Enhanced Genomic Stability and its Effects on Aging and the Epigenome

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Enhanced Genomic Stability and its Effects on Aging and the Epigenome

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Enhanced Genomic Stability and its Effects on Aging and the Epigenome

A dissertation presented

by

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Abstract

The epigenomes of evolutionarily distant species undergo similar alterations during aging, but the upstream causes of these changes are unclear. DNA damage is one potential cause, as epigenetic changes arising from DNA damage are remarkably similar to those observed during aging. To more definitively test if DNA damage is a cause of age-related epigenetic dysfunction, model organisms with enhanced DNA repair should be used to determine whether improved genomic stability mitigates epigenetic changes during aging. Generating such organisms has proven difficult, due to toxicity from overexpressing endogenous DNA repair proteins. I hypothesized that expressing exogenous DNA repair proteins could would be a more viable strategy for generating multiple model organisms with enhanced genomic stability.

In Chapter 2, I identify multiple DNA repair proteins from radioresistant organisms that improve the genomic stability of human fibroblasts exposed to hydrogen peroxide. Two of these proteins- the single-stranded DNA-binding protein SSB and the double-stranded DNA-binding protein Dps1- also enhanced genomic stability in *Saccharomyces cerevisiae*, with SSB also improving genomic stability in *Caenorhabditis elegans*. I discovered that SSB improves genomic stability by enhancing the efficiency of non-homologous end joining, while Dps1 likely improves genomic stability by protecting DNA from free radical damage.

In Chapter 3, I tested whether SSB and Dps1 could preserve the epigenomes of yeast and worms in response to either DNA damage or aging. Both genes mitigated the loss of silencing at

the *HMR* locus caused by DNA damage, a major epigenetic change that occurs during yeast aging. However, only Dps1 was able to extend yeast replicative lifespan. SSB extends the lifespan of worms while also improving healthspan. I discover that worms undergo a global reduction of histone 3 levels with age and following DNA damage, and show that SSB transgenic worms have a delayed onset of this loss.

In Chapter 4, I explore how SSB and Dps1 can be further characterized and utilized in aging research. Taken together, these results suggest that DNA damage is a conserved driver of age-related epigenetic changes, and that enhancing genome stability preserves the aging epigenome.

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Chapter 1: The Roles of Genomic Instability and Epigenetic Dysfunction in Aging

I. Genomic Instability as a Cause of Aging

a. Origins of the DNA Damage Theory of Aging

Aging is characterized by a time-dependent increase in mortality and a decline in physiological functions. Although humans have sought to understand, delay, and reverse aging for thousands of years, it is only during the last fifty years that these efforts have demonstrated tangible progress. During this time, we have uncovered some of the underlying cellular and molecular causes of aging, and have demonstrated that aging is a malleable process whose trajectory can be radically shifted, at least in model organisms [1-2]. As aging is a seemingly universal process, research in this field has employed a diverse array of organisms including yeast [3], fish [4], insects [5], nematodes [6], mammals [7], and even bacteria [8]. Although the average lifespan of these species can differ by more than a thousand-fold [9-11], they remarkably undergo many of the same cellular and molecular changes as they age. Nine of the most commonly observed changes have been branded as the "hallmarks of aging" [12], and it is speculated that some or all of these hallmarks play causal roles in the aging process.

Genomic instability is one of the most well-studied hallmarks of aging. Ironically, a "DNA damage theory of aging" actually predates most research on either DNA damage or aging. In 1958, just five years after the seminal publication of the double-helix model of DNA, physicist Gioacchino Failla first suggested that DNA damage could be an underlying cause of aging [13]. Specifically, Failla believed that aging could be explained by mutagenic events caused by background radiation, ultraviolet light from the sun, and "chemical carcinogens". Failla cited the inverse relationship between lifespan and mutation rate in a small number of species to support this view. Shortly thereafter, a similar theory was independently proposed by

Leo Szilard, who devised a mathematical model whereby aging was driven by DNA damage that leads to "chromosomal inactivation" and subsequent cell death [14].

Although these two models of aging are now recognized as overly simplistic, decades of subsequent research in genetics, molecular and cellular biology, and comparative biology have affirmed a close relationship between genomic stability and aging. Cumulatively, these studies have reached the following five conclusions that demonstrate a likely role for DNA damage in promoting aging:

1) The frequency of many DNA lesions increases with age.
2) The efficiency of most DNA repair pathways declines with age.
3) There is a positive correlation between a species' DNA repair capability and lifespan.
4) Elevating DNA damage levels can reduce lifespan.
5) Interventions that extend lifespan frequently reduce levels of DNA damage.

b. Increased DNA Damage and Decreased DNA Repair Capacity During Aging

Levels of most DNA lesions increase during aging across many species. Analysis of various mouse and human tissues has demonstrated an age-related increase in abasic sites [15], oxidized nucleotides [16-17], double-strand breaks (DSBs) [18-19], and gross chromosomal aberrations [20-21]. Similar age-related increases in DNA damage also occur in other species (**Table 1.1**), demonstrating that genomic instability is a highly conserved characteristic of aging. Research into the mechanisms underlying these increased levels of DNA damage has suggested that a decline the efficiency of many DNA repair pathways is at least partially responsible. Human fibroblasts and tissue extracts show dramatic age-related decreases in the efficiency of multiple DSB repair pathways [19, 53], base-excision repair [54], nucleotide excision repair [55],

Table 1.1: Age-related changes in DNA lesions

Lesion	Organism	Tissue	Change with Age	Reference
Double-strand breaks	Humans	Ovary	Increase	[18]
		PBMCs	Increase	[19]
		HSCs	Increase	[30]
	Mice	Ovary	Increase	[18]
		Brain cortex	Increase	[25]
		Skeletal muscle	Increase	[31]
		Heart muscle	Increase	[31]
		Kidney	Increase	[31]
		Lung	Increase	[31]
		Spleen	Increase	[31]
		Liver	Increase	[32]
	Yeast	N/A	Increase	[22,37]
	Flies	Germ cells	Increase	[23]
	Killifish	Muscle	Increase	[24]
		Brain & retina	Increase	[26]
Single-strand breaks	Humans	PBMCs	Increase	[36]
	Mice	Brain	Increase	[34]
		Brain	No change	[43]
		Liver	Increase	[38]
		Liver	No change	[43]
		Kidney	No change	[43]
	Worms	Whole body	Increase	[35]
Oxidized bases	Human	Skeletal muscle	Increase	[17]
		Brain	Increase	[27, 33]
		CSF	Increase	[40]
		Colon	Increase	[41]
		Pituitary	Increase	[42]
	Mice	Liver	Increase	[16]
		Kidney	Increase	[16]
		Heart	Increase	[16]
		Brain	Increase	[16, 39]
	Yeast	N/A	Increase	[52]
	Worms	Whole body	Increase	[28]
	Flies	Intestinal stem cells	Increase	[29]
Abasic sites	Human	Leukocytes	Increase	[15]
Aneuploidy	Human	Skin fibroblasts	Increase	[20,21]
		Oocytes	Increase	[44]
		Bone marrow	Increase	[47]
		Blood & buccal	Increase	[50]
		Liver	No change	[51]
	Mouse	Oocytes	Increase	[45]
		Brain	Increase	[46]
	Yeast	N/A	Increase	[48,49]

and mismatch repair [56]. This also appears to be a well-conserved phenomenon, as aging also decreases the efficiency of DNA damage repair in rodents [60-62], flies [23, 57-58], worms [59], and yeast [22,63].

c. Positive Correlation Between Lifespan and DNA Repair

While these findings clearly establish that most species experience increased genome instability with age, they don't demonstrate whether DNA damage plays a causal role in aging. More suggestive, though still not definitive, evidence comes from comparative biology studies showing that DNA repair efficiency is positively correlated to species' lifespan. This was first reported in a seminal publication by Hart and Setlow [64], who monitored repair of DNA damage in skin fibroblasts derived from seven species with highly variable lifespans. They found that the repair efficiency of UV-induced DNA damage was logarithmically proportional to lifespan, likely indicating a positive correlation between lifespan and the efficiency of nuclear excision repair. Subsequent studies monitoring repair of UV-induced damage in cells from additional species have confirmed these findings [65, 66]. Furthermore, lifespan has also been found to positively correlate with the efficiency of DSB repair [67] and base-excision repair [68].

The correlated efficiency of multiple DNA repair pathways with lifespan across a large number of species raises the possibility that reducing genome instability is an evolutionarily conserved longevity mechanism. This argument was greatly strengthened by Tian *et al* [67], who directly demonstrated that the efficiency of a DSB repair protein affects lifespan. They found that inter-species differences in DSB-repair efficiency was largely due to differential activity of a single DSB repair protein: SIRT6. This member of the Sirtuin family plays multiple roles in non-homologous end joining (NHEJ) and homologous recombination (HR)- the two major DSB repair pathways-, including stimulating the PARP1 repair enzyme [69], stabilizing the DSB

sensing kinase DNA-dependent protein kinase (DNA-PK) [70], relaxing chromatin to make DSB sites accessible to repair proteins [71], and binding to DSBs and subsequently recruiting the ATM protein kinase [72]. They found that only five amino acid substitutions in SIRT6 were responsible for the differential DSB repair activities between the fibroblasts of mice - a short-lived species with poor DSB repair efficiency - and beavers - a long-lived species with high DSB repair efficiency. Critically, they showed that this discrepancy in SIRT6 activity had a direct impact on lifespan. Flies expressing either wild-type mouse SIRT6 (weak DSB repair activity) or a mutated beaver SIRT6 carrying the mouse amino acids at the five residues critical for SIRT6 activity (also weak DSB repair activity) had a slightly extended lifespan, while a much stronger lifespan extension was achieved by expressing either the wild-type beaver SIRT6 (strong DSB repair activity) or a mutated mouse SIRT6 carrying the beaver amino acids at the five residues critical for activity (also strong DSB repair activity). These findings add support to the argument that DSBs play a causal role in aging, though it should be noted that SIRT6 regulates multiple other processes linked to aging, including inflammation [73], metabolism [74], retrotransposon activity [75], and senescence [76]. Consequently, it is unclear how much of SIRT6-mediated lifespan extension is solely due to its role in enhancing DSB repair.

d. Exacerbating Genome Instability Accelerates Aging Phenotypes

Additional support for a causal role of DNA damage in aging comes from studies of human subjects and model organisms that have experienced elevated levels of DNA damage. Most notable are clinical observations made in patients with progeroid syndromes. These are rare genetic conditions that are commonly referred to as “accelerated aging” due to sharing some, but not all, aging characteristics. These clinical features include shortened lifespans [79-81] and an increased predisposition to age-related diseases [82,83]. Further supporting an association

between progeroid syndromes and aging, tissues and cells from progeroid syndrome patients and corresponding mouse models exhibit accelerated onset of several aging hallmarks, including genomic instability [84], metabolic defects [85], accumulation of senescent cells [86], epigenetic dysfunction [87], and stem cell exhaustion [88]. Intriguingly, the mutations underlying all progeroid syndromes are found exclusively in genes that encode DNA helicases, proteins involved in DSB repair, or structural components of the nuclear lamina. The fact that all of these proteins are essential for maintaining the integrity of the genome further underscores the close relationship between genomic stability and aging. Although some of the key progeroid syndrome phenotypes can't be recapitulated in non-mammalian organisms, mutating or decreasing the expression of the genes underlying progeroid syndromes significantly reduces the lifespan of yeast [89], worms [90,91], and flies [92,93], again suggesting that genomic stability has a potentially universal role in the aging process.

The findings from these progeroid models are further corroborated by clinical and laboratory studies in which DNA damage is transiently elevated, rather than the chronic elevation that occurs in progeroid syndromes. Survivors of pediatric cancers who were treated with chemotherapeutic agents exhibit multiple symptoms of "accelerated aging", including frailty, increased incidences of age-related diseases, and a shortened lifespan [94]. Similarly, survivors of the two 1945 atomic bomb attacks in Japan exhibited a decrease in lifespan that is likely proportional to their radiation exposure levels [95]. These findings are consistent with experimental findings that increasing DNA damage with genotoxic agents decreases the lifespan of yeast [96], worms [91], flies [97], and mice [98]. Furthermore, multiple characteristics of accelerated aging are observed in two mouse models that allow for temporally-controlled induction of DSBs via expression of the restriction endonucleases SacI [32] or I-*PpoI* [99,100] .

Brief expression (~3-4 weeks) of these endonucleases early in life (~3-6 months) leads to a transient elevation in DSB levels, which causes molecular (e.g. acceleration of epigenetic clock), cellular (e.g. increased cell senescence), histological (e.g. decreased muscle mass), and functional (e.g. decreased endurance) phenotypes that are observed during aging. These findings, combined with the aforementioned insights from progeroid syndromes, further highlight genomic stability as a key lynchpin underlying the aging process across species.

e. Lifespan-extending Interventions are Associated with Genome Stability

A final line of evidence suggesting a key role for genome stability in regulating aging comes from studies on the effects of lifespan extending interventions on DNA damage levels and repair efficiency. Calorie restriction (CR) was the first intervention shown to robustly extend lifespan [101], and decades of subsequent studies have validated this finding across numerous species [102-106]. Although there are multiple mechanisms responsible for CR-mediated lifespan extension [107], enhanced genomic stability is a prominent one. CR reduces age-associated accumulation of DNA damage across multiple tissues in mice and rats, including substantial reductions of oxidative DNA damage (8-OHdG) in the brain [16, 108-109], heart [16, 108-110], skeletal muscle [108,109], and liver [16, 109-110]- tissues with high metabolic activities and levels of oxidative stress. CR also significantly extends lifespan and reduces neuronal DSB levels in Ercc1$^{\Delta/-}$ mice, a short-lived strain that is deficient in DSB repair and nucleotide excision repair [111]. These reduced levels of DNA damage are likely due to enhanced DNA repair activities, as CR positively regulates multiple DNA repair pathways, including NHEJ [112], base-excision repair [113], and nucleotide excision repair [62].

Enhanced genomic stability is also associated with some of the most well-validated lifespan extending compounds. This was best demonstrated by Halicka and colleagues [114],

who investigated the molecular and cellular mechanisms underlying seven compounds that extend the lifespan of various model organisms. They found that treatment with each of the seven compounds reduced DSB levels in multiple human cell lines. These results are consistent with findings that some of these compounds- including metformin and resveratrol- have specific roles in the regulation of DSB repair [115,116]. It has yet to be shown if these compounds reduce DNA damage levels during aging in wild-type animals, and this line of investigation would provide more evidence surrounding the role of genomic stability in promoting lifespan extension.

II. Evidence that Genomic Stability is a Driver of Epigenetic Dysfunction

a. Mechanisms through which Genome Stability Affects Aging

Although there is widespread agreement that genomic stability plays an integral role in determining age-related health and lifespan, there is substantial disagreement about the underlying mechanisms. Genomic instability has a variety of deleterious effects on cellular function, and several of these have been proposed as having a causal role in aging (**Figure 1.1**).

One of the first theories of aging suggested that mutations resulting from DNA damage could be a driver of aging. This "mutation accumulation theory of aging" posits that accrual of mutations has deleterious effects that ultimately lead to cellular and organismal decline [117]. Support for this hypothesis largely comes from associations between mutation levels with age and age-related diseases [118], along with the findings that there is a negative correlation between mutation levels and lifespan [119]. However, a number of studies employing different model organisms have cast substantial doubt on the importance of mutations in aging. Two mouse models with defects in transcription-coupled nucleotide excision repair showed a reduction in lifespan but no increase in mutation rate [120], while the mutation rate during yeast

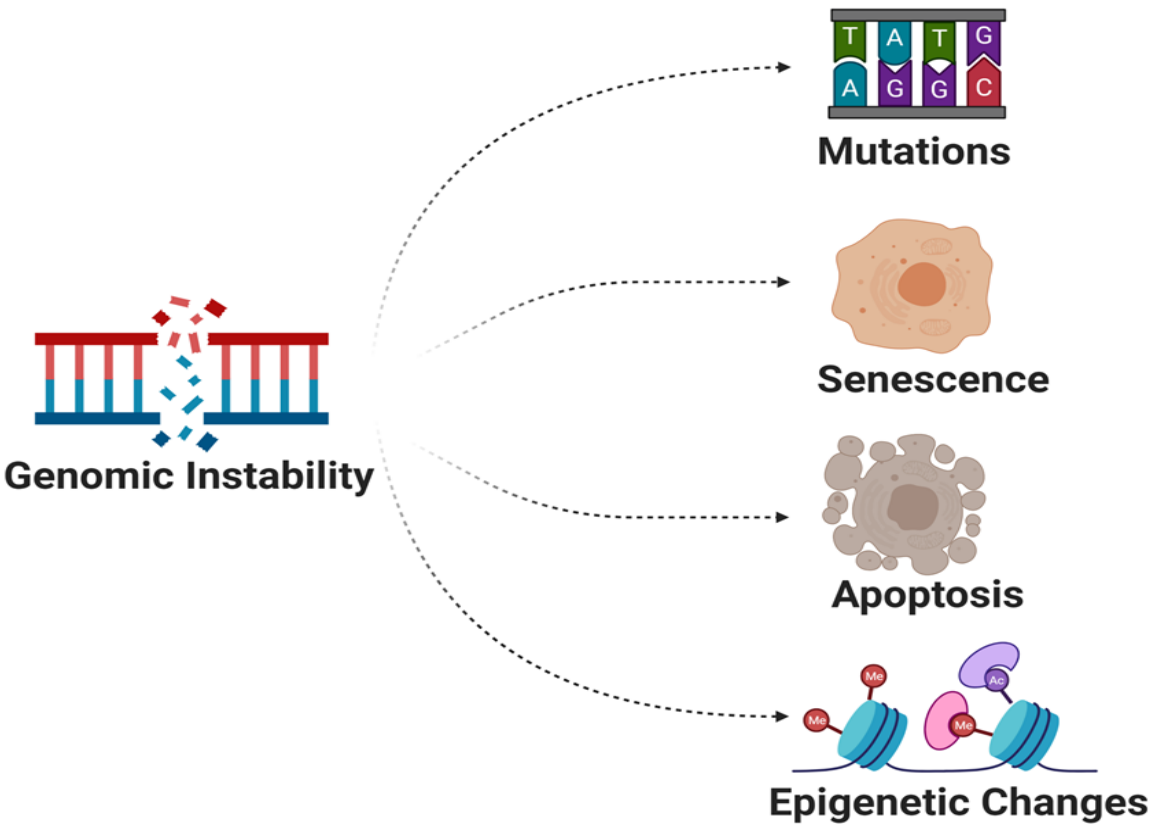

Figure 1.1: Proposed mechanisms by which genomic instability drives aging. Genomic instability has consequences that are deleterious to cell function. Four of these consequences- increased mutations, the onset of senescence, the induction of apoptosis, and changes to the epigenome- have been proposed as causal mechanisms through which genomic instability drives aging.

replicative aging is negligible (averaging less than one mutation per lifespan), and a yeast strain with a highly elevated mutation rate does not undergo premature aging [121]. These and other findings [122,123] uncoupling mutation accumulation with lifespan suggest that mutations are unlikely to play a major, causal role in the aging process.

Another hypothesis is that DNA damage leads to an increase in the number of apoptotic and senescent cells, and that this reduction in healthy cells is a key driver of organismal decline. Levels of apoptotic cells increase in several tissues during mammalian aging, and this mechanism is partially responsible for some prominent age-related physiological changes, including thymic involution [124], loss of myocytes [125], reduced T-cell counts [126], and

sarcopenia [127,128]. Similarly, increased levels of senescent cells have been observed during aging in mouse spleen, small intestine, kidney, liver, lymph nodes, and lymphocytes, [129,130]. Senescent cells are especially deleterious because of the cell non-autonomous mechanisms through which they impair tissues. Most notably, senescent cells secrete an assemblage of inflammatory cytokines, proteases, growth factors, and free radicals that can damage nearby healthy cells, a phenomenon called senescence-associated secretory phenotype (SASP) [131]. There is strong evidence that senescence plays a causal role in aging, as removal of senescent cells extends lifespan and healthspan in wild-type mice [132,133], and senescent cells have been implicated in the pathology of several age-related diseases including osteoarthritis [134], atherosclerosis [135], and type 2 diabetes [136]. While this loss of healthy cells likely plays a causal role in aging, apoptosis and senescence can be induced by numerous mechanisms that are independent of DNA damage, such as tumor-necrosis factor ligand binding [137] and oncogenic stimulation [138]. Consequently, the exact contribution of DNA damage to this process is unknown and should be a subject of further investigation.

b. Epigenetic Dysfunction During Aging

A more recent hypothesis is that genomic instability contributes to aging by causing epigenetic dysfunction, another universally observed hallmark of aging. The epigenome is constantly changing in response to environmental stimuli or changes in cellular conditions, such as infection [139], nutritional conditions [140], and chemical exposures [141]. The epigenome of all common model organisms undergoes dramatic alterations during aging, including changes in nucleosome occupancy, histone modifications, and DNA methylation patterns and levels (**Table 1.2**). Some age-related epigenetic changes are species-or tissue-specific, while others are more widely conserved. One commonly observed epigenetic change is a loss of heterochromatin,

largely due to decreased expression of histones [142-146] and a general decline in repressive histone modifications [145, 158-165]. One consequence of heterochromatin loss observed across species is an age-related increase in expression of repetitive elements [144,174], which can amplify cell dysfunction by causing DNA damage and driving inflammatory responses through the cGAS-STING pathway [175]. Loss of heterochromatin likely plays a causal role in the aging of most species, as preservation of heterochromatin consistently extends lifespan. For example, the median replicative lifespan of yeast can be extended ~30% by countering the age-related loss of nucleosomes through overexpression of Histones 3 & 4 or by deletion of the gene encoding HIR1, which suppresses transcription of histone genes [176]. Similarly, knockdown of the histone methyltransferase *set-26* in *C. elegans* counteracts decreased levels of Histone 3 and the heterochromatin markers H3K27me3 and H3K9me3 during aging, commensurate with a ~20% increase in median lifespan [145]. Although similar aging studies directly altering levels of histones or histone modifying enzymes have not been performed in mammals, preservation of heterochromatin is commonly observed in long-lived mice. For example, the long-lived Ames dwarf mice have higher hepatic and brain levels of the repressive H3K27me3 marker and lower levels of the transcriptional activation marker H3K4me3 at 20 months of age [177]. Similarly, brains of rapamycin-treated and dietary restricted mice exhibit higher H3K27me3 levels were found in the brains of rapamycin-treated and dietary restricted mice [164], two interventions that extend lifespan and improve healthspan [178,179].

