Multigenerational silencing dynamics control cell aging

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Cellular aging plays an important role in many diseases, such as cancers, metabolic syndromes, and neurodegenerative disorders. There has been steady progress in identifying age-related factors such as reactive oxygen species and genomic instability, yet an emerging challenge is to reconcile the contributions of these factors with the fact that genetically identical cells can age at significantly different rates. Such complexity requires single-cell analyses designed to unravel the interplay of aging dynamics and cell-to-cell variability. Here we use microfluidic technologies to track the replicative aging of single yeast cells and reveal that the temporal patterns of heterochromatin silencing loss regulate cellular life span. We found that cells show sporadic waves of silencing loss in the heterochromatic ribosomal DNA during the early phases of aging, followed by sustained loss of silencing preceding cell death. Isogenic cells have different lengths of the early intermittent silencing phase that largely determine their final life spans. Combining computational modeling and experimental approaches, we found that the intermittent silencing dynamics is important for longevity and is dependent on the conserved Sir2 deacetylase, whereas either sustained silencing or sustained loss of silencing shortens life span. These findings reveal that the temporal patterns of a key molecular process can directly influence cellular aging, and thus could provide guidance for the design of temporally controlled strategies to extend life span.

Significance

Aging is an inevitable consequence of living, and with it comes increased morbidity and mortality. Novel approaches to mitigating age-related chronic diseases demand a better understanding of the biology of aging. Studies in model organisms have identified many conserved molecular factors that influence aging. The emerging challenge is to understand how these factors interact and change dynamically to drive aging. Using multidisciplinary technologies, we have revealed a sirtuin-dependent intermittent pattern of chromatin silencing during yeast aging that is crucial for longevity. Our findings highlight the important role of silencing dynamics in aging, which deserves careful consideration when designing schemes to delay or reverse aging by modulating sirtuins and silencing.

Author contributions: Y.L., M.J., R.O., I.S.T., L.P., J.H., and N.H. designed research; Y.L. and M.J. contributed equally to this work.

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indicates reduced silencing (24, 25) (Fig. 1C). We observed that cells with the rDNA reporter gene exhibit weak fluorescence; in contrast, cells carrying the same reporter at the \textit{URA3} locus, which is not subject to silencing, show very high fluorescence. In addition, deletion of \textit{SIR2}, which is required for rDNA silencing (18), yields significantly increased reporter expression at the rDNA, but not at \textit{URA3} (\textit{SI Appendix}, Fig. S1E).

Using the microfluidic device and the reporter, we tracked the dynamics of rDNA silencing throughout the entire life spans of individual cells (Figs. 1D and 2). We found intermittent fluorescence increases in most cells, indicating sporadic silencing loss during aging. About half (~46\%) of the cells, during later stages of aging, continuously produced daughter cells with a characteristic elongated morphology until death (Fig. 2, blue arrows). These

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**Fig. 1.** Tracking the dynamics of chromatin silencing in single aging cells using a microfluidic device. (A) The design layout of the device. “Cell traps” contains cell traps for single mother cells. (B) A schematic of multiple, parallel cell traps. Mother cells stay at the bottom of finger-shaped chambers and daughters are pushed out. Details of the device are provided in \textit{SI Appendix}. (C) Illustrative schematic of the reporter for chromatin silencing. A GFP reporter under a strong constitutive promoter (\textit{TDH3}) is inserted at an NTS1 repeat within the rDNA. Chromatin silencing at the rDNA is reflected by fluorescence intensity: low, silenced; high, decreased silencing. Details of the reporter are provided in \textit{SI Appendix}. (D) Time-lapse images of a single cell trap throughout an entire life span. Arrows point to the mother cell.

