

## Effects of an unusual poison identify a lifespan role for Topoisomerase 2 in *Saccharomyces cerevisiae*

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### ABSTRACT

A progressive loss of genome maintenance has been implicated as both a cause and consequence of aging. Here we present evidence supporting the hypothesis that an age-associated decay in genome maintenance promotes aging in *Saccharomyces cerevisiae* (yeast) due to an inability to sense or repair DNA damage by topoisomerase 2 ( $\gamma$ Top2). We describe the characterization of LS1, identified in a high throughput screen for small molecules that shorten the replicative lifespan of yeast. LS1 accelerates aging without affecting proliferative growth or viability. Genetic and biochemical criteria reveal LS1 to be a weak Top2 poison. Top2 poisons induce the accumulation of covalent Top2-linked DNA double strand breaks that, if left unrepaired, lead to genome instability and death. LS1 is toxic to cells deficient in homologous recombination, suggesting that the damage it induces is normally mitigated by genome maintenance systems. The essential roles of  $\gamma$ Top2 in proliferating cells may come with a fitness trade-off in older cells that are less able to sense or repair  $\gamma$ Top2-mediated DNA damage. Consistent with this idea, cells live longer when  $\gamma$ Top2 expression levels are reduced. These results identify intrinsic  $\gamma$ Top2-mediated DNA damage as a potentially manageable cause of aging.

### INTRODUCTION

The longevity of an organism is determined by a confounding combination of genes and environment. Lifespan assays in model organisms have identified numerous longevity genes—those that when mutated or when over- or underexpressed affect mean and/or maximum lifespan—with primary roles in many cell processes, including genome maintenance, metabolism, mitochondrial function, and oxidative stress [1, 2]. The lifespan of *Saccharomyces cerevisiae* (yeast) is measured using chronological or replicative models, both of which respond similarly to environmental interventions such as caloric restriction and oxidative stress [3]. While there is no correlation between the chronological and replicative lifespans of natural yeast isolates [4], the two methods have yielded overlapping

sets of longevity genes, and both have identified longevity pathways or genes with relevance to mammalian aging. The standard method for quantifying replicative lifespan (RLS) is slow and tedious because it requires the manual microdissection of daughter cells from their mothers, but see [5, 6]. In this study we describe improvements to the high throughput capable Death of Daughters (DeaD) RLS proxy assay [7], and employ it in a screen for small molecules that shorten RLS without affecting proliferation or viability.

Here we characterize LS1, a RLS shortening molecule that acts by poisoning yeast topoisomerase 2 ( $\gamma$ Top2).  $\gamma$ Top2 is an essential enzyme that generates transient double strand breaks (DSBs) in order to relieve positive and negative DNA supercoils during replication, transcription and DNA repair, and to separate tangled

(concatenated) chromosomes prior to mitosis [8]. Top2 poisons stabilize Top2-DNA covalent complexes (Top2ccs) in which the 5'-phosphate ends of the double strand break are linked to the enzyme via phosphotyrosine ester bonds. If left unrepaired Top2ccs disrupt transcription and replication and lead to genome instability, senescence and cell death [9, 10]. Top2 poisons include widely used chemotherapeutic drugs, but are also found in foods and in the environment [11]. DNA abasic sites, alkylated bases, and UV-induced lesions are also capable of stabilizing Top2-DNA adducts [11]. This is relevant to the potential role of Top2 in aging because all of these base modifications and lesions accumulate in aging cells and tissues [12]. Genetic programs that recognize and repair Top2ccs, including specific tyrosyl phosphodiesterases and nucleases [13-15] and DNA repair systems [16, 17], support the notion that Top2ccs are a normal fact of life. Chemotherapeutic Top2 poisons kill dividing (cancer) cells by overloading these remediation mechanisms with large numbers of Top2ccs.

The simplest explanation for our results is that LS1 is selectively toxic to aging cells that have a diminished capacity to repair Top2ccs. In support of this hypothesis we show that reducing *TOP2* expression is sufficient to extend RLS. Previous evidence in support of the DNA damage theory of aging is based on observations and experiments that link DNA damage to reductions in longevity. Our results show that native levels of DNA damage by  $\gamma$ Top2 are a direct cause of aging.

## RESULTS

### Death of Daughters (DeaD) assay recapitulates aspects of replicative aging

The DeaD assay is a high throughput proxy for the standard RLS microdissection assay, which is slow and labor intensive [7]. The DeaD assay is based on the W303R derived strain K6001 that was engineered to study mother cell specific mating type switching [18, 19]. As described in the Supplementary Data we reconstructed and greatly improved the performance of the original DeaD strain (Supplemental Figs. S1, S2 & S3). DeaD strains contain two chromosomal copies of the essential *CDC6* gene, one under the control of the inducible *GALI* promoter and the other under the control of the mother-specific *HO* promoter. In galactose-containing medium, both mothers and daughters express *CDC6*, predominately from the *GALI* promoter, and divide exponentially. In glucose-containing medium, daughter division is strongly inhibited due to the lack of expression of *CDC6* from either promoter, and the growth rate and saturation point of the culture becomes limited by the reproductive

capacity of the mother cells rather than nutrient limitation.

The DeaD assay recapitulates lifespan shortening and extension associated with under- and over-expression of *SIR2* (Fig. S3). The DeaD assay also recapitulates lifespan shortening by a number of gene deletions previously shown by microdissection assay to shorten RLS, including *sir2 $\Delta$* , *sgs1 $\Delta$* , *rad9 $\Delta$* , *rad51 $\Delta$* , *rad52 $\Delta$* , *phb1 $\Delta$* , and *isw1 $\Delta$*  (Fig. S4), but does not show RLS extension in many strains containing gene deletions known to extend RLS (including *tor1 $\Delta$*  and *hxx2 $\Delta$* ). Therefore, in this report we focused on the demonstrated utility of the DeaD assay to report RLS shortening.

### High throughput screen for small molecules that shorten replicative lifespan

Small molecules that reduce DeaD cell growth under restrictive conditions, but minimally affect growth under permissive condition, are candidate probes that target longevity factors. An essential criterion for meaningful RLS shortening is a lack of toxicity at doses where statistically significant RLS shortening is observed. It is not interesting to shorten "apparent" RLS simply by making cells sick.

The DeaD assay was used to screen a 138,758 compound NIH small molecule library for those that reduced growth in glucose (restrictive) without significantly affecting growth in galactose (permissive) (see PubChem Assay #AID 804). 759 active compounds were reassessed under both restrictive and permissive conditions in a 10-point 2-fold dilution series ranging from 0.098 - 50  $\mu$ M (see PubChem Assay #AID 849). The top 44 DeaD lifespan-shortening compounds were ranked according to their ratios of restrictive growth/permissive growth (Supplementary Methods). Here we describe studies on the biological activities and mechanism of action of LS1 (6H-Indolo [2,3-b] quinoxaline: SID 4264584).

### LS1 is a potent lifespan shortener

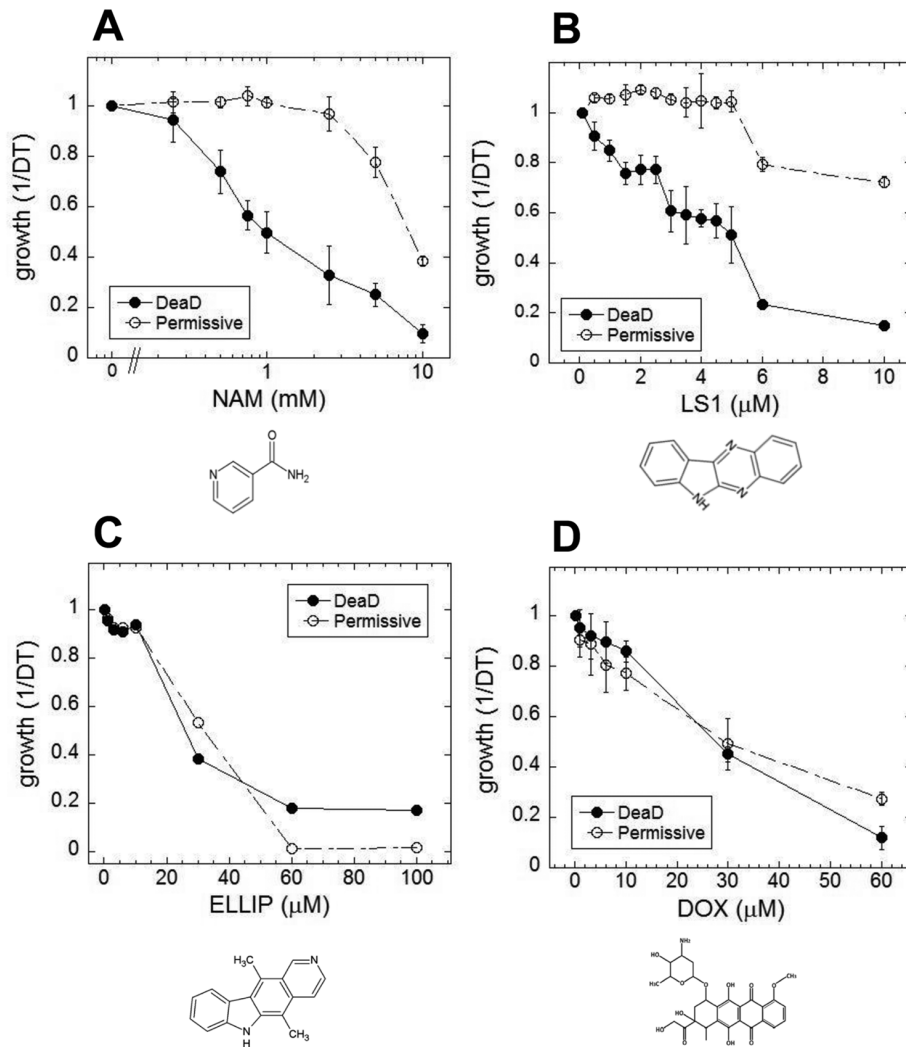
In yeast, nicotinamide (NAM) shortens RLS largely due to the compound's activity as a feedback inhibitor of Sir2, though it inhibits other yeast sirtuins that affect lifespan and also prevents lifespan extension by caloric restriction by a sirtuin-independent mechanism [20-22]. As shown in Fig. 1A, NAM shortens DeaD lifespan at concentrations ( $IC_{50}$  = 1 mM) where it has no discernable effect on permissive growth. Similar to NAM, LS1 also exhibits dose-dependent DeaD lifespan shortening (DeaD  $IC_{50}$  = 5  $\mu$ M) at concentrations where permissive growth is unaffected (Fig. 1B), though it is

~200-fold more potent than NAM. The DeaD assay RLS shortening activity of 1  $\mu\text{M}$  LS1 was confirmed by standard microdissection assay (Fig. 2).

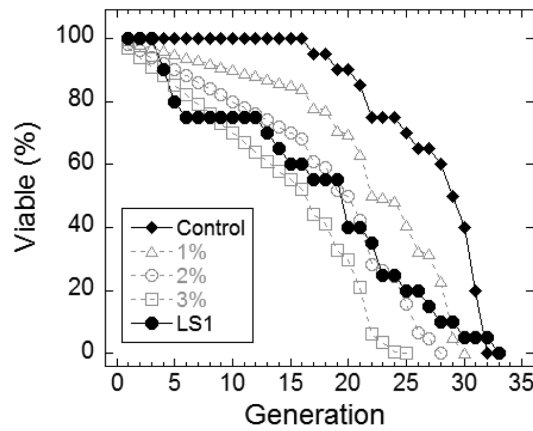
### LS1 acts independently from the role of Sir2 in ERC accumulation

The RLS of yeast is normally limited by the age-dependent accumulation of extrachromosomal rDNA circles (ERCs) [23]. ERC formation requires Fob1 and is suppressed by Sir2 activity [24, 25]. Thus *fob1* $\Delta$  cells are resistant to the lifespan shortening effects of *SIR2* deletion and NAM [24]. To test whether LS1 acts in this

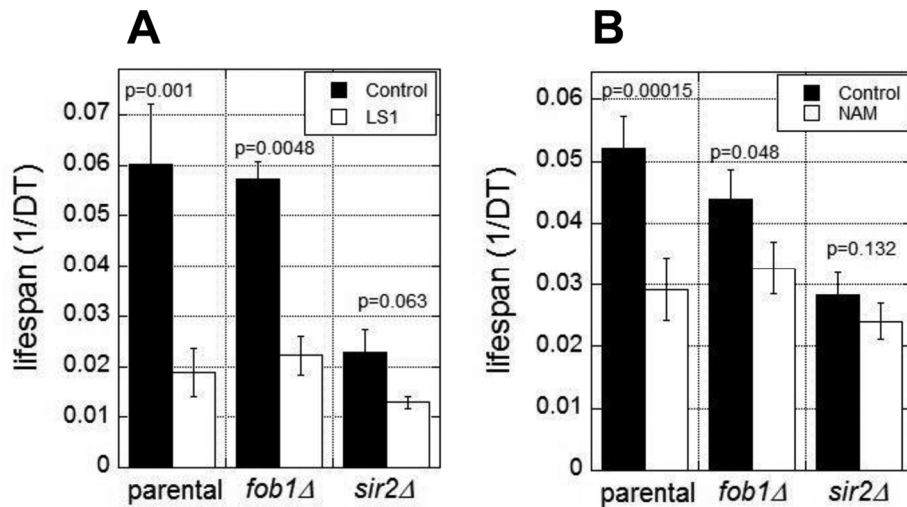
pathway, for example, by directly or indirectly inhibiting Sir2, we assessed the effects of LS1 in *fob1* $\Delta$  and *sir2* $\Delta$  strains. As shown in Fig. 3A, LS1 effectively reduced DeaD lifespans in both *fob1* $\Delta$  and *sir2* $\Delta$  strains. In contrast, NAM, which acts predominately by inhibiting Sir2, shows significantly less DeaD lifespan shortening activity in *fob1* $\Delta$  and *sir2* $\Delta$  strains (Fig. 3B). The modest lifespan reduction by NAM in *fob1* $\Delta$  and *sir2* $\Delta$  strains may be due to its' sirtuin-independent RLS-shortening activities [22]. We conclude that LS1 shortens RLS by an ERC- and Sir2-independent mechanism.



