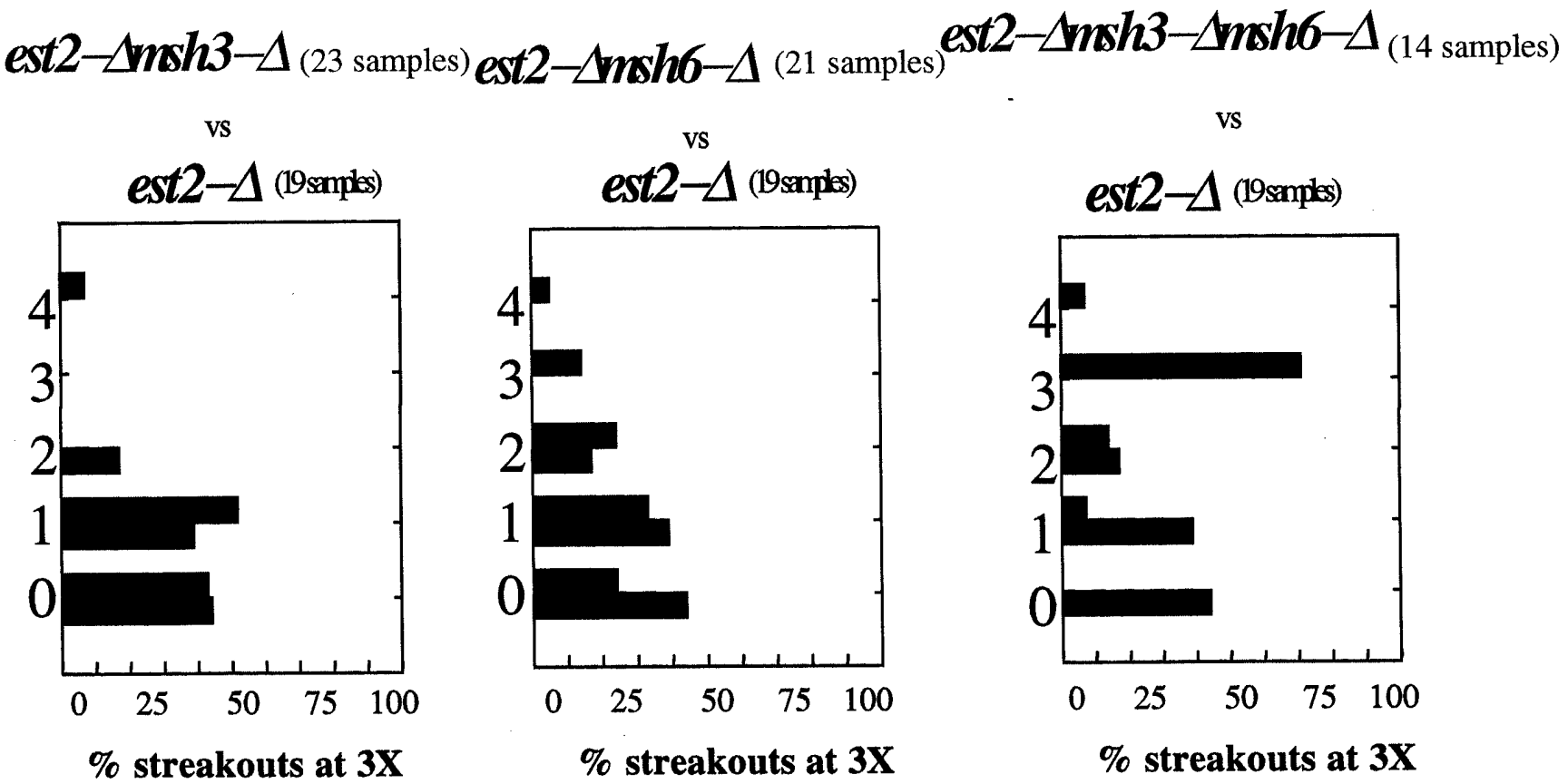
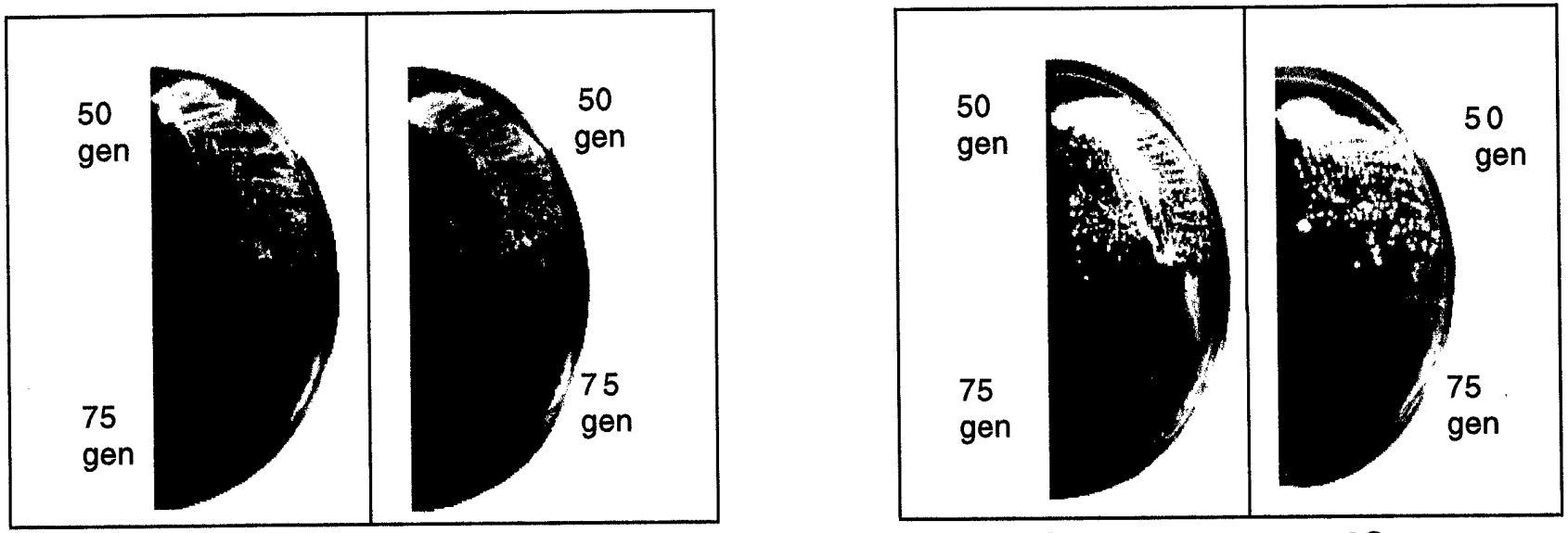