**Fig. 2.** Dynamic patterns of silencing loss during aging. (A) Dynamics of silencing loss in cells aging with elongated daughters. (Top) Representative images of cell aging and death with elongated daughters. Blue arrows point to daughter cells, white arrows point to the living mother cell, and the red arrow points to the dead mother cell. (Bottom) Single-cell color map trajectories of reporter fluorescence. Each row represents the time trace of a single cell throughout its life span. Color represents the normalized fluorescence intensity as indicated in the color bar, which is used throughout to allow comparisons between different conditions. Cells are sorted based on their RLSs. (B) Dynamics of silencing loss in cells aging with rounded daughters. (C) Daughter cell morphology is correlated with the silencing state of mother cells. Color map trajectories of representative mothers are aligned with the morphology (round vs. elongated) of daughters produced throughout their life spans. A cross-correlation analysis of daughter morphology and mother silencing dynamics is shown in \textit{SI Appendix}, Fig. S2.
cells also exhibited a sustained and dramatic increase in fluorescence, indicating sustained loss of silencing in aged cells (Fig. 2A, color map). In contrast, the other half of the cells, at later phases of their life spans, continuously produced small round daughter cells (Fig. 2B, blue arrows) with sharply increased cell cycle length. These cells had a shorter average life span than the other aging type (with a mean RLS of 18 compared with 24) and did not show sustained silencing loss during aging (Fig. 2B, color map). These two distinct types of age-associated phenotypic changes suggest different molecular causes of aging in isogenic cells (8, 9, 14).

Previous studies showed that the aging phenotype with small round daughters could be related to an age-dependent mitochondrial dysfunction (9, 12), but the molecular mechanisms underlying the other aging type characterized by elongated daughters remain largely unclear. Our results revealed that the sustained rDNA silencing loss, which can lead to genome instability (18), is specifically associated with the aging phenotype featured by elongated daughters. In support of this, young mother cells can also sporadically produce a few elongated daughters, the occurrence of which correlates with the transient silencing loss during the early phases of their life spans (Fig. 2C). In addition, cross-correlation analysis revealed a ∼140-min time delay between the occurrence of silencing loss in mother cells and the production of elongated daughters (SI Appendix, Fig. S2). This temporal order suggested a potential causal relationship between silencing loss and the elongated daughter phenotype. In this work, we focused our analysis on the dynamics and heterogeneity of the type of aging process with sustained silencing loss and elongated daughters.

To exclude the possibility that the observed fluorescence patterns are caused by age-associated global effects on gene expression (26), we simultaneously monitored two distinguishable fluorescent reporters inserted at the rDNA and at URA3. Whereas the rDNA reporter showed early sporadic and late sustained induction of fluorescence, the reporter at URA3 exhibited relatively constant fluorescence during aging (SI Appendix, Fig. S3A). To confirm that the observed silencing dynamics are not specific to the NTSI region, we measured the reporter response at another non-transcribed spacer region of rDNA (NTS2) and observed similar dynamic patterns as those found at NTS1 (SI Appendix, Fig. S3B).

Together, these results validate that the reporter responses during aging. We observed very rare events of recombination or extrachromosomal rDNA circles of the reporter gene, which can be easily distinguished from silencing loss in single-cell time traces (SI Appendix, Fig. S3C). We have excluded those cells from the analysis.

To evaluate how dynamic patterns of chromatin silencing influence cell aging, we quantitatively analyzed the time traces of silencing loss in individual cells producing elongated daughters before death. With very diverse life spans ranging from 9 to 48 generations, all the cells show sustained silencing loss toward later stages of aging. Most cells also exhibit early sporadic waves of silencing loss, each of which spans multiple cell divisions (Fig. 3A and Movie S2). This unprecedented long-wavelength dynamics is distinct from most previously characterized molecular pulses, which are on timescales faster than or close to a cell cycle (5). We further dissected each single-cell time trace into two phases: an early phase with sporadic silencing loss and a late phase with sustained silencing loss (Fig. 3B, “Intermittent Phase” and “Sustained Phase”). The length of Intermittent Phase (or the number of silencing waves) is highly variable among cells (coefficient of variation, 0.63; SI Appendix, Fig. S4A) and correlates closely with final life span, suggesting the longevity of a cell is largely determined by the time it stays in this phase (Fig. 3C, Left). Long-lived cells generally have a longer Intermittent Phase and produce more silencing waves than short-lived cells (Fig. 3A and SI Appendix, Fig. S4B and D). In contrast, the length of Sustained Phase is more uniform among cells (coefficient of variation, 0.29; SI Appendix, Fig. S4A) and shows little relationship with life span, suggesting sustained silencing loss defines cell death within a relatively constant period of time (Fig. 3C, Right).