**Figure 1. Small molecule effects on DEAD lifespans.** Growth rates of DEAD strain (BB579) in the presence of increasing concentrations of nicotinamide (NAM) (A), LS1 (B), ellipticine (ELLIP) (C) and doxorubicin (DOX) (D) under nonpermissive (●) and permissive (○) conditions.



**Figure 2. LS1 shortens replicative lifespan by standard microdissection assay.** Microdissection in the presence and absence of  $1\mu\text{M}$  LS1 was performed as described in Materials and Methods. LS1 treated ( $\bullet$ ) FY839 cells had an average lifespan of 16.1 generations, a 41.5% drop from the untreated sample ( $\circ$ ). Graph shows combined data from two independent FY839 colonies. Superimposed over the experimental data are modeled data showing the effect on the lifespan of the untreated sample of adding 1% ( $\Delta$ ), 2% ( $\circ$ ) and 3% ( $\square$ ) non-age-related cell death/generation.



**Figure 3. Lifespan shortening activity of LS1 is independent of 2μ circles and Sir2.** DEAD assays were performed in the presence and absence of  $5\mu\text{M}$  LS1 (A) or  $1\text{mM}$  NAM (B) in parental (BB579), *fob1Δ*, or *sir2Δ* strains as described in Materials and Methods. Data represent the average of three biological replicates. Error bars represent standard deviation.

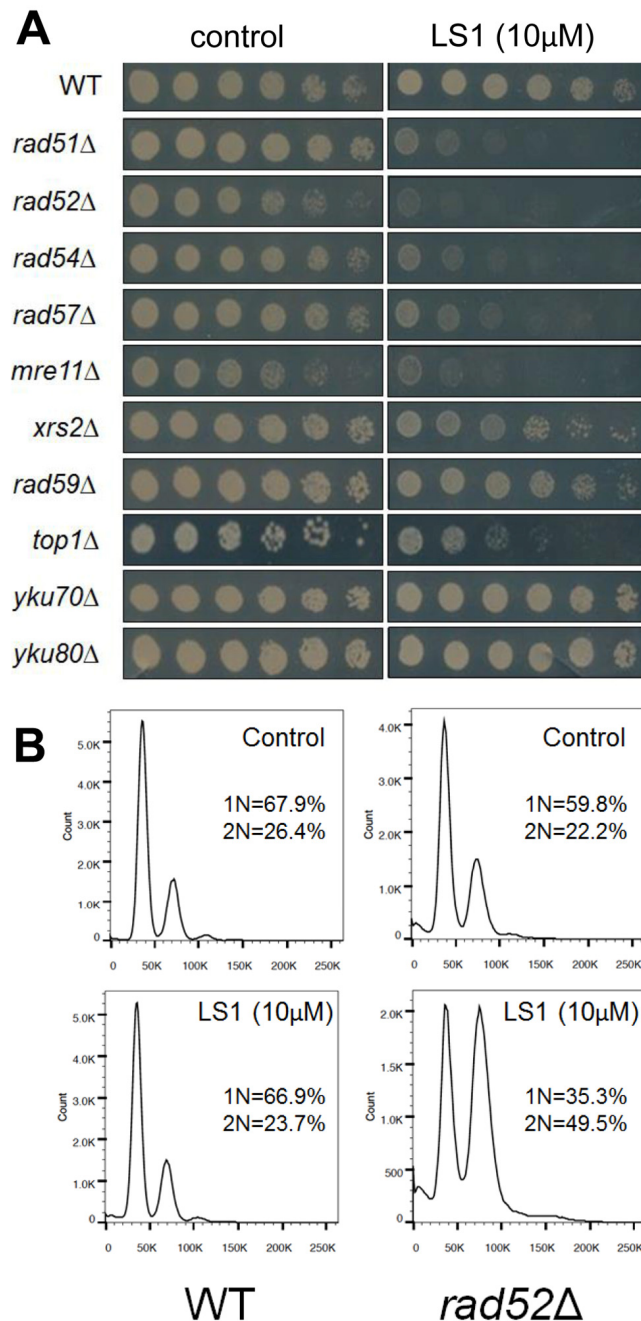
### LS1 is toxic in cells lacking homologous recombination

To gain insight into the mechanism of action of LS1 we performed a genome-wide chemical-genetic screen for deletions of nonessential genes that caused slow growth in the presence of LS1. This screen identified a set of genes belonging to the *RAD52* epistasis group required

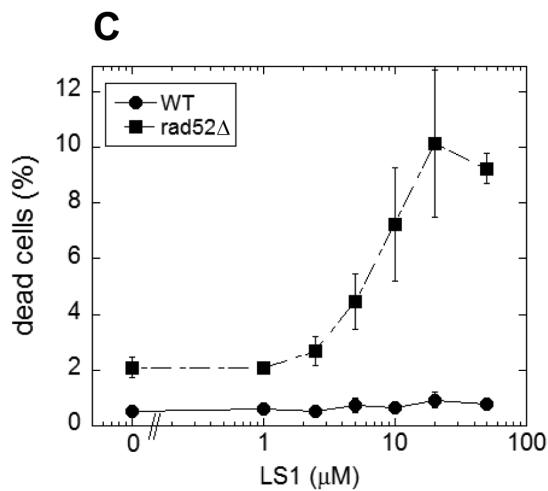
for homologous recombination. Specifically, LS1 caused a slow growth phenotype in *rad51Δ*, *rad52Δ*, *rad54Δ*, *rad57Δ*, and *mre11Δ* cells. LS1 had no vegetative growth effect on *yku70Δ* or *yku80Δ* cells that are defective in DSB repair by nonhomologous end joining (NHEJ) (Fig 4A). Thus HR, but not NHEJ, is necessary for resistance to LS1. FACS analysis revealed that LS1-treated *rad52Δ* cells tended to arrest growth at G2/M (Fig. 4B). Parental

cells treated with LS1 did not show a cell cycle phenotype. The growth defect of *top1Δ* cells in the presence of LS1 was of interest, since the only class of DNA damaging agents that have been reported to result in enhanced growth defects in *top1Δ* mutants are Top2

poisons [26]. *top1Δ* mutants are hypersensitive to Top2 poisons because these cells rely on Top2 for all topoisomerase functions that are normally shared by Top1 and Top2 [9, 10, 27]. Thus the sensitivity of *top1Δ* cells is fully consistent with LS1 being an  $\gamma$ Top2 poison.



**Figure 4. Synthetic genetic interactions of LS1 with RAD52 epistasis group deletions.** A comprehensive screen of the non-essential deletion collection revealed synthetic growth defects of LS1 together with deletions of genes required for homologous recombination. (A) Three-fold dilutions (starting at 0.1 OD<sub>600</sub>) of log phase growing cultures of parental (BY4741) and deletion strains were plated at 30°C for 48 h onto SCD agar containing 0.1% DMSO vehicle (control) without or with 10 $\mu$ M LS1. (B) DNA content by flow cytometry of LS1 treated parental BY4741 and *rad52Δ* cells. Log phase liquid cultures were treated with 0.1% DMSO vehicle (control) without or with 10 $\mu$ M LS1 for 6h followed by fixation with ethanol and propidium iodide staining as described in Materials and Methods.

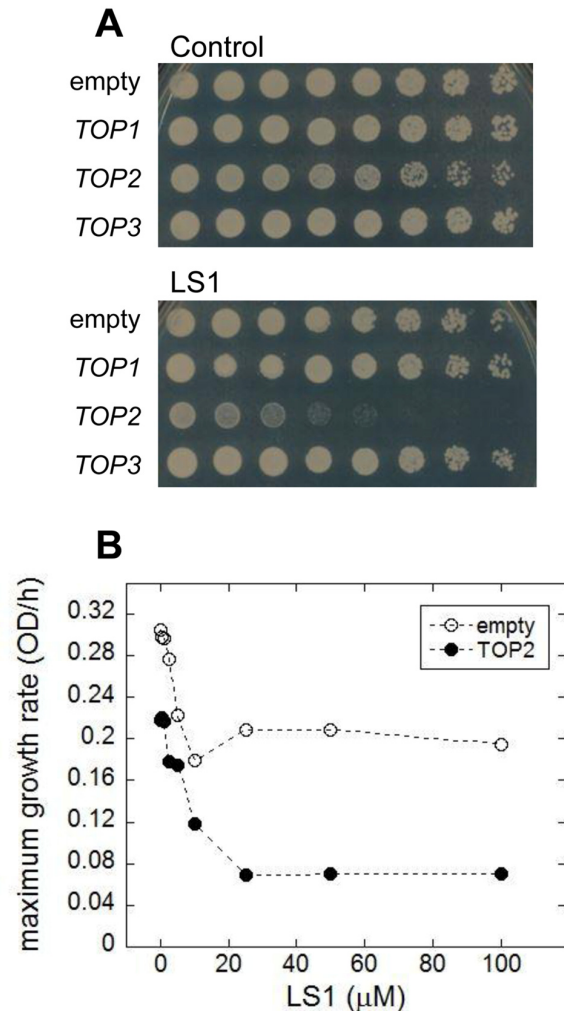


**Figure 4. Synthetic genetic interactions of LS1 with RAD52 epistasis group deletions.** (C) Quantitative effect of increasing concentrations of LS1 on cell viability in log cultures of *RAD52* and *rad52Δ* cells. Quantitation of cell viability is described in Materials and Methods.

The synthetic growth defect of LS1 in *rad52Δ* cells is quantified in Fig. 4C and shows that the slow growth phenotype of *RAD52*<sup>+</sup> cells at very high LS1 concentrations is not due to cell death (Fig. 1B). Interestingly, these data show that in the absence of LS1 *rad52Δ* log cultures contain a steady state level of ~2% dead cells compared with less than 0.5% in *RAD52*<sup>+</sup> cultures (Fig. 4C). Although a death rate of ~2% per generation would be undetectable by standard methods used to quantify growth, these levels are amplified in RLS studies. Adding 1%, 2%, or 3% non-age-associated cell death/generation reduces the mean RLS of parental cells by 19%, 31% and 45%, respectively (Fig. 2). Therefore, the observed ~30% reduction in mean RLS caused by 1 μM LS1 could be explained if LS1 killed ~2% of cells/generation. In fact, LS1 had an insignificant effect on the numbers of dead cells in exponentially dividing cultures, which carry about 0.5% dead cells/generation, even at concentrations as high as 50 μM (Fig. 4). Steady state levels of age-independent cell death in log phase *rad52Δ* cultures could be responsible for the significant fraction of the published short RLS of this and other purportedly short-lived strains [28]. Numbers of nonviable *rad52Δ* cells increased dramatically during stationary phase (not shown), consistent with the report that *RAD52* is required for full chronological lifespan [29]. We conclude that the shorter RLS of LS1-treated cells is not due to low levels of non-age-related cell death, as may be the case for strains such as *rad52Δ*.

