Inactivating histone deacetylase HDA promotes longevity by mobilizing trehalose metabolism

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Histone acetylations are important epigenetic markers for transcriptional activation in response to metabolic changes and various stresses. Using the high-throughput SEquencing-Based Yeast replicative Lifespan screen method and the yeast knockout collection, we demonstrate that the HDA complex, a class-II histone deacetylase (HDAC), regulates aging through its target of acetylated H3K18 at storage carbohydrate genes. We find that, in addition to longer lifespan, disruption of HDA results in resistance to DNA damage and osmotic stresses. We show that these effects are due to increased promoter H3K18 acetylation and transcriptional activation in the trehalose metabolic pathway in the absence of HDA. Furthermore, we determine that the longevity effect of HDA is independent of the Cyc8-Tup1 repressor complex known to interact with HDA and coordinate transcriptional repression. Silencing the HDA homologs in C. elegans and Drosophila increases their lifespan and delays aging-associated physical declines in adult flies. Hence, we demonstrate that this HDAC controls an evolutionarily conserved longevity pathway.

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Aging is a complex biological phenomenon associated with functional declines and accumulated damages or abnormalities at all levels of an organism, from organs and tissues to cellular macromolecules. Understanding the molecular underpinning of aging is vital to intervene against human health risks, because aging is a major risk for numerous human diseases that are the principal cause of death. The single-celled budding yeast \textit{Saccharomyces cerevisiae} is an important model for cellular and organismal aging due to its short lifespan and the power of its available genetic tools. Several longevity-associated molecular pathways, such as TOR signaling and sirtuin activities, have been identified and extensively characterized using yeast, and these evolutionarily conserved pathways have been invaluable to the biological understanding of aging. Continued study of the molecular mechanisms that affect yeast lifespan may lead to treatments for age-related diseases in humans and delay the onset of aging.

Yeast replicate by asymmetrical cell division. In each cell cycle event, a small daughter cell, referred to as a bud, separates from the mother. One yeast cell can undergo a limited number of cell divisions before entering a permanently arrested state. This replication capacity is called replicative lifespan (RLS). Traditionally, measuring RLS involves physically separating daughter cells from their mothers on agar plates, using a manual micromanipulator equipped with a fiber-optic needle. Because of extensive labor required, an RLS screen using a yeast knockout collection was only recently completed using manual microdissection. Thanks to the tremendous effort made by the authors, several important and conserved aging regulating pathways were identified through this screen, such as ribosome biogenesis. However, due to the amount of work required to perform such experiments, data were only obtained from five individual cells in the majority of strains. This tedious and low-throughput experimental method is not compatible with routine individual cells in the majority of strains. This tedious and low-throughput experimental method is not compatible with routine screening approaches to yeast aging research. Recently, several groups developed microfluidic-based methods to tackle this problem. Microfluid-based technology allows significantly shorter experiment times, from 3–4 weeks to merely 3 days, yet the throughput of this method is still limited by the necessity to manually count cell division times. Therefore, strategies are required to take advantage of the power of yeast genetics and apply unbiased and high-throughput genetic screening approaches to yeast aging research. To date, there have been few advancements on this front. One example, the High-Life technique is based on mother cell enrichment and flow-cytometry, however, due to special requirements of the yeast genotype during mother cell enrichment, this method is more suitable for discovering lifespan-affecting chemical compounds in a limited number of yeast strain genotypes. Another screening method developed by Sen et al. utilized old cell sorting techniques and an oligonucleotide chip array of a barcoded histone mutant collection; however, the method provided no statistical comparison with traditional low-throughput methods, and the barcode microarray did not have a dynamic range or throughput comparable to sequencing. Therefore, we believe that developing and carefully validating high-throughput yeast RLS screening methods is vitally important. Ideally, such methods would be based on accurate quantification and be easy to apply on different types of yeast mutant collections.

In this work, we set out to develop a high-throughput screening method, SEQuencing-Based Yeast replicative Lifespan screen (SEBYL), to identify previously unknown aging regulators in budding yeast. By using SEBYL with a yeast knockout collection, we identified 285 long-lived gene deletions. Notably, a significant portion of these had extended lifespans in previous classical experiments. To demonstrate our method’s ability to discover previously unidentified genes and pathways involved in aging, we focused on characterizing one aging regulator that was identified from the screening, histone deacetylase complex HDA. We found that HDA regulates aging via stress response pathways, particularly in the DNA damage stress response. The presence of the HDA complex inhibits expression of trehalose metabolism genes, which protect against stress. When the HDA complex is mutated, trehalose genes are de-repressed, enhancing the stress response, and eventually promoting longevity. In summary, SEBYL is a robust method that saves time and energy and can discover aging regulating genes using various preexisting yeast mutant collection resources.

**Results**

**The SEBYL experimental pipeline.** We developed an improved high-throughput yeast RLS screen method, SEBYL, based on an existing protocol. First, all strains in a barcoded yeast mutant collection were pooled together, labeled with NHS-LC-biotin, and cultured overnight. Four consecutive rounds of old cell purification experiments harvested cells with advanced replicative age. Over successive sorting of old mother cells, long-lived mutants are expected to be enriched in old fractions, whereas short-lived mutants will be depleted, as reported previously. To account for growth defects and improve the accuracy of lifespan predictions, young cell populations are also collected at each sorting for barcode sequencing to determine cell cycle times for each mutant during each growth period. We utilized this improved screening design with the yeast knockout (YKO) collection that contains 4828 viable single gene deletion mutants. In this library, each individual gene is replaced by the selection marker kanMX4, which is flanked by unique UPTAG and DNTAG barcode sequences. We collected sorted fractions of young and old cells from the first, second, third, and fourth rounds of sorting, as well as an unsorted fraction, for analysis. The unsorted fraction represents the initial relative distribution of all the mutants in the pool. Whereas the relative enrichment for each mutant in the old cell fractions is influenced by both age and growth rate, the relative enrichment in the young cell fractions reflect only the growth rate. Hence, incorporating both young and old cell fractions in the lifespan modeling can accurately estimate relative difference in lifespan. The YKO lifespan screen was performed in three biological replicates. For each sorting, we (1) counted the number of cells present in each sample, and (2) extracted genomic DNA from each sample and performed barcode sequencing analysis to determine the relative abundance of each mutant strain. A modified Gompertz nonlinear regression model was used to estimate RLS for 3437 deletion strains from the YKO library.