We further quantified the rise time of each fluorescence increase in single aging cells (the duration of silencing loss; Fig. 3B, t1 and t2) and found a significant difference between the durations of early sporadic and late sustained silencing loss: a sporadic silencing loss on average lasts for ∼300 min, whereas sustained silencing loss lasts for ∼2,200 min until death (Fig. 3D). Moreover, sporadic waves of silencing loss show modest effects on the basal silencing level during aging, as indicated by the trough levels of silencing loss pulses in single-cell time traces (SI Appendix, Fig. S4 B and C), yet do not contribute additively onto sustained silencing loss to inducing cell death (SI Appendix, Fig. S4E).

Together, our analyses reveal that cells undergo spontaneous silencing loss during aging. The early phase of aging features a reversible process, in which cells can effectively reestablish silencing and produce nondetrimental short waves of silencing loss. The late phase is irreversible: aged cells cannot reestablish silencing (27), resulting in sustained silencing loss and death. Individual cells may have different intrinsic capacities to maintain the reversible phase, and thereby the ultimate life span.

To provide a quantitative framework for understanding aging dynamics, we developed a simple phenomenological model. The model postulates that an aging cell can be in one of two states: state 0 is the silenced state in which it produces normal daughters, and state 1 is the silenced state in which it produces elongated daughters (Fig. 4A). The transitions between the states are characterized by probabilities p01 and p10 that depend linearly on the cell age (Fig. 4B). We also assume that in the silencing loss state (state 1), a damage factor D accumulates uniformly, and the probability of cell death is proportional to D, and therefore to the number of generations a cell spends continuously in state 1 (Fig. 4C). In the silenced state (state 0), D is set to zero. We fit the model only using the experimental data on phenotypic changes and simulated this model stochastically. The model reproduced the main statistical properties of age-dependent phenotypic changes and RLS remarkably well (Fig. 4 C–F). We also generated individual cell state trajectories (Fig. 4G) that qualitatively and quantitatively reproduce the data in Fig. 2A. To predict how silencing dynamics influence aging, we further simulated the effects of an induced silencing loss. Whereas a short pulse of silencing loss does not affect life span, a sustained silencing loss dramatically shortens life span (Fig. 4 H and I). To evaluate these predictions, we set out to modify silencing dynamics using genetic or chemical perturbations.

Chromatin silencing at the rDNA is primarily mediated by the lysine deacetylase Sir2, encoded by the best-studied longevity gene to date, which is conserved from bacteria to humans (18). To examine the role of Sir2 in regulating silencing dynamics, we monitored the aging process of sir2A cells. We observed that sir2A cells do not exhibit sporadic silencing loss; instead, most cells show sustained silencing loss throughout their life spans (Fig. 5A), indicating that the intermittent silencing dynamics is dependent on Sir2-mediated silencing reestablishment. Most (∼70%) sir2A cells continuously produce elongated daughters until their death, in accordance with the observed correlation between silencing loss and elongated daughters. Furthermore, in sir2A cells, sustained silencing loss leads to cell death within a relatively uniform time frame, strikingly resembling the Sustained Phase in WT cells (Fig. 5A, red dashed line). These results suggest Sir2 promotes longevity by generating intermittent silencing dynamics and delaying entry into the Sustained Phase. We also examined sgf73Δ, a mutant with an extended longevity (28), and observed intermittent silencing dynamics during aging and elongated daughters at the late phase of aging. This long-lived mutant shows more silencing loss pulses than WT, consistent with the possibility that the intermittent silencing dynamics promotes longevity (SI Appendix, Fig. S5).