Together, these results suggest that the lack of toxicity of LS1 toward young cells at concentrations where it

exhibits strong lifespan shortening activity in old cells could be due to an age-associated decrease in the capacity of older cells to sense and repair LS1-induced DNA damage. It follows that the lifespan shortening activity of LS1 could be explained by an age-dependent decline in DNA repair systems [2, 30]. We hypothesize that LS1 is selectively toxic in older cells that have diminished capacity to efficiently either sense or repair LS1-induced DNA damage.



**Figure 5. Overexpression of TOP2 confers hyper-sensitivity to LS1.** (A) Effect of LS1 on BY4741 transformed with high copy number Yeast Tiling collection plasmids encoding *TOP1*, *TOP2* or *TOP3* (*pTOP1*, *pTOP2*, and *pTOP3*). Threefold-dilutions of log phase cells were patched onto YPD medium containing vehicle DMSO (control) or 10μM LS1. Overexpression of *TOP2* was confirmed by quantitative western blot using secondary antibodies conjugated to infrared excitable fluorescent dyes and developed using the LI-COR fluorescent imaging system (not shown). (B) Quantitative effect of LS1 concentrations on growth of *TOP2* overexpressing strain compared to cells containing the empty vector. Maximum growth rates were calculated from triplicate growth curves generated using a BioScreen C system and BGFit webserver as described in Materials & Methods.

## LS1 is a TOP2 poison

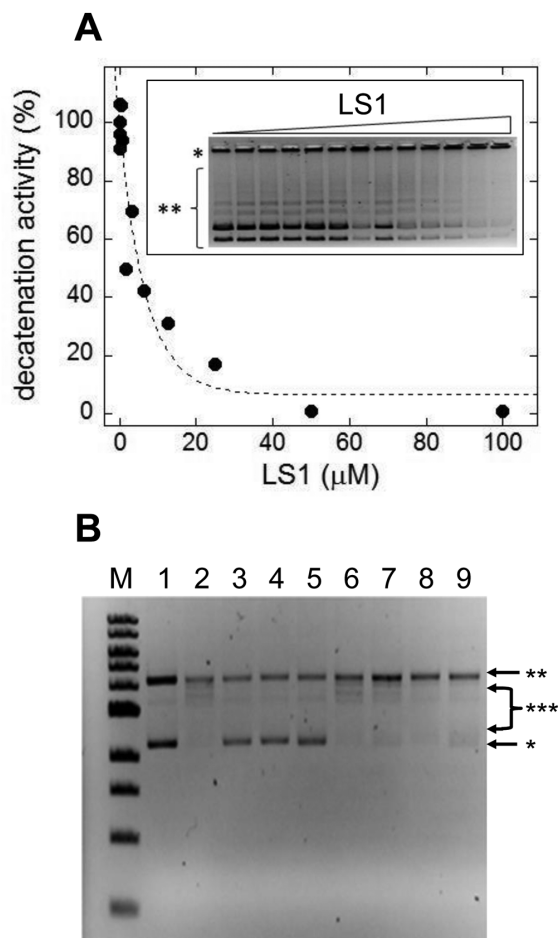
LS1 is basically the unsubstituted scaffold of ellipticine, a Top2 poison [31, 32]. If LS1 were simply a weak Top2 poison, then other Top2 poisons dosed at subtoxic concentrations might also reduce RLS. Two structurally distinct Top2 poisons, ellipticine and doxorubicin, were tested using the DeaD assay for RLS shortening activity. Ellipticine and doxorubicin shorten apparent DeaD lifespan simply by virtue of their being cytotoxic (Fig. 1C & D).

We performed a simple *in vivo* test for yTop2 poisoning by LS1. Because yTop2 participates directly in the formation of cleavage complexes, cells expressing higher levels of yTop2 have the potential to form greater

numbers of cleavage complexes and are, therefore, more sensitive to poisons [26]. Consistent with LS1 being a Top2 poison, over-expression of *TOP2*, but not *TOP1* or *TOP3*, increased LS1 toxicity (Fig. 5A). The dose-dependent toxicity of LS1 in cells expressing native or many-fold higher levels of yTop2 is also consistent with a Top2 poisoning mechanism (Fig. 5B). Interestingly, even at high concentrations LS1 did not completely inhibit growth in cells expressing native levels of yTop2. This is interesting because yeast *TOP2* is an essential gene, so strong inhibition should either be lethal or at least cause strong growth defects. It is possible that the cell's repair and remediation pathways cope with a limited number of LS1-induced Top2ccs and, in the steady state, regenerate a sufficient pool of Top2 to perform essential roles. In contrast, doxorubicin and ellipticine completely inhibited growth of cells expressing native *TOP2* levels (Fig. 1C & D). Higher concentrations LS1 had even greater inhibitory effects on the growth of cells overexpressing *TOP2*, indicating that presumptive remediation pathway(s) can be overwhelmed (Fig. 5B).

Based on these *in vivo* results we directly tested LS1 for inhibition of Top2 enzyme activity. Fig. 6A shows that LS1 inhibited kinetoplast DNA decatenation by human Top2 $\alpha$  ( $IC_{50} = 3 \mu M$ ). Unlike some Top2 poisons, including ellipticine and doxorubicin, LS1 did not detectably intercalate into DNA (Fig. 6B). This finding is consistent with a previous study concluding that LS1 does not bind DNA in contrast to other quinoxaline analogs [33]. A lack of DNA binding or intercalation ability does not by itself explain the lack of toxicity of LS1, since etoposide, another well studied, nonintercalating Top2 poison is cytotoxic.

Top2 poisons induce the accumulation of DSBs, either by promoting the formation of Top2cc adducts or by slowing their re-ligation, making them aberrantly long-lived. Parenthetically, bisdioxopiperazines such as ICRF-193 promote non-covalent but tightly bound Top2 which are toxic because they interfere with transcription and other DNA metabolic processes; however, because the toxicity of these molecules is not enhanced by mutations in the *RAD52* pathway it is unlikely that DSBs are generated [34, 35]. In support of the conclusion that LS1 is a true Top2 poison, we show that



**Figure 6. LS1 is a nonintercalating TOP2- $\alpha$  inhibitor.** (A) Effect of LS1 on decatenation of *C. fasciculata* kinetoplastid DNA (kDNA) by purified human TOP2- $\alpha$ . Inset shows a negative image of the ethidium bromide stained agarose gel separating kDNA species. Quantitation of decatenated kDNA was done with GelQuant.NET 1.8.2 software and results were plotted using KaleidaGraph 4.1.0 software. (B) A DNA topoisomerase I (Top1) DNA unwinding assay was used to assess the ability of LS1 to intercalate as indicated in Methods. Known Top2 poisons that either intercalate (doxorubicin) or do not (etoposide) were included as controls. An *E. coli*-compatible plasmid (puc18) exhibiting both supercoiled (\*) and nicked/relaxed (\*\*) forms was used as the substrate (lane 1). In the absence of an intercalator (lane 2) Top1 converts the plasmid to fully nicked/relaxed (\*\*) or intermediate relaxed forms (\*\*\*). Intercalation was assessed for DOX (10 $\mu M$  or 50 $\mu M$ ; lanes 3-4), ELLIP (100 $\mu M$ ; lane 5), 100mM ETOP (lane 6) and LS1 (10 $\mu M$ , 50 $\mu M$ , or 100 $\mu M$ ; lanes 7-9).

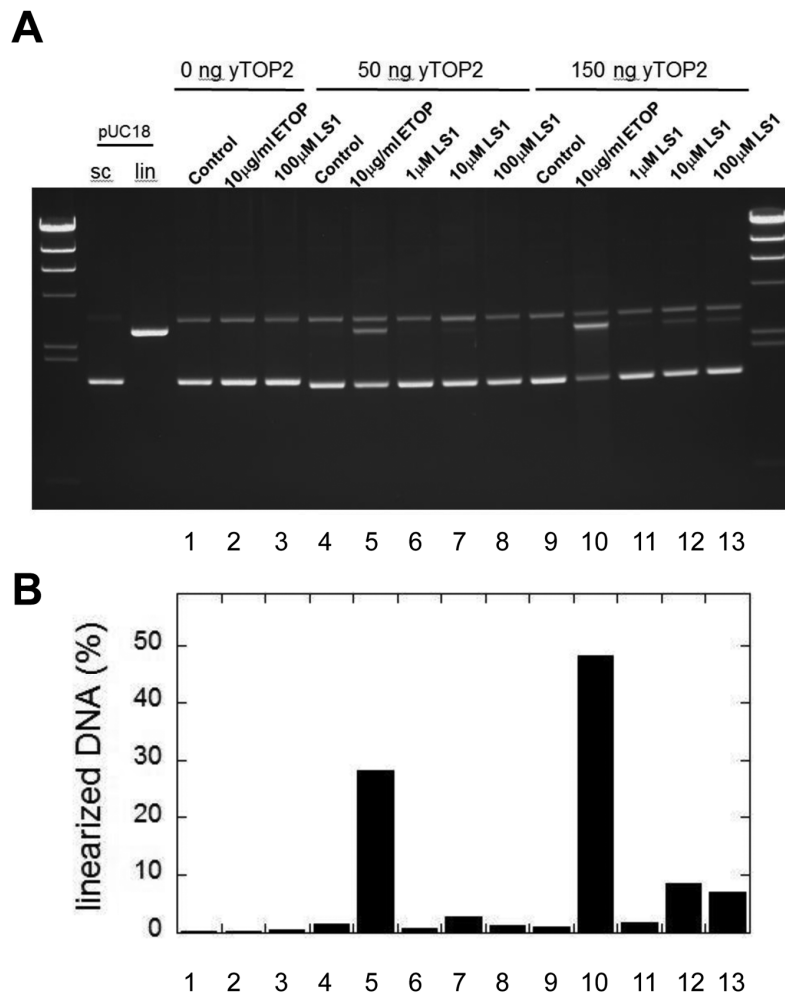
LS1 promotes the *in vitro* formation by yTop2 of DSBs in plasmid DNA (Fig. 7A & B), albeit less efficiently than etoposide.

### LS1 enhances the potency of chemotherapeutic Top2 poisons in yeast and cancer cells

We hypothesized that LS1 induces the formation of Top2cc adducts that, in the absence of robust homologous recombination, cause cell death by the same mechanism—DNA damage—as classic Top2 poisons. However, unlike other Top2 poisons, LS1 is not toxic at concentrations where it has strong effects on RLS (Fig. 1B). We reasoned that LS1-Top2ccs might be more prone to lethal poisoning by a second more toxic poison. Thus LS1 could at least transiently target Top2 to DNA, effectively increasing the target population for attack by lethal Top2 poisons. As a test we quantified 0, 1 & 5  $\mu\text{M}$  etoposide-induced cell death in the presence

and absence of 0 -100  $\mu\text{M}$  LS1 in yeast cells expressing native or overexpressed levels of *TOP2*. In cells expressing native levels of Top2, etoposide exhibited little toxicity with or without LS1 (Fig. 8A). As expected from a Top2 poison, etoposide exhibited a strong dose-dependent toxicity in cells overexpressing *TOP2* (Fig. 8B). A physiologically relevant concentration of 10  $\mu\text{M}$  LS1, which alone caused less than ~2-fold cell death in *TOP2* overexpressing cells, enhanced 1 or 5  $\mu\text{g/ml}$  etoposide toxicity by 10 and 8-fold, respectively. We hypothesize that LS1 enhances etoposide toxicity by transiently stabilizing Top2ccs that serve as good targets for etoposide poisoning.

Based on the enhancement of etoposide cytotoxicity in yeast, we asked if LS1 might enhance the toxicity of Top2 poisons to human cancer cells. Doxorubicin is a frontline chemotherapeutic for both solid and liquid tumors. Enhancers have been sought because lifetime



**Figure 7. LS1 induces double strand breaks.** (A) A pUC18 plasmid linearization assay was used to determine if LS1 is capable of promoting formation of Top2cc containing double strand breaks. Assays were performed as described in Materials and Methods. (B) Percent linearized DNA quantified with GelQuant.NET 1.8.2 software.