Table 1.2: Age-related changes to the epigenome

Epigenome Change	Marker	Organism	Tissue	Age-related change	Reference
Histone Levels	H3 & H4	Human	Senescent cells	Decline	[142]
	H1 & H2	Mouse	Muscle stem cell	Decline*	[143]
	H3	Yeast	N/A	Decline	[144]
		Worm	Whole body	Decline	[145]
		Fly	Whole body	Decline	[146]
Histone Variants	H3.3	Human	Brain	No change	[147]
			Senescent fibroblasts	Increase	[148]
		Mouse	Brain	Increase	[147,149]
			Liver	Increase	[149]
			Kidney	Increase	[149]
			Heart	Increase	[149]
	H2A.Z	Human	Senescent fibroblasts	Increase	[149]
		Mouse	Brain	Increase	[150]
	H2A.J	Human	Epidermis	Increase	[151]
			Senescent fibroblasts	Increase	[152]
		Mouse	Epidermis	Increase	[152]
			Hair-follicle stem cell	Increase	[152]
			Senescent fibroblasts	Increase	[152]
	MacroH2A	Human	Senescent fibroblasts	Increase	[153]
		Mouse	Lung	Increase	[153]
			Skeletal muscle	Increase	[153]
		Baboon	Skeletal muscle	Increase	[153]
Histone Modifications	H3K4me3	Human	Brain	Increase**	[154]
		Mouse	Quiescent stem cells	Decrease**	[143]
		Yeast	N/A	Increase	[155]
		Fly	Head	Increase	[156]
		Worm	Whole body	Increase**	[157]
	H3K9me2	Fly	Whole body	Decrease	[146]
	H3K9me3	Human	Hematopoietic stem cells	Decrease	[158]
			Mesenchymal stem cells	Decrease	[159]
			HGPS fibroblasts	Decrease	[160]
		Mouse	Brain	Increase	[161]
			Brain	Decrease	[162]
			Hematopoietic stem cells	Decrease	[158]
			Muscle stem cells	Decrease	[163]
		Worm	Whole body	Decrease	[145]
		Fly	Whole body	Increase	[156]
		Killifish	Skeletal muscle	Increase	[24]

	H3K27me3	Human	HGPS fibroblasts	Decrease	[160]
		Mouse	Brain	Decrease	[164]
			Satellite cells	Increase	[143]
		Worm	Whole body	Decrease	[165]
		Fly	Skeletal muscle	Increase	[166]
		Killifish	Skeletal muscle	Increase	[24]
			Brain	Increase	[167]
	H3K36me3	Worm	Whole body	Decrease	[145]
	H3K56Ac	Human	Senescent fibroblasts	Decrease	[142]
		Yeast	N/A	Increase	[168]
	H4K16Ac	Human	Liver	Decrease	[169]
		Mouse	Liver	Decrease	[169]
			Kidney	Decrease	[169]
		Yeast	N/A	Increase	[168]
DNA methylation	5mC	Human	Senescent fibroblasts	Decrease	[170]
			Hematopoietic cells	Decrease**	[171]
			Leukocytes	Decrease	[172]
			Lung fibroblasts	Decrease	[173]
		Mouse	Brain	Decrease	[173]
			Liver	Decrease	[173]
			Small Intestine	Decrease	[173]

*Transcriptional change. Unknown if protein expression is altered.
**Site-specific, rather than global, changes.

Changes in the DNA methylation landscape is another epigenetic change that is especially important for mammalian aging. Methylation of cytosine's carbon 5 generates 5-methylcytosine (5mC), a modification that regulates gene expression in certain contexts. Most significantly, methylation of CG dinucleotides (CpGs) in or near promoters, and particularly the transcriptional start site, serves to repress gene expression [180]. An early study on DNA methylation revealed a reduction in 5mC levels in repetitive genomic loci in aged cow thymus and heart [181]. Subsequent studies found global loss of 5mC during aging in several tissues of mice [182], rats [183], and humans [171], in addition to human fibroblasts during continual passaging *in vitro* [184]. These findings suggest that global hypomethylation is a conserved feature of mammalian aging. Intriguingly, a longitudinal study analyzing global DNA methylation levels from blood cells in "middle/advanced age" subjects found that a decrease in

global methylation was associated with worsening frailty status [185], linking this epigenetic change with a key aging phenotype.

In addition to this global hypomethylation, aging is associated with highly site-specific DNA methylation changes that include both loss and gain of methyl groups. Remarkably, a subset of these site-specific changes correlate exceeding well with chronological age [186]. This has resulted in the development of several DNA methylation "clocks", which are the most accurate biomarkers of aging currently known. While some clocks are tissue- or species-specific, others retain high activity across tissues or species [186,187]. Recently, a "universal DNA methylation clock" has been developed which accurately (R^2>0.96) predicts chronological age across 59 tissues derived from 128 mammals [188]. In addition to their ability to predict chronological age, these clocks also reflect age-related health. Methylation clocks correlate closely with clinical frailty scores [189], cognition [190], and are accelerated in patients with age-related diseases including Alzheimer's disease [191], type two diabetes [192], and cancer [186]. Furthermore, these clocks "tick" at a slower rate in long-lived mice and in mice and non-human primates subjected to interventions that extend lifespan [193,194]. The robustness of these clocks in predicting biological age and health status across species suggests that the underlying causes of aging are highly conserved and that the epigenome plays a key role in regulating the aging process.

c. Evidence that Genomic Instability is a Driver of Epigenetic Dysfunction

One tantalizing, unresolved question is why the epigenomes of evolutionarily diverse species with vastly different lifespans undergo many of the same changes with age. Though there are likely multiple drivers of epigenetic dysfunction during aging, genomic instability has emerged as one of the more likely culprits due to documented connections between genomic

instability age-related epigenetic changes (**Figure 1.2**). One of the first lines of evidence suggesting this is that DNA damage in yeast accelerates the onset of epigenetic changes commonly observed during aging. Two major epigenetic changes that occur during yeast aging are desilencing of sub-telomeric regions and silent mating loci, the latter leading to sterility [196,197]. Both of these epigenetic changes result from the redistribution of chromatin factors involved in maintaining silencing, including members of the Sirtuin family. Inducing DSBs in yeast through either genotoxic agents or expression of a restriction endonuclease leads to desilencing of both telomeres and the silent mating loci through a similar redistribution of the Sirtuin family members SIR2 and/or SIR3 to sites of DSBs [198,199]. This raises the possibility that many age-related epigenetic changes might be caused by redistribution of chromatin modifiers to sites of DSBs, which increase in frequency during aging [22, 37].

Intriguingly, a highly similar pattern of redistribution of chromatin modifiers is observed during aging and following DSB induction in mammals. Aging of the neocortex in mice is associated with derepression of many genes kept transcriptionally silent by SIRT1, the yeast SIR2 homologue. Induction of DSBs through H_2O_2 treatment is sufficient to cause desilencing of many of these same genes due to SIRT1 relocalization to sites of DSBs, similar to what is reported in yeast [199]. Furthermore, DNA damage in both yeast and mammals causes derepression of repetitive elements [75,200] another epigenetic change that is widely observed during the aging of distantly related species. Redistribution of both yeast SIR2 and mammalian SIRT1 is dependent on the DNA damage response (DDR), as this redistribution is blunted without the activities of PI3-kinase related protein kinases (MEC1 for yeast and ATM for mammals) [198,199]. Since the components of the DDR are widely conserved, it's likely that the epigenetic changes that occur in response to DNA damage are highly conserved as well.

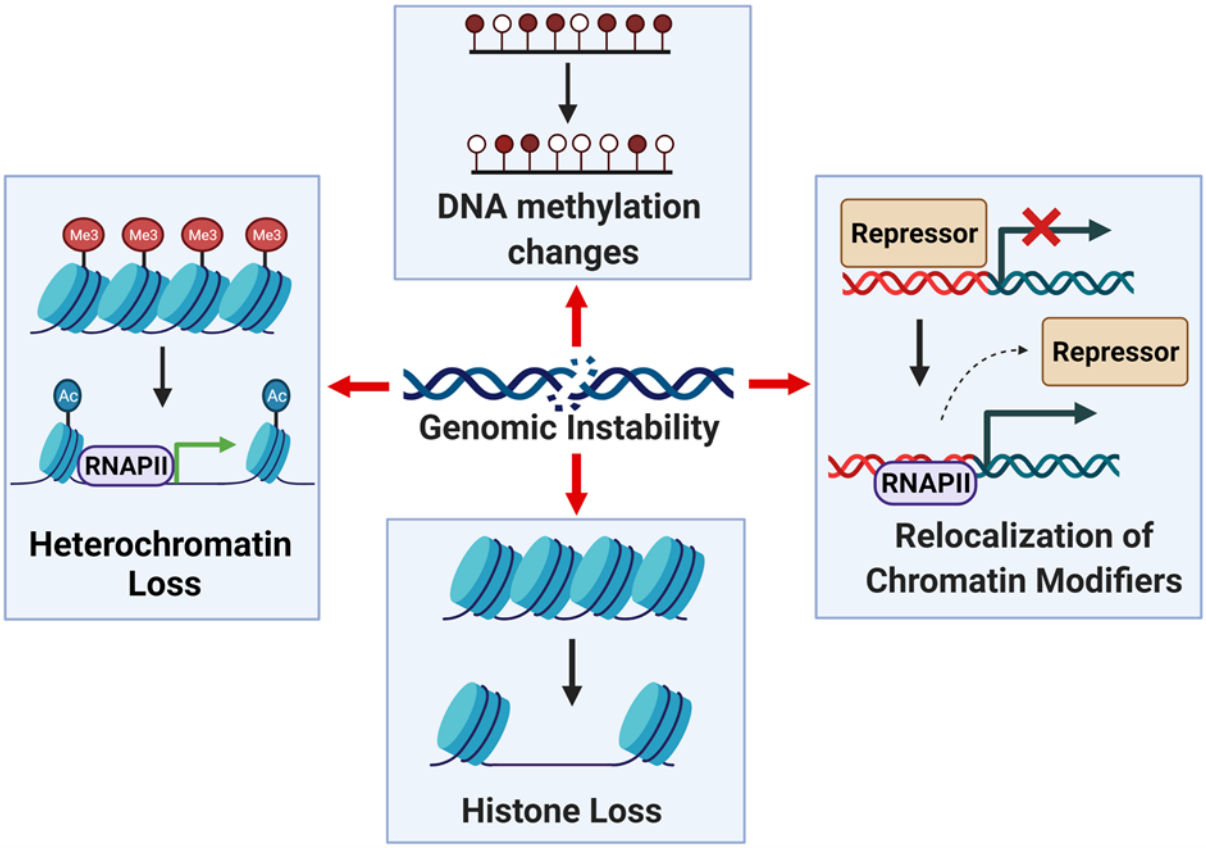

Figure 1.2: Genomic instability accelerates several age-related signs of epigenetic dysfunction. Inducing genomic instability across several different model organisms results in long-lasting epigenetic changes, several of which occur during the aging process. Prominent among these are DNA methylation changes that accelerate the DNA methylation clock, relocalization of chromatin-binding factors that alters gene expression, loss of histones, and an overall loss of heterochromatin resulting from histone loss and changes to histone posttranslational modifications.

Adding further evidence that DNA damage is a likely driver of epigenetic decay during aging, two recent mouse models demonstrated that DSBs are sufficient to accelerate multiple aspects of epigenetic aging. In one model, DSBs were induced in four to six-month-old mice by expression of the I-*PpoI* restriction endonuclease for three weeks. Tissues of these mice analyzed at 15 months of age showed multiple signs of exacerbated epigenetic aging, including increased expression of repetitive elements in the muscle and liver, acceleration of the DNA methylation clock in skeletal muscle, and a skeletal muscle gene expression profile that closely resembled

that of mice nine months older (15 vs. 24 months) [99]. Similar results were obtained *in vitro* using fibroblasts isolated from these mice. Inducing I-*PpoI* expression for one day in these fibroblasts moderately elevated DSB levels, yet was sufficient to cause long-term changes to the epigenome that mimicked age-related changes. Prominent changes included redistribution of the histone marks H3K27me3, H3K27ac, and H3K56ac, reduced expression of histone genes, increased transcription of repetitive LINE-1 elements, acceleration of the DNA methylation clock, and an overall gene expression profile that closely resembled that of fibroblasts derived from older mice [100]. Importantly, the cells and mice from this model of accelerated epigenetic aging displayed accelerated aging phenotypes, including increased frailty, decreased grip strength, decreased endurance, and impaired learning and memory in the mice, along with an early onset of senescence in the fibroblasts. These results were corroborated by a separate study in which the restriction endonuclease SacI was expressed specifically in the livers of three month old mice for 9 days. Livers of these mice showed epigenetic and histological evidence of accelerated aging as quickly as one month later, including gene expression profiles that matched those of older animals, increased cell senescence, and increased karyomegaly [32].

Taken together, all of these findings strongly suggest that genomic instability is a universal driver of the age-related epigenetic dysfunction observed across species.

III. Strategies for Enhancing Genomic Stability

a. Background and overexpression of endogenous DNA repair proteins

Most investigations of the role of DNA damage in aging have looked for correlations between DNA damage and age-related outcomes, or have sought to exacerbate DNA damage and see if this is sufficient to accelerate cellular, molecular, and physiological aspects of aging. Correlative studies, while informative, cannot provide definitive evidence for a causal role of

DNA damage in aging. This is especially true since many of the aging hallmarks, not just genomic instability, have compelling correlations with aging. For example, while the lifespan of species correlates with the efficiency of several DNA repair pathways [64-68], it also correlates well with proteostasis [201] and the rate of telomere shortening [202]. While more definitive conclusions can be drawn from studies that exacerbate genomic instability, this approach generates DNA damage levels that vastly exceed endogenous levels. Consequently, it is unclear if the findings of these studies are an accurate depiction of the role of genomic instability in aging.

An alternative strategy is to enhance genomic stability and see how this affects lifespan, healthspan, and cellular and molecular changes during aging. Since this would specifically lower endogenous DNA damage levels, it would lead to a much clearer understanding of the role of DNA damage in aging. While the utility of this strategy has been widely recognized [203], it has proven exceptionally difficult to enhance genomic stability. The most sensible and common approach to improving genomic stability has been to overexpress key DNA repair proteins. This strategy seems particularly well-suited to enhancing genomic stability during aging, since the expression levels of several DNA repair proteins decrease during aging, coinciding with decreased DNA repair efficiency [204,205].

Unfortunately, overexpression of endogenous DNA repair proteins has largely failed to improve genomic stability, particularly in mammals. One common problem is that overexpression of endogenous DNA repair proteins results in apoptosis. This has been observed following overexpression of the mismatch repair proteins MSH2 and MHL1 [206], the WRN helicase [207], and the RAD51 recombinase [208]. Although the exact mechanism behind this phenomenon is unknown, it is likely that overexpression of these proteins disrupts the carefully

regulated stoichiometry between DNA repair proteins that are involved in the same complexes or repair pathways. This rationale could also explain why overexpression of some DNA repair proteins actually increases genomic instability. For example, overexpression of either the base excision repair glycosylase NTHL1 or a catalytically inactive variant increases DSBs in nontransformed human cells [209], while overexpression of mismatch repair proteins in yeast increases mutation rates and sensitizes cells to genotoxic agents [210]. Another common finding is that overexpression of individual DNA repair proteins is often insufficient to enhance the efficiency of repair. Mao *et al* [211] found that human fibroblasts had a decreased efficiency of HR repair during replicative aging, coinciding with lower expression levels of Rad51, Rad51C, Rad52, and NBS1- key proteins involved in this DSB repair pathway. However, overexpression of each of these proteins didn't improve HR efficiency, and overexpression of Rad52 alone, or overexpressing all of the proteins together, actually decreased HR efficiency.

The most notable exception to these findings is that overexpression of SIRT6 is sufficient to enhance the efficiency of HR and NHEJ [69]. Though the reason for this exception isn't completely understood, it could be due to SIRT6's activities being far upstream in DSB repair pathways [69-72]. However, SIRT6 is not an ideal candidate for understanding how specific enhancement of DSB repair affects aging, as SIRT6 plays pivotal roles in other longevity-linked processes [73-76].

b. Overexpression of exogenous DNA repair proteins

Expressing exogenous (i.e. "from other species") DNA repair proteins is an alternative strategy for improving genomic stability. Although this strategy has received little attention compared to overexpression of endogenous proteins, there are reasons to believe that this is a more promising approach. First, expressing an exogenous protein with no homolog is likely to

avoid the issues of stoichiometric imbalance that occur when overexpressing endogenous DNA repair proteins that are known to work in complexes. Second, overexpressing an exogenous protein has the potential to add new DNA repair or protection mechanisms to cells and organisms, whereas overexpressing endogenous proteins can only add to the efficacy of existing mechanisms. Finally, exogenous proteins have the potential to be orders of magnitude more efficient at DNA repair or protection than endogenous mechanisms, meaning that even a low level of overexpression of an exogenous protein could dramatically lower the level of endogenous DNA damage. In contrast, the impact of endogenous proteins is constrained by the extent to which the protein can be safely overexpressed. The trade-off is that exogenous proteins have a higher likelihood of being non-functional when expressed in another species, particularly if they require other proteins for their activity that do not have an appropriate homolog expressed in that species.

The potential of this approach is best illustrated by mice that express photolyases from the plant *Arabidopsis thaliana.* Photolyases are a class of enzymes that directly repair cyclobutene pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP), helix distorting lesions that are caused by ultraviolet (UV) light exposure. These enzymes are largely phylogenetically conserved, with the exception of placental mammals. Mice expressing the *A. thaliana* photolyase have improved repair of UV-induced DNA damage, as evidenced by an increased rate of CPD and 6-4PP removal, decreased mutagenic events, reduced apoptosis levels, and decreased incidence of skin cancer [212,213]. This improvement is the result of the many advantages photolyases have in comparison to the nucleotide excision repair pathway used by placental mammals. These include a far quicker rate of lesion repair (occurring in less than a

nanosecond!), the ability to repair damage themselves without the need for dozens of other proteins, and the avoidance of strand breaks as part of the repair process [214].

Mammalian genomic stability has also been improved by expressing exogenous DNA repair and protection proteins from organisms that are highly resistant to multiple forms of DNA damage. Dsup is a double-stranded DNA-binding protein that is unique to Tardigrades- known informally as "water bears"- a phylum comprised of microscopic animals that are highly resistant to multiple genotoxic agents. Human cells expressing Dsup have dramatically enhanced genomic stability, as evidenced by a ~50% reduction in DSB levels following treatment with H_2O_2 or gamma irradiation, concurrent with increased survival and decreased apoptosis [215,216]. This improved genomic stability results from Dsup binding to the genome in a nucleosome-like structure and protecting it from hydroxyl radicals [217]. This is the first evidence suggesting that some of the key mechanisms underlying the extraordinary genomic stability of "extremophiles" can be transferred to other species by expression of exogenous proteins.

Surprisingly, this method of improved genomic stability is not limited to eukaryotes, as proteins from bacterial extremophiles can also safeguard mammalian genomes. One of the most impressive examples comes from mice that express PprI, one of the key regulators of the DDR in the radioresistant bacterium *Deinococcus radiodurans*. Mice expressing this transgene had substantially improved survival when exposed to lethal doses of gamma irradiation [218]. Furthermore, they exhibited fewer adverse side effects from radiation exposure, including reduced lymphopenia and decreased levels of apoptotic splenocytes and thymic cells. PprI resulted in increased expression across several tissues of the RAD51 recombinase, which aids in the repair of DSBs caused by radiation. This replicates one of the key mechanisms of PprI in its native organism, where PprI causes transcriptional upregulation of the RAD51 homolog RecA

following radiation exposure [219]. This is a remarkable finding, especially since mice have no identified PprI homolog and PprI would have to be transported into the nucleus to directly stimulate transcription of RAD51, a process that isn't required in bacteria which lack a nucleus.

Collectively, these findings suggest that expression of exogenous DNA repair and protection genes- and in particular ones from radioresistant extremophiles- is a promising strategy for enhancing the genomic stability of mammals and potentially other organisms. Organisms expressing these proteins could then be used to examine whether improved genomic stability impacts age-related epigenetic decay, lifespan, and healthspan.

IV. Summary

Genomic instability and epigenetic dysfunction are two hallmarks of aging that are widely observed across species. Changes to the epigenome likely play a pivotal role in the aging process, as epigenetic markers like DNA methylation patterns are highly accurate biomarkers of aging and interventions that slow or reverse age-related changes to the epigenome frequently extend lifespan. Although the underlying causes of epigenetic dysfunction are unknown, genomic instability is a suspected culprit due to the highly similar changes that occur to the epigenome during aging and following the induction of DNA damage. However, these similarities are from experiments in which DNA damage levels are highly elevated, and it is unknown if they accurately recapitulate the epigenetic changes that occur under endogenous levels of DNA damage. The hypothesis that genomic instability is a driver of age-related epigenetic decline could more definitively be tested by engineering organisms with enhanced DNA damage prevention or repair capacities and testing if the epigenomes of these organisms are better preserved during aging. Despite many attempts, it has proven difficult to generate organisms with improved genomic stability, as overexpression of endogenous DNA repair

proteins is frequently toxic. There is emerging evidence that expressing DNA repair or protection proteins from other species might be a more fruitful strategy for enhancing genomic stability, but this strategy has not been thoroughly explored.

In chapter two of this thesis, I explore this strategy by identifying and characterizing novel proteins from the radioresistant bacterium *Deinococcus radiodurans* that enhance genomic stability in mammalian cells, yeast, and worms. In chapter three, I use transgenic yeast and worms expressing these proteins to test the hypothesis that improved genomic stability extends lifespan, improves healthspan, and stabilizes the epigenome following DNA damage and during aging.