SEBYL yields reproducible high-quality results. First, we validated the repeatability of three parallel experiments by calculating the correlation coefficient of barcode counts between each biological replicate. We confirmed high correlation in populations ranging from youngest (S1O) to oldest (S4O; Fig. S1A). Even after incorporating all nine data points and extensive modeling, the correlation coefficients for calculated RLS scores were still reasonable, in the range of 0.75–0.83 among all three biological replicates, especially when considering the numerous complicated cell sorting steps in each of the week-long screening experiments. Our data consistency is either comparable or better than the reported chronological lifespan screens (Figs. 1B and S1B, S1C). The wild-type strain had very similar lifespan curves across three experiments (Fig. 1C), and two control strains included in the screen, short-lived \textit{sir2Δ} and long-lived \textit{fob1Δ}, both showed...
the expected lifespan phenotypes (Fig. 1D). From these data, we conclude that the screen is reproducible and technically sound.

**Lifespan estimation by SEBYL is concordant with published data.** Because a previous RLS screening of the YKO library was performed by manual dissection\(^6\), we can comprehensively assess the accuracy of SEBYL by comparing our results to the manual method. Due to the enormous amount of time and effort required to perform a manual screen, McCormick et al.\(^6\) designed the experiment to start with five mutant cells and measured the RLS of additional cells only after data from the initial five cells in the manual screen yielded statistically significant results. As such,
RLS data from the majority of mutant strains (~70%) remained inconclusive. Presumably, the inclusion of more mother cells in the manual RLS measurement would decrease the false positive and false negative discovery rates, so that the measured lifespan would approach the actual population average. We therefore categorized manual screening results by the number of mother cells that were counted in the experiments and compared how many long-lived mutants (defined as the top 20% longest RLS) were present in both manual and SEBYL datasets. When we included all available mutants in the manual screen, we found 23% overlap. But as cell number increased, the proportion of overlap increased drastically, reaching 53.6% when only including strains that had more than 100 cells assessed for RLS in manual screen (Fig. 1E).

We then took the long-lived and short-lived mutants identified by manual screening and determined how they ranked in our experiment. As expected, long-lived strains were enriched in the highest-ranking fractions of SEBYL results (Fig. S1D), whereas the opposite was true for short-lived strains (Fig. S1E). Additionally, we performed the same analysis using the RLS dataset from the Saccharomyces Genome Database, which includes results from both high-throughput and classical experiments, and we observed a similar enrichment of long-lived strains in the fraction that contained the highest-ranking mutants (Fig. S1F). We believe these results confirm the ability of SEBYL to yield RLS data comparable to manual measurements.

**Age-related morphological changes.** We obtained RLS data for 3437 deletion strains, 2294 of which did not have conclusive RLS results from previous manual screening (<5 cells counted). SEBYL results can be used for functional analysis of preexisting high-throughput datasets to identify or characterize aging-related factors. Yeast cell morphology is a general indication of their well-being, and a previous study indicated that cell size is negatively correlated with RLS. Thus, we wanted to see if other yeast morphological traits could indicate RLS. We took advantage of the Saccharomyces cerevisiae Morphological Database, which contains comprehensive quantitative records for morphological features of all available YKO strains. We compared each morphological trait between top the 10% and bottom 10% of long-lived strains to see if the average scores were significantly different (see Supplementary Data 1 for complete analysis results). A few traits emerged as positive indicators of longevity and three examples are: actin localized at bud site (A110), nuclear roundness (DCV184), and nuclear diameter in budded cells (DCV178; Fig. 2A). Accumulation of actin at the bud neck and increased nuclear diameter are indications that cells are actively undergoing cell division. Although the biological significance of nuclear roundness is not entirely understood, a deformed nucleus usually indicates that cells are not healthy.

For negative indicators of RLS, we identified several traits associated with nuclear abnormality as well as cell size, such as having no nuclei in the cell (D204), large bud size (C125), or large mother cell size (C11–1; Fig. 2B). An altered number of nuclei potentially indicates polyploidy and aneuploidy, which are detrimental for the normal aging process. Abnormal nucleus number could also result from mis-segregation, which becomes more evident as cells undergo replicative aging. A larger size for either daughter or mother cells is also suggested to negatively correlate with lifespan, in agreement with previous reports.