To further test predictions of the model and examine the causative roles of silencing dynamics on aging, we exposed cells to
nicotinamide (NAM), an inhibitor of Sir2 (29), to chemically disrupt silencing with physiologically relevant durations. In response to a 1,000-min NAM input mimicking the Sustained Phase, the majority of cells cannot recover from silencing loss (Fig. 5B). All (100%) of the treated cells, although young, continuously produce elongated daughters and die within a similar time frame to sustained silencing loss in sir2 mutant or WT cells (Fig. 5B). The cells also show an elevated DNA damage response, as reported by the induction of RNR3 (30, 31) (Fig. 5B, Inset), and have a significantly shortened life span (mean RLS, 12), comparable to that of sir2 mutants. These results suggested that sustained silencing loss causes the elongated daughter phenotype and accelerates cell death in young cells. In contrast, in response to a 240-min NAM input, mimicking the sporadic silencing loss, most cells exhibit a synchronized silencing loss followed by effective silencing reestablishment on the removal of NAM (Fig. 5B). This short-term silencing loss does not induce the DNA damage response (Fig. 5C, Inset) and does not affect life span, in accord with the sporadic silencing loss in the Intermittent Phase of naturally aging cells. These perturbation experiments validate the model's predictions.
and confirm that a prolonged duration of silencing loss triggers an irreversible process sufficient to induce cell death, whereas short-term silencing loss is fully reversible and does not promote aging or death.

Finally, we considered the role of the intermittent pattern of silencing, as opposed to continuous chromatin silencing. To this end, we used nicotinic acid (NA), an activator of Sir2 (32), to prevent sporadic silencing loss in aging cells. As shown in Fig. 5D, most cells show continuous silencing with few sporadic losses of silencing. The repression of silencing loss during aging results in the absence of the elongated daughter phenotype in the majority (∼98%) of cells. Importantly, this sustained chromatin silencing also results in significantly shortened life span (mean RLS, 11), suggesting constant chromatin silencing can activate a different aging or death pathway. Furthermore, we confirmed that the effects of NA on life span are not likely a result of direct cellular toxicity (32), but are instead primarily mediated through Sir2 (32) (SI Appendix, Fig. S6), our results suggest the intriguing possibility that chromatin silencing may be a double-edged sword with both anti-aging and proaging functions, probably depending on target genomic regions. whereas sustained rDNA silencing loss increases heterochromatin instability, sustained chromatin silencing may repress genes that benefit longevity; either state would accelerate cell aging and death. The intermittent silencing dynamics driven by Sir2 allow the cell to periodically alternate between the two states, avoiding a prolonged duration in either state and maintaining a time-based balance important for longevity.

**Discussion**

Further analysis will continue to identify downstream targets that mediate the negative effects of sustained silencing on life span. For example, potential candidates include genes in other heterochromatic regions, such as subtelomeric genes that encode metabolic enzymes or have mitochondrial functions (33), or Sir2-repressed genes with prolongevity functions (34), or critical processes influenced by rDNA transcription, such as ribosomal biogenesis, a potential regulator of yeast aging (35, 36). Interestingly, a recent study (37) demonstrated that aggregation of a cell-cycle regulator, but not the previously reported loss of silencing at HM loci (38), causes sterility in old yeast cells. This work, together with our findings here, suggests chromatin silencing at various genomic regions might undergo different age-dependent changes, probably because of their specific silencing complexes. For example, whereas the silencing at HM loci is regulated by a protein complex containing Sir2, Sir3, and Sir4 (39), a different complex containing Sir2, Net1, Cdc14, and Nan1 is required for the silencing at the rDNA (40, 41). Furthermore, it has been shown that rDNA and HM silencing have different sensitivities to NAM or genetic perturbations (42–44), implying different regulatory modes at these loci. We anticipate that further systems-level analysis will enable a more comprehensive understanding of chromatin regulation during aging. Another interesting question for future investigation is the fate of elongated daughters. One possibility is that the production of these abnormal daughters may serve as a rescue strategy to alleviate damage accumulation in mother cells. Future technological advances that allow life span tracking of selected daughter cells would enable us to examine the silencing dynamics, life span, and aging type decision of these elongated daughters and their daughters.