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Chapter 2: Identification of Genes that Enhance Genomic Stability Across Species

I. Statement of Work

All experiments described in this chapter were designed by myself and David Sinclair, with input from dissertation committee members Raul Mostoslavsky, Stephen Elledge, Vadim Gladyshev, and Bruce Yankner. Notably, Bruce Yankner was the first to suggest that *C. elegans* would be an ideal model for testing the effects of SSB on lifespan and healthspan, while all committee members recommended the generation of Dps1 mutants to test its mechanism of action. I conducted all experiments except for the generation of transgenic worms. Joe Zullo (Yankner Lab) designed and conducted the microinjections that generated all transgenic worms, using plasmids I generated and provided. Xiao Tian (Sinclair Lab) helped conduct the HR and NHEJ efficiency assays, specifically helping me optimize the transfection protocol. While I conducted the yeast rDNA recombination experiments, Brianah McCoy and Qiurui Zeng (both of the Sinclair Lab) helped in the blinded scoring of colonies. Jae Hyun Yang (Sinclair Lab) helped in the design of the experiments measuring DSB repair in I-*PpoI*-inducible fibroblasts and provided the cells for this experiment. Roger Chang (Silver Lab) provided the glycerol stocks of *D. radiodurans* from which genomic DNA was extracted. Patrick Griffin (Sinclair Lab) constructed and validated the Cell Profiler program I used to analyze DSB foci. I performed all of the other analysis of the data in this chapter, and generated all of the graphs and images.

II. Abstract

Fully understanding if and how endogenous levels of DNA damage contribute to the aging process has been hampered by an inability to specifically improve genomic stability across model organisms. The most widely attempted method, overexpression of endogenous DNA repair proteins, has encountered frequent failures due to either an inability to improve DNA repair or by inducing cell death. Expression of exogenous DNA repair proteins has yielded some success, but this strategy has not been thoroughly explored. Here, I report the identification of proteins from the radioresistant bacterium *Deinococcus radiodurans* that enhance genomic stability across mammalian cells, yeast, and worms. Using H_2O_2 resistance as a proxy for improved genomic stability, I screened a library of the key genes involved in maintaining *D. radiodurans* genomic stability. Six genes improved H_2O_2 survival across multiple human cell lines. Two of these genes- encoding the single-stranded DNA binding protein SSB and the double-stranded DNA binding protein Dps1- also improved H_2O_2 survival in *S. cerevisiae*. SSB also improved survival to multiple genotoxic agents in *C. elegans*. I validated that SSB and Dps1 protect the genome by showing a reduction in DSBs in human cells following H_2O_2 treatment. Using DSB reporter assays, I find that SSB improves genomic stability by enhancing the efficiency of NHEJ in mammalian cells and yeast. This was further corroborated by increased repair of DSBs induced by a restriction endonuclease. Analysis of Dps1 mutants show that its protective effects required both DNA binding and sequestration of Fe^{2+} ions, suggesting that Dps1 protects the genome from hydroxyl-mediated DNA damage. Overall, these results demonstrate that proteins from radioresistant organisms can be highly effective at improving genomic stability across species. These characteristics make these proteins, and in particular SSB and Dps1, ideal tools for understanding how improved genomic stability affects aging.

III. Introduction

Protecting the integrity of the genome is essential for the survival of all organisms. The genome is constantly being damaged by a variety of endogenous and exogenous forces, including ultraviolet rays, reactive oxygen species (ROS) and other free radical byproducts from cellular metabolism, alkylating agents, and radiation. It is speculated that protection of genetic material was one of the major challenges that faced the first life forms [1], particularly since the early Earth was exposed to high levels of radiation. Consequently, it is not surprising that organisms have evolved several highly conserved mechanisms for protecting and repairing the genome. Archaea, bacteria, and eukaryotes share many similar DNA repair pathways, including mismatch repair, nucleotide excision repair, base excision repair, homologous recombination, and non-homologous end joining [2,3]. Not only are the steps of these repair pathways quite similar, but there are also substantial structural similarities between the key proteins involved in these processes.

Despite the highly conserved nature of these repair pathways, there is substantial variation in the efficacy of DNA repair and protection mechanisms between species, even between closely related ones. A study of fibroblasts derived from 18 rodent species found an approximately five-fold difference in their ability to repair UV-induced DNA damage and up to a two and a half-fold difference in their ability to repair DSBs [4]. A separate study reported a ~50-fold difference in the repair efficiency of UV-C and H_2O_2 damage among rodents [5]. Even more substantial discrepancies are discovered when comparing genomic stability among more evolutionarily distant species. The most common method for assessing genomic stability across divergent species is by comparing levels of DNA damage and cell death following exposure to different doses of genotoxic agents. Comparing the reported LD_{50} of different organisms reveals a

staggering difference in sensitivities to various genotoxic agents: a ~3,000-fold difference to gamma irradiation [6,7], a >200-fold difference to UV-C [8,9] , and a >100-fold difference to H_2O_2 [10,11].

One conspicuous finding from these studies is that a select group of species are able to survive extraordinarily high doses of multiple genotoxic agents. These species primarily belong to specific phylums across all three domains of life and live in a variety of different habitats, suggesting a genetic, rather than environmental, basis for their enhanced resilience. Although there is still some debate about the mechanisms that underlie these unusual phenotypes [12,13], there is strong evidence that enhanced DNA protection and repair mechanisms play a large role. Supporting this, mutating DNA repair genes greatly sensitizes these organisms to genotoxic agents [9,14]. More concrete evidence comes from a directed evolution study in *E. coli,* a bacterium with unremarkable radioresistance. This approach uncovered that the gamma irradiation LD_{50} to *E. coli* could be greatly enhanced by only making point mutations in *recA*, *dnaB*, and *yfjk*, three genes involved in DNA repair and metabolism. These three mutations conferred a level of radioresistance of *E. coli* that was very comparable to that of *D. radiodurans*, likely the most radioresistant organism on the planet [13].

Genomic instability is a suspected cause of aging due to its increased frequency with age [15,16] , the positive correlation between DNA repair capabilities and lifespan [4,17], and the reduced lifespan of organisms with elevated DNA damage [18-20]. However, the most definitive test of this hypothesis would be to evaluate if improving genomic stability leads to lifespan extension. This has proven difficult to achieve, as overexpressing endogenous DNA repair proteins, surprisingly, does not typically improve genomic stability [21-23]. However, genomic stability has been improved by expressing exogenous DNA repair proteins from organisms

resistant to high levels of genotoxic agents. *E. coli* expressing either PprA or PprI, two genes essential for maintaining genomic stability in *D. radiodurans*, have enhanced resistance to both gamma irradiation and H_2O_2 [24,25]. Both of these genes are unique to the *Deinococcus-Thermus* phylum, indicating their ability to enhance genomic stability does not require other *D. radiodurans*-specific proteins. Further showing that these genes can maintain activity across different species, PprI enhances DSB repair in mammalian cells [26] and in mice [27] by stimulating expression of RAD51, similar to its ability to stimulate transcription of RecA in *D. radiodurans* [28]. Mammalian genomic stability is also improved by expressing Dsup, a protein unique to radioresistant Tardigrades. Dsup coats the genome of human cells and reduces DSB levels following exposure to gamma radiation [29].

These findings suggest that exogenous DNA repair and protection proteins are promising tools for enhancing genomic stability across species. Enhancing genomic stability across multiple species would allow us to test whether DNA damage has a causal role in aging. Here, I identify and characterize DNA repair and protection proteins from the radioresistant bacterium *D. radiodurans* that can enhance genomic stability across several species, including the common aging models *S. cerevisiae* and *C. elegans*. Organisms expressing these proteins can be used in future studies to test how improved genomic stability affects cellular and molecular hallmarks of aging, healthspan, and lifespan.

IV. Results

D. radiodurans genes enhance mammalian genomic stability

To identify exogenous genes that enhance genomic stability, I evaluated whether genes from the radioresistant bacterium *Deinococcus radiodurans* could enhance the genomic stability of human cell lines. *D. radiodurans* was chosen because:

1) It has extraordinarily high resistance to several different genotoxic agents [30], suggesting exceptional genomic stability.

2) Many of the genes underlying this phenotype have been identified and extensively characterized (**Table 2.1**).

3) The *Deinococcus-Thermus* phylum contains several unique genes encoding DNA repair or DNA-binding proteins that are essential for its remarkable resistance to genotoxic agents [31,32].

A literature review identified 24 genes that are strongly implicated in *specifically* maintaining genomic stability in *D. radiodurans* (**Table 2.1**). Genes were considered to have a "specific" role in maintaining genomic stability if they had a documented or highly suspected role in preventing or repairing DNA damage. This criterion helps exclude pleiotropic genes that might have several effects unrelated to genomic stability. In addition to these 24 *D. radiodurans* genes, I included the *dsup* gene from the radioresistant eukaryote *Ramazzottius varieornatus*, as it has already been shown to enhance genomic stability in human cells [29].

These 25 genes were cloned into a mammalian expression vector containing an in-frame nuclear localization sequence (NLS) from the human *c-myc* gene and a V5 tag (**Fig 2.1a**). I first tested whether these genes could be successfully expressed and localized to the nucleus of human cells. This was of particular concern since these genes were not codon optimized, and the GC content of many of these genes exceeds 75%, reflecting the GC-rich nature (67%) of the *D. radiodurans* genome [33]. All 25 genes were successfully expressed at high levels in transiently transfected HEK293T cells, with all genes showing nearly exclusive nuclear localization (**Figure 2.1b**).

Table 2.1: *Deinococcus radiodurans* genes implicated in genomic stability

	Organism	Gene	Function
1	*D. radiodurans*	DdrA	ssDNA binding protein
2		DdrB	
3		SSB	
4		RecN	DNA recombination after damage
5		RecO	
6		RecF	
7		RecA	Homologous recombination
8		RuvB	
9		RuvC	
10		DdrC	Unknown- Induced by DNA damage
11		DdrD	
12		Dps1	Binds DNA, prevents damage from genotoxins
13		Dps2	
14		PprA	Binds broken DNA ends
15		IrrE	Regulator of DNA repair
16		RqkA	Coordinates DSB Repair
17		TerF	Improves radioresistance
18		DRB0067	Protects against oxidative damage
19		Mfd	DNA repair during stalled transcription
20		HU	DNA binding protein
21		DR2162	Unknown- Predicted role in DNA repair
22		DR0428	Improves radioresistance
23		MutM	Excises oxidized nucleotides
24		DR0756	Crosslink repair
25	*R. varieornatus*	Dsup	Binds DNA, prevents damage from genotoxins

To test if any of these genes enhance genomic stability, I conducted an H_2O_2 viability screen in HEK293T cells. This assay was chosen because oxidative stress is a common source of endogenous DNA damage, is implicated in several aging and age-related diseases [34-36], and H_2O_2 treatment accelerates age-related epigenetic decay in both yeast and mammalian cells [37]. This screen identified five *D. radiodurans* genes, along with the *R. varieornatus* gene *dsup*, that significantly enhanced viability (**Fig 2.1c**). We further validated these findings by demonstrating that these six genes also enhanced H_2O_2 viability in primary human fibroblasts (**Fig 2.1d**).

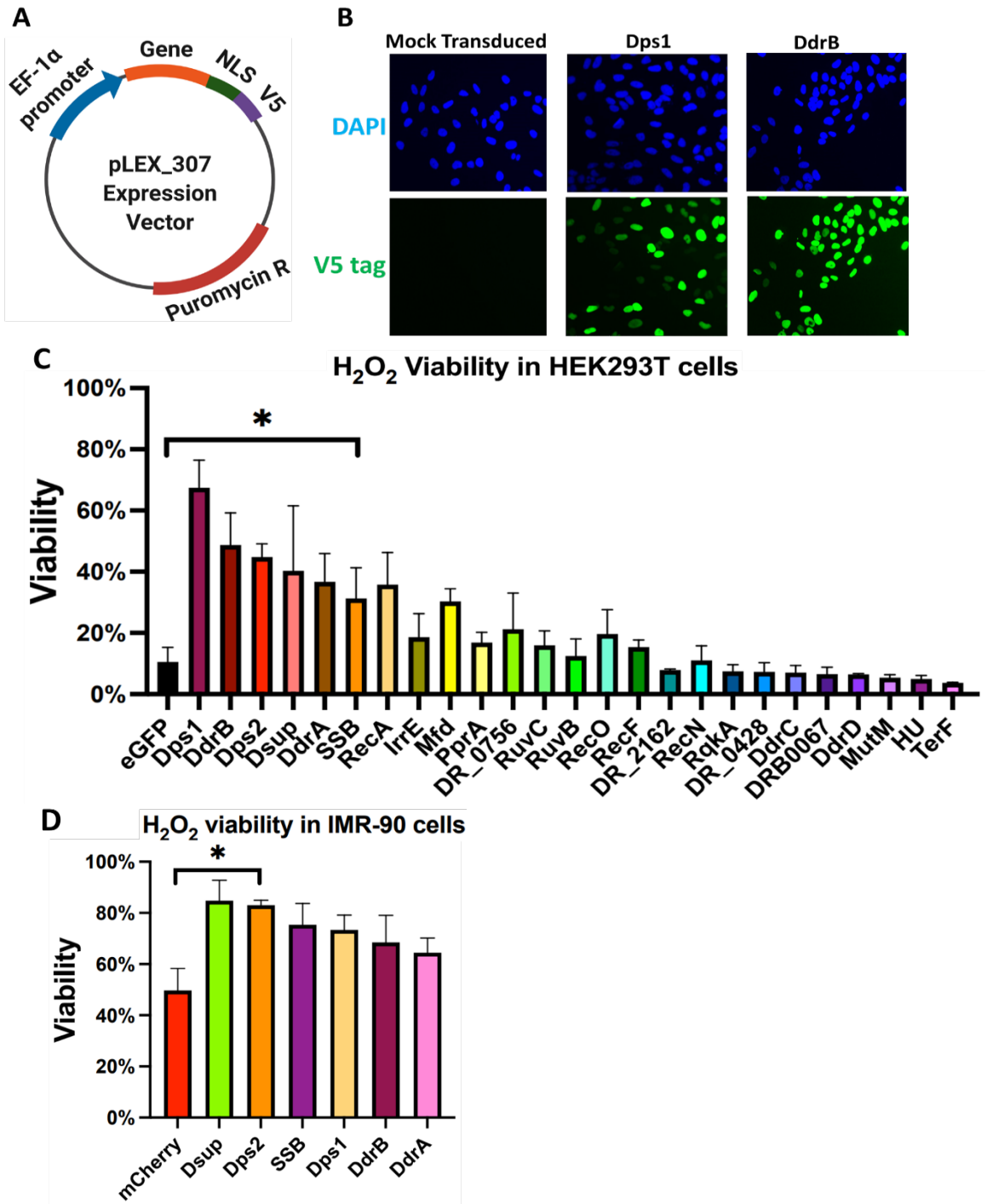


Figure 2.1: Expression and H_2O_2 viability screening in human cell lines. (A) Overview of expression vector features used to express *D. radiodurans* transgenes in human cells. (B) ICC of two D. radiodurans genes expressed in HEK293T cells. These are representative of expression and localization of all 24 transgenes. (C) H_2O_2 viability screen in HEK293T cells. Data depicts average viability + SEM from 3 experiments analyzed using a one-way ANOVA. (D) Testing hits from HEK293T cells in an H_2O_2 viability assay in primary IMR-90 human fibroblasts. Data depicts average viability + SEM from 3 experiments analyzed with a one-way ANOVA.

Ssb and *dps1* enhance the genomic stability of yeast

Since my goal was to identify genes that enhance genomic stability across multiple organisms, I next tested whether these genes affect genomic stability in the fungi *Saccharomyces cerevisiae*. Yeast strains were generated that successfully expressed each of the six genes that enhanced viability in human cell lines (**Fig 2.2A**). Two of these six genes- *ssb* and *dps1*- enhanced H_2O_2 viability in yeast (**Fig 2.2B-C**), suggesting that they may bolster genomic stability. To further test this possibility, I tested how these genes impact the stability of the rDNA locus. The yeast rDNA locus is a highly repetitive region comprised of 150 – 200 copies of the 9.1 kb rDNA sequence. Instability at this locus leads to the formation of extrachromosomal rDNA circles, which is the major cause of yeast replicative aging [38,39]. rDNA stability was monitored using an *ade2* strain containing a single *ADE2* gene integrated in the rDNA locus. This *ADE2* can be lost through recombination, resulting in the formation of "half-sector" colonies that are half red and half white (**Fig 2.2D**). This reporter was validated by inducing genomic instability with H_2O_2. As expected, H_2O_2 increased rDNA recombination rates (~4-fold, **Fig2.2E**), while overexpression of the rDNA stabilizing gene *SIR2* suppressed recombination (**Fig2.2F**). Three *D. radiodurans* genes- *ssb*, *dps1*, and *ddrB*- also significantly lowered rDNA recombination rates, suggesting they enhanced rDNA stability (**Fig2.2F**).

Ssb enhances DSB repair while Dps1 shields the genome from DNA damage

I chose to further study *ssb* and *dps1*, as these were the only two genes that enhanced genomic stability in all mammalian and yeast cell assays. *Ssb* encodes a single-stranded DNA binding protein that is involved in DNA replication and double-strand break (DSB) repair [40]. *D. radioruans* SSB shares significant homology with other bacterial single-stranded binding proteins, although it is the only known bacterial single-stranded binding protein that contains

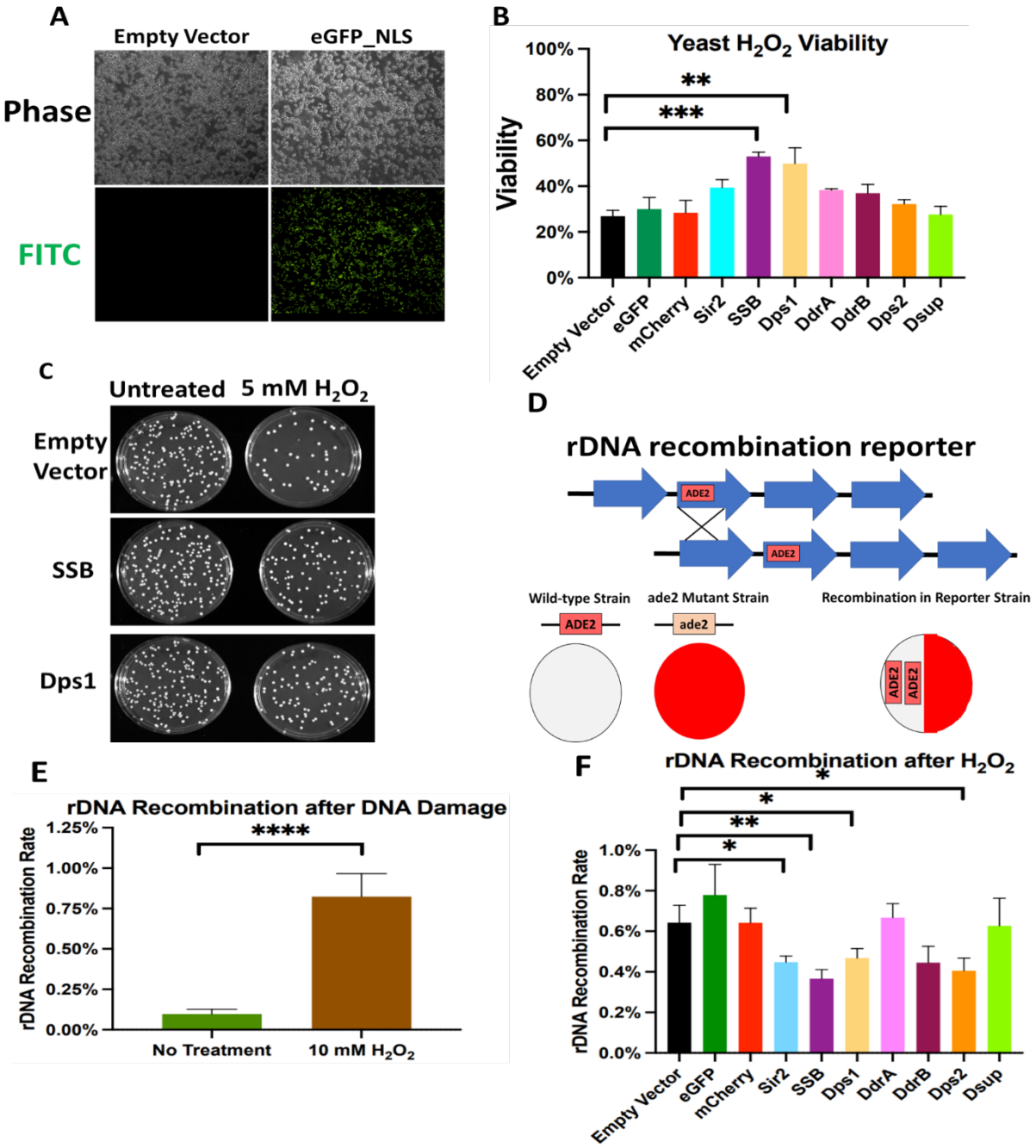


Figure 2.2: *D. radiodurans* genes improve genomic stability in yeast (A) ICC demonstrating transgene expression. (B) H_2O_2 viability of yeast exposed to 5 mM H_2O_2. Data represents average survival + SEM of three different experiments, with each experiment using a unique isolate from each group. Data analyzed with a one-way ANOVA. (C) Representative colony images from H_2O_2 viability assay (D) Schematic of rDNA recombination reporter assay. (E) rDNA recombination rates with H_2O_2 treatment. Data represents average recombination rate + SEM from ten different isolates tested across three different experiments. Data analyzed using student's t-test. (F) Average rDNA recombination rate + SEM in yeast expressing transgenes. At least five different isolates were tested for each group across at least three different experiments. Data analyzed with a one-way ANOVA.