**Novel long-lived deletion mutants indicate unexplored mechanisms of aging.** We identified 285 putative long-lived and 288 putative short-lived mutants (one standard deviation above and below the mean, see Supplementary Data 2 for complete results of SEBYL screen). The long-lived candidates clustered into several functional groups, including ribosome proteins, tyrosine phosphatase, glycosylation genes, endosome, mating, and chromatin regulatory genes. Ribosome proteins and chromatin regulators are well-documented roles in aging, and also appeared as clusters in a published manual microdissection screen (Fig. 3A). We also discovered a less-characterized cluster enriched with tyrosine phosphorylation regulators in our network analysis. This cluster included six tyrosine phosphatase genes, Ptp2, SIW14, OCA1, OCA2, OCA4, and OCA6. Of these phosphatases, Ptp2 is known to target the MAP Kinase Hog1, a major stress regulator in budding yeast, and also play a role in osmolarity stress response. The molecular functions of the other candidate phosphatases are less well characterized, but all of them except OCA6 have extended chronological lifespans, according to a previous study, suggesting loss of function in these tyrosine phosphatases may lead to enhanced resistance against external stress, which tends to extend chronological lifespan.

To further identify and validate the long-lived deletion mutants, we chose 15 long-lived deletion strains and performed a microfluidics-based RLS measurement. These strains had no conclusive RLS data from the manual screen and ranked among the highest in our candidate list. We confirmed that 12 out of the 15 strains were significantly longer-lived than wild type (Fig. 3B), with similar results from microdissection experiments (Fig. S2A), further supporting the reliability of our screening method. It should also be noted that these genes represent several pathways that are poorly understood in the context of aging regulation. Among these pathways, MFA1 and DIT2 are involved in sexual reproduction; PCL1 and RAX1 are regulators of cytokinesis. We believe future studies of these long-lived candidates could reveal previously unknown molecular mechanisms of aging.

**Mutations in the HDA complex lead to longevity.** Among candidate mutants with the greatest lifespan extensions, we found deletion strains of two subunits of histone deacetylase complex HDA: hda1Δ and hda2Δ. There are four classes of histone lysine deacetylases: Class I, II, III, and IV, which are classified by sequence similarity. HDA is a Class-II histone deacetylase complex made up of three subunits: the catalytic subunit Hda1 and accessory factors Hda2 and Hda3. HDA complex specifically deacetylates acetylated lysine on H3, but has less activity on histone H4. Curiously, a previous study found no significant lifespan change in an hda1Δ mutant. However, SEBYL results, coupled with microfluidic and microscopic dissection validation, suggested that mutations of all three HDA components would lead to robust lifespan extension (Fig. 4A, B).

To reveal the mechanism behind hda1Δ mediated RLS extension, we analyzed the transcriptome by RNA-sequencing (RNA-seq). GO analysis showed significant upregulation of several carbohydrate metabolism related pathways, such as glucose import, glycogen metabolism, etc. (Fig. 4C, see Supplementary Data 4 for complete list). To better understand the molecular process represented by these changes, we compared the hda1Δ transcriptome to a previously published dataset containing the transcriptome of 1400 single gene deletion strains, to find mutants with highly similar transcriptomes as that of hda1Δ. The most similar mutants we identified were tup1Δ, cyc8Δ, and several factors involved in DNA damage repair (DDR), such as homologous recombination factor Rad50 and DNA helicase Sgs1 (Fig. 4D).

Tup1 and Cyc8 form the Tup1/Cyc8 transcription repression complex in budding yeast. The complex represses expression of several stress response genes, including genes that regulate glucose and oxygen usage, DNA damage, osmotic stress, and...
other signals. Previous studies showed that the Tup1/Cyc8 complex recruits Hda1, and another class I histone deacetylase, Rpd3, to target genes, inducing promoter deacetylation and transcription inactivation, explaining the transcriptional similarity between hda1Δ and tup1Δ/cyc8Δ strains. Additionally, it was reported that rpd3Δ could also extend lifespan, similar to hda1Δ. To test whether the longevity phenotype of hda1Δ was indeed mediated by Tup1/Cyc8-dependent gene inactivation, we used epistasis analysis to determine whether hda1Δ mutation could still extend RLS in a TUP1 deletion background. In contrast to this prediction, we observed that TUP1 deletion did not extend lifespan on its own, but actually slightly shortened RLS, and deletion of HDA1 extended lifespan even in cells lacking TUP1 (Fig. 4E). These results indicate that the effect of hda1Δ on lifespan is likely independent of TUP1 function. Apart from replicative lifespan (RLS), chronological lifespan (CLS) defines the time yeast are able to survive after nutrient depletion and cell cycle cessation, which is believed to be tightly connected with the stress resistance ability of cells. Since Tup1/Cyc8 complex is a known repressor of stress response, we tested whether the CLS of tup1Δ and hda1Δ mutants showed any difference. We confirmed that only hda1Δ extended CLS, further supporting the conclusion that TUP1 deletion has little consequence on lifespan outcome (Fig. 4F).

Ruling out Tup1/Cyc8, we shifted our focus to DDR pathways, because DDR mutants have highly similar transcriptomes to that of hda1Δ. Inhibition of histone deacetylase activity by HDAC inhibitors is reported to hinder the DNA damage response; thus, it is possible that hda1Δ leads to a defective DDR, causing genotoxic stress and activating downstream stress response genes. Another possibility is that HDA1 deletion does not affect DDR directly, but upregulates downstream adaptive responses activated by narrow toxic environments. To narrow down possibilities, we used a growth assay to ask how HDA mutation affects cellular resistance to DNA damaging chemicals. The growth assay showed that all three HDA mutant strains had increased resistance to the genotoxic drugs methyl methanesulfonate (MMS) and camptothecin (CPT; Fig. 4G), similar increase of MMS resistance in hda1Δ was also seen in study conducted by another group. Additionally, we confirmed that growth of hda1Δ is less inhibited in MMS containing liquid media compared to wild type by measurement of growth curve (Fig. 4H–J). This result strongly favors the second hypothesis that HDA1 deletion upregulates downstream adaptive responses. Additionally, we found that the stress resistance of the rpd3Δ strain was not nearly as strong as in HDA mutants. Although a lifespan extension effect from rpd3Δ is previously reported, this finding was not repeatable in our own experiments and other published results using the BY4741 background. These findings are evidence that indicate potential different functions of HDA and Rpd3 in lifespan regulation.