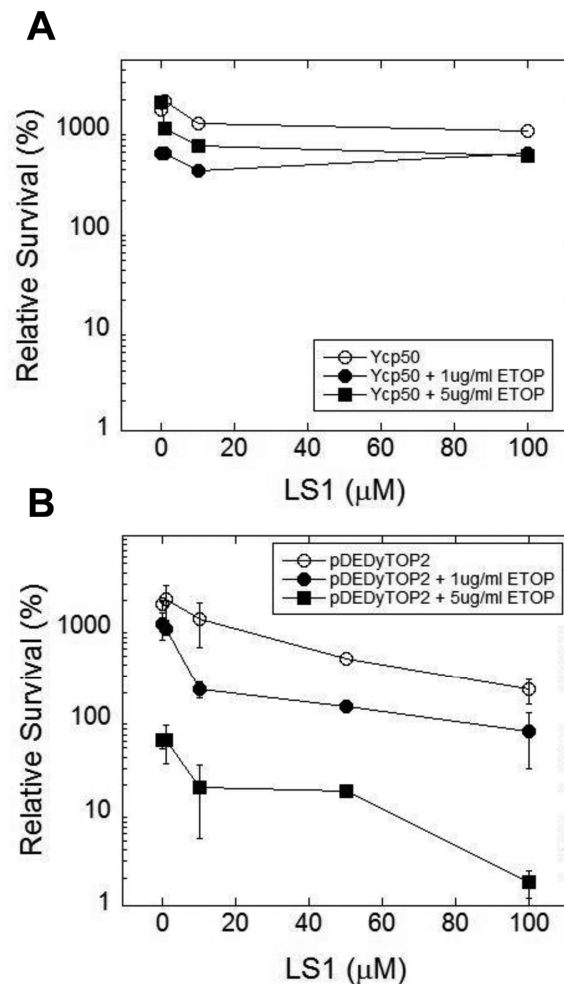


doses of doxorubicin are limited by cardiotoxicity [36, 37]. As a test of principle, human HT1080 fibrosarcoma cells were incubated with or without increasing concentrations of doxorubicin. As shown in Fig. 9A & B, LS1 alone is nontoxic to HT1080 cancer cells, but it enhances cell killing by doxorubicin up to fivefold. In contrast, LS1 did not enhance the toxicity of doxorubicin to noncancerous HCA2T human primary foreskin fibroblasts (Fig. 9C). LS1 also enhanced etoposide toxicity in HT1080 cells (data not shown). The fact that LS1 enhanced the toxicity of Top2 poisons in both yeast and human cancer cells indicates that it acts by poisoning Top2 in both species. The stimulatory interaction between LS1 and two chemically distinct Top2 poisons supports our conclusion that the physiological target of LS1 is Top2. We considered the possibility that LS1 might increase

the potency of doxorubicin and etoposide by inhibiting multidrug resistance (MDR) pumps such as P-glycoprotein. Inhibition of P-glycoprotein increases intracellular concentrations of a broad range of xenobiotics, including vinblastine, resulting in greater drug potencies [38]. Since LS1 did not increase the toxicity of vinblastine in HT1080 cells (Fig. 9A, right panel) we conclude that the enhancement of doxorubicin toxicity by LS1 is not due to direct or indirect inhibition of MDR pumps.

### Reducing Top2 activity extends RLS

The above results are consistent with the hypothesis that LS1 enhances the potency of Top2 poisons in both yeast and human cancer cells by increasing the effective concentration of Top2 and its intrinsic DNA damage-

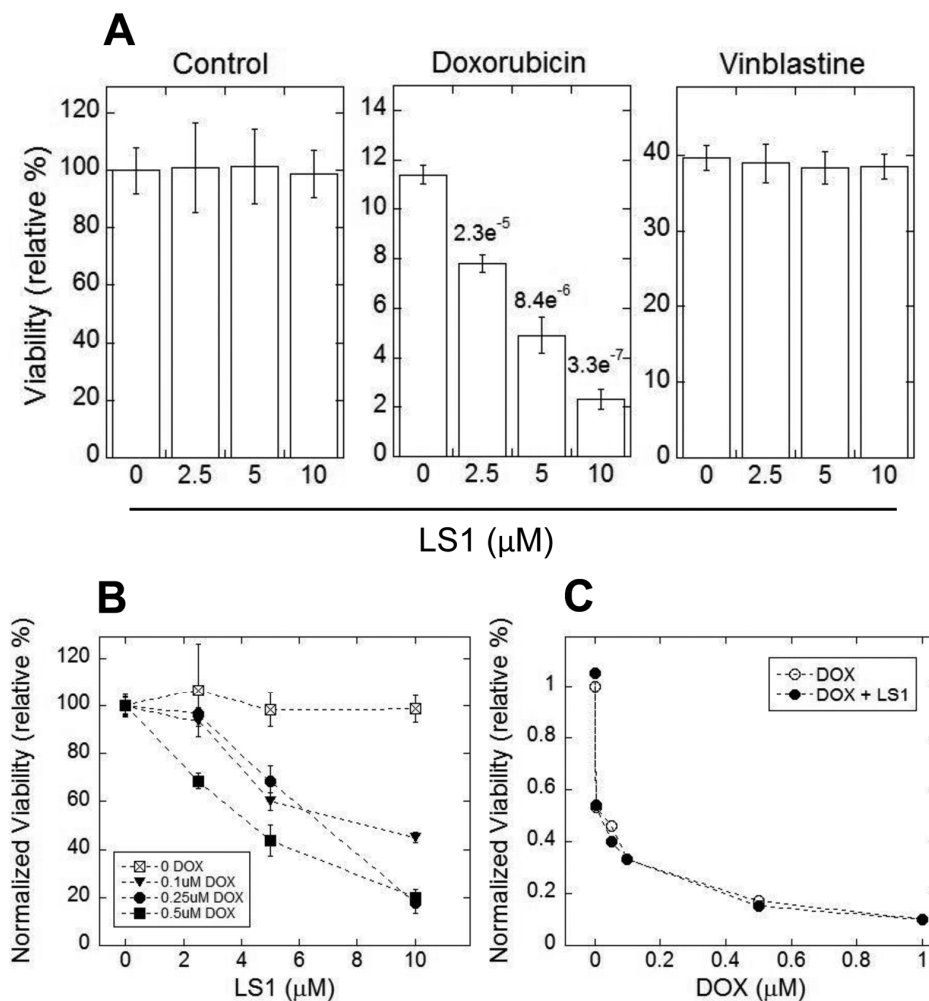


**Figure 8. LS1 enhances killing of yeast by etoposide.** LS1 enhances etoposide (ETOP) cytotoxicity in cells overexpressing *TOP2*. (A) Cells expressing native levels of *TOP2* are not sensitive to ETOP in the presence of LS1. Cells contained an empty CEN plasmid Ycp50. (B) Overexpression of *TOP2* (YCPpDED1-TOP2) increased sensitivity to ETOP in the presence of LS1. Yeast strain YMM10 [80], which contains gene deletions in several drug efflux pumps, was used to promote ETOP sensitivity.

causing activity. This model suggests that reducing  $\gamma$ Top2 activity could reduce intrinsic levels of DNA damage and actually extend RLS. Investigating the effects of reducing  $\gamma$ Top2 levels must take into account that *TOP2* is an essential gene in yeast, and that both its' strong over- and under-expression cause growth defects [39, 40]. The first approach we took was to quantify DeaD lifespans of novel hypomorphic *top2* point mutants that were isolated by virtue of being resistant to LS1 (Fig. 10A), but exhibited no growth defects (not shown). These point mutations mapped to diverse sites within the protein (Fig. S6). Cell extracts from a number of these strains showed several-fold reduced  $\gamma$ Top2 decatenation activities (Fig. 10B). As

shown for one, *top2-28* (Fig. 10C), all of these mutants conferred resistance to doxorubicin, ellipticine and etoposide. The properties of these mutants are consistent with previously characterized hypomorphic alleles that, as would be expected from general loss of function mutations, confer resistance to multiple Top2 poisons [41-43].

As shown in Fig. 11A, *top2-28* cells, which express reduced  $\gamma$ Top2 activity (Fig. 11B), exhibited a significantly longer DeaD lifespan when compared to cells expressing wild type *TOP2* (Fig. 11A). To validate and extend these DeaD assay results, we performed microdissection assays on a strain encoding an under-



**Figure 9. LS1 enhances doxorubicin killing of HT1080 fibrosarcoma cells.** (A) Cytotoxicity was measured in HT1080 cells for LS1 (left panel), DOX (middle panel), or vinblastine (right panel). (B) LS1 enhances killing of HT1080 cells at several concentrations of DOX. Experiments were performed as in (A). (C) Effect of LS1 on a primary human foreskin fibroblast cell line HCA2T that has been immortalized by transformation with the catalytic subunit of telomerase. HT1080 and HCA2T cells were seeded at low density. After 24 hours, cells were treated with DMSO (vehicle control) or DMSO containing DOX at the indicated concentrations without or with 10 μM LS1. DMSO and DOX data were based on the average of 6 biological replicates. Vinblastine data was based on three biological replicates. Average values and standard deviation of the mean are plotted where available. P-values determined by student t-test are shown for DOX.

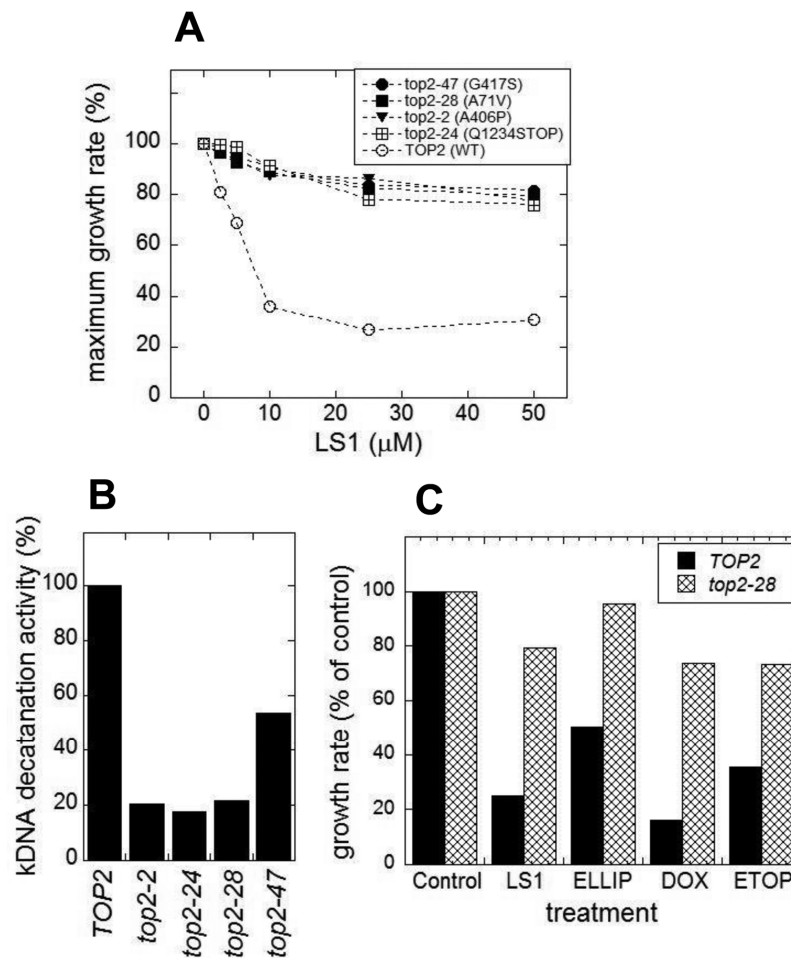
expressing *TOP2-DAmP* gene [44]. This strain expresses native *TOP2* at approximately 30% wild-type levels (Fig. 11C), but exhibited no apparent growth defect (not shown). Since the haploid strain containing the *TOP2-DAmP* allele was generated from the heterodiploid and compared to the haploid strain containing the *TOP2* wild-type allele, the strains were isogenic except at the *TOP2* locus. As shown in Fig.11D, constitutively reducing yTop2 levels extended both mean and maximum RLS.

The budding index of the terminally senescent mothers from the microdissection assays revealed that *TOP2-DAmP* cells tended to arrest more frequently as unbud-

ded (G1) cells (Fig. 11E). Low budding indexes of senescent mother cell populations indicate robust growth control at G1/S checkpoints, and correlate with longer microdissection [28] and microfluidics-based RLS assays [45-47]. Therefore, wild type levels of yTop2, which are presumably tuned to optimize proliferative growth, are deleterious in aging cells—a phenomenon known as antagonistic pleiotropy [48, 49].