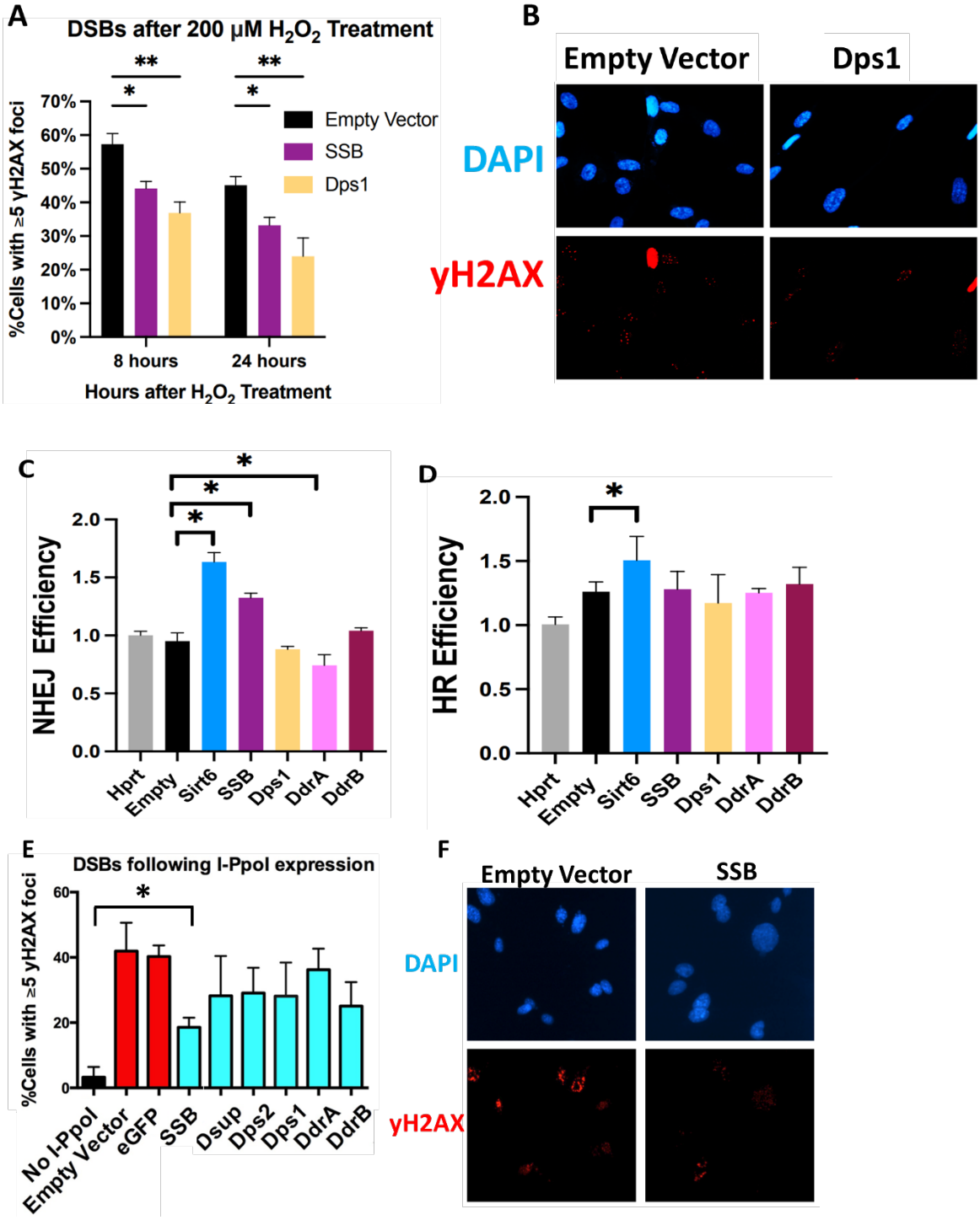
more than one OB-fold, the ssDNA binding domain [41]. SSB plays a vital role in the radioresistant phenotype of *D. radiodurans*, as reducing SSB expression by ~50% leads to a 93% decline in the LD_{50} for gamma radiation [32]. *Dps1* encodes a double-stranded DNA-binding protein that belongs to the bacterial family of "DNA-binding proteins from starved cells". These proteins are upregulated in response to a variety of stressors including starvation, oxidative stress, and exposure to high concentrations of certain metals [42]. *D. radiodurans* Dps1, like other members of the Dps family, contains an N-terminal DNA-binding domain followed by several alpha helix motifs. These alpha helices contain a total of four amino acids that are involved in the binding of Fe^{2+} ions. Dps1 monomers pack together to form a dodecameric structure containing a large central cavity that can bind up to 500 Fe^{2+} ions [43]. Dps proteins protect DNA from oxidative damage by binding DNA, and thus protecting it from free radical damage, and by sequestering Fe^{2+} ions, which are required to produce free radicals through the Fenton reaction.

I wanted to understand the mechanisms through which SSB and Dps1 protect the genomes of mammalian and yeast cells. Since both enhanced H_2O_2 viability, I hypothesized that they might reduce DSB levels. DSBs are the most deleterious form of DNA damage that has been implicated as one of the main causes of H_2O_2 -mediated death [44]. Primary human fibroblasts expressing SSB or Dps1 had significantly reduced DSB levels both 8 and 24 hours following H_2O_2 treatment (**Fig 2.3A-B**), supporting my hypothesis that they are improving genomic stability.

I next wanted to uncover the specific mechanisms through which SSB and Dps1 were reducing DSB levels. One potential mechanism would be by improving the efficiency of DSB repair. This hypothesis was tested using two human primary fibroblast lines used for monitoring

the efficiency of one of the two major DSB repair pathways: either non-homologous end joining (NHEJ) or homologous recombination (HR). These reporter cell lines were previously used in screens to identify endogenous proteins that enhance DSB repair, finding that only overexpression of SIRT6 could improve the efficiency of DSB repair [45,46]. Human fibroblasts expressing SSB have improved NHEJ efficiency, while fibroblasts expressing Dps1 or the other *D. radiodurans* genes identified in my original viability screen have no improved efficiency of either DSB repair pathway (**Fig 2.3C-D**). Critically, SSB expressing fibroblasts maintained the same level of HR efficiency as control cells. This suggests that SSB's enhancement of NHEJ activity does not merely reflect a shift from HR towards NHEJ, and indicates that SSB is improving the overall ability of a cell to repair DSBs.

To further test SSB's ability to enhance DSB repair, I evaluated if SSB could affect the repair of endonuclease-induced DSBs. Our lab has previously reported the development of a murine fibroblast cell line through which DSBs can be induced by the tamoxifen-controlled expression of the restriction endonuclease I-*PpoI* [47]. Fibroblasts expressing SSB showed a significant reduction in DSB levels one day after I-*PpoI* induction (**Fig2.3E-F**), adding further evidence that SSB enhances the efficiency of DSB repair. I further tested whether SSB could be having the same effect in yeast. NHEJ efficiency in yeast can be evaluated by monitoring the transformation efficiency of a plasmid that has been linearized *in vitro*, as yeast that fail to repair this plasmid will not grow under certain selective conditions (**Fig2.3G**) [48,49]. Yeast stably expressing SSB displayed improved repair of the *in vitro* linearized plasmid, suggesting that SSB also improves NHEJ efficiency in yeast (**Fig2.3H**).


A
DSBs after 200 µM H_2O_2 Treatment
%Cells with ≥5 γH2AX foci
70%
60%
50%
40%
30%
20%
10%
0%
**
*
**
*
Empty Vector
SSB
Dps1
8 hours
24 hours
Hours after H_2O_2 Treatment
B
Empty Vector
Dps1
DAPI
γH2AX
C
NHEJ Efficiency
2.0
1.5
1.0
0.5
0.0
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Hprt
Empty
Sirt6
SSB
Dps1
DdrA
DdrB
D
HR Efficiency
2.0
1.5
1.0
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Hprt
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Sirt6
SSB
Dps1
DdrA
DdrB
E
DSBs following I-PpoI expression
%Cells with ≥5 γH2AX foci
60
40
20
0
*
No I-PpoI
Empty Vector
eGFP
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DdrA
DdrB
F
Empty Vector
SSB
DAPI
γH2AX

Figure 2.3 (Continued)

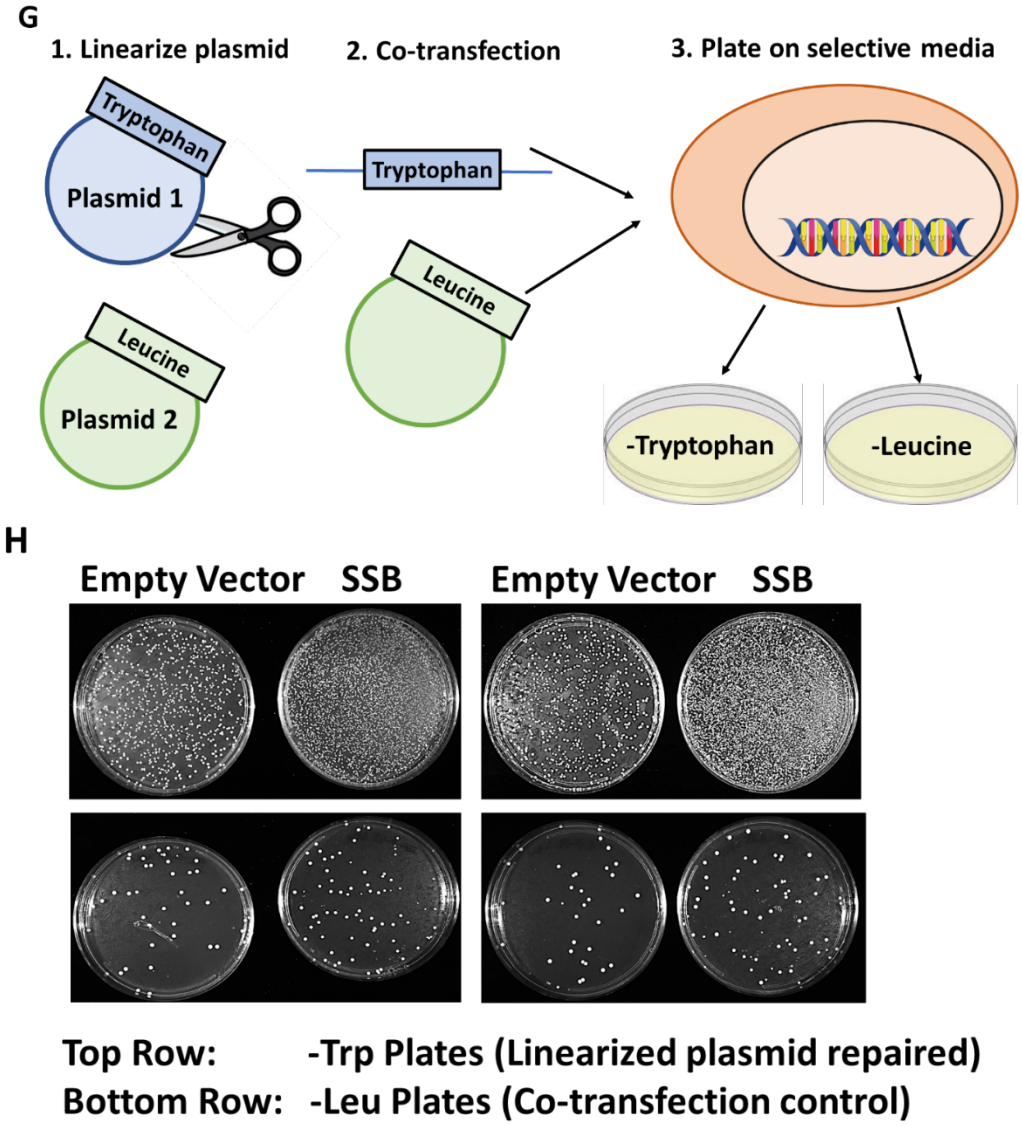

Figure 2.3: SSB and Dps1 promote genomic stability, with SSB enhancing NHEJ
(A) DSB levels following H_2O_2 treatment in human fibroblasts. Data represents averages taken from three experiments with three replicates per experiment and analyzed with a one-way ANOVA. (B) Representative image of yH2AX foci in SSB expressing cells 8 hours after H_2O_2 treatment. (C) Efficiency of NHEJ and (D) HR in human fibroblast reporter cells stably expressing transgenes of interest. Data represents average repair efficiency from three different experiments and analyzed with by a one-way ANOVA. (E) DSB levels 24 hours after I-*PpoI* induction in mouse ear fibroblasts expressing transgene of interest. Data represents average percentage of cells + SEM across three different experiments and analyzed with a one-way ANOVA. (F) Representative image of yH2AX foci in SSB and control fibroblasts 24 hours after I-*PpoI* induction. (G) Schematic of assay to assess NHEJ efficiency in yeast. (H) Colony formation in Scrambled or SSB expressing yeast following the transformation procedure depicted in G. Representative images from two experiments are shown. A third experiment was conducted and showed similar results.

While Dps1 also reduced DSB levels following H_2O_2 treatment, it did not have any effect on DSB repair efficiency. I hypothesized that Dps1 could be reducing DSB levels in mammalian cells by binding to the genome and sequestering Fe^{2+} ions, as these are the two mechanisms through which Dps-family member proteins protect DNA *in vivo* and *in vitro* [42,50]. To test this, I generated three Dps1 mutants that lacked the DNA-binding domain, lacked one of the four Fe^{2+} binding sites, or lacked all 4 Fe^{2+} binding sites (**Fig2.4A**). All Dps1 mutants exhibited a significantly reduced ability to protect human fibroblasts from H_2O_2 -mediated death. This suggests that Dps1 enhances genomic stability by protecting DNA from free-radical-induced DNA damage (**Fig2.4B**).

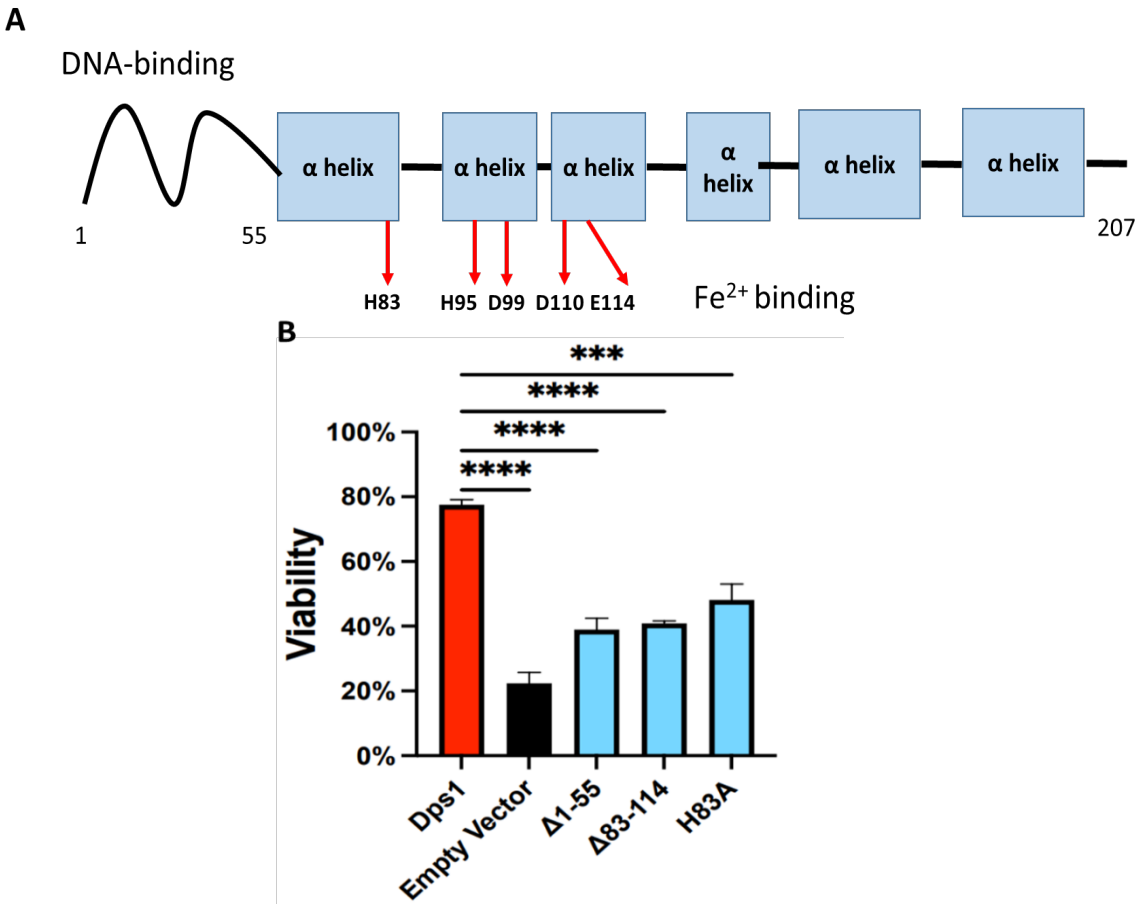

Figure 2.4: Dps1 protection requires DNA-binding and Fe^{2+} sequestration. (A) Domains of Dps1 monomer highlighting DNA-binding domains and the five Fe^{2+} sequestration residues. (B) Average H_2O_2 viability + SEM of Dps1 mutants in HEK293T cells. Data represents three different experiments analyzed with a one-way ANOVA.

Ssb enhances genomic stability of *C. elegans*

I next tested whether SSB or Dps1 could enhance the genomic stability of *C. elegans*. While three SSB transgenic worm lines were established, along with two GFP and two empty vector control lines, no viable Dps1 lines were generated following two separate attempts. The NLS tagged transgenes localized predominantly, but not exclusively, to the nucleus (**Fig 2.5A**). To examine if SSB improved genomic stability, I tested if SSB transgenic worms had altered viability in response to the genotoxic agents H_2O_2 and ultraviolet light, as increased resistance to both of these agents is associated with interventions that extend longevity [51-54]. SSB transgenic worms had substantially higher resistance to H_2O_2 treatment (**Fig 2.6B**), consistent with my previous findings in mammalian and yeast cells. SSB expression also led to a modest increase in the lifespan of worms exposed to UV-C. In addition to this increased survival, SSB worms exposed to non-lethal doses of UV-C and H_2O_2 appeared to maintain higher levels of motility, further suggesting increased resistance to DNA damage. Collectively these results suggest that SSB transgenic worms likely have enhanced genomic stability.

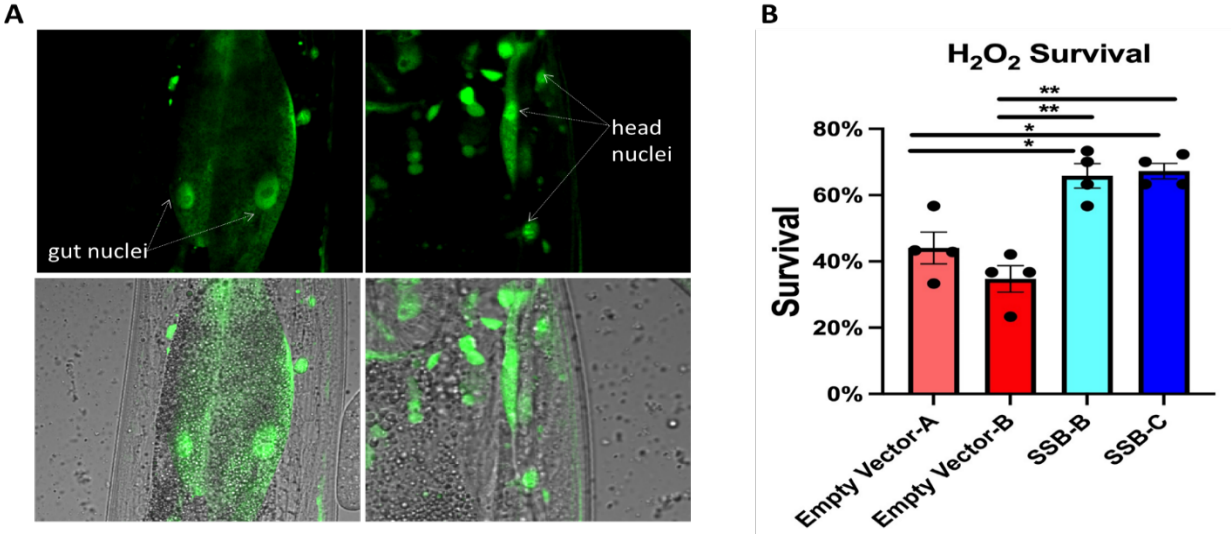

Figure 2.6 SSB transgenic worms have increased genomic stability. (A) SSB expression in transgenic *C. elegans*, highlighting probable nuclei in the gut and head. (B) H_2O_2 survival in C. elegans 8 hours after H_2O_2 treatment. Data represents average survival +/- SEM from three different experiments analyzed with a one-way ANOVA.

V. Discussion

To the best of my knowledge, this is the first genetic screen identifying exogenous proteins that enhance genomic stability. One of the surprising results was the high frequency (5/23) of screened *D. radiodurans* genes that enhanced H_2O_2 viability in human cells. This 22% hit rate is much higher than the 2% hit rate reported from a genome-wide screen identifying endogenous genes that could improve H_2O_2 viability in human fibroblasts [55]. Part of this discrepancy is likely due to my screen specifically targeting genes involved in genomic stability, which is known to be a key factor in surviving high levels of oxidative stress [56]. Consequently, it would be expected that this targeted approach would have a higher hit rate than an unbiased, genome-wide screen. However, this could also reflect an improved ability of exogenous proteins, particularly from organisms with remarkable stress resistance, to improve genomic stability and viability in other species. Additional gain-of-function studies screening larger numbers of exogenous genes will provide more quantitative evidence that clarifies whether proteins from stress-resistant organisms are especially capable at improving genomic stability.

Another noteworthy finding is that all six genes that improved H_2O_2 viability have very similar functions: either binding single- or double-stranded DNA. Based on my findings on the mechanisms of Dps1-mediated protection, along with previous findings about the dsDNA binding protein Dsup [29], it is likely that these dsDNA binding proteins help prevent DNA damage. On the other hand, ssDNA-binding proteins are more likely to aid in DNA repair. This is supported by the finding that SSB enhanced NHEJ efficiency in human cells while expression of DdrA, an ssDNA-binding protein structurally similar to the HR factor RAD52 [57], had a negative impact on NHEJ efficiency. DNA strand breaks caused by H_2O_2 treatment results in high levels of ssDNA that is susceptible to digestion by exonucleases, and preservation of these

ends is essential to prevent extensive degradation of the genome and for aiding in DSB repair [58]. The high hit frequency of ssDNA-binding proteins could indicate that binding and protection of single-strand ends is one of the critical factors for ensuring survival following high levels of oxidative stress. The lack of hits outside of DNA binding proteins might also indicate that *D. radiodurans* proteins with other functions in maintaining genomic stability, such as those involved in resolving Holliday junctions during HR [59], require other proteins that are not present when expressed in other organisms. This is one of the major drawbacks of an exogenous screening approach, as many proteins, especially those involved in DNA repair, operate in multi-protein complexes and would be unlikely to function in organisms that are evolutionarily distant from their native species.