Figure 5. Telomerase-independent survival enhancement effect of mutations in mismatch repair genes is *RAD52*-dependent



est1-Δ rad52-Δ

est1-Δ rad52-Δ msh2-Δ

est2-Δ rad52-Δ

est2-Δ rad52-Δ mlh1-Δ

Figure 6. There is LITTLE or NO increase in the extent of Y' amplification in *msh2-Δ*, *mlh1-Δ*, and *pms1-Δ* survivors

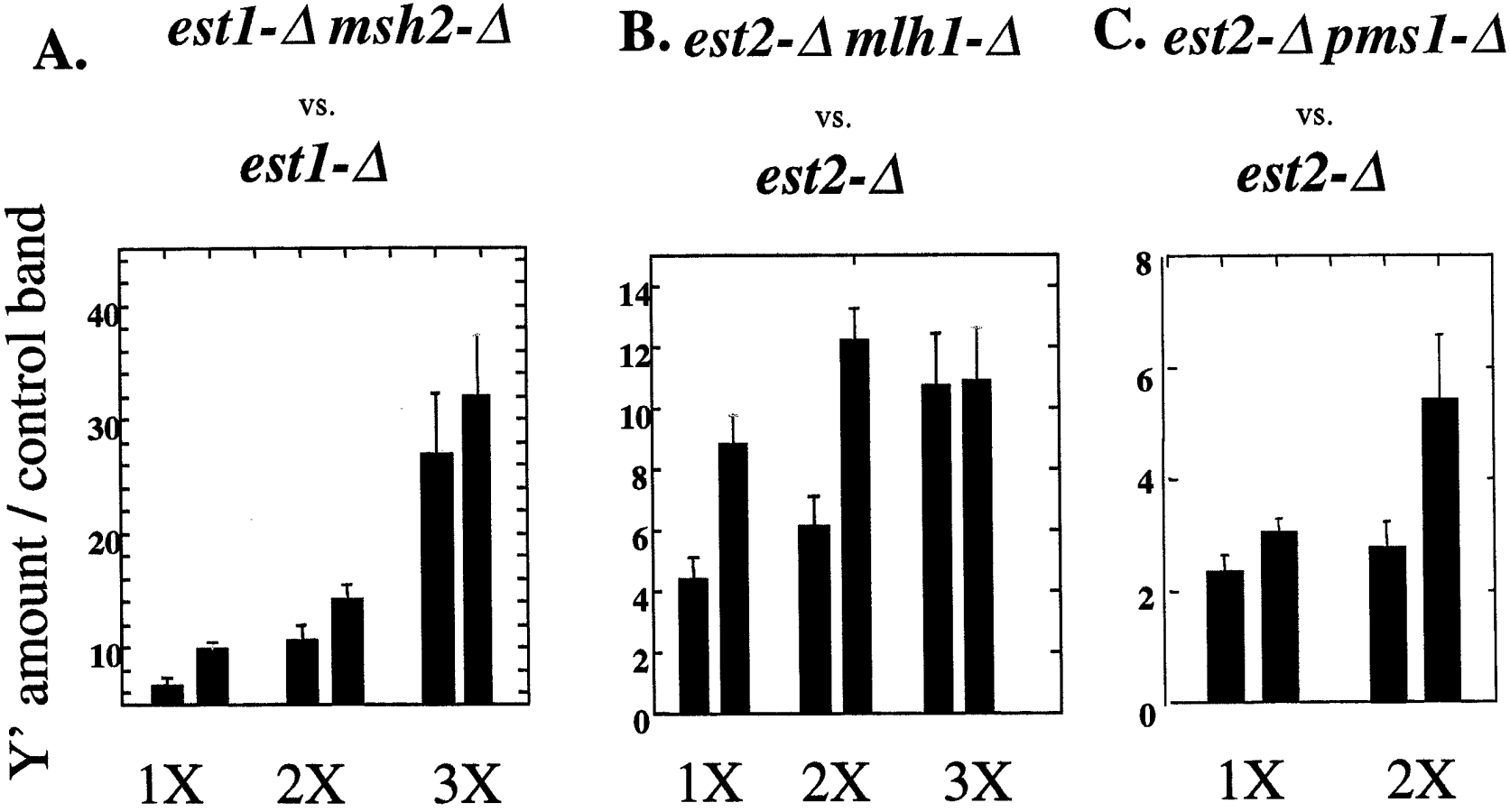


Figure 7. Y' amplification effects of *msh3-Δ* and/or *msh6-Δ* in survivors

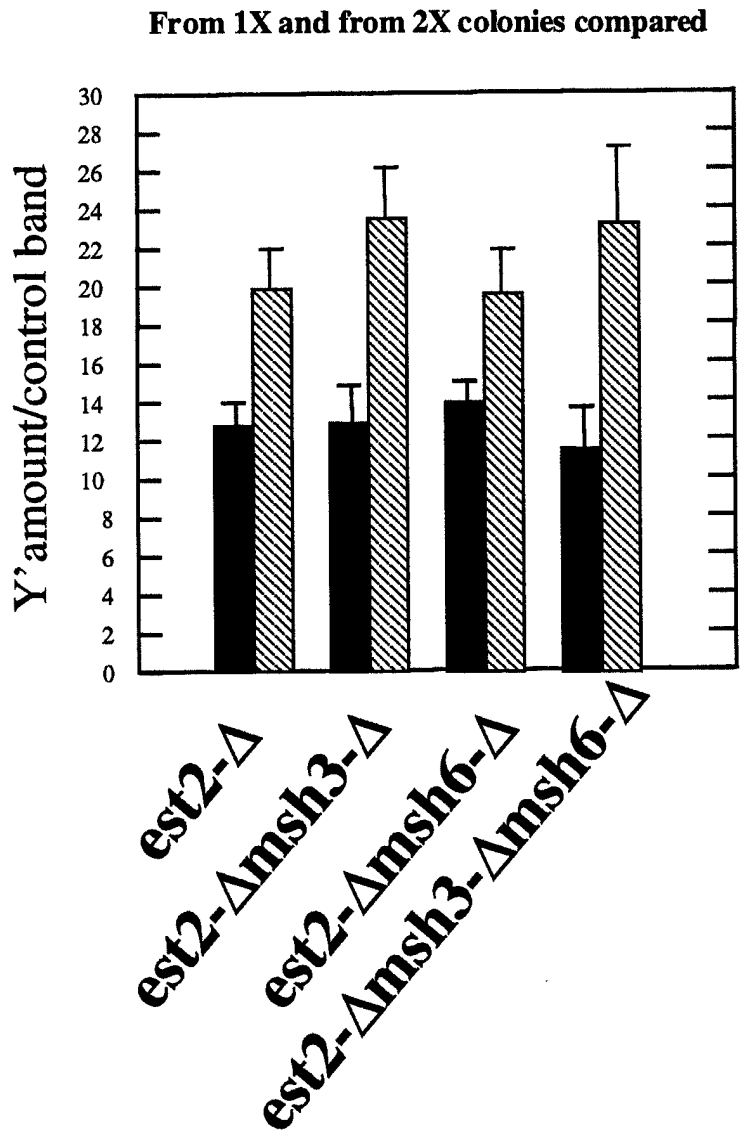


Figure 8. Mutations in *RAD1, RAD10* and *EX01* have no survival enhancement effect