## DISCUSSION

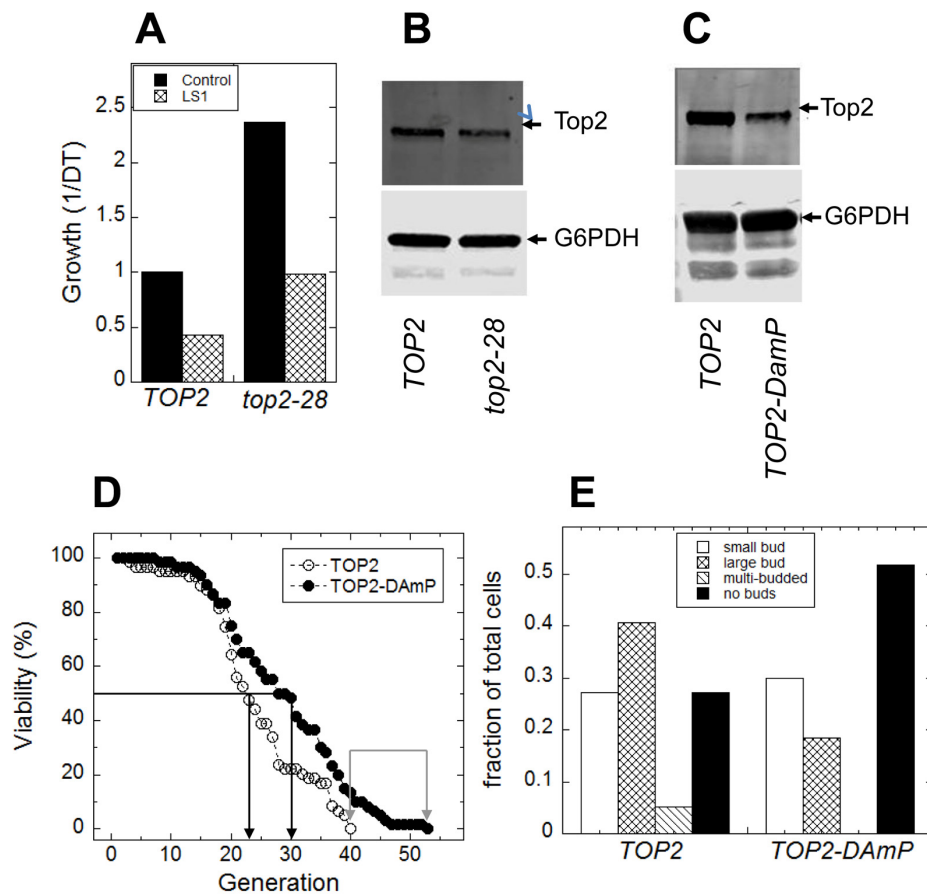
We describe novel biological properties of a drug-like molecule identified in a high throughput Dead assay screen for compounds that shorten yeast RLS. LS1 was



**Figure 10. *top2* hypomorphic mutants confer resistance to LS1 and other Top2 poisons.** LS1 resistant *top2* mutants were selected as described in Methods and Methods using the host JN394 *top2ts2-4 rad52Δ* strain in combination with hydroxylamine mutagenesis of the YCPDED1-*TOP2* plasmid that complements the chromosomal *top2ts2-4* allele. (A) LS1 sensitivity of four representative LS1 resistant *top2* alleles. Growth rates were determined using the BioScreen C system and fit using the BGFit web-server as described in Materials and Methods. LS1 resistant *top2* alleles also confer resistance to other Top2 poisons. (B) Cell extracts from strains LS1 resistant *top2* alleles exhibit reduced Top2 activity using the kDNA assay. (C) *top2-28* cells exhibit increased resistance to multiple Top2 poisons, including LS1, ellipticine (ELLIP), etoposide (ETOP) and doxorubicin (DOX). All poisons were used at a concentration of 50μM and growth rates were compared to control (0.1% DMSO) and determined as in panel (A).

originally described as the unsubstituted scaffold used in the development of less cytotoxic analogues of ellipticine [32]. *In vivo* and *in vitro* evidence show that LS1, like ellipticine, is a Top2 poison, albeit weaker and significantly less cytotoxic. LS1 exhibits none of the cytotoxicity to cancer (or normal) cells that ellipticine and other chemotherapeutic Top2 poisons show. LS1 inhibits the *in vitro* decatenation activity of human TOP2- $\alpha$ , and induces  $\gamma$ Top2 to generate stable DSBs (Top2ccs) in plasmid DNA. Like known Top2 poisons, LS1 is toxic to cells overexpressing *TOP2* and to cells deleted for *RAD52* epistasis group genes. The latter synthetic interaction indicates that homologous recombination is needed to repair Top2ccs formed in the presence of LS1, and suggested the hypothesis that LS1 is toxic to aging cells, which, like young *rad52* $\Delta$  cells, may exhibit a diminished capacity to recognize and/or repair Top2ccs.

A key finding in our study is that yeast cells live longer when expressing reduced levels of  $\gamma$ Top2. We propose that the Top2 poison activity of LS1 accelerates aging by presenting higher than normal Top2cc levels to aging cells with declining genome maintenance systems [14-17]. It follows that decreasing  $\gamma$ Top2 levels would extend RLS by reducing DNA damage to levels below that which cause normal rates of senescence. The idea that Top2 is a natural source of DNA damage is not new. Top2-mediated DNA damage has previously been implicated in physiologically normal processes [11]. In mammals, TOP2 $\beta$ -induced DSBs are necessary for the regulated transcription of neuronal early-response genes [50] and for TOP2 $\beta$  mediated androgen-induced DNA rearrangements in prostate cancer [51]. *C. elegans top-2* is also responsible for a striking increase in DNA damage during zygotic genome activation [52]. The massive induction of transcription that accompanies



**Figure 11. Reduced Top2 activity extends replicative lifespan.** (A) Dead cells expressing *top2-28* displayed a roughly two-fold increase in lifespan both in the absence or presence of LS1 compared to cells expressing native *TOP2*. (B) Anti-Top2 immunoblot of cell extracts from *TOP2* and *top2-28* expressing Dead strains, using anti-G6PDH as the loading control. (C) Anti-Top2 immunoblot of cell extracts from *Top2-DamP* and *TOP2* strains using anti-G6PDH as the loading control. (D) Microdissection RLS assays of isogenic haploid strains expressing either *Top2-DamP* or *TOP2*, obtained by sporulation the heterodiploid. (E) Budding indexes of terminal mother cells taken from the microdissection RLS assays of *Top2-DamP* and *TOP2* strains shown in panel C.

entry of larval primordial germ cells into the cell cycle triggers widespread DNA damage and activates DNA damage checkpoints that slow entry into mitosis. Remarkably, reducing *top-2* expression both reduced levels of DSBs in germ line cells and accelerated their entry into mitosis, presumably by precluding the need for the DNA damage checkpoint. This phenomenon provides a precedent for our hypothesis that a reduction in  $\gamma$ Top2 expression decreases DNA damage in old cells and extends RLS. As suggested by an apparent loss of checkpoint control and increased rates of loss of heterozygosity (LOH) in the daughters of old mothers [53, 54], old yeast cells proceed through mitosis before damaged chromosomes can be repaired.

Proving that a molecule selectively shortens RLS is challenging because even low levels of toxicity—either cytotoxic or cytostatic—will reduce apparent RLS by arresting cell division irrespective of age. An effective lifespan shortening agent should have little effect on the physiology of log-phase cells, ~87% of which are 3 generations or younger, even when the target is an essential housekeeping factor like  $\gamma$ Top2. As is the case with many drugs that target physiologically important targets, the dose-dependence of lifespan shortening compounds is critical to limiting the toxic side effects associated with targeting essential longevity factors. LS-1 does become toxic at very high doses, possibly due to over-inhibition of  $\gamma$ Top2 or off-target effects, or a combination of both. The initial DeaD screen included both permissive and nonpermissive assays that provided a filter to exclude molecules such as doxorubicin and etoposide that are toxic under permissive conditions (Fig. 1C & D). In contrast, LS1 and NAM reduce DeaD lifespan at concentrations that have no apparent effects on permissive growth. Besides having no effect on growth rate, LS1 has no discernable effects on either the cell cycle or viability. The viability criterion is important because even low levels of nonage-associated cell death cause significant decreases in apparent RLS using the microdissection assay. For example, *rad52* $\Delta$  cells, which had previously been argued to be short-lived [28], died in log phase at a rate of ~2%/generation. A 2% cell death rate has a negligible impact on proliferative growth, but reduces mean RLS by ~30%. LS1 has no detectable effect on cell viability even at concentrations 70-fold higher than were sufficient to reduce RLS by ~30%. We conclude that LS1 is a bona fide lifespan-shortening probe that is ~200-fold more potent than NAM.

An age-associated decline in genome maintenance creates a situation where otherwise manageable levels of DNA damage become toxic. We propose that LS1 is selectively toxic to older cells that have lost the capacity to mitigate  $\gamma$ Top2-induced DNA damage, either by

failing to properly repair Top2ccs or by failing to efficiently activate the DNA damage checkpoint and stalling the cell cycle until the damage can be repaired. As described above, older diploid yeast mother cells switch to a state of high genome instability that continues to produce high levels of LOH in daughters until the mothers die [53, 54]. LOH in the daughters of older mother cells is primarily due to damage-prone breakage induced repair (BIR) of DSBs. Moreover, unlike young cells that delay the cell cycle in order to repair DSBs, old mothers that produce daughters with LOH do not exhibit cell cycle delay or arrest, consistent with a loss of the DNA damage checkpoint [53, 54]. These studies argue that LOH in aging yeast arises not from an increase in the rate of DNA damage, but rather in the loss of genome maintenance systems needed to repair damage. Increased genomic instability in the form of LOH also rises dramatically during chronological aging. Interestingly, although chronological and replicative lifespans of natural isolates are not correlated [4], variability among natural isolates in the lag between the rise in LOH and loss of viability during chronological aging does correlate with the RLS of natural isolates, and argues that the capacity to resist genome instability contributes to natural variation in RLS [55-57]. LS1 does not affect chronological lifespan (M. Weinberger and W. Burhans, personal communication), consistent with the notion that Top2 poisoning by LS1 is selectively toxic to replicating cells, presumably by interfering with genome maintenance during and following DNA replication.

Are our results consistent with what others have observed in aging yeast? Whereas Hu et al. [58] observed numerous genome rearrangements in older mother cells, Kaya et al. [55] sequenced the genomes of colonies produced by daughters of individual old mother cells and found only low numbers of mutations, effectively ruling out the accumulation of mutations in the mothers as the cause of senescence. Our results are consistent with both of these studies. A single unrepaired DSB in a mother cell that has lost the capacity to either sense or repair the damage can be lethal. This type of damage would not manifest itself as DNA damage in the genomes of daughter cells. Accordingly, in the case of  $\gamma$ Top2-mediated DNA damage, it is not the accumulation of mutations that leads to senescence, but rather as few as a single catastrophic DSB—those not transmitted to daughters because the mothers die—that lead to age-associated cell death.

Our observation using microdissection RLS assays that terminal mothers under-expressing *TOP2* exhibit a higher frequency of arrest as unbudded G1/S cells compared to normal cells is consistent with improved

checkpoint function. Consistent with the idea that normal levels of  $\gamma$ Top2 cause RLS-limiting DNA damage and loss of growth control, Delaney et al. [28] reported that mutations that cause defects in genome stability genes tend to shorten RLS and increase the proportion of mothers that senesce as budded cells. Moreover, there is a strong statistical correlation between RLS and the budding index of terminal mothers, the latter of which can vary between ~25-70%. Since not all mothers senesce as budded cells, it is unlikely that DNA damage and loss of growth control is the only mechanism that limits RLS in yeast. Thus we conclude only that  $\gamma$ Top2-induced DNA damage contributes to aging.

Beyond studies in yeast, genome instability or defects in genome maintenance are strongly correlated with aging [2, 12, 59, 60]. Strong evidence in support of the DNA damage theory of aging includes progeroid syndromes in humans and rodents that are associated with mutations that affect genome maintenance (reviewed in [2]). Additional evidence comes from studies showing that DNA repair systems decline in older animals [61-63]. The specific role of DSBs in aging is supported by the appearance of age-associated phenotypes in mouse liver following the selective induction of DSBs in that tissue [64]. Age-associated defects in growth signaling, resulting in inappropriate entry into S-phase before DNA damage can be sensed and repaired, or before adequate stores of nucleotides are available, can lead to catastrophic events such as replication fork collapse and irreparable arrest at G2/M [65]. But these observations do not address whether DNA damage is cause or consequence of aging. What has been lacking in support of the DNA damage theory of aging are cases, such as we show in the case of  $\gamma$ Top2, where lifespan is extended by reductions in DNA damage or by increases in repair systems or DNA damage checkpoints. Some evidence, such as the extended lifespan of male mice that overexpress SIRT6 [66], which is known to increase expression of DNA repair systems [67], are confounded by the pleiotropic roles of SIRT6 [68]. Similarly, RLS extension in yeast by overexpressing histones, which normally become depleted by about 50% in aging yeast, results in a global increase in transcription, suggests that this form of decline in genome maintenance plays a role in aging and, importantly, can be mitigated [69].