HDA-dependent longevity is mediated via trehalose metabolism. We found no change in DDR pathways genes by gene ontology analysis (Fig. 4C), bringing up the question of how disrupting the HDA complex can contribute to enhancement of cellular resistance to genotoxic stress. As carbohydrate metabolism genes were significantly upregulated in hda1Δ, we aimed to identify the specific pathway that was involved. We analyzed five metabolic pathways that involved carbohydrates as substrates or products: glycolysis, tricarboxylic acid (TCA) cycle, myo-inositol metabolism, glycogen metabolism, and trehalose metabolism. We found that only glycogen and trehalose pathways were significantly upregulated in both hda1Δ and two DDR mutant strains: rad50Δ and sgs1Δ (Fig. 5A). Both glycogen and trehalose are conserved storage carbohydrates; glycogen plays a vital energy storage role conserved from budding yeast to humans, whereas trehalose also serves as storage carbohydrate in other fungi, plants, nematodes, and insects, although it is not synthesized in mammals. Notably, trehalose has long been known to protect cells from extracellular stress: in budding yeast, trehalose protects against desiccation, freezing, high ethanol concentration, and osmotic and oxidative stresses, and was recently proposed to play a vital function in adaptation to stress by maintaining viscosity homeostasis. Trehalose has a similar protective role in the nematode C. elegans, and its metabolism is essential for survival of the fruit fly D. melanogaster. Although trehalose is not synthesized in mammals, mouse consumption of trehalose increases longevity in some contexts, by enhancing autophagy in neuronal tissue. Neither glycogen nor trehalose are reported to function in the response to DNA damage.
Fig. 3 Gene interaction network analysis and microfluidic validation of SEBYL results. A Functional clustering of 285 long-lived deletion mutants. Circles represent long-lived deletions; edges are published physical protein–protein interactions. Overrepresented categories are highlighted in designated colors. B RLS of designated strains of both α and α mating types combined. Average RLS is noted in parenthesis. *p-value calculated by two-sided Mann-Whitney U test. ns not significant, **p < 0.01, ***p < 0.001. See Supplementary Data 3 for all statistical analysis on RLS experiments. See also Fig. S2.
damage, but levels of both acutely increase upon genotoxic stress\textsuperscript{43}. Using a published dataset\textsuperscript{44}, we confirmed that all glycogen and trehalose metabolism genes were upregulated upon MMS treatment (Fig. 5B), confirming that metabolism of these two carbohydrates responds to genotoxic stress. Furthermore, we used real-time PCR to verify that glycogen and trehalose synthesis enzymes were significantly upregulated in hda1\textsuperscript{Δ}, but not tup1\textsuperscript{Δ} mutants (Fig. 5C). Previous RNA-seq data also support this conclusion (Fig. S3A)\textsuperscript{45}. Uridine diphosphate glucose (UDP-glucose) is a substrate for both trehalose and glycogen metabolism\textsuperscript{46}. We found that overexpression of the UDP-glucose pyrophosphorylase gene \textit{UGP1} extended yeast
lifespan, and this extension was epistatic with hda1Δ (Fig. 5D), suggesting an active role for glycogen and/or trehalose metabolism in mediating the hda1Δ longevity phenotype.

Because syntheses and hydrolyses were both upregulated in an hda1Δ mutant, it is unclear how intracellular glycogen and trehalose levels respond to HDA gene deletion. We measured glycogen and trehalose levels with established methods and confirmed that, although levels of neither carbohydrates were significantly different under normal growth condition, both glycogen and trehalose levels were drastically increased in hda1Δ mutant under treatment with MMS (Fig. 5E). This result strongly suggests that the accumulation of either one or both carbohydrates contributes to the enhanced genotoxic resistance of HDA mutants. To further determine if one of these two molecules plays more important role in stress resistance, we tested whether trehalose or glycogen was involved in genotoxic resistance in HDA mutants. We found that deletion of glycogen synthase GSY2 had no epistatic effect on hda1Δ mutants (Fig. 5F). In contrast, knockout of TPS2 or TPS3, the phosphatase and regulatory subunit of trehalose synthase respectively, completely suppressed the longevity phenotype of hda1Δ (Fig. 5G, H). We also found that the long-lived UGP1OE strain to have significantly upregulated trehalose level, while the trehalose level is decreased significantly in TPS2 mutant (Fig. 5I). Furthermore, we found that hda1Δ cells also have higher resistance to high concentration of sodium chloride (Figure S3B), consistent with the fact that trehalose protects cells from osmotic stress. These findings led us to conclude that disruption of HDA function increases trehalose gene expression and its level under stress, and that extended lifespan in hda1Δ cells was mediated by trehalose metabolism, not glycogen.