Finally, SSB's enhancement of NHEJ efficiency across species highlights the potential of exogenous proteins to generate unique phenotypes that might be difficult to achieve through more conventional approaches. Several researchers have attempted to enhance DSB repair through overexpression of numerous endogenous repair genes, with only one candidate, SIRT6, demonstrating improved efficiency [45,46]. One possible explanation is that SSB is acting through a unique mechanism that dramatically improves one or more steps in the NHEJ repair pathway, and that such a level of extraordinary improvement is unlikely to be achieved by merely increasing expression levels of endogenous proteins. A similar phenomenon was previously reported in mice expressing a plant photolyase, with these transgenic mice showing massively accelerated repair of UV-induced lesions [60,61]. Future investigations on the specific mechanisms through which SSB participates in NHEJ will help clarify why it is one of the only proteins that can improve NHEJ and may also provide insights into other proteins or strategies that could bolster DSB repair.

These findings cumulatively suggest that genomic stability of mammalian cells, yeast, and worms can be enhanced by expression of SSB and Dps1. Consequently, organisms expressing these proteins would be excellent model systems for testing the effect of improved genomic stability on aging, helping us understand how endogenous levels of DNA damage affect cellular function and whether it has a causal role in determining lifespan or healthspan.

VI. Materials & Methods

Plasmid Generation

A glycerol stock of *Deinococcus radiodurans* R1 was generously donated by the lab of Pam Silver (Harvard Medical School). Genomic DNA was extracted from saturated *D. radiodurans* cultures, and genes of interest (Table 1) were PCR amplified to contain the full open reading frame (excluding stop codon) along with flanking *attB1* and *attB2* sequences (Primers in **Table 1.3**). *Dsup* from *Ramazzottius varieornatus* was amplified from a plasmid generously provided by the laboratory of Takekazu Kunieda (The University of Tokyo). *Gfp*, *mcherry*, and *sir2* were amplified from plasmids belonging to the Sinclair Lab. The "Empty Vector" control used in all experiments was generated by amplifying a 59-nucleotide sequence synthesized by IDT that doesn't encode for any protein. Amplified PCR products were purified (Omega Biotek #6492-02) and subsequently cloned into the Gateway Donor vector pDONR223 (Addgene #2395) using the Gateway BP Clonase Enzyme Mix (ThermoFisher #11789100). These donor vectors were then purified (Omega Biotek D6942-02), and subsequently cloned into a modified Gateway Entry vector pLEX307 (Addgene #41392) using the Gateway LR Clonase Enzyme Mix (ThermoFisher #11791100). This vector was modified to contain a 27-nucleotide sequence encoding a nuclear localization signal from *c-myc* 5' to the attR2 site, generating a vector we named "pLEX307_NLS". Expression of all transgenes in mammalian cells was accomplished using

pLEX307_NLS. Transgene expression in *S. cerevisiae* was accomplished using the pAG306GPD-ccdB plasmid (Addgene #14140). Genes were amplified from their pLEX307_NLS vector (Primers in **Table 1.3**) to contain the open reading frame, *c-myc* NLS, and V5 tag, flanked by *attB1* and *attB2* sites. These amplicons were PCR purified, cloned into the pDONR223 entry vector, and then subcloned into the pAG306GPD-ccdB Gateway Entry plasmid. Plasmids for transgene expression in *C. elegans* were generated following the same protocol as described for yeast, except pCFJ150 (Addgene #19329) was used as the Gateway Entry plasmid.

Cell Culture

293T cells (ATCC #CRL-3216) and IMR-90 cells (ATCC #CCL-186) immortalized with hTERT were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown in a 37°C incubator with 5% CO_2. The I-*PpoI*-inducible fibroblasts were harvested from the ears of 3-month old ICE mice. These cells were cultured in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin, and 0.1 mM beta-mercaptoethanol. ICE fibroblasts were grown in a 37°C incubator with 3% O_2 and 5% CO_2. All cells were maintained at 20-80% confluency, with media being changed at least every three days. H_2O_2 (Sigma Aldrich #H1009) and 4-OHT (Sigma Aldrich #H7904) were added at the indicated concentrations directly to the culture medium. hTERT IMR-90 cells stably expressing the transgenes of interest were generated by transducing hTERT IMR-90 cells in 6-well plates at ~40% confluency with lentivirus encoding the transgenes in a pLEX307_NLS vectors. 500 µL of lentivirus was added to 1.5 mL of growth media per well, along with 5 µg/mL of Polybrene (Fisher Scientific #NC9840454). The following day the media was removed, cells were washed three times with PBS, and growth media was replaced. Three days after transduction, cells were

split with growth media containing 2 μg/mL Puromycin (Thermo Fisher Scientific #A1113802). Cells were incubated for three days, with media changed every day with fresh growth media containing Puromycin.

Yeast Strains

Yeast strains are listed in **Table 1.4**. Yeast expressing the transgenes of interest were generated by transforming the appropriate strain with expression vectors that had been linearized within the URA3 site. For transformation, yeast strains were streaked out from glycerol stocks onto YPD plates, incubated at 30°C for two days, and then single colonies were picked into YPD media. 50 mL cultures of logarithmically growing yeast cells (OD_{600}= 0.500 – 0.700) were resuspended in 10 mL of a 1X lithium acetate (1X LiOAc) buffer containing 0.1 M LiOAc, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Cells were centrifuged, supernatant removed, and resuspended in 1 mL of 1X LiOAc buffer. 100 μL of yeast cells, 36 μL of 1M LiOAc, 10 uL of 10 mg/mL salmon sperm DNA (ThermoFisher Scientific #15632011), and up to 1.5 μg of linearized plasmid was then added to a microcentrifuge tube. 280 μL of 50% PEG-3350 was then added, the tube vortexed, and incubated for 30 minutes at 30°C, rotating at 45 RPM. DMSO was added to a final concentration of 10%, vortexed, and heat shocked at 42°C for 15 minutes. Cells were then placed on ice for 5 minutes, centrifuged, and resuspended in 200 μL of TE buffer (pH 8.0). 125 μL of resuspended cells were transferred to CSM-URA plates. These plates were placed in a 30°C incubator for three nights. Resulting yeast colonies were subsequently picked, grown in CSM-URA media, and frozen down as glycerol stocks.

Worm Strains

N2 Bristol *Caenorhabditis elegans* worms were used for all experiments. Worms were cultured at 20°C on NGM plates with OP50 bacteria. Transgenic worms were generated by microinjection

of pCJF150 expression plasmids into the gonads of N2 worms. For experiments, worms were synchronized by conducting a 5-10 hour egg lay simultaneously with all of the relevant strains. Progeny from these worms were then used for experiments.

Immunocytochemistry

Cells were grown to 40-80% confluency in 8-well glass chamber cover slides (ThermoFisher #155409PK). Media was removed, cells were washed twice with 500 µL of PBS, and then fixed with 4% formaldehyde for 15 minutes. Cells were then washed twice again with PBS and permeabilized with 0.2% TritonX-100 in PBS (PBS-T). Cells were washed twice again, blocked with 5% BSA in PBS-T. For localization of transgenes, cells were incubated for one hour with a FITC-conjugated V5 antibody (ThermoFisher #R963-25) in 5%BSA PBS-T at 1:1000. For detection of DSB foci, cells were incubated for one hour with an γH2AX antibody (Cell Signaling #2577) diluted 1:1000 in 5%BSA PBS-T, washed three times with PBS, and then incubated with an HRP-conjugated anti-Rabbit IgG antibody (VWR #NA934) diluted 1:5000 in 5% BSA PBS-T. Cells were washed three times with PBS, and then incubated for 10 minutes with DAPI in PBS at 1:10,000. Cells were then washed twice more with PBS and imaged.

Mammalian Cell H_2O_2 Viability

H_2O_2 viability was assessed in 293T cells using the MTT viability assay [62]. 293T cells were seeded in 24-well tissue culture plates at 175,000 cells/well and grew overnight. The next day, cells were transfected with 650 ng of pLEX307_NLS plasmids containing the transgene of interest. Transfections were done in Optimem reduced serum media (ThermoFisher #31985062) with plasmid DNA incubated in Optimem along with PEI transfection reagent. Transfection mixes were added to the seeded cells, cells were incubated overnight, and the following day the media was removed and fresh growth media was added. The following day, media was removed

and growth media containing 1150 μM of H_2O_2 was added to the cells. Cells were incubated overnight. The following day, media was removed, and replaced with 1 mL of fresh growth media. 200 μL of MTT solution (5 mg/mL MTT in PBS) was added, and cells were placed back in the incubator for 3.5 hours. Media was then carefully removed, and 1 mL of MTT solvent (4 mM HCl, 0.1% NP-40 in isopropanol) was added to cells. Solvent was mixed with the cells thoroughly, followed by a 20 minute incubation while rocking. Absorbance was then monitored at 590 nM using a plate reader.

H_2O_2 viability was assessed in hTERT-IMR90 cells using Calcein-AM (VWR #206700). Cells stably expressing the transgenes of interest were plated at 10,000 cells/well in 96-well tissue culture plates. The following day, media was removed and exchanged with growth media containing 450 μM H_2O_2. Cells were incubated overnight, and the following day media was removed, cells were washed once with PBS, and 500 μL of Calcein-AM dissolved 1:400 in phenol-red free skeletal muscle cell growth media (PromoCell #C23060) was added. Cells were placed back at 37°C for 30 minutes, after which media was removed, cells were washed once with PBS, and fluorescence was monitored using a plate reader (excitation = 490 nm, emission = 520 nm).

Yeast H_2O_2 Viability

Yeast were grown overnight in CSM-URA media in a 30°C shaking incubator. The following day, cultures were backdiluted into 11 mL of CSM-URA media at OD_{600}=0.3. Cultures grew until OD_{600}=0.6, at which point each individual culture was split into two 5 mL cultures. 5 mM of H_2O_2 was added to one of these cultures, no H_2O_2 was added to its counterpart, and tubes were put back in the incubator for 90 minutes. A small volume of each culture was diluted ~1:25,000 in diH_2O, and 100 μL of this dilution was plated onto YPD plates and spread with autoclaved

beads. Plates were put in a 30ºC incubator for three days, after which colonies were counted and viability was calculated by dividing the number of colonies resulting from the 5 mM H_2O_2 -treated culture by the number of colonies resulting from the culture that did not receive H_2O_2.

Worm H_2O_2 and UV-C Viability

For H_2O_2 viability, 3 day old worms were harvested from their NGM plates by washing with M9 buffer, worms were washed twice with 15 mL of M9 buffer, and resuspended to a final volume of 1 mL. This 1 mL suspension of worms in M9 buffer was then added to 1 mL of M9 buffer in a 6-well tissue culture plate. 1 mL of M9 buffer with 3 mM H_2O_2 was then added to these wells, for a final H_2O_2 concentration of 1 mM. Worms were incubated for 8 hours, after which survival was assessed. Worms were scored as dead if no movement was detected after they had been prodded twice with a platinum wire. For UV-C viability, 3 day old worms were picked onto NGM plates without OP50 and exposed to the appropriate dose of 254 nm UV-C using a UV-Stratalinker 1800. Worms were then carefully transferred back to an NGM plate with OP50, and survival was monitored each day.

Yeast rDNA recombination

Yeast were grown overnight in CSM-URA media in a 30ºC shaking incubator. The following day, cultures were backdiluted with CSM-URA media to OD_{600}=0.3. Cultures grew until OD_{600}=0.5 – 0.7. 5 mL of each culture was then transferred into a new tube, and 1 mM of H_2O_2 was added if needed. Cultures were then incubated for 90 minutes in the 30ºC shaking incubator. A small volume of each culture was diluted ~1:25,000 in diH_2O, and 500 µL of this dilution was plated onto 150 mM YPD plates not supplemented with adenine, and spread with autoclaved beads. Plates were put in a 30ºC incubator for three days. Plate identities were blinded, and total

colonies and number of half-sector colonies were counted. Plates were unblinded, and recombination frequency was calculated by calculating the percentage of half-sector colonies.

Yeast NHEJ Efficiency Assay

YDS106 strains with an integrated empty vector or SSB vector were used in this experiment. Three independent cultures were prepared for each strain. Strains were transformed with 500 ng of EcoR1-linearized pRS314 plasmid and 25 ng of circularized pRS315. Transformed cells were plated onto CSM-TRP plates and CSM-LEU plates, and plates were incubated at 30^{o}C for three days. Pictures of the resulting colonies were then taken. For negative controls, both strains were transformed without the addition of plasmids, and plated onto CSM-TRP and CSM-LEU plates.

Mammalian HR and NHEJ Efficiency Assay

The human fibroblasts, DsRed expression plasmid, and I-SceI expression plasmid used in this experiment were generously donated by the Gorbunova Lab. These cells are hTERT-immortalized human fibroblasts isolated from neonatal foreskin that contain a stably integrated copy of the NHEJ and HR reporter cassettes, as previously described [45,46]. Cells at ~50% confluency were electroporated using the CZ167 pre-programmed protocol on a 4D Nucleofector X Unit device (Lonza Biosciences #AAF-1002X). Cells were electroporated with a mixture containing 5 µg of an I-SceI expression plasmid, 5 µg of the appropriate pLEX307_NLS expression plasmid, and 50 ng of a DsRed expression plasmid. Three days after transfection, cells were harvested and GFP+ and DsRed+ cells were quantified by flow cytometry using a LSR-II Analyser (BD). Efficiency of HR and NHEJ was calculated by comparing the GFP+:DsRed+ ratio in each group to the GFP+:DsRed+ ratio of the empty vector control.

DSB Repair in ICE Cells

Repair of I-*PpoI* DSBs was evaluated in ear fibroblasts from 3 month old ICE mice ($Cre^{-/+}$ I-$PpoI^{-/+}$) that stably expressed transgenes of interest. Corresponding fibroblasts from 3 month old CRE mice ($Cre^{-/+}$ I-$PpoI^{-/-}$) were used as controls for spontaneous DSB levels in the presence of 4-OHT. When cells were ~50% confluent, growth media was replaced with fresh growth media containing 0.5 μM 4-OHT to induce I-*PpoI* expression. The following day, media was removed, cells were fixed, and immunocytochemistry was conducted using a γH2AX antibody (Cell Signaling #2557). Cells were then imaged on a camera-equipped Nikon Eclipse Ti microscope using NES Elements software. Captured images were automatically analyzed using a Cell Profiler pipeline designed for the quantification of γH2AX foci.

Data Analysis

All statistical analysis performed using student-t tests, one-way ANOVAs, or two-way ANOVAs using GraphPad Prism 9.3.0. Dunnett's test was applied to all one-way ANOVAs, and Tukey test applied to all two-way ANOVAs to control for multiple comparison testing. Asterisks signify the following: * ($p<0.05$), **($p<0.01$) ***($p<0.001$), ****($p<0.0001$).

Table 1.3: Primers used for amplification of *D. radiodurans* genes

Gene	Forward Primer	Reverse Primer
DdrA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGC TGAGCGATGTCCAGAAACGACTGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAACGGCGTT TCTTCTTCC
DdrB	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTTGC AGATTGAATTTATCACC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAACGGCGT TTCTTCTTCC
DdrC	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGTA CTGGAGACCCCTC	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTCAAAAAC ATAATCTGTGCTAGAATATC
DdrD	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATA CCCTGAAAAAAGCTGGAACGATG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCTGCCGGG GTGTTTTC
Dps1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACGA AGAAAAGCACCAAGAGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTCGAGGCGC TCGTCGTC
Dps2	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCGTC ATTCTGTGAAAACTGTTGTGGTC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGGCCACGC AGCGGCAC
DR_0179	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCAAC AGCAGACAGGCGGGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCACCGTGG GCGGCCAG
DR_0199	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGACA TGAAGAAGTTGATGAAGCAGATG	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAGCCGGGC AGTCCCAG
DR_0428	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGGG CCAGGCCGTACC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAGAAGACGT TGAGCGATGTTCG
DR_0756	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCG TCTCCGCCGCC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAGGTAAGCC AGTTCCCAGTCCTTG
DR_1769	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCTG ACCCCGCTGCCCG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGGCGCACG ACGCCCGG
DR_2162	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTCT CGCCGCCGAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTCCTGCCT GACCTGCC
DR_2444	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCG ACCTGCCATCTCTGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCGCGCACG ACTTCCAG
DR_A0282	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTTCAT GAAGAGCAAGGCCGCCGGCTC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGACGGGTG GTGAAATCGTTGACCTC
DR_B0067	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCTG GTAAGCGCGCCG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGGCTTCCA AGTCCATCGAAAG
Dsup	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCAT CCACACACCAATCATC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTCCTCTTCC GTCCTCCAGC
eGFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGA GCAAGGGCGAGGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTACAGC TCGTCCATGCCGAG
Empty	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGACTG AGGGTCACTGATCTACTCAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTTACACTG ATCGACGTCCTTGC
HU	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACGA AAAAGTCTACCAAGGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCAGGTTGCCC TTGAGGGTG
IrrE	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCCA GTGCCAACGTCAGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTGCAGCG TCCTGCGGC
mCherry	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGA GCAAGGGCGAGGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTACAGC TCGTCCATGCCGC
Mfd	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCC TTAGCGCTACCCCCAAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCGAAGTAC CCCAGCACCTCAATC
Mnth	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATT CCCGTTCTCCCAGCCTGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCCCCCAGC AGCTCCCAC
MutM	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCCG AACTGCCGGAAG	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCGCTCCGG TCTGTTCTG
PprA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCAA GGGCTAAAGCAAAAGACCAAAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTCTCGCGC AGGCCGTG
RadA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGAGC GGGTGGCGGTGCTG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGCCAAACG GCTTTCACGGCTTC
RecA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGCA AGGACGCCACCAAAGAAATCTCC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCTTCGGCG GCTTCGGG
RecF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGG ATGTGCGTCTCTCGG	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCCGTGCCC TCCGCTTG
RecN	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCC GCAAGGCCCGTAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCAGCCAGC AACTCGCG
RecO	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCGCT CACGCACCGCC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTCAGCACC GGCACGCC
RecR	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAT ATCCGCCTTCCCTCGTGTCCCTC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGGGATGCG GGCACCG
RqkA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCGC TGACCCCTGGAACCC	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCTTCCTGCT CGCTGCG
RSR	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGA ACTTGCTCCGTGCCATCAAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACCTCGCCC CGCGCAAAAG
RuvA	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTG CTTACTTGTCCGGCGTGGTGCGT	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCGCAGCCGC CCGAGGGC
RuvB	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACTG CCCCCGAGAATCTGG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTCAGAAAG ATGCCGTTGCCGTC
RuvC	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGGG TTCTGGGGATTGACCCCG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGCCGCAGC GGGGC
Sir2	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCA TCCCACATATGAAATACGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAGGGTTTTG GGATGTTCATCTGATG
SSB	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCCC GAGGCATGAACCAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAGGGCAGG TCGTCTTCTTCCGGCGG
TerF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAGTG CACAGGTTAGGGATGGAAGAC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATTCGGTTG CTCTTGGCCAGGTTG
UvsE	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACCT CGGCCTGTGAAGC	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTCTTTCTTGA ACGGCGCCATC

Table 1.4: Yeast strains used in this study

Strain ID	Genotype
PSY316AT	MATα, ura3-53 leu2-3, 112 his3-Δ200 ade2-1,01 can1-100 ADE2-TEL V-R
YDS970	PSY316 MATα, ura3-53 leu2-3, 112 his3-Δ200 ade2-1,01 can1-100 ADE2-TEL V-R, HMR::GFP
W303	W303 Mata, ade2-1, leu2-3, 112, can1-100, trp1-1, ura3-52, his3-11, 15,
YDS106	W303 Mata, ade2-1, leu2-3, 112, can1-100, trp1-1, ura3-52, his3-11, 15, RDN1::ADE2, RAD5

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Chapter 3: Effect of Enhancing Genomic Stability on Aging and Epigenetic Integrity

I. Statement of Work

All experiments described in this chapter were designed by myself and David Sinclair, with input from dissertation committee members Raul Mostoslavsky, Stephen Elledge, Vadim Gladyshev, and Bruce Yankner. I conducted all experiments, with the following exceptions. Joe Zullo (Yankner Lab) solely conducted one of the three *C. elegans* longevity experiments described in this chapter. Chris Perry (Sinclair Lab) assisted me in conducting the remaining two *C. elegans* longevity experiments, scoring survival for approximately half of the days of each experiment due to COVID-related restrictions preventing me from entering the lab on certain days. All worm strains used in this experiment were generated by Joe Zullo, as previously stated in the "Statement of Work" for Chapter two of this dissertation. Brianah McCoy (Sinclair Lab) assisted me in conducting the HMR desilencing assays in response to heat shock and galactose-induced EcoR1. Yeast replicative lifespan analysis was conducted by Ruofan Yu and Wei Liu of Weiwei Dang's Lab at Baylor College of Medicine using strains I generated. They also performed the replicative lifespan analysis. I performed all of the other data analysis and generated all of the figures shown in this chapter.

II. Abstract

Genomic instability and epigenetic dysfunction are two widely conserved hallmarks of aging. It is unclear if genomic instability plays a causal role in aging, largely due to the inability to test whether enhanced genomic stability affects lifespan or healthspan. Likewise, it is unknown if DNA damage is a driver of age-related epigenetic dysfunction, although both DNA damage and aging lead to remarkably similar epigenetic changes. Here, I investigate these questions using yeast and worms that have enhanced genomic stability through the expression of the *D. radiodurans* proteins Dps1 or SSB. In yeast, SSB and Dps1 mitigate desilencing of the *HMR* silent-mating loci, a key hallmark of age-related epigenetic dysfunction, in response to DNA damage. Dps1 extends yeast replicative lifespan, but SSB surprisingly shortens replicative lifespan. This suggests that SSB is detrimental under conditions of endogenous levels of DNA damage, despite being beneficial under conditions of elevated DNA damage. Conversely, SSB expression extends median lifespan in worms by ~20%. SSB transgenic worms also have an improved healthspan, displaying increased locomotion during aging and decreased accumulation of autofluorescent pigments. Furthermore, I find that SSB transgenic worms display increased levels of histone 3 (H3) and H3K27me3 during middle and late ages, marks which are progressively lost during *C. elegans* aging. This H3 and H3K27me3 loss is accelerated by inducing DNA damage early in life, suggesting that genomic instability might be a driver of this loss. Taken together, these results indicate that enhanced genomic stability can extend lifespan and improve healthspan, and that this improvement is correlated with increased epigenetic integrity. However, these findings also highlight that effective strategies for improving genomic stability are species-specific.