The antagonist pleiotropy (AP) theory of aging posits that the normal activities of some genes are beneficial during development and reproduction, but, for whatever reason(s), promote senescence later in life [48, 49]. The mutation accumulation (MA) theory posits that aging results from the accumulation of mutations that are selectively deleterious only late in life [48, 49]. Both

theories depend on the assumption that AP genes or MA alleles escape natural selection because their phenotypes manifest post-reproductively. Our results indicate that yeast *TOP2* is an AP gene. If we assume that DNA damage by  $\gamma$ Top2 occurs at approximately constant rates throughout yeast lifespan, then the AP properties of *TOP2* require an age-associated decline in genome maintenance similar to that which produces LOH in the daughters of old mother cells. This scenario is inconsistent with the MA theory of aging because most of these deleterious mutations would be deleterious whether they occurred early or late in life. In any case, Kaya et al. [55] did not find a significant accumulation of age-associated mutations. Thus our results with  $\gamma$ Top2 support the AP theory of aging.  $\gamma$ Top2 activity is required for proliferation in young cells, but it becomes toxic late in life. Thus  $\gamma$ Top2 is indirectly antagonistically pleiotropic as its effect on longevity depends on an age-associated decline in genome maintenance. If correct, the AP effects of *TOP2* could be mitigated late in life, either by moderately reducing *TOP2* expression to levels that do not affect proliferation, which we have shown to be possible, or by enhancing repair or damage sensing and checkpoint systems later in life. In addition to  $\gamma$ Top2, other DNA cleaving or modifying enzymes that have potential to cause DNA damage with AP properties such as Topoisomerase 1 [70, 71] may contribute to aging and age-associated diseases.

## MATERIALS AND METHODS

### Strains and LS1 chemical-genetic interactions

Negative chemical-genetic interactions with LS1 were initially determined by replica plating spots of similarly diluted mid-log cultures of the Yeast deletion collection library (GE Life Sciences; [72, 73]) on SCD and SCGal media lacking or containing 20 $\mu$ M LS1. Any patch showing less growth was confirmed more thoroughly by replica plating a series of spot dilutions of log-phase (OD<sub>600</sub>=1.0) cultures on media containing either DMSO (0.1%) in the absence or presence of various concentrations of LS1. To test if LS1 is a topoisomerase poison, high copy number plasmids from the Yeast Tiling Collection (Thermo Scientific) containing *TOP1*, *TOP2*, and *TOP3* genes were isolated and transformed into the parental yeast strain BY4741 using Li-acetate/PEG with *LEU2* as the selectable marker. [74].

### Low to moderate throughput liquid culture DeaD assays

Strains carrying the three DeaD assay loci (*GAL1::CDC6*, *HO::CDC6*, and the partial deletion of

the *ASH1* promoter) are grown two days on YP/2% galactose agar plates. A single colony of each strain is suspended in water and used to inoculate small (1-3 mL) cultures in SC<sub>RafGal</sub> (SC/ 1.9% raffinose/ 0.1% galactose, USBiologicals, Swampscott MA) for overnight growth to early log phase (to ensure early log phase by the next day, we typically do parallel inoculations at calculated optical densities (600nm) of 0.0001, 0.00003, and 0.00001). After overnight growth, one culture of each strain is used to inoculate SC/2% glucose and SC<sub>RaffGal</sub> at a calculated OD<sub>600</sub> of 0.0001. Using a BioScreen C plate reader (Growth Curves USA, Piscataway NJ), cultures are grown at 30°C for 3 days, with an optical density (600 nm) reading taken every 20 minutes.

To calculate “DeaD lifespan,” we use the restrictive (i.e. SC/2% glucose) optical density reading at 60 hours, normalized to the estimated number of doublings that have occurred. To estimate the number of doublings, we use the actual number of doublings in permissive (i.e. SC<sub>RaffGal</sub>) medium in the first 24 hours, plus 36 times the calculated number of doublings per hour derived from the culture growth from hour 18 to hour 24. This assumes that the restrictive growth medium does not become limiting during the course of the assay, an assumption that is supported by restrictive optical density readings that are typically in the range of 0.1 to 0.3 at 60 hours. Growth rates expressed as the reciprocal of the doubling time (1/DT).

### **TOP2 mutagenesis and isolation of LS1-resistant yeast**

A plasmid (YCpDED1-yTOP2) containing yeast *TOP2* gene under the control of DED1 promoter and strain JN394top2-4 (*MATa ura3-52 leu2 trp1 his7 adel-2 ISE2 rad52::LEU2 top2-4*) containing a temperature sensitive allele of *TOP2* in a *rad52Δ* background were employed [26]. YCpDED1-yTop2 plasmid was maintained by URA selection. Hydroxylamine mutagenesis of the plasmid was performed as previously described [41, 75]. Mutations in the plasmid copy of the yeast *TOP2* gene that retained function yet were resistant to LS1 were obtained by selection at the restrictive temperature (35°C) where the chromosomal *TOP2* ts2-4 allele is non-functional by including 20μM LS1 in the growth media. The resistance of individual strains carrying *TOP2* mutations was checked by plating spot-dilutions on SCD-URA plates containing either DMSO carrier or DMSO with 20μM LS1 added. To rule out potential mutations in the strains as a cause of LS1 resistance, strains were cured for the *pDED1-yTOP2* mutagenized plasmid by treatment with 5-fluoroorotic acid (5-FOA), and subsequently tested for LS1 resistance. Reciprocally,

*pDED1-yTOP2* mutagenized plasmids were also rescued from strains showing resistance to LS1, transformed into naïve JN394t2-4 and re-tested for resistance to LS1. Growth in liquid media (SCD-URA) containing various concentrations of LS1 was measured in a Bioscreen C instrument (Growth Curves USA; Piscataway, NJ). Relative growth rates ( $\Delta$ O.D.600/h) of JN394t2-4 containing *pDED1-yTOP2* (wild-type or mutant derivatives) were measured for up to 60h after an initial inoculum of .005 OD<sub>600</sub> units. Growth data were fit to obtain maximum growth rates using the BGFit webserver [76].

### **Inhibition of human Topoisomerase 2-alpha (hTop2-α)**

LS1 inhibition of hTop2-α was determined by measuring the effect of LS1 on the ability of hTop2-α to decatenate kinetoplast DNA from *Crithidia fasciculata*. Human Top2-α was added to 0.2μg of kDNA (both were purchased from Topogen, Inc; Buena Vista, CO) in the presence of 1% DMSO (vehicle control) without or with increasing concentrations of LS1 as indicated. Samples were incubated at 37°C for 15min, and then reactions were terminated by the addition of an SDS buffer. Samples were resolved by 1% agarose 1xTAE gel and stained with ethidium bromide. Decatenated versus intact kDNA species were quantified by densitometry using GelQuant.NET1.8.2 software. Final % products were plotted using KaleidaGraph software.

### **Tissue culture cell growth**

All cell lines were grown in monolayer at 37°C in 3% O<sub>2</sub>, 5% CO<sub>2</sub> and 97% relative humidity in HERA Cell 240 incubators on treated polystyrene cell culture plates (Corning). Immortalized human fibroblast cells (HCA2T) were maintained in MEM (ATCC) supplemented with 15% FBS (Gibco) and 1x Pen/Strep (Gibco). Human fibrosarcoma cells (HT1080) were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco), 1x Pen/Strep (Gibco) and 1x nonessential amino acids (Gibco).

### **Tissue culture cell survival**

HT1080 and HCA2T cells were split to a density of  $1 \times 10^5$  cells per well of a six well plate 24 hours prior to treatment with the indicated concentration of DMSO (Sigma), Doxorubicin (Sigma), Vinblastine (Sigma) and LS1. All drugs were concurrently applied to the cells. Cell survival was measured 48 hours after treatment by counting the adherent cells in each group using a Z2 particle counter (Beckman Coulter). The ratio of adherent drug-treated cells to adherent cells treated with

DMSO represents the raw survival. Experiments using doxorubicin were repeated six times; experiments using vinblastine were performed in triplicate.

### Western blotting

Anti- $\gamma$ Top2 polyclonal antibodies were purchased from Topogen (Buena Vista, CO). For the *TOP2* tiling experiments, protein extracts were prepared using the rapid boiling method [77]. When cells were grown in minimal media, protein extracts were prepared using the glass bead method [78]. Secondary antibodies conjugated to infrared dyes (LI-COR; Lincoln NE) were employed with an infrared gel scanner (LI-COR; Lincoln NE) for quantitation of the western blots.

### DNA intercalation assay

DNA Topoisomerase I (Top1) –based assay was used to assess the ability of LS-1 to intercalate. This approach relies upon linking number changes in supercoiled plasmid DNA induced by the intercalating molecule [79] which can be compared to DNA that was not treated. After treatment with the putative intercalator, linking number changes can be inferred after relaxation with DNA Top1. If intercalation occurred, upon removal of Top1, the final linking number (or state of supercoiling) will be distinct compared to DNA that was not treated (control). Molecules that fail to intercalate appear similar to control samples. DNA was incubated with DMSO alone or DMSO containing the indicated amount of doxorubicin, etoposide, or LS-1 for 15min at 25°C, followed by an additional 1h with added Top1. Next, DNA was extracted with phenol and chloroform and precipitated with ethanol. Samples were resolved by electrophoresis through 1% agarose 1xTAE gel and species were quantitated by densitometry with GelQuant.NET1.8.2 software.

### CONFLICTS OF INTEREST

The authors have no conflict of interests to declare.

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## SUPPLEMENTARY MATERIAL

### Construction and characterization of an improved DeaD strain

K6001 is a W303-derived strain that was originally constructed for use in the study of mother cell-specific mating type switching [72, 73]. K6001 encodes two integrated copies of the essential gene *CDC6*, independently regulated by the repressible *GAL1* promoter and the mother cell-specific *HO* promoter, respectively (*GAL1:Ub:CDC6* and *HO:CDC6*). Previously, we exploited this strain as an alternative to the standard microdissection method of quantifying replicative lifespan (RLS), which is labor intensive and slow [74]. When K6001 cells are grown permissively in galactose-containing media (raffinose/galactose), *GAL1:CDC6* is expressed both in mother and daughter cells and the culture grows exponentially. When expression of the *GAL1:CDC6* gene is repressed by glucose, only the mother-cell-specific expression of *HO:CDC6* remains to support growth. Since *HO* expression is largely restricted to mother cells, daughters generally do not divide and growth of the culture is limited by the RLS of the initial cohort of mother cells. Since the success of this strategy depends on the conditional Death of Daughter cells, we call it the “DeaD” assay. Although promising, K6001 as a DeaD strain exhibited significant limitations. Its mother cells cease division on glucose after an average of only 3-4 divisions [74]. K6001 also has a short average lifespan under permissive conditions, presumably due to its W303 background (17 generations for K6001 [74], similar to 20.8 generations for W303R [18]). Early tests of the assay showed a reduction of DeaD lifespan by deletion of *SIR2* [74] but these tests ignored the fact that the *HO:CDC6* fusion is haploid-specific [75]. Deletion of *SIR2* prevents silencing of the mating type information at *HML* and *HMR* and thus renders cells pseudo-diploid [76, 77]. After a switch to glucose, *sir2Δ* mutants will thus be unable to express either copy of *CDC6* (see below for mechanism of HO gene expression) and will die rapidly, as was observed [74]. When the opposite mating type information was deleted from this strain, allowing *HO:CDC6* expression to sustain mother cells in glucose, no difference in DeaD lifespan was observed between *SIR2* and *sir2Δ* cells (data not shown). Combined with the other limitations, this finding indicated that the strategy exemplified by K6001 would have to be modified before it could be used as a platform for studying replicative lifespan.

We began by switching the parental background from W303 to the longer-lived S288C background [78]. For galactose-specific *CDC6* expression, we chose the strategy employed in K6001: a ubiquitin:*CDC6* fusion

driven by the *GAL1* promoter. The N-terminal ubiquitin fusion allows modification of the amino terminus of Cdc6 to reduce protein stability and tighten control over Cdc6 activity. The ubiquitin moiety is co-translationally removed by ubiquitin processing proteases, and the amino acid serving as the new amino terminus of Cdc6 determines its half-life according to the N-end rule [79].