Mutation of Class-II histone deacetylases shows preserved lifespan phenotypes. Class-II histone deacetylases are conserved from yeast to humans. In mammals, there are six members of Class-II HDAC: HDAC4, 5, 6, 7, 9, and 10, and their roles in neuronal physiology and immunity were identified in mice. However, evidence for Class-II HDAC activity in aging is still lacking. As such, we asked whether the effect of HDA mutation was conserved during evolution. Because mammals do not synthesize trehalose, we tested whether Class-II HDAC mutants had similar lifespan phenotypes in the nematode Caenorhabditis elegans and the insect Drosophila melanogaster. C. elegans has 5 Class-II HDACs: hda-4, hda-5, hda-6, hda-10, and hda-11. We found that one of these genes, hda-6, significantly extended lifespan when knocked-down by RNAi at both L1 and L4 developmental stages (Fig. 6A, B). Since Hda1 regulates stress resistance in yeast, we next tested whether hda-6 deficiency could result in higher stress resistance in worms as well. Indeed, we confirmed that worms with hda-6 knockdown were more resistant to heat and UV treatment (Fig. 6C–D). Moreover, we found that one of the trehalose synthase gene in worm, tps-1, was activated to a higher level in the hda-6 KO strain under UV and heat treatments (Fig. 6E).

In order to test whether silencing of HDAC6, the Drosophila homolog of yeast HDAI, could increase adult fly lifespan, we used a ubiquitous driver tubulin-Gal4 (tub-Gal4) to knockdown the expression of HDAC6 in all the fly cells. However, the embryos died before hatching to 1st instar larvae, suggesting that HDAC6 is required for early embryo development in Drosophila (Supplementary table S1). To overcome this problem and test the effects of HDAC6 knockdown in aging adult flies, we applied the Gal80 system with tub-Gal4, tub-Gal80ts. This method allows us to keep the expression of HDAC6 unchanged during embryonic development, and larvae and pupa stages by keeping the flies in 18 °C, and then knockdown HDAC6 expression after adult fly emergence by keeping newly hatched adult flies in 25 °C, at this temperature the Gal80ts allele lost its inhibition of Gal4, allowing the expression of HDAC6 RNAi to silence the expression of HDAC6 in adult flies.

We found that HDAC6 silencing in adult flies increased the adult lifespan by almost 25% (Fig. 6F). In the control group without HDAC6 RNAi, half of the adult flies died at day 50 and all of them died before day 60. With the HDAC6 silencing in adult flies, more than 60% flies are alive at day 60. Half of the adult flies died at day 65, and all of them died around day 80. We also compared the climbing ability in different aged flies (Fig. 6G). At day 5, there was no difference between control and HDAC6 silencing flies. At day 40, the climbing ability in control flies dramatically reduced, while HDAC6 silencing flies still have normal physical strength, suggesting that silencing of HDAC6 in adult flies not only expands lifespan, but also delays aging-associated physical function decline, at least in Drosophila. In conclusion, these results suggest that the previously uncharacterized functions of Class-II HDACs in aging regulation may be evolutionarily conserved.
To detect H3K18 acetylation, we confirmed an increase in promoter acetylation at trehalose metabolism genes (Fig. 7G). These changes were not visible on genes not activated by HDA1 deletion, such as glycolysis pathway genes, suggesting that HDA mediated histone deacetylation is selective. Gene ontology analysis showed a significant increase in H3K18 acetylation at promoters of genes in stress response, drug response, and pentose transport categories (Fig. S4B), which is consistent with...
the increased stress resistance and upregulation of carbohydrate transportation we observed (Fig. 4C, G). Furthermore, these H3K18 acetylation increases in hda1Δ correlate well with elevated expression of these genes (Fig. S4C), confirming that HDA regulates the expression of target genes through H3K18 acetylation.

As a histone deacetylase, the HDA complex is expected to physically interact with gene promoters and deacetylate histones. Indeed, there is demonstrated physical interaction between the HDA complex and target promoters. Since it is a transcriptional repressor for stress resistance genes, we asked whether genotoxic stress could remove the HDA complex as part of stress adaptation. Surprisingly, Myc-tagged Hda1 protein recruitment to TPS1 and TPS2 promoters increased under MMS treatment, whereas there was no significant change at the control gene RPS9A promoter (Fig. 7H). Thus, the level of HDA recruitment under genotoxic stress positively correlated with trehalose gene expression levels, which is consistent with the fact that removal of the HDA complex enhances genotoxic resistance.

Since histone deacetylases have higher affinity to acetylated histones than unacetylated ones, it is likely that increased recruitment of the HDA complex during genotoxic stress is caused by increased recruitment of histone acetyltransferases and hence histone acetylation levels.
Fig. 7 The lifespan phenotypes of HDA mutants are mediated by H3K18 acetylation. A RLS of WT, hda1Δ, H3K9Q, and hda1Δ H3K9Q. B RLS of WT, hda1Δ, H3K14Q, and hda1Δ H3K14Q. C RLS of WT, hda1Δ, H3K23Q, and hda1Δ H3K23Q. D RLS of WT, hda1Δ, H3K27Q, and hda1Δ H3K27Q. E RLS of WT, hda1Δ, H3K18Q, and hda1Δ H3K18Q. F RLS of WT, hda1Δ, H3K18R, and hda1Δ H3K18R. G Metagene plot and heatmap of H3K18 acetylation on trehalose (left) and glycolysis (right) promoters as detected by ChIP-Seq. H ChIP-qPCR of Hda1-myc binding on TPS1, TPS2, TPS3, and ACT1 promoters, under designated time after MMS treatment. Error bars represent SEM, n = 3. p-value calculated by two-sided T-test. I RLS of WT, hda1Δ, spt3Δ, and hda1Δ spt3Δ. J Mechanism of HDA mediated suppression of trehalose metabolism and its effect on yeast aging. See also Figure S4.
During stress conditions, stress response genes are acetylated by the SAGA complex in budding yeast. SAGA is an evolutionarily conserved histone acetyltransferase complex that acetylates K9, K14, K18, K23, and K27 on histone H3. Using data from a previous study, we found that MMS treatment enriched SAGA component Spt3 at promoter regions of trehalose genes. We also confirmed that the lifespan extension phenotype of hda1Δ depends on a functional SPT3 gene. These findings suggest that genotoxic conditions cause the SAGA complex to be increasingly recruited to trehalose gene promoters and creates the acetylated chromatin that facilitates HDA complex recruitment.