Dynamics-based regulation is an emerging theme in biology, the role of which has been increasingly appreciated in many biological processes across a wide range of organisms (3, 4, 45–49). Our analysis here uncovered the significant role of silencing dynamics in cellular aging and opened the possibility of designing temporally controlled perturbations to extend life span. For example, although constant NA exposure shortens life span, it might be possible to design dynamic regimes of NA treatment to specifically prevent sustained silencing loss, and thereby delay aging. Given the observed single-cell heterogeneity in silencing and aging dynamics (Figs. 2 and 3), future efforts will focus on technologies that enable distinct real-time NA treatments to individual cells based on their silencing states.

**Materials and Methods**

**Strains and Plasmids Construction.** Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for manipulation of DNA were used throughout. The yeast strains used in this study were generated from the BY4741 (MAT a his3Δ1 leu2Δ1 met15Δ0 ura3Δ0) strain background. Strain and plasmid information is provided in SI Appendix, Tables S1 and S2.

**Microfluidic Device for Yeast Aging Studies.** In designing a microfluidic device for studying aging in budding yeast, the viability of the cells, efficiency of cell trapping, and robustness of the device were our primary concerns. The robustness of the device is affected by clogging resulting from excess cells around the traps and at the waste port, which can interfere with mother cell life span and retention. Supplying media through ∼20-μm-tall main channels readily allowed excess cells to be washed away and prevented clogging.
Therefore, a critical feature of our device is its two-layer design, making it extremely robust over the course of our experiments, which takes more than 80 h. This is a unique feature compared to recently published devices that are all single-layer (10, 13, 14). The device was optimized for using continuous gravity-driven flow during operation, with the three-inlet design also facilitating media switching experiments. Cell loading efficiencies and final retentions until cell death are -93% and 75%, respectively. See SI Appendix for further details on the device and its validation.

Live-Cell Imaging and Analysis. Time-lapse imaging experiments were performed using a Nikon Ti-E inverted fluorescence microscope with Perfect Focus, coupled with an EMCCD camera (Andor iXon x3 DU987). The light source is a spectra X LED system. Images were taken using a CFI plan Apo- chromat Lambda DM 60x oil immersion objective (NA 1.40 WD 0.13MM). The microfluidic device was placed on the motorized microscope stage (with Encoders) within a 30 °C incubator system. The flow of medium in the device was maintained by gravity and drove cells into traps. Waste medium was collected to measure flow rate, which was about 2.5 mL/day. Images were acquired every 15 min for a total of 80 or more hours. Images were processed and quantified with a custom MATLAB code. Cell divisions of each mother were scored and categorized based on their aging phenotypes, characterized by the morphologies of later daughters they produced. Mothers continually producing elongated daughters at the last few generations were categorized as “aging with elongated daughters.” Deteriorating mothers continued producing rounded daughters through the last few generations were categorized as “aging with rounded daughters.” Dynamic patterns of reporter fluorescence have been shown in jet rainbow color maps. The inferno versions of the color maps to provide continuous luminance visualization of the data have been included in SI Appendix, Fig. S8.

Detailed methods and the development of the phenomenological model are given in SI Appendix.

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Supporting Information

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**Movie S1.** Time-lapse movie tracking the replicative aging of individual yeast cells throughout their entire life spans. Mother cells were trapped at the bottom of finger-shaped chambers 1, 3, 4, 5, and 6 (from left to right) throughout their entire life spans, following each division until the mother's death. The original mother cell in chamber 2 was pushed out by its daughter cells in the middle of the experiment, and was therefore lost for further analysis. Mother cells in chambers 1, 3, 5, and 6 produced elongated daughters at the late phases of aging, preceding their deaths, whereas the mother cell in chamber 4 produced round daughters before its death.

**Movie S2.** Time-lapse movie showing multigenerational silencing waves of an aging mother cell. The movie on the left shows the fluorescence intensity changes during the aging process of a mother cell with the rDNA silencing reporter (encircled). (Right) Real-time quantification of fluorescence intensity, indicating the silencing state of rDNA: decreased fluorescence indicates enhanced silencing, whereas increased fluorescence indicates reduced silencing. Vertical dashed line represents each division time of the mother cell in which the distance between two adjacent dashed lines indicates the cell cycle length. In the movie, from 0 min to 1,050 min, the mother cell underwent budding toward the bottom of the finger-shaped chamber and newborn daughter cells were pushed out through the small opening at the bottom of the chamber. From 1,050 min to 3,015 min, the mother cell changed budding direction, producing daughter cells upward until its death. This movie shows that during the aging process, the mother cell exhibits waves of silencing loss, each of which spans multiple cell divisions.