We cloned the *GAL:Ub:CDC6* fusion from K6001 to a plasmid vector. DNA sequencing revealed a tyrosine codon at the beginning of *CDC6*, rather than the expected arginine [73]. Multiple independent clones from two separately obtained K6001 isolates gave the same result. According to the N-end rule, tyrosine is less destabilizing than arginine [79]. Rapid turnover of *GAL1*-expressed Ub-Cdc6 is required for efficient death of daughters after a shift to glucose. Arginine, which is maximally destabilizing [79], is thus the desired N-terminal residue. All of the clones also carried a conservative mutation in the ubiquitin moiety: arginine 74 to lysine. We used PCR mutagenesis to correct this mutation and to change the N-terminal residue of Cdc6 to either arginine or methionine. We then integrated these alleles, and the original K6001 allele, in place of the endogenous *CDC6* in the S288C-derived strain Y7092 [80]. Since the *GAL:Ub:CDC6* allele is the only source of *CDC6* in these strains, all cells arrest when transferred to glucose, and the efficiency of this arrest is a function of the stability of Cdc6. Cells expressing arginine at the N-terminus of Cdc6 achieved the most efficient growth arrest. (Fig. S1A). This allele was incorporated into all subsequent DeaD strains.

The *HO:CDC6* allele in K6001 is an imprecise fusion of the open reading frame of *CDC6* to the *HO* promoter: the fusion leaves in place more than 90bp of the *CDC6* 5' untranslated region, and all of the 3' *CDC6* untranslated sequences. This construction excludes the 3' UTR of *HO*, which appears to play a role in mother-cell-specificity of *HO* expression [81]. For our new DeaD strain, we therefore created a precise replacement of the *HO* open reading frame with *CDC6*, leaving the large *HO* promoter and the *HO* 3' untranslated region in place. This *HO:CDC6:HO* fusion was integrated into a strain already carrying *GAL1:Ubr:CDC6* to create DeaD strain BB573. BB573 mother cells have improved survival on glucose compared to K6001 mother cells: mean RLS increased from 3.4 to 8.3 generations ( $p=5.94 \times 10^{-13}$ , Fig. S1B). However, 8.3 generations is still much shorter than the 26-28 generation mean lifespan of normal SC288c-derived cells [78].

To further improve mother cell survival in glucose, we sought to increase mother cell expression of *HO:CDC6*. Insight into expression of this fusion can be gained from studies of normal *HO*. In cells that express the wild-type

HO endonuclease, mother cells switch mating type at a rate of ~70% [82]. Inhibition of switching in the remaining ~30% of mothers is dependent on the transcriptional repressor Ash1, since deletion of *ASH1* increases mother cell switching to 95-100% [72, 82]. These results indicate that Ash1 is normally incompletely excluded from mother cells. Ideally, Ash1 would be completely partitioned into the incipient daughter cell (the bud), leaving none in the mother cell to repress *HO* expression.

*ASH1* is expressed at the end of mitosis, when the transcription factors Swi5 and Ace2 enter the nucleus and promote ASH1 expression by binding to four putative Swi5/Ace2 binding sites (predicted by the nucleotide sequence kGCTGr, where “K” is G or T and “R” is A or G, [83] in the *ASH1* promoter. *ASH1* expression is dramatically decreased in *swi5 ace2* double mutants [72, 84]. We deleted the Swi5/Ace2 binding sites, either *in toto* or in pairwise combinations, in BB573 and assessed the effect on mother and daughter cell survival on glucose by pedigree analysis (Fig. S2). Deletion of the 1<sup>st</sup> two Swi5p binding sites (*ASH1-Δ12*) caused too drastic a drop in Ash1: daughter cell survival dramatically increased (represented by tall bars in Fig. S2 panel B), compare with parental strain BB573, Fig S2 panel A). This effect was caused to a lesser extent by deletion of the middle two sites (*ASH1-Δ23*, Fig. S2 panel C) or of all four binding sites (*ASH1-Δ14*, Fig S2 panel D). In contrast, deletion of the 3<sup>rd</sup> and 4<sup>th</sup> Swi5p binding sites produced a strain with enhanced mother cell survival without increased daughter cell “escape” (Fig S2 panel E). This strain, *ASH1-Δ34*, was renamed BB579 and it and its derivatives were used in all subsequent work. BB579 has a mean survival of 11.3 generations on glucose (longer than its parental strain, BB573,  $p=1.77 \times 10^{-7}$ ) corresponding roughly to a model combining Gompertzian senescence with a stochastic death rate of 7.5% (Fig. S3 panel A).

Yeast cells become sterile near the end of their lifespan due to de-repression of the silent *HM* mating type loci and the resulting pseudo diploidy [85]. De-repression of the *HM* loci in a DeaD strain will result in failure to express haploid-specific *HO:CDC6*, as discussed above, and which might cause premature death in aging cells and an artificially short lifespan. To investigate the potential benefit of preventing pseudo diploidy by deleting one of the two mating type loci, *HMR*, we deleted *HMR* in *SIR2* and *sir2Δ* BB579 cells. We performed microdissection (Fig. S3 panels A and B) and DeaD lifespan assays (Fig. S3 panel C) of parental BB579 (*HMR*), *hmrΔ*, *sir2Δ* and *hmrΔ sir2Δ* strains. Deletion of *SIR2* alone produced an extremely short apparent RLS, presumably due to repression of HO expression resulting from pseudo diploidy, and deletion

of *HMR* and *SIR2* together gave an intermediate phenotype (Fig. S3 panels A and C) in both the microdissection and DeaD lifespan assays. None of these deletions had significant effects on permissive growth in galactose, although the *hmrΔ sir2Δ* strain exhibited a minor growth defect (Fig. S3 panel D). By microdissection RLS assay, deletion of *HMR* slightly extended RLS: mean RLS increased from 11.3 to 12.1 generations ( $p=1.5 \times 10^{-2}$ ), and maximum increased from 32 to 45 (Fig. S3 panel B). Deletion of *SIR2* in *hmrΔ* BB579 cells shortened RLS to a mean of 8.6 generations ( $p=1.45 \times 10^{-4}$ ), with a maximum of 33 generations (Fig. S3 panel A).

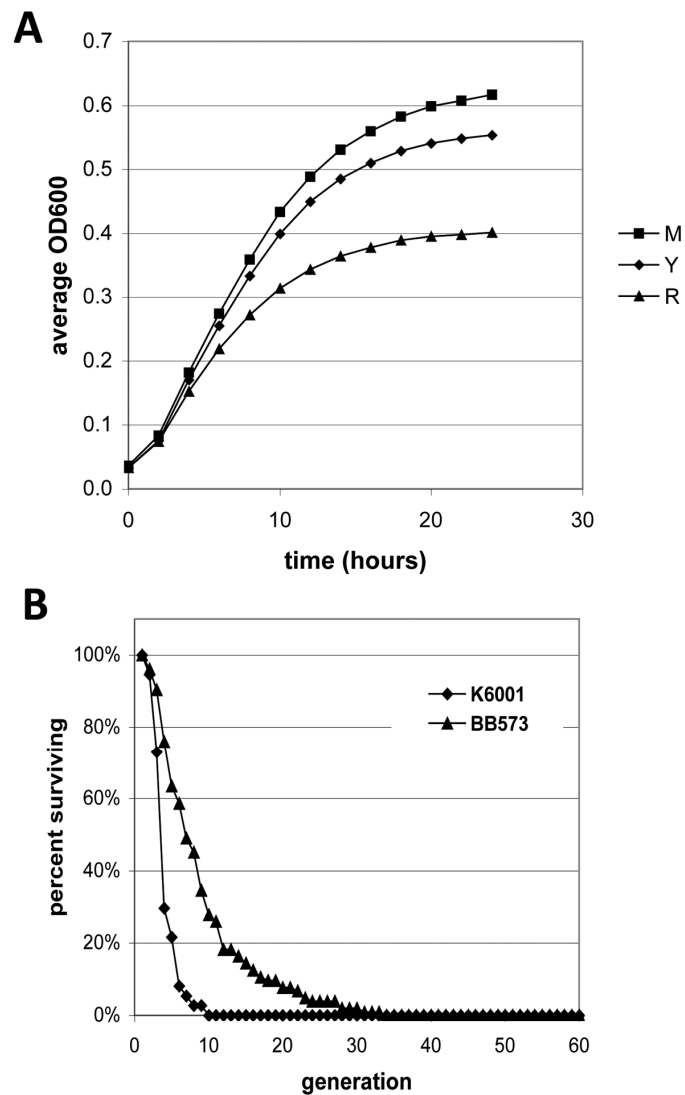
The above results demonstrate that *SIR2* strains have longer DeaD assay lifespans than *sir2Δ* strains in the improved BB579 background. To test this relationship further, and to determine whether the DeaD assay is sensitive not only to lifespan shortening but also to lifespan extension, we asked whether we could detect a range of effects on RLS by altering the expression level of *SIR2* with a series of promoter fusions. Replicative lifespan varies with *SIR2* expression level in yeast, since deletion of *SIR2* shortens lifespan and an extra copy of *SIR2* extends it [18]. There is likely to be an upper limit to lifespan extension by up-regulation of *SIR2*, however, since *SIR2* overexpression from the *GAL1* promoter causes toxicity and elevated rates of chromosome loss [86]. For our test, we used PCR-based integration to replace the endogenous *SIR2* promoter with the *CYC*, *ADH*, *TEF*, or *GPD* promoter [87] in an *HMR*-deleted BB579 derivative. DeaD assay lifespan of the resulting strains paralleled the predicted promoter strength (*CYC*<*ADH*<*TEF*<*GPD*, [87]), with strains carrying the strongest two promoters showing extension of DeaD assay lifespan (Fig. S4 panel C).

As expected if strong overexpression of *SIR2* is toxic, the *GPDpr:SIR2* fusion, which we expect to be more strongly expressed than the *TEFpr:SIR2* fusion [87], confers no additional advantage for lifespan (Fig. S4 panel C). None of these deletions had significant effects on permissive growth in galactose, although the *hmrΔ sir2Δ* strain exhibited a minor growth defect (Fig. S3 panel B).

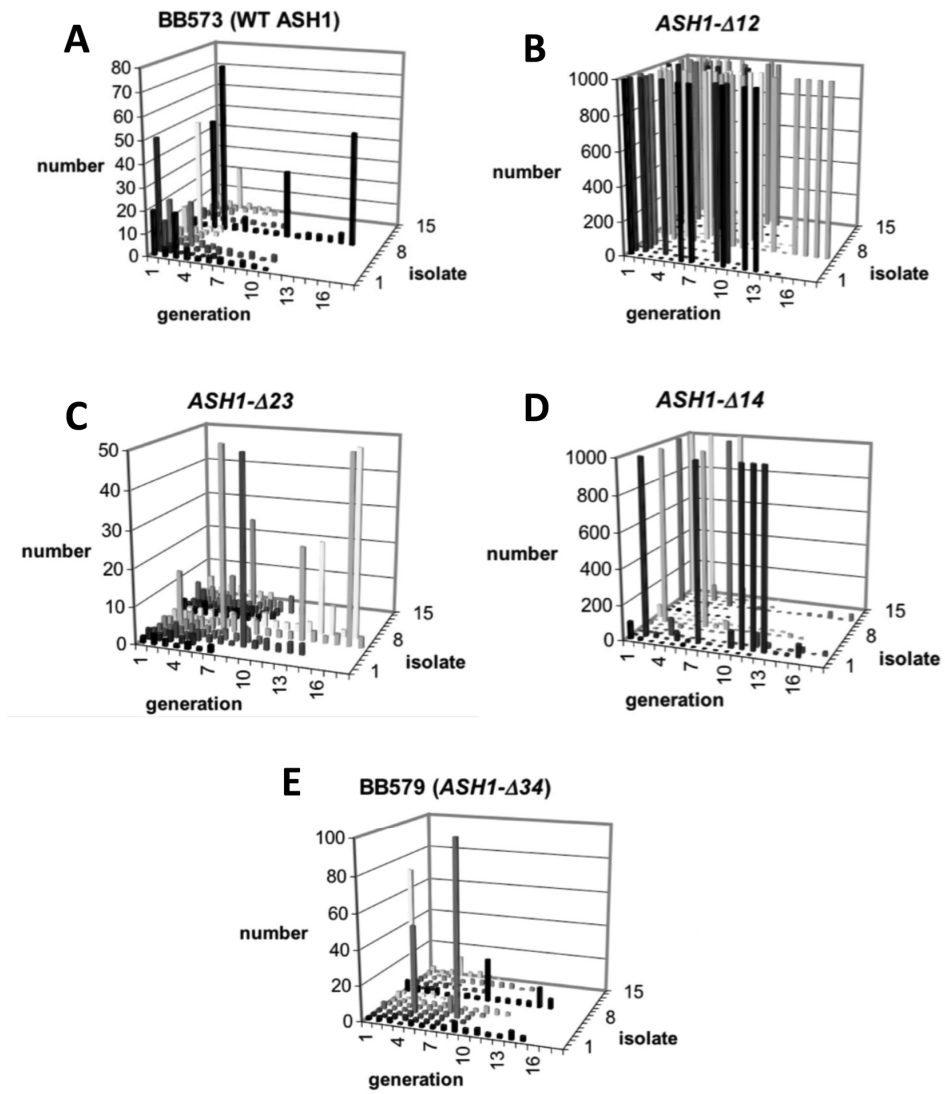
In a parallel set of experiments, we replaced the endogenous *SIR2* promoter with the same promoter series in a wild-type S288C strain and measured replicative lifespan using the standard microdissection assay. The results (Fig.4S Panel D) are a striking parallel to the DeaD assay findings. The *TEF* promoter extended mean lifespan by a robust 45%, while the stronger *GPD* promoter showed a lesser extension of 22%. We conclude that improvements to K6001 engineered into BB579 allow recapitulation of key

features of yeast aging using high throughput capable liquid growth-based assays, including lifespan shorte-

ning and extension by under- and overexpression of *SIR2*.