**Discussion**

In this study, we developed a barcode sequencing-based technique, SEBYL, to perform high-throughput screening for yeast mutants with an altered RLS. We demonstrated that SEBYL yielded results that are consistent with classical experiments, yet taking <1 month to perform. Our screen also provided data for strains with previously inconclusive RLS data that had been generated by classical screening and identified several long-lived candidates not previously reported. We conclude SEBYL to be fast, reliable, and suitable for RLS screens of many different barcoded yeast mutant collections.

Using SEBYL, we identified a previously unknown regulator of aging, the histone deacetylase complex HDA. We showed that strains with defective HDA function were more resistant to various stressors, especially genotoxicity. This resistance depended on the metabolism of storage carbohydrate trehalose, of which metabolism genes were significantly upregulated in hda1Δ cells, potentially protect yeast cells from genotoxic stress. We also demonstrated that genotoxic stress caused the SAGA complex to bind the trehalose promoter. This binding likely induced histone acetylation, which in turn mediated HDA recruitment to target locations. Based on these results, we propose the following model: Under genotoxic stress, the HDA complex is recruited to trehalose promoters by acetylated histones, which inhibits expression of trehalose genes via promoter deacetylation. When HDA is non-functional, the acetylation at promoters of trehalose genes increases and activates expression of trehalose genes. Increased trehalose gene expression promotes stress resistance, thus extending the lifespan of yeast cells (Fig. 7).

**SEBYL is a fast and reliable approach to RLS screening in budding yeast.** Microdissection and microfluidic-based RLS measurements are not truly high-throughput, due to the requirement for manual cell counting. In both methods, determining cell division events requires judgement by eyesight, which is time and energy consuming. Compared to previously published yeast RLS screening approaches, the SEBYL method has the following advantages: (1) A considerably faster process where all benchwork can be finished in only 2 weeks; (2) SEBYL does not require additional instruments, such as an automated flow cytometer. Only the reagents for old cell sorting and next-generation sequencing access are needed; (3) Because SEBYL uses the entire pool of yeast collection, results are based on an unbiased screen of all mutants present in one collection; (4) SEBYL does not require strains with a specific genetic background. All barcoded yeast collections can be used; (5) SEBYL is demonstrably reproducible and generates lifespan data comparable to results obtained from both microdissection and microfluidic methods. Here, we demonstrate the use of SEBYL on yeast gene knockout collection and proved it to be a valid way to complement existing methodologies of RLS screening.

Apart from identifying genes involved in aging regulation, SEBYL also generates large datasets for bioinformatic analysis. Bioinformatic studies using large-scale datasets are increasingly important for characterizing gene functions and molecular pathways. Here we demonstrated that SEBYL screening data could identify cell morphology traits associated with both longer and shorter lifespans. Due to technical difficulties of large-scale lifespan screening, there is still a lack of comprehensive RLS data collections. We anticipate that the development of SEBYL will facilitate generation of RLS datasets and provide valuable resources for bioinformatic studies of aging biology.

**HDA regulates aging through trehalose metabolism independent of Tup1/Cyc8.** The Tup1/Cyc8 complex is transcriptional co-repressor complex with functional similarity to metazoan corepressor protein such as Groucho protein in Drosophila, and Transducin-like Enhancer of Split (TLE) family in mammalian cells. Previous study shows Tup1/Cyc8 complex acts as an inhibitor of various stress resistance genes by recruiting histone deacetylase Rpd3 and HDA complex on target promoters. In this study, we provide evidence that inactivation of HDA complex could lead to increased stress resistance and lifespan extension independent of Tup1/Cyc8 function: First of all, removal of TUP1 gene was not able to extend replicative lifespan, nor was it able to suppress RLS extension brought by hda1Δ (Fig. 4E); Secondly, tup1Δ and rpd3Δ strains did not show significantly enhanced genotoxic resistance as seen in three HDA mutants (Fig. 4G). Furthermore, the significant induction of trehalose metabolism genes was also not recapitulated in tup1Δ mutant (Fig. 5C and D).

These lines of evidence suggest that just like class I histone deacetylase Rpd3, HDA may also have distinct target profiles depending on different enzymatic complexes it resides in. In this study, we identified the trehalose metabolism genes to be especially important targets of HDA complex mediated deacetylation. Trehalose levels increase drastically under stress, which has been proposed to be an important protectant to organisms. We found that abrogating HDA releases the inhibition of trehalose metabolism genes (Fig. 7G), and therefore leads to higher resistance to various source of stress (Figs. 4G and S2B). Though we are not able to decipher how increased trehalose benefits stress response, a recent study shows that trehalose is essential for maintaining cellular viscosity homeostasis, making cells adapt better to different environmental conditions. Further study on detailed mechanisms behind the protective functions of trehalose may lead to novel intervention strategies for aging and age-related diseases.

**Class-II histone deacetylases may have additional roles in aging in higher eukaryotes.** This study contains the first direct evidence to suggest that deletion of Class-II deacetylase HDA extends lifespan, such effect is also conserved from unicellular budding yeast to metazoan C. elegans and D. melanogaster. We determined that at least in budding yeast, the effect of hda1Δ on lifespan is mediated through released inhibition of trehalose metabolism genes, which leads to enhanced stress resistance and extended lifespan.