III. Introduction

Genomic instability has long been implicated in aging largely due to increased accumulation of DNA damage with age [1-3] and due to multiple lines of experimental and clinical evidence showing that elevated DNA damage levels reduce lifespan and healthspan [4-8]. However, it is still uncertain if DNA damage plays a causal role in aging. The most rigorous test of this hypothesis would be to generate organisms with enhanced genomic stability and evaluate whether this impacts lifespan or healthspan. Despite many efforts, generating such organisms has proven exceptionally difficult, largely due to negative consequences from overexpressing endogenous DNA repair proteins [9-11].

Another related and unresolved question is how DNA damage contributes to cellular and organismal aging. Hypotheses have included increasing mutation rates, causing apoptosis, triggering cell senescence, and exacerbating inflammation [12]. Though there are varying degrees of supporting evidence for each of these theories, all of these factors could plausibly play a role in DNA damage contributing to aging. An intriguing hypothesis that has been the subject of several recent investigations [13-15] is that DNA damage leads to permanent changes to the epigenome. This hypothesis links genomic instability and epigenetic dysfunction, two of the most commonly observed aging hallmarks across species [16]. Epigenetic changes play key roles in DNA damage signaling and repair, particularly during DSB repair. Successful DSB repair requires substantial alterations to the epigenome, including eviction of nucleosomes [17], acetylation of histone tails to facilitate chromatin relaxation and allow access for DNA repair enzymes [18], ubiquitination of chromatin [19], phosphorylation of Ser139 on H2A to recruit DSB repair proteins [20], relocalization of chromatin modifiers to DSB sites [21], and erasure of DNA methylation marks at damage sites [22].

Although these changes are thought to fully reset after DSB repair, there is emerging evidence that some epigenetic changes persist long after [23]. For example, HR-mediated repair of DSBs in mammalian cells leads to long-lasting changes in DNA and histone methylation patterns around the damaged site, and these changes are even stably transmitted to daughter cells [24]. In addition to epigenetic changes arising from DNA repair, persistent accumulation of DNA lesions themselves can result in substantial changes to the epigenome. The oxidative DNA lesion 8-oxo-dG reduces expression of nearby genes through several mechanisms, including impaired transcription factor binding [25] and increased stalling of RNA pol II [26]. Notably, this DNA lesion accumulates with age across many species and tissues [27-30], and is responsible for the age-related downregulation of many important genes in the human cortex [30].

The hypothesis that DNA damage is a driver of epigenetic dysfunction during aging is further supported by evidence that elevating DNA damage levels across species accelerates epigenetic changes that naturally occur during aging. For example, DNA damage causes the derepression of repetitive elements in both yeast [31] and mammalian cells [21], leads to heterochromatin loss in yeast [32] and mammalian cells [15], and accelerates DNA methylation clocks in mice [14] and humans [33]. However, these findings are all derived from studies in which DNA damage levels are greatly elevated, making it unclear if endogenous levels of DNA damage are sufficient to cause age-related epigenetic changes.

Here I use yeast and worms with enhanced genomic stability, achieved through expression of the *D. radiodurans* DNA-binding proteins Dps1 and SSB, to investigate the connection between DNA damage, epigenetic stability, and aging. I test whether enhanced genomic stability affects

the epigenome in response to DNA damage and aging, whether it improves healthspan, and whether it extends lifespan.

IV. Results

SSB and Dps1 mitigate loss of HMR silencing following DNA damage

I first tested whether SSB or Dps1 could mitigate epigenetic dysfunction caused by DNA damage in yeast. One of the classical examples of epigenetic dysfunction during yeast aging is loss of silencing at the silent mating type loci *HMR* and *HML* [34]. DNA damage causes a similar loss of silencing at the *HMR* locus [21], likely resulting from the relocalization of silencing factors, such as Sirtuin proteins SIR2/3/4, away from the silent mating loci and to sites of DNA damage [21, 35]. I used a yeast strain harboring a GFP reporter at the *HMR* locus (**Fig 3.1A**) to test if SSB or Dps1 can mitigate this loss of silencing. In the absence of DNA damage, yeast expressing SSB or Dps1 had no change in *HMR* desilencing, indicating that SSB and Dps1 did not affect the regulation of this loci under normal culture conditions. However, SSB and Dps1 significantly reduced *HMR* desilencing following H_2O_2 treatment, indicating that they helped preserve the epigenome following DNA damage (**Fig 3.1B**). It's unlikely that this preservation of the epigenome is deleterious, as I previously reported that yeast expressing SSB or Dps1 have increased survival following H_2O_2 treatment.

To further explore the connection between DNA damage and epigenetic stability, I tested if SSB or Dps1 could suppress *HMR* desilencing caused by EcoRI-induced DSBs. Expression of EcoRI led to *HMR* desilencing, albeit not quite as dramatically as following H_2O_2 treatment. Yeast expressing SSB had a moderate, but not statistically significant, reduction in *HMR* desilencing, while Dps1 had no effect (**Fig3.1C**). These findings are consistent with my previous findings on the mechanisms through which SSB and Dps1 promote genomic stability. Since SSB

enhances NHEJ efficiency, it is expected to enhance genomic stability by accelerating repair of DSBs formed by either EcoRI or H_2O_2. Dps1 likely binds to the genome and prevents hydroxyl-mediated DNA damage. Consequently, Dps1 is expected to promote genomic stability following H_2O_2 treatment, but not expected to impact the formation or repair of DSBs caused by restriction endonucleases.

While it seems probable that SSB and Dps1 are suppressing *HMR* desilencing by promoting genomic stability, it is possible that this epigenetic preservation is caused by another mechanism. One alternative possibility is that SSB or Dps1 are enhancing H_2O_2 viability and mitigating HMR desilencing by causing a non-specific increase in yeast stress resistance, rather than by preserving genomic stability. To test this possibility, I subjected yeast to heat shock, a stressor which leads to protein misfolding, aggregation, and epigenetic dysfunction [36]. Heat shock increased *HMR* desilencing, but this epigenetic dysfunction was not rescued by either SSB or Dps1 (**Fig3.4D**). In contrast, yeast overexpressing the *HMR* silencing factor SIR2 had reduced desilencing following both heat shock and H_2O_2 treatment. Since SIR2 is one of the major *HMR* locus silencing factors, it is expected that its overexpression would likely mitigate all causes of *HMR* desilencing. The inability of SSB and Dps1 to reduce *HMR* desilencing by heat shock strongly suggests that these proteins preserve epigenetic integrity specifically by enhancing genomic stability.

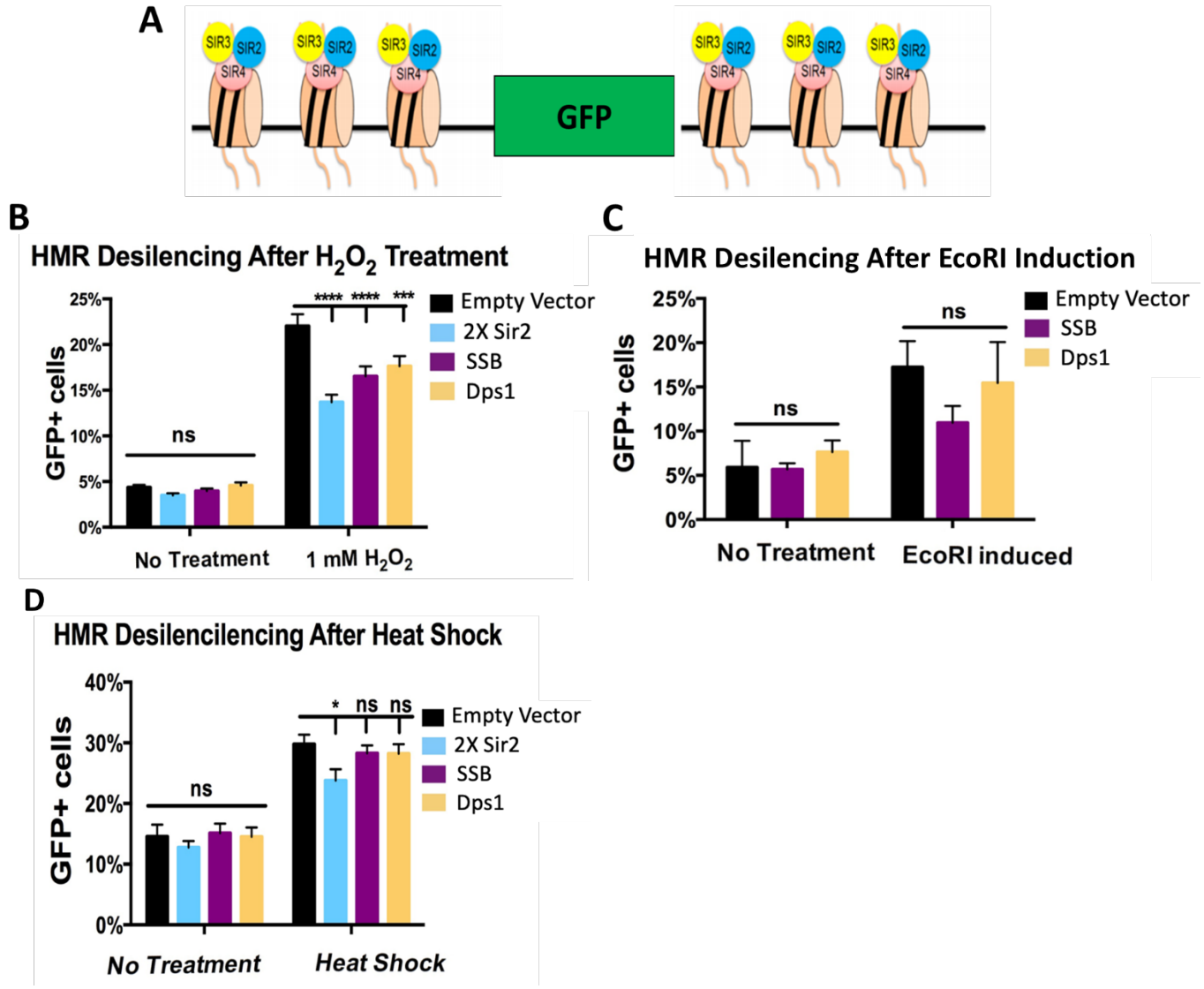


Figure 3.1 SSB and Dps1 suppress HMR desilencing caused by specific mechanisms of DNA damage. (A) Schematic representing this reporter assay. A gene encoding GFP was stably integrated at the HMR locus, which is normally kept silenced by several factors, including members of the SIR family of proteins. (B) HMR desilencing following H_2O_2 treatment, (C), EcoR1 Expression or (D) heat shock. Data shown for B-D represent the average percent of GFP-positive cells + SEM of five different yeast isolates, with each isolate tested in three different experiments for each assay. All data was analyzed by two-way ANOVA.

Dps1 extends yeast replicative lifespan

Next, I wanted to test if SSB or Dps1 extends yeast lifespan. Yeast have two distinct modes of aging; chronological lifespan- which tracks how long yeast remain viable in stationary

culture- and replicative lifespan- which monitors how many daughter cells are produced. Genomic stability is closely connected to yeast replicative aging [37], but its importance in chronological aging is largely unknown. I tested if rDNA instability, one of the main signatures of genomic instability during replicative aging, was altered during chronological aging. Rates of rDNA recombination were virtually identical in chronologically "young" and "old" cells (**Fig 3.2A),** indicating that rDNA stability is unaltered during chronological aging. Furthermore, yeast cultured under low glucose conditions- an intervention that substantially extends chronological lifespan [38]- showed no changes in rates of rDNA recombination (**Fig 3.2A)**. These findings suggest that rDNA instability is not associated with yeast chronological aging, potentially suggesting that genomic stability is not a major driver of this mode of aging. Neither SSB nor Dps1 had any effect on the chronological lifespan of yeast, which would be expected if genomic instability was not a driver of chronological aging (**Fig 3.2B-C**).

In contrast, enhanced genomic stability is well-connected to lifespan extending interventions in yeast replicative aging [37]. Yeast expressing Dps1 had a ~20% extension of median replicative lifespan, while yeast expressing SSB had a ~10% reduction in median lifespan (**Fig 3.2D).** Consequently, although both SSB and Dps1 promoted genomic stability under conditions of H_2O_2 treatment, it appears that only Dps1 is beneficial for longevity under normal culture conditions, while SSB antagonizes longevity. Experiments are ongoing to determine how Dps1 impacts levels of DNA damage and epigenetic dysfunction during yeast aging.

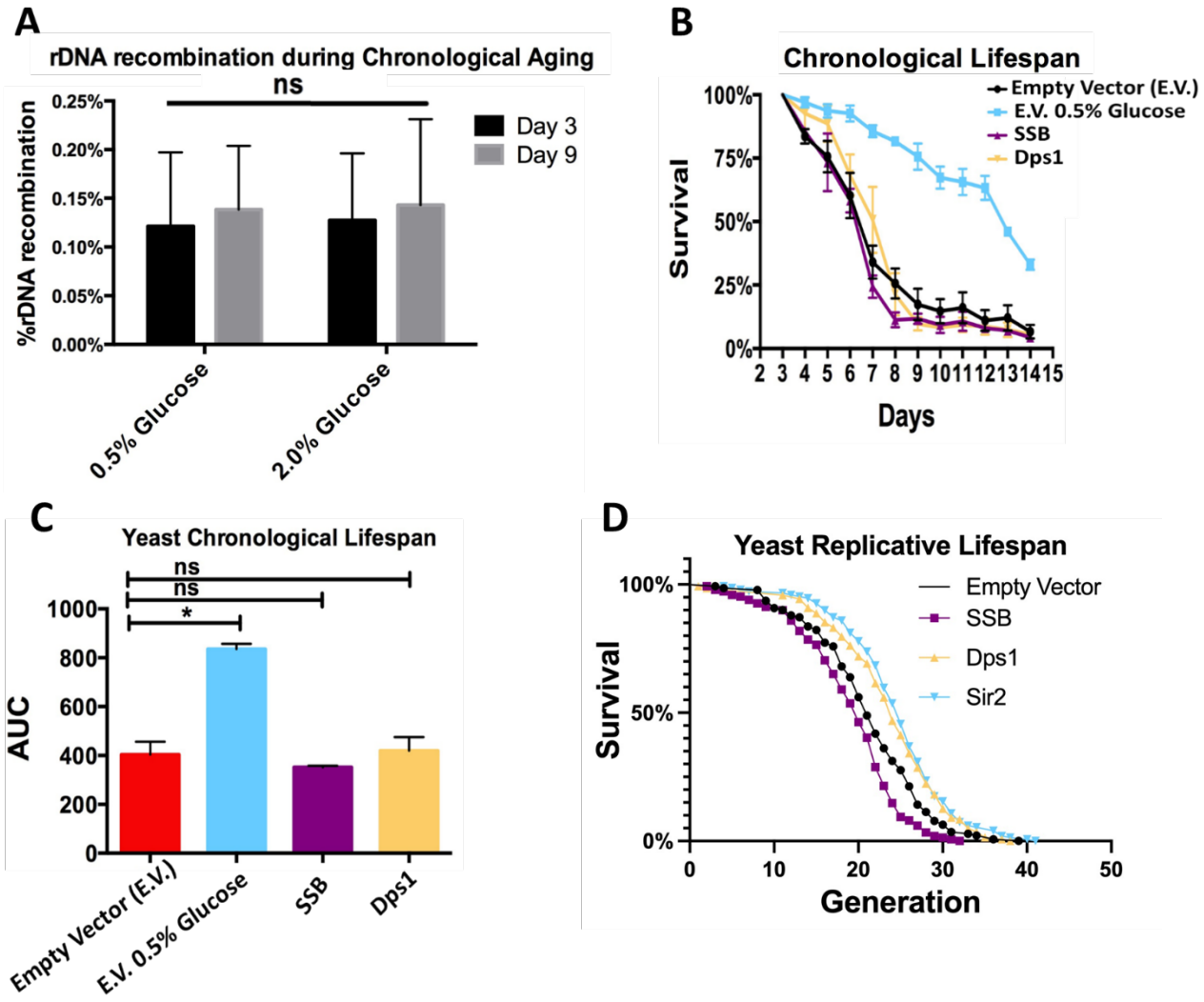


Figure 3.2: Chronological and replicative lifespan in transgenic yeast and genomic instability in the chronological aging model. (A) rDNA recombination rate during chronological aging. Data represents the average rDNA recombination rate + SEM from three different experiments and analyzed by a two-way ANOVA. (B) Chronological lifespan of transgenic yeast. Data represents the chronological lifespan of three isolates from each group +/- SEM. (C) Quantification of the area under the curve (AUC) of the chronological lifespan data shown in B. Data was analyzed with a one-way ANOVA. (D) Replicative lifespan of transgenic yeast. Data represents the mean replicative lifespan from at least 100 mother cells of each genotype.

SSB improves healthspan and extends lifespan in worms

Like many multicellular organisms, *C. elegans* experience a progressive loss of health during aging [39]. Some interventions that affect aging in *C. elegans* alter both lifespan and

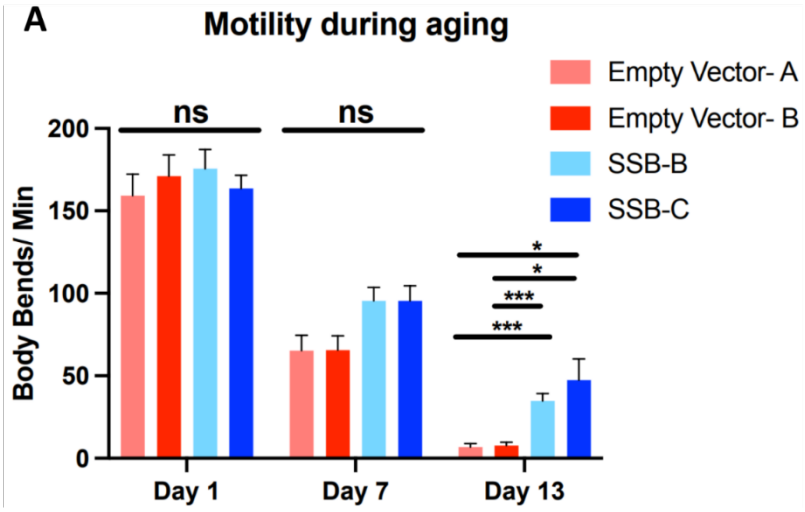
healthspan, while others only change one of these parameters [40]. Loss of motility is one of the most commonly observed healthspan parameters that declines with age and is one of the earliest observable changes in *C. elegans* health [39]. SSB transgenic worms had no difference in motility compared to control lines in early adulthood. However, SSB expressing worms experienced a more gradual loss of motility during aging, resulting in significantly higher movement in "elderly" worms (**Fig 3.3A)**.

To further explore the effect of SSB on healthspan, I monitored changes in autofluorescence during aging, another commonly used healthspan marker [41]. *C. elegans* increasingly autofluoress under several different excitation wavelengths during aging. Although there is dispute around the underlying mechanisms of this phenomenon, with accumulation of lipofuscin pigment or advanced glycation end products commonly proposed reasons [41,42], the degree of autofluorescence frequently reflects age-related health [42]. Similar to motility, SSB transgenic and control worms displayed no difference in autofluorescence during early adulthood (**Fig 3.3B**), but old SSB transgenic worms had lower levels of autofluorescence indicating an improved healthspan.

As a final evaluation of healthspan, I tested whether SSB alters reproduction. Worm fecundity peaks in early adulthood (~2 days old) and then gradually declines until ceasing around one week of age [43]. Some interventions that impact healthspan or lifespan also alter reproductive behavior [44]. Frequently, these interventions lower fecundity or delay the onset of progeny production, suggesting a trade-off between reproductive success and aging [44]. SSB transgenic worms displayed no difference in reproductive behavior, with both total fecundity (**Fig 3.3B-C)** and the kinetics of progeny production (**Fig 3D)** virtually identical to that of

control strains. Taken together, these findings indicate that SSB improves healthspan during aging, and that this improvement is not a result of altered reproductive behavior.

To further test if SSB has an impact on aging, I evaluated whether SSB affected lifespan. SSB transgenic worms had an extended median lifespan of ~20%, while maximal lifespan was extended ~15% (**Fig 3.3E-F**). The effect size was similar when comparing SSB transgenic worms to control lines containing either an empty vector or expressing GFP, indicating that exogenous transgene expression alone was not responsible for lifespan extension.