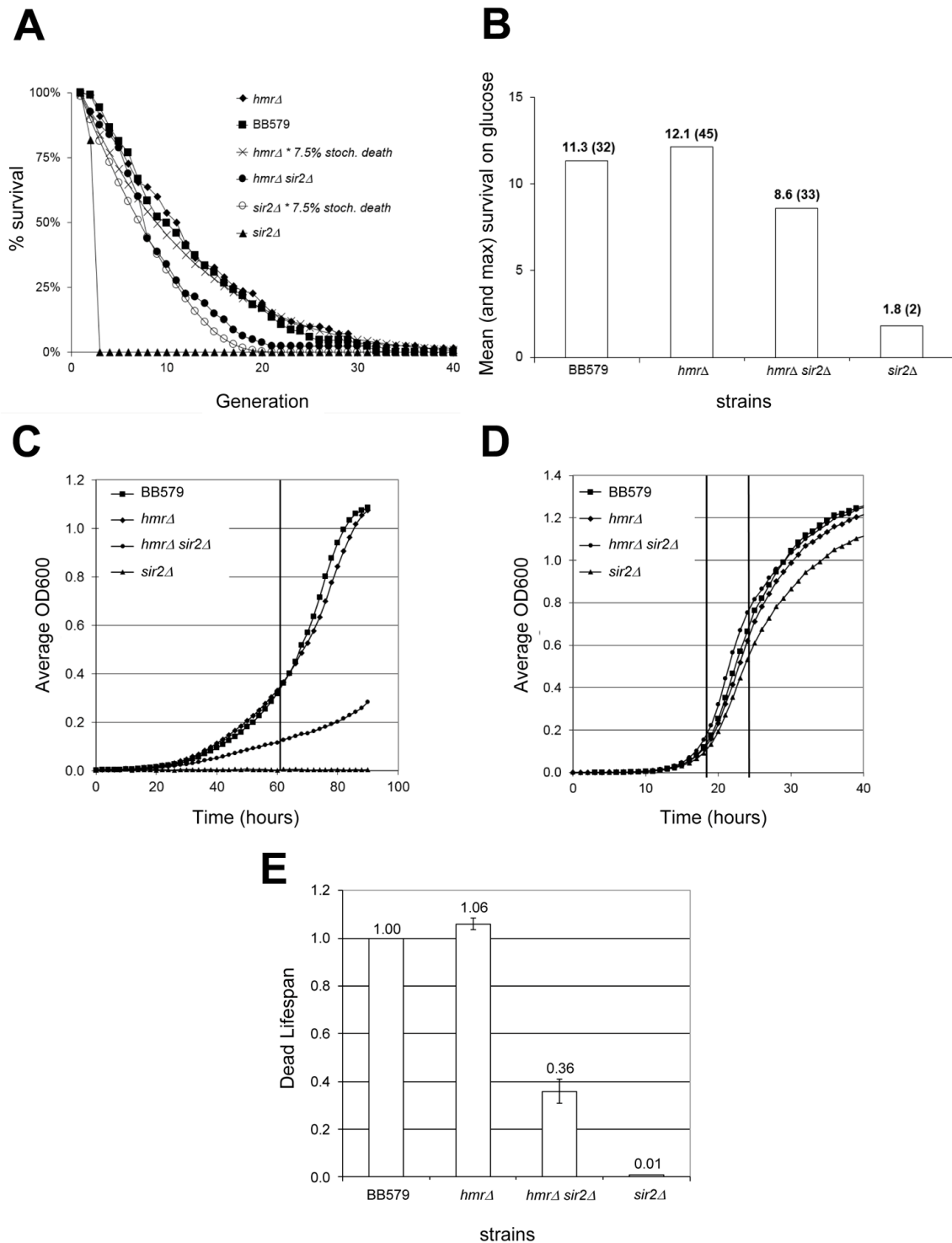


**Figure S1. Characterization of improved DeaD strain BB573.** (A) Arrest of *GAL:Ub:CDC6* strains in glucose is dependent on the N-terminal residue of Cdc6p. Cells were transferred to glucose at time 0 and growth arrest was monitored by measuring the culture OD600. The letter indicates the N-terminal Cdc6p residue: M: methionine, Y: tyrosine, or R: arginine. (B) BB573 mother cells have improved survival on glucose compared to K6001 mother cells: mean RLS increased from 3.4 to 8.3 generations ( $p=5.94 \times 10^{-13}$ ).

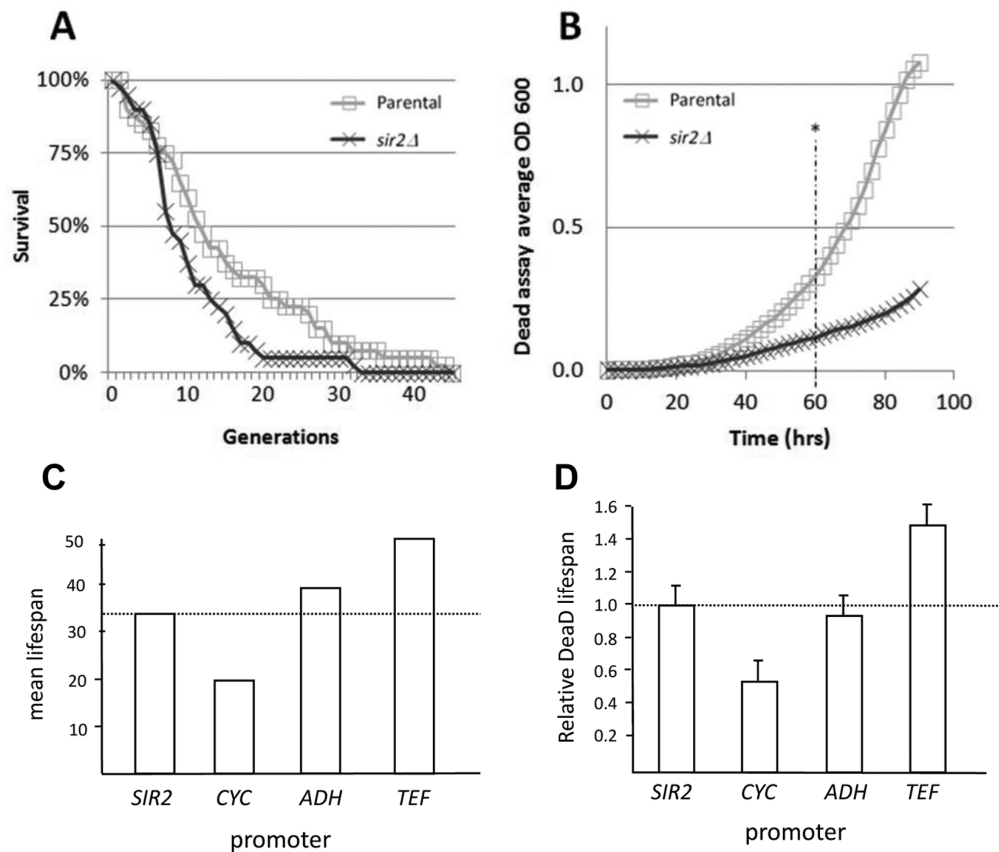


**Figure S2. Pedigree analysis of BB573 and derivatives carrying *ASH1* promoter deletions.** Strains were grown in a galactose-containing medium to mid-log phase and arrayed on glucose (SCD) plates for microdissection. Divisions of mother cells are represented on the x axis, and divisions of daughter cells by the height of the bars.

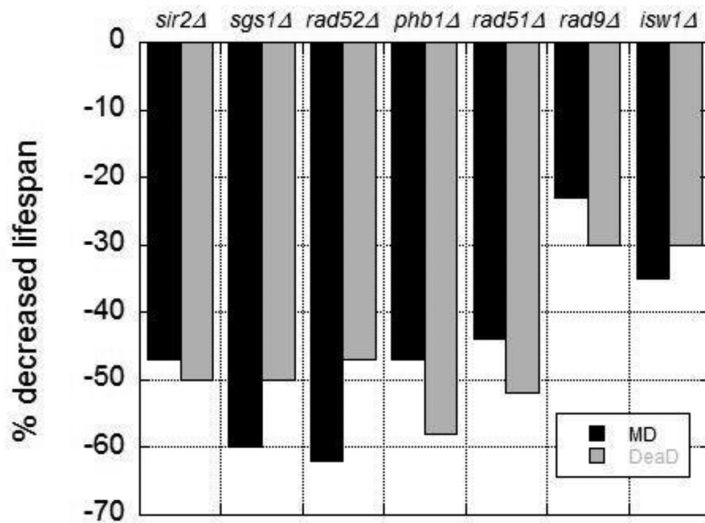




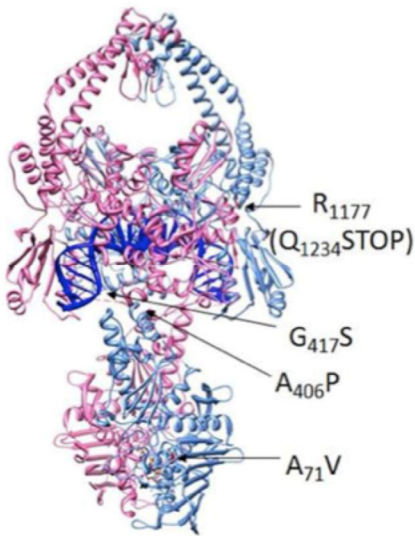
**Figure S3. The effect of *hmr* and *sir2* deletions on mother cell survival and Dead lifespan in new Dead assay strain BB579.** (A) Survival of BB579 and its *hmr* $\Delta$  *hmr* $\Delta$ *sir2* $\Delta$ , and *sir2* $\Delta$  derivatives on glucose. Strains were grown to mid-log phase in a galactose-containing medium and arrayed on glucose (SCD) plates. Mother cell survival was determined by microdissection. For comparison, models of BY4741 (“x”) and its *sir2* $\Delta$  derivative (open circles) with a 7.5% rate of stochastic death are included. (B) Mean and maximum replicative lifespans from (A). (C) Representative restrictive liquid cultures of the same four strains. (D) Parallel permissive liquid cultures. (E) Relative Dead assay lifespans of the four strains, using data from (C) and (D) together with two more independent experiments. Error bars are +/- one standard error of the mean of the three experiments.



**Figure S4. DeaD assay recapitulates microdissection replicative lifespan measurement.** (A) In the traditional microdissection replicative lifespan method the DEAD strain deletion of *SIR2* results in a drop in average lifespan from 15.5 generations ( $\square$  Parental) to 10.5 generation ( $\times$  *sir2Δ*), a 32.25% decrease. (B) In the DeaD assay the average lifespan decrease (\* measured at 60hrs) from optical density of 0.331 ( $\square$  Parental) to 0.116 ( $\times$  *sir2Δ*), a 65% drop in lifespan. (C) & (D) When *SIR2* expression levels are varied by promoters of varying strength from low (*CYC*), normal (*ADH*) and high (*TEF*), relative and mean lifespan as reported by the DeaD assay also show reduced, normal, and extended levels.



**Figure S5. DeaD assay reports replicative lifespan for several strains that show reduced lifespan in the traditional microdissection assay.** Yeast strains were obtained from the gene deletion collection. Replicative lifespan (%) for both microdissection and DeaD assay were determined as described in Methods.



**Figure S6. Map of LS1 resistant hypomorphic *top2* alleles.** UCSF Chimera was used to display the X-ray structure of yeast Top2 and map the location of each of the LS1 resistant hypomorphic alleles onto the protein structure. The dispersed locations of each mutation are consistent with other *top2* alleles previously found to confer broad resistance to Top2 poisons such as DOX or ETOP.

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