Post-translational modification (PTM) of histones changes significantly during aging, and is characterized by a general opening of chromatin structure; histone PTMs are thus proposed to play important roles in aging regulation. Histone acetylation and deacetylation are vital to transcriptional regulation, but these processes are also heavily implicated in cellular senescence and organismal aging. Histone deacetylases (HDACs) are extensively studied with regard to their functions in the aging process: Sirtuins, one of the most studied groups of anti-aging factors, are nicotinamide adenine dinucleotide dependent Class-III lysine deacetylases. The anti-aging activity of the sirtuin protein family...
is evolutionarily conserved from yeast to mouse and regulates various pathways, including gene silencing, nutrition sensing, and stress response. Class I deacetylases, such as yeast Rpd3 and human HDAC1, are also proposed to affect aging by various mechanisms.

Unlike class I HDACs and sirtuins, the function of Class-II HDACs in aging is relatively unknown and the existing data is sometimes contradictory. For example, the reduction of HDAC4 is shown to facilitate clearance of pathogenic protein that aggregates in a Huntington disease model and antagonizes neuron degeneration. In contrast, another study reported HDAC4 overexpression delays cellular senescence via SIRT1 sumoylation. Because we observed increased lifespan phenotypes after ablating HDA in multiple model organisms, it will be interesting to see whether inhibiting any of the Class-II HDACs affects lifespan in mammals. Additionally, the relationship between Class-II HDACs, trehalose metabolism and aging still needs to be explored in greater detail. Although we provided evidence supporting that Hdal regulates aging through trehalose metabolism in the budding yeast, it remains to be seen whether such mechanism stays conserved throughout evolution. In conclusion, the roles of Class-II HDACs play an important role in aging regulation, and hence could be feasible targets to combat age-related diseases because multiple known HDAC inhibitors are already used in treating cancer.

Methods

Yeast deletion strain pool construction and old cell isolation. Yeast library was pinned to YPD plates and grown for 3 days, then scraped into 50 ml centrifuge tube with YPD and 200 μg/ml G418 (Thermo Fisher Scientific). Genomic DNA was extracted from young and old fractions using standard methods and the barcodes UPTAG and DNTAG were quantified by next-generation sequencing using the Illumina HiSeq 2500 platform. Sequencing of three biological replicates was performed using the Illumina HiSeq 2500 platform.

Chronological lifespan measurement. Yeast cells were inoculated in YPD and incubated for 3 days as starting culture, with three replicates for each strain. At designated time point (every 5 or 6 days apart), a sample of yeast culture were collected and diluted 106 folds, then spread onto YPD plates. Number of colonies were counted and relative colony-forming units (CFU) compared to day 0 culture were calculated.

Yeast growth assay. Yeast cells were grown overnight at 30 °C, diluted to OD600 of 0.1, then potted in fivefold serial dilutions on designated agar plates. Control plates (SC) were incubated at 30 °C for 24 h and all other drug plates for 48 h. Plates were scanned with Epson Perfection V500 Photo scanner.

RNA extraction, reverse transcription PCR, and real-time quantitative PCR analysis. Yeast cells with total OD600 of 1 were lysed in QiAzo1 buffer (QIAGEN) with 0.5-μm zirconia/silica beads (BioSpec) for 4 cycles. Total RNA was purified with miRNeasy Mini Kit (QIAGEN). Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using 1 μg of purified RNA. Real-time PCR was performed with ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) and data analysis performed by QuantStudio Real-Time PCR software. Yeast gene expression data were normalized with ACT1 and analyzed with the Gen5 software.

Trehalose and glycogen measurement. Measurement of glycogen and trehalose were performed according to published protocol. Briefly, 109 cells were harvested and washed with 1 ml ice-cold water. Cells were resuspended in 125 μl of 0.25 M Na2CO3 and incubated at 95 °C for 3 h. pH was adjusted to 5.5 with 7.5 μl 1 M acetic acid and 300 μl 0.2 M NaAcetate, pH 5.2 then separated into two tubes for glycogen and trehalose measurement. For glycogen, 20 ml/mg solution of Aspergillus niger α-amylolucosidase (Sigma) was added and incubated at 57 °C overnight. For trehalose, 15 ml of 0.2 M NaAcetate and 3 μl of porcine trehalase (Sigma) was added and incubated at 37 °C overnight. Released glucose was measured with the Glucose assay kit (Sigma-Aldrich) and read by BioTek Synergy 2 Multi-Mode Reader, with the data analyzed using the Gen5 software.

Yeast RNA interference and lifespan measurement on Caenorhabditis elegans. RNAi plates (standard NGM plates with 25 μg/ml carbencicilin and IPTG 1 mM) were freshly made before beginning of experiment. The worms were maintained for two generations before experimental and moved to RNAi plates at L1 or L4 stage. Lifespan score was performed every other day. At least 120 worms were included per RNAi treatment. Worms used in this study is N2, sirNA targeting hda-6 used in this study is F41H10.6 in Vidal library.

Caenorhabditis elegans RNA collection and purification. Approximately 500 N2 worms at day 3 after RNAi treatment were collected for RNA extraction. The worms were left on hda-6 RNAi Fudr plates when they reach L4 stage, on everyday 330RNAi food was added to prevent worm from staying. The worms were growing at 20 °C for 3 days and harvested, then lysed in in QIAzol buffer (QIAGEN) with 1.0-μm zirconia/silica beads (BioSpec) for 4 cycles. Total RNA was purified with miRNeasy Mini Kit (QIAGEN). Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using 1 μg of purified RNA.