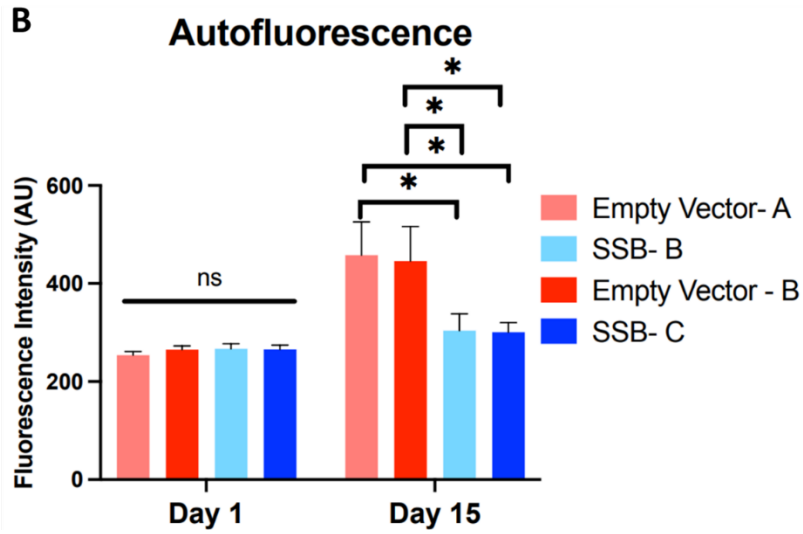


Figure 3.3 (Continued)

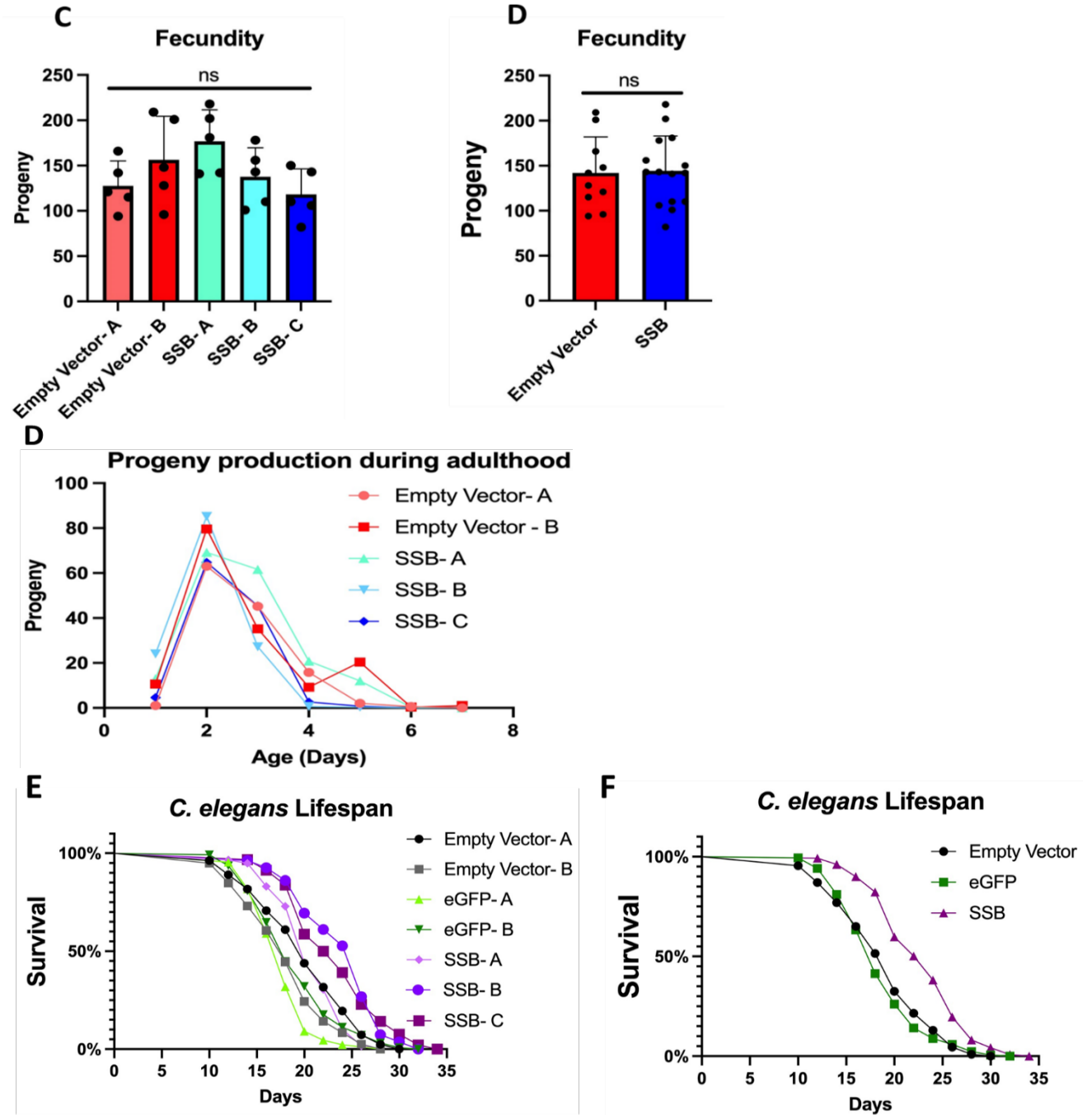


Figure 3.3: Improved healthspan and lifespan of SSB transgenic worms.
(A) Age-related changes in motility as measured by body-bends and (B) autofluorescence. Data from A and B depicts average + SEM from 10 different worms per timepoint/group and analyzed with a two-way ANOVA. (C) Total fecundity in individual (n=5 per line) and (D) combined *C. elegans* strains. Data represents average progeny + SEM and was analyzed by a one-way ANOVA. (D) Average progeny production of *C. elegans* lines during aging (n=5 for each timepoint). (E) Lifespan of Empty Vector, eGFP, and SSB individual lines. (F) Combined lifespan curves for the individual lines shown in E.

SSB mitigates loss of H3 caused by aging or DNA damage

I next wanted to test the hypothesis that SSB transgenic worms have enhanced epigenomic stability during aging. One of the most commonly reported changes to the epigenome of aging worms is a global loss of the heterochromatin marker H3K27me3 [45,46]. I first tested whether the N2 *C. elegans* lines used in this study undergo age-related loss of H3K27me3. This was of particular importance since all previous reports of age-related loss of H3K27me3 were performed in germline-deficient worms [45,46], whereas the worms used in this study have an intact germline. These worms also exhibited an age-related decrease in H3K27me3 levels, although this was largely a result of reduced histone 3 (H3) levels rather than specific loss of the H3K27me3 marker (**Fig 3.4A**). A decline in H3 levels during worm aging has previously been reported in a germline deficient worm strain [46], with no previous reports of H3 changes in worms with an intact germline.

Next, I tested if the loss of H3 and H3K27me3 could be driven by DNA damage. Age-related epigenetic changes in yeast, mammalian cells, and mice can be accelerated by inducing DNA damage [14,15,21], but there is very little evidence for this in worms. To test this possibility, DNA damage was briefly induced in early adult (Day 3) worms through a 1-hour treatment with H_2O_2. This DNA damage event accelerated the loss of H3 during aging with Day 9 levels of H3 that were ~40% lower than that of age-matched controls (**Fig 3.4B**). Similar to our observations during aging, H3K27me3 expression declined at very similar levels to global H3. This suggests that H3K27me3 is not specifically being lost in our strains due to either aging or DNA damage, but is declining as a consequence of a global reduction in total H3.

Finally, I tested whether SSB affected the observed changes in H3. SSB transgenic worms had a smaller loss of H3 and H3K27me3 levels after H_2O_2 treatment, further suggesting

that enhancing genomic stability mitigates epigenetic dysfunction caused by DNA damage. SSB worms also maintained higher H3 and H3K27me3 levels during aging, suggesting that SSB also protects against age-related epigenetic changes (**Fig 3.4C**). Taken together, these findings suggest that DNA damage is likely a contributing factor to epigenetic dysfunction during *C. elegans* aging. They also suggest that SSB helps preserve the epigenome during damage and aging, suggesting a causal relationship between DNA damage, epigenetic integrity, and aging.

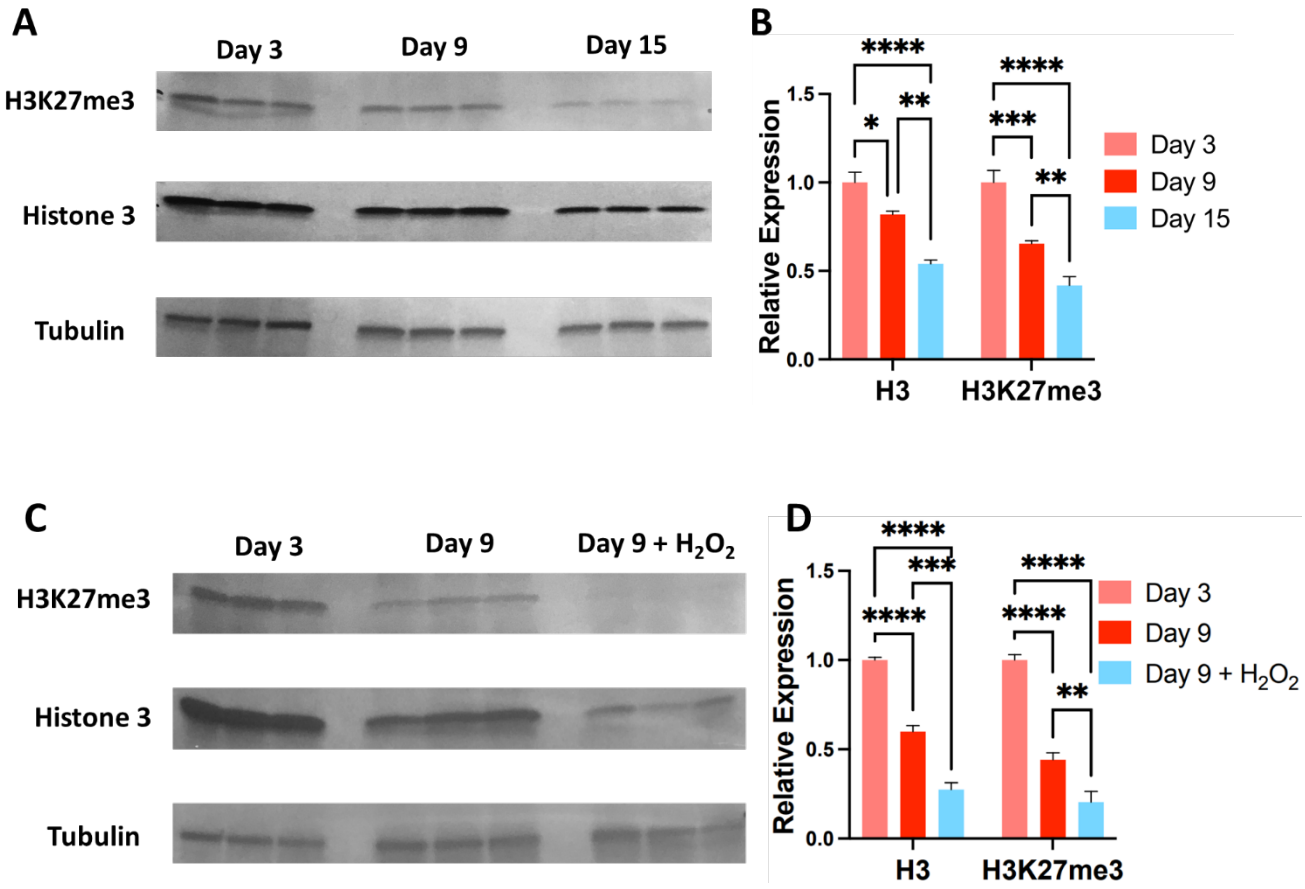


Figure 3.4 (Continued)

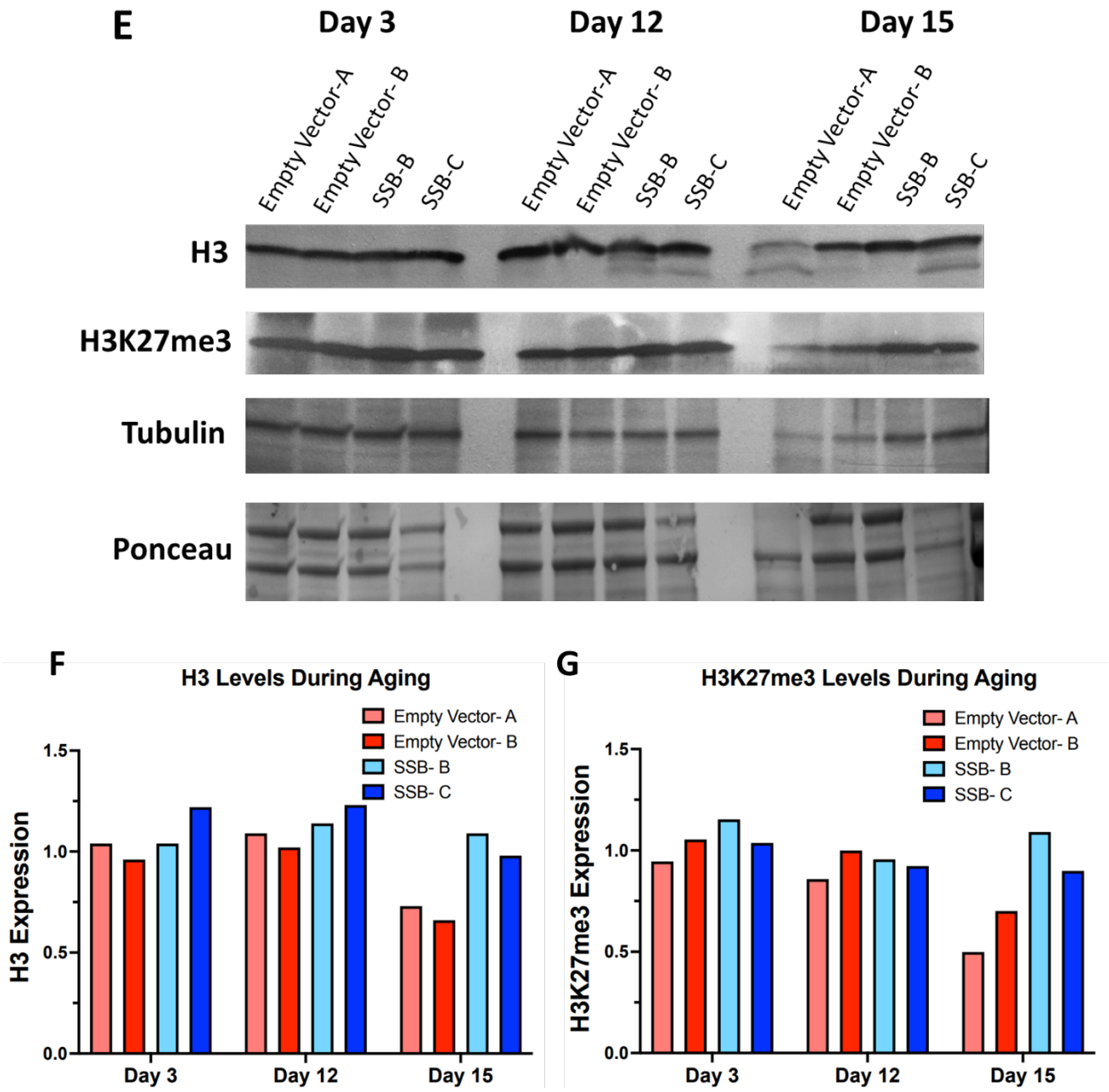


Figure 3.4: Aging and DNA damage drive decreased Histone 3 expression, which is rescued by SSB. (A-B) Changes in H3 and H3K27me3 during *C. elegans* aging and (C-D) following an early-life exposure to H_2O_2. Immunoblots in both A and C show whole-body lysates from worms collected across three separate experiments and run on the same gel. B and D represent the average intensity +SEM of H3 and H3K27me3 across the three experiments. Data was analyzed by a two-way ANOVA. (E) Immunoblot and (F-G) quantification of H3 and H3K27me3 levels from two Empty Vector and two SSB transgenic lines during aging.

V. Discussion

To the best of my knowledge, this is the first evidence that specifically bolstering genomic stability preserves the integrity of the epigenome. Epigenetic dysfunction was reduced in both yeast and worms during acute DNA damage and during worm aging, suggesting that genomic stability is one of the key lynchpins underlying epigenetic integrity. I am currently following up on these findings by testing if enhanced genomic stability also suppresses epigenetic dysfunction in mammalian cells. Specifically, I will investigate if SSB's enhanced repair of I-*PpoI*-induced DSBs in mouse fibroblasts can stabilize the epigenome. These DSBs are reported to accelerate several markers of epigenetic aging, including the acceleration of the DNA methylation clock, reduced histone 3 and histone 4 expression, and global and site-specific changes in H3K27 posttranslational modifications [15]. Additionally, I am testing if SSB or Dps1 can mitigate the desilencing of SIRT1-repressed genes that occurs following H_2O_2 treatment in mammalian cells [21]. This mechanism bears a striking resemblance to the H_2O_2 -mediated desilencing of the silent mating loci in yeast, which results from the redistribution of the SIRT1 orthologue, Sir2, and other Sirtuin family members following DNA damage.

One of the surprising findings from this study is the divergent effects of SSB and Dps1 on yeast replicative lifespan, with Dps1 extending and SSB shortening lifespan. Yeast expressing either of these proteins displayed multiple phenotypes that are associated with increased longevity, including increased H_2O_2 resistance [47,48], decreased rDNA instability [49,50], and decreased *HMR* desilencing [21]. It is likely that the different lifespan effects are due to the different mechanisms through which SSB and Dps1 are protecting the genome. My experiments in Chapter 2, along with previous findings on Dps family proteins [51,52], suggest that Dps1 binds to the genome and protects it from hydroxyl-mediated damage. Hydroxyls and other free

radical byproducts of cellular metabolism have been implicated in yeast replicative aging [53,54] and can damage the genome by resulting in single-strand breaks, DSBs, or oxidative DNA lesions, with the latter two forms of damage increasing during yeast aging [1,28]. Counteracting oxidative stress through either antioxidant treatments [55,56] or through interventions that decrease ROS production [57,58] extends replicative lifespan, suggesting a likely role for oxidative damage in this mode of aging. Dps1 likely extends lifespan by binding to the genome and lowering levels of free radical-induced damage. This theory is currently being tested by evaluating whether yeast expressing Dps1 have lower levels of 8-oxo-dG or DSBs during aging. This proposed mechanism of action would also explain why Dps1 enhanced viability, decreased rDNA instability, and decreased *HMR* desilencing in H_2O_2-treated yeast.

In contrast, my experiments in Chapter 2 demonstrate that SSB improves NHEJ efficiency in yeast, making this the likely explanation for why SSB transgenic yeast are more resistant to H_2O_2. NHEJ activity is critical for yeast under supraphysiological levels of DNA damage. This is supported by the finding that *yku70* mutant strains, which have impaired NHEJ activity, have increased DSB levels following H_2O_2 treatment [59] and decreased viability following H_2O_2 treatment or restriction endonuclease expression [59,60]. However, yeast predominantly rely on the HR pathway under normal culture conditions, with NHEJ accounting for as little as 0.1 – 0.3% of DSB repair in wild-type haploid cells [61,62]. I suspect that SSB might be antagonizing longevity in yeast by interfering with HR-mediated repair, potentially by competing with HR factors like RPA1 for single-stranded DNA. Consequently, SSB would increase genomic instability under non-stressed culture conditions, leading to the observed decrease in replicative lifespan. However, this tradeoff between NHEJ and HR efficiency might

be beneficial under conditions of massively elevated DNA damage, especially since NHEJ is a quicker method for DSB resolution [63,64].

This model would also explain why SSB extended lifespan and improved healthspan in *C. elegans*. In contrast to yeast, DSB repair in *C. elegans* somatic tissues appears to occur almost exclusively through NHEJ [65]. HR is unlikely to play a consequential role in DSB repair in the soma, highlighted by the finding that multiple *C. elegans* mutants (*mre-11*, *rad-50*, *rad-51, rad-54*) with impaired HR activity display no difference in the frequency of deleterious phenotypes that occur in the somatic tissues of irradiated worms [66]. Therefore, bolstering NHEJ activity in this model organism would be less likely to result in genomic instability, even if there is a tradeoff with HR activity. HR plays a dominant role in DSB repair in the *C. elegans* germline [66], with impaired HR activity resulting in increased chromosomal fragmentation in germ cells and impaired fecundity [67]. However, no difference in reproductive activity was seen in SSB transgenic worms. This could indicate that SSB expression in *C. elegans* does not impair HR efficiency- a finding I reported in Chapter 2 in SSB-expressing human fibroblasts- or that it does not impair HR activity to a sufficient degree to reduce fecundity. Alternatively, SSB expression might be much lower in the germline as compared to the soma, as the *C. elegans* germline has enhanced silencing mechanisms for extrachromosomal DNA [68].

These findings support the hypothesis that genomic instability is an underlying cause of aging across species. They also suggest that the integrity of the aging epigenome is partially determined by the stability of the genome. Enhancing genomic stability is therefore a promising strategy for mitigating epigenetic dysfunction, improving healthspan, and extending lifespan. However, these findings also warn that the specific mechanisms through which genomic stability

can be successfully enhanced might be species- or tissue-specific, particularly when modifying DSB repair activity.

VI. Materials and Methods

Immunoblotting

For H_2O_2-treatment, worms were washed from NGM plates on Day 3 using M9 buffer and incubated in M9 media containing the indicated H_2O_2 concentration for one hour. After this, worms were washed three times with 15 mL of M9 buffer before being transferred back to NGM plates. All immunoblots were conducted with *C. elegans* whole-body lysates. Protein was quantified using the BCA Protein Assay Kit (ThermoFisher #23225) and 5 – 40 μg of total protein was loaded per lane. Proteins were separated via SDS-PAGE and transferred to a 0.45 μm nitrocellulose membrane. The following primary antibodies were used: H3K27me3 (Sigma Aldrich #07-449), total H3 (Abcam #1791), and alpha-tubulin (Millipore Sigma #T5168). The following secondary antibodies were used: HRP-conjugated anti-Rabbit IgG antibody (VWR #NA934) and HRP-conjugated anti-Mouse IgG antibody (VWR #NA934). Bands were generated using Ashersham ECL chemiluminescent reagents (Sigma Aldrich, #RPN2209).

Yeast Lifespan

Yeast replicative lifespan experiments were conducted in the *S. cerevisiae* BY4742 strain. Experiments were conducted in the laboratory of Dr. Weiwei Dang at the University of Baylor College of Medicine. The experimental protocol and analysis were conducted as previously described [69]. Briefly, yeast cultures were loaded onto a microfluidic device comprised of a PDMS chip containing 16 parallel microfluidic channels to allow for media introduction and 8,320 single-cell traps for trapping mother cells. The chip was placed on an inverted microscope equipped with an incubator system kept at 30°C. Yeast cultures in YPD media were passed

through the chip and time-lapsed microscopy was used to monitor the production of daughter cells. YPD media was flowed through the chip throughout the experiment to remove daughter cells. Time-lapse images were analyzed to record the total number of daughter cells produced by each mother over 96 hours. The RLS of at least 100 mother cells was assessed for each group. Yeast chronological lifespan experiments were conducted in the Sinclair Lab in the YDS106 strain (Table S2). Yeast were grown to saturation in YPD media containing either 2% or 0.5% glucose in a 30°C shaking incubator. Upon reaching saturation and at each of the following timepoints, an aliquot of each culture was removed, diluted ~1:25,000 in diH_2O, and 200 µL of each dilution was plated on four 100 mm YPD dishes and spread with autoclaved beads. Plates were placed in a 30°C incubator for three days, after which plates were blinded and colonies were counted.

HMR Desilencing Assay

Experiments took place in the *S. cerevisiae* YDS970 (Table S2) strain expressing the indicated transgenes. Yeast cultures were grown overnight in CSM-URA media in a 30°C shaking incubator. The next morning, cultures were backdiluted to OD_{600} = 0.2 in 5 mL and grew at 30°C for another 30 minutes. For H_2O_2 treatment, 5.1 µL of 30% H_2O_2 was added to achieve a final concentration of 10 mM. These tubes were then incubated at 30°C for 30 minutes, centrifuged, washed with 10 mL of autoclaved H_2O, and resuspended in 5 mL of CSM-URA media. Cells were put back in 30°C shaker for four hours, after which GFP^+ cells from each culture were quantified using a FACSCanto (BD). For heat shock treatment, cells were grown for 30°C for one hour after backdilution, followed by growth at 42°C for 45 minutes. Cultures were then transferred back to 30°C for two hours followed by quantification of GFP^+ cells using the FACSCanto.