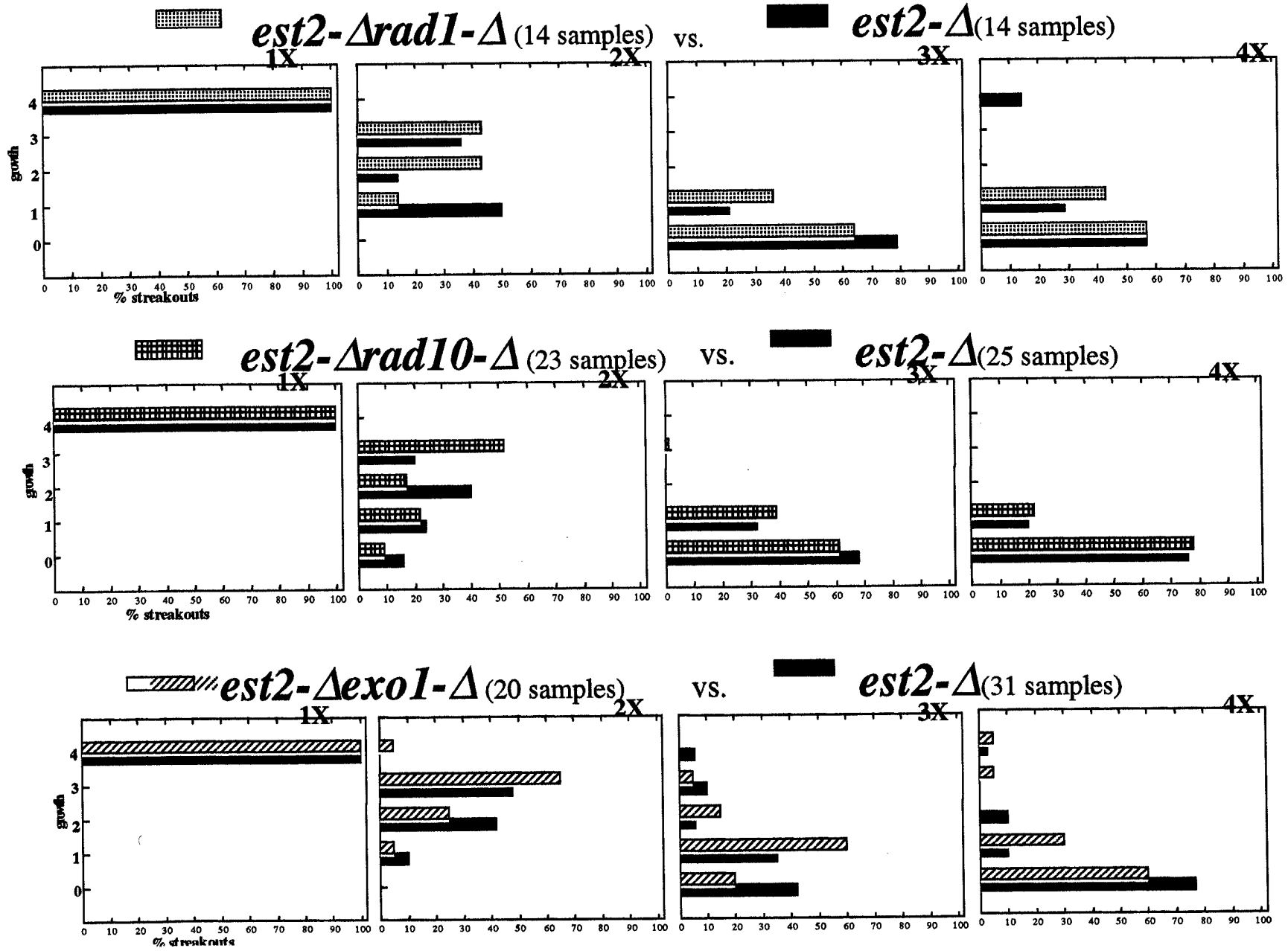


Table 1. *RAD52* is required for the maintenance of survivors

*est1⁻rad52⁻*pRAD52.URA3 stable survivors	5-FOA resistance frequency (# on 5-FOA / # viable)
survivor #1	2×10^{-3}
survivor #2	5×10^{-4}
survivor #3	1×10^{-3}
survivor #4	2×10^{-3}
survivor #5	2×10^{-3}
survivor #6	1×10^{-3}
survivor #7	4×10^{-4}
survivor #8	4×10^{-4}
survivor #9	1×10^{-6}
survivor #10	1×10^{-6}
AVERAGE	1×10^{-3}
Control (wildtype pRAD52.URA3)	3×10^{-1}