Caenorhabditis elegans heat treatment. N2 worms were grown on NGM plates with HB1019 E. coli containing the empty vector pL4400 (control) and hda-6 RNAi construct at 20 °C. For each replicate, 32 L4 worms were transferred to fresh plates;
when they reached day 1 of adulthood, these worms were incubated in 37°C. Live worms were counted after 12 h.

**Caenorhabditis elegans UV treatment.** N2 worms were grown on NGM plates with HB109 E. coli containing the empty vector pL4400 (control) and hda-6 RNAi construct at 20°C. Fifty L4 worms were transferred to fresh plates; when they reached day 1 of adulthood, these worms were exposed to 0.08 J/m² UV. Immediately after exposure, worms were transferred to fresh plates and continued to be grown at 20°C. Live worms were counted and transferred to fresh plates every 2–3 days for the duration of the experiment.

**Drosophila melanogaster strains.** Flies were reared on standard food at temperature described in the experiment for UAS-Gal4 strains. The following strains were used in this study: tub-Gal4/Tm3, tub-Gal4, tub-Gal80ts/CyO, and HDAC6-RNAi (BL-31053 and BL-34072). All these fly lines were obtained from the Bloomington Drosophila Stock Center.

**Drosophila melanogaster adult survival assay.** Drosophila embryos and larvae were kept in 18°C to avoid developmental lethality. Once adult flies emerged, adult male flies were transferred to 25°C to deactivate Gal80ts, which allows the expression of UAS-HDAC6 RNAi hairpin to silence the endogenous HDAC6 expression. Adult male flies were then maintained in vials at 25°C in groups of 10 per vial and passed to a new vial every 3 days. A total of 300 flies were analyzed with 100 flies for each genotype group.

**Drosophila melanogaster climbing assay.** Climbing male flies were monitored by analyzing their performance to climb 6 cm within 14 s. A successful attempt was scored as 1, and failure to reach the top as 0. Each fly was assessed five times to calculate the average climbing score. 40 flies per age/genotype group were analyzed.

**Chromatin immunoprecipitation and ChiP-seq library preparation.** Yeast culture of OD between 0.6 and 0.8 were harvested and cross linked with 37% formaldehyde to final concentration of 1% and incubate at room temperature for 10 min. Cross-linking was stopped by adding 2.5 M glycine to minimize cross-linking. Chromatin immunoprecipitation (ChiP) assay was performed using True MicroChip Kit (Diagenode) with Bioruptor, then centrifuged to collect supernatant. Chromatin immuno- precipitation Kit (Qiagen) was used for preparing ChiP DNA. RNAi hairpin to silence the endogenous HDAC6 expression. Adult male flies were then maintained in vials at 25°C in groups of 10 per vial and passed to a new vial every 3 days. A total of 300 flies were analyzed with 100 flies for each genotype group.

**Yeast strains and media.** All strains used in this study except for Yeast Knockout collection are listed in Table S4. The yeast knockout collection (mating type alpha) was obtained from Dharmarao (YSC1054). StandardYPD medium (1% yeast extract, 2% peptone, and 2% glucose, with 1.5% agar included for solid medium) was used for all yeast experiments unless otherwise noted.

**Statistics and data processing.** Statistics. Yeast RLS p-values were determined using the Wilcoxon rank-sum test. Statistical significance of all other data not specified above was calculated using an unpaired equal variance two-tailed Student’s t test.

**Barcode sequencing data analysis.** Barcode sequencing data were analyzed by a blast-based decode method using a home-made python script. Reads were first trimmed by cutting the 3’ and 5’ adapters. Then trimmed barcodes were aligned using blastn with the following parameters: -task mgblast -word_size 7 -reward 2 -penalty -3. Custom blastn database was created by makeblastdb using pre-defined barcode-mutant annotation file. Barcodes that represent specific mutants were counted from the best blastn matches with up to three differences. Uptag and Dntag were counted separately. The count data was then used for the following yeast mutant lifespan estimation.

**Statistical Model of SEBYL method.** We developed a method to estimate yeast mutant lifespan from barcode sequencing counts, with the idea that mutants depleted from the old cell fraction were predicted to have shorter lifespan and those enriched in the old cell fraction were predicted to have a longer lifespan. The barcode counts were generated from nine samples/libraries: the unsorted (USC), young and old cell fraction from the first (S1Y, S1O), second (S2Y, S2O), third (S3Y, S3O), and fourth (S4Y, S4O) of cell sorting.

**ChiP-seq data processing.** Read alignment was performed using TopHat2 package. Subsequent processing was performed using an in house script where signal extraction scale69 were first applied to normalize each sample for signal scale, samples were then normalized again with trimmed mean of M-values (TMM) method. Metagene plot and heatmap of histone acetylation markers were generated by deepTools package.

**RNA-seq data processing.** Read alignment was performed using TopHat2 package. Subsequent processing was performed using an in house script where signal extraction scale69 were first applied to normalize each sample for signal scale, samples were then normalized again with trimmed mean of M-values (TMM) method. Metagene plot and heatmap of histone acetylation markers were generated by deepTools package.
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**Author contributions**

Conceptualization by W.D., R.Y., and X.C.; Methodology by W.D., R.Y., X.C., J.Z., B.W., W.L., E.C., H.L., M.J., L.Q., and Z.H.; Investigation by R.Y., X.C., L.S., B.W., J.Z., Z.H., M.K., and W.D.; Writing: Original Draft by W.D., R.Y., and X.C.; Writing: Review & Editing by R.Y., X.C., L.S., B.W., Z.H., M.K., and W.D.; Funding Acquisition by W.D., M.J., L.Q., M.K., Z.H., and R.Y.; Supervision by W.D., M.K., and Z.H.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information**

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