Worm Motility

Worm motility was assessed during a time-course of an aging population of the four indicated worm strains. On the indicated days, ten worms from each group were randomly picked off of solid NGM plates and placed into M9 buffer mounted on glass coverslips. Worms were allowed to equilibrate for one minute, after which videos capturing movement were recorded using a camera-equipped Nikon Eclipse Ti microscope. At the conclusion of the experiment, videos were blinded and the number of body bends per minute was scored. Videos were then unblinded and the data was analyzed using a Two-way ANOVA.

Worm Reproductive Behavior

Worms were age-synchronized using a simultaneous 2 hour-egg lay for all strains. Five of the resulting offspring of each strain were individually placed on NGM plates 60 hours after the conclusion of the egg lay. Worms were kept on NGM plates for 24 hours, after which they were transferred to a new NGM plate. This was repeated throughout the 7-day egg lay. The resulting progeny on each plate were counted 60 hours after the adults had been removed.

Worm Autofluorescence

On the indicated days, worms from each group were randomly picked off of solid NGM plates and placed into M9 buffer containing 40 mM NaN_3 as an anesthetic. Worms were anesthetized for 5 minutes, after which images were taken using the “phase” and “DAPI” settings of the camera-equipped Nikon Eclipse Ti microscope. Image files were blinded, followed by analysis of DAPI intensity. After the data was recorded, files were unblinded and the data was analyzed using a Two-way ANOVA.

Data Analysis

All statistical analysis performed using one-way ANOVAs or two-way ANOVAs using GraphPad Prism 9.3.0. Dunnett's test was applied to all one-way ANOVAs, and Tukey test applied to all two-way ANOVAs to control for multiple comparison testing. Asterisks signify the following: * ($p<0.05$), **($p<0.01$) ***($p<0.001$), ****($p<0.0001$).

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Chapter 4: Future Studies and Applications of Enhanced Genomic Stability

I. Future Characterization of SSB and Dps1 in Promoting Genomic Stability

In Chapter two of this dissertation, I demonstrate that SSB and Dps1 from *D. radiodurans* enhance genomic stability across multiple species. SSB enhances genomic stability in mammalian cells, yeast, and worms by improving NHEJ efficiency, leading to decreased DSB levels following H_2O_2 treatment or expression of a restriction endonuclease. Dps1 reduces DSB levels following H_2O_2 treatment, likely by binding to and protecting genomic DNA from free radicals. Although the effects of SSB and Dps1 are not entirely unique, what makes these proteins valuable research tools is the likely specificity with which they improve genomic stability. Enhancing the efficacy of DSB repair is quite difficult, with only the overexpression or increased activity of SIRT6 as a reliable method for achieving this goal across species [1-3]. However, SIRT6 regulates many other processes linked to longevity, including inflammation [4], metabolism [5], and retrotransposon activity [6]. These multiple activities make SIRT6 an ideal longevity gene candidate, but a less than ideal candidate for testing how specifically enhancing genomic stability impacts aging. Although I can't be certain that *D. radiodurans* SSB doesn't have any functions outside of preserving genomic stability, there is currently little evidence that it or other bacterial SSBs play a significant role in processes other than DNA damage repair and replication [7]. This makes SSB a more precise tool for studying the consequences of enhancing genomic stability during aging. Similarly, Dps1 expression is just one of many known methods for reducing free radical damage to the genome, including treating cells with antioxidants [8,9] However, these treatments also protect lipids and proteins from free radical damage. In contrast, Dps1 is localized to the nucleus and binds DNA, making it less likely to have protective effects beyond increased genomic stability. However, future studies will be needed to determine whether Dps1 expression has other effects, such as reducing oxidation of nuclear proteins.

There are several future lines of investigation that could be conducted to better characterize and understand the effects and functions of SSB and Dps1. One ongoing effort is to better understand the extent to which these proteins alter genomic stability during aging. Both proteins showed great potential in their ability to reduce high levels of acute DNA damage induced through treatment with genotoxic agents, but it is still unknown if and how they affect endogenous levels of DNA damage. To that end, the Sinclair Lab is testing how levels of DSBs and 8-oxo-dG change during the replicative aging of yeast expressing SSB or Dps1 and in SSB transgenic worms. It will be particularly interesting to see if SSB transgenic yeast experience higher levels of DNA lesions during aging, as this would support my hypothesis that their shortened lifespan is due to an impaired ability to repair DSBs under endogenous levels of DNA damage. This data will provide a more comprehensive understanding of the effects of Dps1 and SSB on genomic stability and could provide more quantitative information about how changes in DSB levels affect lifespan.

Another intriguing line of investigation is understanding the specific mechanism through which SSB enhances NHEJ efficiency. This is particularly vital because bolstering DSB repair has been historically difficult to achieve. Consequently, understanding the mechanisms underlying SSB's effects on NHEJ could reveal new strategies for upregulating the efficiency of DSB repair. One way to further understand SSB's mechanism of action is to conduct a pull-down assay at various timepoints following DSB induction. This would be especially informative if SSB interacts with canonical NHEJ proteins (e.g. Ku70/80 or DNA-PKcs), and would support a role for SSB's direct participation in NHEJ. Although single-stranded DNA-binding proteins are traditionally thought to only play a key role in the HR pathway of DSB repair, the human single-stranded DNA-binding protein RPA enhances NHEJ repair in an *in* vitro DNA end-joining assay

[10], suggesting that this class of DNA repair proteins might play an unknown role in NHEJ. Alternatively, SSB might not play a direct role in NHEJ, but could enhance its efficiency by changing the expression or localization of DSB repair proteins or by altering DNA damage response (DDR) signaling. These possibilities could be explored by probing for expression levels of key NHEJ proteins and monitoring activation of the DDR in SSB expressing yeast or mammalian fibroblasts following DSB induction.

Finally, future work should address whether the activities of *D. radiodurans* SSB and Dps1 are unique, or if similar phenotypes can be achieved using related orthologues. One of the main reasons *D. radiodurans* genes were selected for this screen is that this organism has unique proteins that enable it to survive extraordinarily high levels of DNA damage [11,12]. Although some of these unique proteins, such as DdrA and DdrB, protected human fibroblasts from H_2O_2 -mediated cell death in my initial screen, only SSB and Dps1 enhanced genomic stability across multiple experiments. SSB and Dps1 both belong to a large family of proteins. The SSB family of proteins has orthologues in prokaryotes, eukaryotes, and archaea, while Dps1 orthologues are found in prokaryotes and archaea, but are absent from eukaryotes [13,14]. *D. radiodurans* Dps1 is structurally very similar to other members of the Dps family of proteins, except that it contains a channel through which Fe^{2+} can be released. *In vitro* studies show that this channel is detrimental for genome stability, as mutant Dps1 variants lacking this channel have enhanced protection of naked DNA from hydroxyl-mediated damage [15]. Therefore, it's possible that this mutated Dps1 or orthologues from other species would be better suited for enhancing genomic stability of other species.

SSB is clearly critical for maintaining genomic stability, as the radioresistant phenotype of *D. radiodurans* is absent in strains that express ~50% less SSB, with these strains having a

~93% reduction in LD_{50} for gamma irradiation [16]. Notably, *D. radiodurans* SSB is the only known bacterial single-stranded DNA-binding protein that contains two OB-folds, the canonical ssDNA-binding domain associated with this family of proteins [17]. It is unclear if this unique structure confers differential activity that would be difficult to achieve with other single-stranded DNA-binding proteins. To test this, single-stranded DNA-binding proteins from other organisms, such as *E. coli*, could be tested for their ability to enhance NHEJ in mammalian cells and yeast. It would also be worthwhile to test if expressing a nuclear-targeted variant of the human mitochondrial single-stranded DNA binding proteins SSBP1 or SSBP2 affects NHEJ, as these are the endogenous single-stranded DNA binding proteins that are structurally most similar to those of prokaryotes.

II. Understanding the Interplay Between Genomic and Epigenomic Stability

In Chapter 3 I show that SSB protects worms against signs of epigenetic dysfunction during aging. Specifically, SSB protects against a decline in H3 levels, which I observed during aging and which is accelerated by DNA damage. Work is currently ongoing to further explore how SSB and Dps1 affect epigenetic integrity during aging and following DNA damage. For example, the Sinclair Lab is evaluating how SSB alters the epigenome in murine fibroblasts after endonuclease-induced DSBs. Induction of the I-*PpoI* endonuclease for one day is sufficient to cause long-lasting changes to the epigenome that closely mirror age-related epigenetic changes [18]. Since SSB reduces DSB levels in these cells, this will be an ideal system for further testing if enhancing DSB repair can stabilize the epigenome. Specifically, future work should address how SSB affects the DNA methylation clock, transcriptional and proteomic changes in histone expression, H3K27-associated posttranslational modifications, and gene expression patterns, which are all altered in this system and during aging [18].

Another obvious line of investigation is further characterization of how SSB changes the aging epigenetic landscape in *C. elegans*, and whether Dps1 alters the aging epigenome in yeast. It will be especially informative to see how the preserved H3 levels of SSB transgenic worms influence changes in gene expression. A global loss of heterochromatin is a common characteristic of aging epigenomes [19], including in worms [20]. A reduction in histone levels is one of several mechanisms through which this heterochromatin is lost, and this loss contributes to age-related changes in gene expression [21]. Performing RNA-Seq on aging populations of SSB transgenic worms would demonstrate how SSB affects these gene expression patterns. Specifically, SSB may protect against the desilencing of repetitive elements that is observed during the aging of worms and many other species [22]. Both DNA damage [23] and loss of heterochromatin [24] result in desilencing of repetitive elements, so the enhanced genomic stability and elevated H3 levels of SSB transgenic worms could mitigate this desilencing. Furthermore, high-resolution H3 ChIP-SEQ could be conducted to more precisely map where nucleosomes are being preserved in SSB transgenic worms. This data could be used to determine if particular gene expression changes are associated with, and possibly caused by, the loss of H3. Very similar experiments can be conducted in replicatively aging cultures of yeast expressing Dps1, as yeast experience very similar age-related epigenetic changes, including a global loss of H3, desilencing of repetitive elements, and changes in gene expression patterns, some of which are driven by loss of H3 [21,24].

A more complicated future line of investigation would be to determine how critical the epigenetic preservation offered by SSB (and potentially Dps1) are to its impacts on lifespan and healthspan. As previously mentioned, there are several mechanisms other than epigenetic dysfunction through which genomic instability can impact aging, including increasing rates of

mutagenesis and inducing senescence or apoptosis. I hypothesize that the extended lifespan and improved healthspan of SSB transgenic worms is likely due to enhanced preservation of the epigenome. Although SSB transgenic worms have preserved H3 levels during aging, this finding is merely correlative. Experiments that uncouple the epigenetic effects of SSB from its effects on genomic stability would provide a more rigorous test of my hypothesis. For example, H3 could be partially knocked down in SSB transgenic worms via RNAi at the age when H3 levels normally decline in wild-type worms, followed by an analysis of healthspan and lifespan in these worms. Furthermore, we could test if the epigenetic effects observed in SSB transgenic worms are themselves sufficient to influence aging, such as by evaluating the healthspan and lifespan of worms overexpressing H3. Similar experiments in yeast demonstrated that declining H3 levels were a regulator of lifespan and age-related gene expression, as H3 overexpression robustly extended lifespan and resulted in a "younger" transcriptome [25]. Although no single experiment is likely to provide irrefutable evidence that DNA damage-induced epigenetic dysfunction is a major driver of aging, the cumulative evidence from these proposed experiments can add substantial evidence for or against this hypothesis, while also providing further insights into the causes and consequences of specific epigenetic changes during aging.

III. Potential Applications of SSB and Dps1 in Aging Research

In these studies, I used SSB and Dps1 as tools to investigate whether improved genomic stability impacts lifespan, healthspan, and the integrity of the epigenome. Since SSB and Dps1 operate through different mechanisms and improve genomic stability across several different model organisms, they are ideal tools for future studies on the consequences of DNA damage in aging and age-related diseases. Specifically, they could be used to investigate whether oxidative DNA damage has a causal role in age-related diseases. Increased levels of oxidative DNA

damage are found in several age-related diseases, including type 2 diabetes [26], cardiovascular disease [27], rheumatoid arthritis [28], and Alzheimer's Disease [29]. Although there is evidence that combatting oxidative stress can be beneficial in mouse models of some of these diseases [30-33], it is unclear if damage to the genome specifically plays a role in pathogenesis. SSB and Dps1 transgenic cells or animal models would likely lower endogenous DNA damage levels in these models, and consequently would help uncover the cellular and molecular consequences of genomic instability in these diseases. They may even provide evidence for whether or not therapies addressing genomic instability has therapeutic potential.

SSB and Dps1 would be particularly useful tools in understanding the role of oxidative DNA damage and DSBs in the aging brain and in neurodegenerative disorders. 8-oxo-dG lesions are found at higher levels in older brains [34], and are elevated in the brains of Alzheimer's patients [35]. There is substantial evidence that these lesions play a role in driving molecular and histological features of brain aging and Alzheimer's Disease. Accumulation of 8-oxo-dG during human brain aging and in the 5XFAD mouse model of Alzheimer's Disease is responsible for downregulating genes that play critical roles in memory, learning, and neuronal survival [34,35]. Critically, pharmacological stimulation of HDAC1, a canonical histone deacetylase, enhances removal of 8-oxo-dG lesions and improves cognition in both aged mice and 5XFAD mice [35]. This highlights a potential causal role for 8-oxo-dG accumulation in impairing cognition, although this result is confounded by HDAC1 having other key roles outside of regulating 8-oxo-dG removal, including regulating gene expression [36] and controlling inflammatory responses [37]. Since Dps1 is highly effective at preventing oxidative DNA damage and has no known role outside of this activity, it could provide more reliable evidence about the role of oxidative DNA damage in regulating cognition in the contexts of aging and neurodegenerative disorders.

Alzheimer's Disease is also associated with increased DSB levels [39]. This is partly due to accumulation of $\alpha\beta_{1\text{-}42}$ peptide, which increases DSB formation by increasing oxidative stress [40] and simultaneously impairing DSB repair [41]. Neuronal DSBs cause several deleterious effects at the molecular level, including dysregulation of gene expression [42] and cell death [43]. These DSB-mediated effects could aid the pathogenesis of Alzheimer's Disease, as elevating neuronal DSB levels exacerbates many key clinical features of this disorder, including impaired memory and learning and decreased motor functions [44,45]. However, this evidence does not tell us whether DSBs play a causal role in the onset or progression of Alzheimer's Disease. As with the connection between genomic instability and aging, the best test of this hypothesis would be to specifically lower DSB levels in an Alzheimer's mouse model and observe how this affects disease pathogenesis. SSB would be an ideal candidate for this experiment, especially since NHEJ activity is impaired in the brains of Alzheimer's patients [46], and SSB enhances NHEJ efficiency in murine and human cells. In addition to exploring SSB's effect in Alzheimer's Disease mouse models, it would be informative to test if SSB affects cognitive function in the Sinclair Lab's ICE mice model, where DSBs are transiently elevated early in life through controlled expression of the I-*PpoI* endonuclease. These mice experience accelerated cognitive decline which includes reduced short-term memory, long-term memory, and altered ambulatory activity [47]. SSB accelerates DSB repair in ear fibroblasts derived from these mice, and thus would make an excellent candidate for testing if accelerated repair is sufficient to reduce cognitive decline.

IV. Future Potential of Exogenous Genomic Screening

Functional genomic screens are an invaluable tool for uncovering genes underlying a

biological activity or producing a desired phenotype. It was through a mutagenesis screen that it was first uncovered that aging is a genetically malleable process, with specific mutations in *C. elegans* resulting in a greater than 50% extension of median lifespan [48]. The current toolbox for conducting genomic screens has expanded considerably in recent years, with overexpression vectors, RNAi, TALENs, CRISPR, CRISPRi, and CRISPRa all commonly used techniques for either loss-of-function or gain-of-function screens. These techniques are largely restricted to probing endogenous genes, except for overexpression vectors which can express both endogenous and exogenous genes.

Expression of exogenous genes has resulted in some of the biggest advancements in biological research, and also shows promising therapeutic potential. GFP and other fluorescent proteins were among the first and most widely adopted exogenous genes to have a critical impact on biomedical research [49]. These fluorescent proteins have become critical tools for monitoring protein localization, protein-protein interactions, changes in gene expression, as well as labeling cells, tissues, and macromolecules [50]. Other exogenous proteins, like β-galactoside from *E. coli*, are used to monitor viral replication or infectivity and gene expression across different model organisms [51]. During the last twenty years, a small number of exogenous proteins have been utilized for much more sophisticated activities. Expressing light-activated ion channels from algae and bacteria in the brains of living animals, a technique called optogenetics, has enabled us to precisely regulate the activities of specific neuronal cell populations [52]. This technique has led to several breakthroughs in mice, including enhanced understanding of the neural circuits underpinning behaviors like fear conditioning [53]. Most recently, we have witnessed the rapid and widespread exogenous expression of CRISPR-Cas proteins from bacteria for a myriad of functions, including precise genome editing [54,55], fine-tuned control of gene

expression [56], recording cellular events and exposures [57], and detection of specific mutations or pathogen infections [58]. In addition to these research applications, CRISPR technology has tremendous therapeutic potential and is currently being investigated in multiple clinical trials involving cancer immunotherapy [59], monogenic blood and eye disorders [60,61], and bacterial infections [62].

Despite the large impact that exogenous proteins have made in biomedical research, there are surprisingly few efforts directed at the identification of novel exogenous proteins for research purposes. Most of these efforts consist of functional metagenomic screens, in which genomic DNA is extracted from the microorganisms within a specific environment (e.g. soil sample), sheared, cloned into an expression vector, and then functionally screened in a host organism-usually a species of bacteria or yeast. While this is a powerful technique that can theoretically screen the genomes of thousands of different species, the applications of this technology have been largely limited to specifically identifying a limited number of enzymes with activities that have important industrial or agricultural uses [63,64]. To the best of my knowledge, no functional metagenomics screen has been conducted with the goal of identifying genes with novel functions that would make them valuable tools for biomedical research.

Beyond the research presented in this dissertation, I am aware of only a few published reports that have addressed biological research questions by identifying exogenous proteins with novel functions. In one example that is highly relevant to this work, researchers tested the hypothesis that *C. elegans* lifespan could be extended by accelerating the breakdown of their *E. coli* food source via overexpression of a lysozyme [65]. Notably, *C. elegans* lifespan was not extended by overexpression of the endogenous worm lysozyme Lys-1, but expression of the Lyz lysozyme from zebrafish led to a robust lifespan extension of 30%. The researchers speculate

that the zebrafish lysozyme might have enhanced activity or some unknown differential activity compared to the endogenous lysozyme, leading to the discrepancy in lifespan. This finding highlights the potential of exogenous genes to outperform endogenous genes due to novel activities. In another example, multiple proteins from extremotolerant organisms were recently reported to prevent apoptosis in human cells [66]. These proteins were identified following a screen of ~300 proteins from tardigrades, nematodes, and the Chinese giant salamander. Further analysis of the underlying mechanisms uncovered a widespread ability of these proteins to form condensates and sequester caspase-7, suggesting that this is a conserved mechanism through which certain proteins can protect cells. This work represents the largest genomic screen of exogenous genes in mammals currently known.

All of these findings highlight the utility of exogenous proteins in biomedical research. To build upon these findings, more large-scale exogenous genomic screens should be conducted to identify novel genes that impart a desired phenotype (e.g. extended lifespan or increased stress tolerance). One of the advantages of this approach is that the search space is virtually unlimited; the UniProt database of protein sequences contains approximately 190 million entries [62], and this likely represents only a tiny fraction of all biological proteins. Although it is currently not feasible to screen such a large number of candidates, the advent of ultra-low cost DNA synthesis has made it possible for academic labs to screen thousands or tens of thousands of candidate genes individually, and this number can be much higher for pooled screening approaches. This endeavor could be aided by a collective effort to construct overexpression libraries of exogenous genes that are affordable to researchers. This is similar to previous efforts that have resulted in the availability of affordable RNAi, CRISPRi/a, and cDNA libraries that are the workhorses of endogenous genomic screens. Additionally, exogenous genomic screening could be enhanced by

expanding the available resources for functional metagenomic screening. Functional metagenomic screens are not as precise as most gene overexpression approaches, as they typically involve cloning randomly sheared DNA fragments into expression vectors. This inevitably results in screening many truncated proteins and a high likelihood of screening only a fraction of an organism's total genome. However, the trade-off is that metagenomic screens enable users to screen the genomes of thousands of organisms from a given environmental sample, including organisms lacking a sequenced genome. A robust toolkit already exists for conducting functional metagenomic screens in bacteria [68] and yeast [69], and expanding this toolkit to conduct screens in other organisms, particularly in human and murine cells, would greatly expand the feasibility of conducting large-scale exogenous genomic screens.

One key issue regarding the development of exogenous genomic screening systems is deciding which organism's genes should be used. While the ideal choice is likely to be specific for the phenotype or function being screened for, research presented in this dissertation along with findings from other studies [66,70] suggest that the genomes of extremotolerant organisms are particularly fruitful reservoirs of proteins with useful applications. Extremotolerant organisms include species that can survive unusually harsh conditions, including high and low temperatures, and high levels of DNA damage, metal concentrations, salinity, acidity, or alkalinity. Surviving in these harsh environments frequently requires novel proteins that are highly unique to these organisms [71,72] or protein variants that contain unusual activity or features compared to orthologues from non-extremotolerant organisms [70,73]. These proteins would be especially useful in screens seeking to enhance stress resistance, as highlighted by the ability of SSB and Dps1 to protect multiple species against oxidative stress. This potential was further underscored by a recent publication in which a ribosomal protein (RpS23) variant found

in hyperthermophilic archaea was expressed in several model organisms. This protein increased the fidelity of protein synthesis in yeast, worms, and flies, resulting in lifespan extension in all three organisms [70]. With the increasing throughput and declining cost of DNA synthesis, and a rapidly growing synthetic biology toolkit, exogenous genomic screens are primed to become a vital technology for the discovery of novel proteins that can be used as tools to answer crucial biomedical research questions.

V. References

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