**Other Supporting Information Files**

5I Appendix (PDF)
Materials and Methods:

Strains and plasmids construction

Standard methods for the growth, maintenance and transformation of yeast and bacteria and for manipulation of DNA were used throughout. The yeast strains used in this study were generated from BY4741 (MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) strain background. Strain and plasmid information is provided in Tables S1 and S2.

To make the nuclear reporter for single cells, a IRFP::KanMX fragment was PCR amplified and integrated into the C-terminus of NHP6a at the native locus by homolog recombination. To make sir2Δ mutants, a CgHIS3(1) fragment was amplified to replace the SIR2 open reading frame by homolog recombination. Similarly, the SGF73 ORF was replaced with CgHIS3. The URA3 ORF is deleted in the BY4741 background. To add a mutated URA3 gene (ura3-1) back to its native locus, a CgURA3(1) fragment was amplified and inserted at URA3, then CgURA3 was replaced with a ura3-1 allele from the W303 strain.

To make the NTS1 silencing reporter, a Xho1-NTS1-EcoRI fragment from pDM704 (2) was ligated into the pRS306 plasmid vector. A EcoRI-P_{TDH3}-GFP-EagI fragment contains 680 bp of TDH3 promoter and GFP ORF was made by fusion PCR and then ligated into pRS306 and the plasmid pRS306 with Xho1-NTS1-EcoRI fragment to get plasmids NHB0206 and NHB0200. Plasmids NHB0213 and NHB0214 were constructed in the same way. The NTS2 fragment for plasmid NHB0214 was derived from plasmid pDM312(2). Yeast strains with rDNA silencing reporter were generated by transformation with NHB0200 cut with HindIII to integrate at NTS1, NHB0213 cut with HindIII to integrate at NTS1, NHB0214 cut with SmaI to integrate at NTS2, and NHB0206 cut with StuI to integrate at ura3-1. All transformations were performed with the lithium acetate method (3), and integration was confirmed by PCR.

Quantification of bud scar numbers

Yeast cells were inoculated into 2 ml of synthetic complete medium (SC, 2% dextrose) and cultured overnight at 30°C. 2 µl of saturated culture was diluted into 20 ml of fresh SC medium and grown at 30°C overnight. To evaluate the bud scar distribution of yeast cells, a cell culture with OD600 ~ 1.0 was sampled and stained with 5 µg/ml WGA conjugates (Alexa Fluor 647 conjugate, Thermo Fisher) for 10 min at 37°C. Cells were washed twice with PBS after incubation then examined with a Leica TCS SP8 Stimulated Emission Depletion (STED) super-resolution microscope using a Cy5.5 channel to visualize the bud scars.

Design and fabrication of the microfluidic device for yeast aging studies

In designing a microfluidic device for studying aging in budding yeast, the viability of the cells, efficiency of cell trapping and robustness of the device were our primary concerns. Multiple designs were built and tested to optimally satisfy these criteria. The robustness of the device is affected by clogging due to excess cells around the traps and at the waste port, which can interfere with mother cell lifespan and retention. Supplying media through ~20 µm tall main channels readily allowed excess cells to be washed away and prevented clogging. Therefore, a critical feature of our device is its two-layer design, making it extremely robust over the course of our experiments that takes more than 80 hours. This is a unique feature compared to recently
published devices that are all single-layer (4-6). The device was optimized for using continuous gravity-driven flow during operation, with the three-inlet design also facilitating media switching experiments. The height and width of cell traps were optimized for cell loading and retention. Cell loading efficiencies and final retainions until cell death are approximately 93% and 75% respectively. See Quantification of single-cell traces below for a consideration of cell retention. We quantified the widths of cells in the culture population immediately before loading and cells that are just loaded into the device, respectively. As shown in Fig. S1B, the distributions of cell widths are similar for the population before loading and for the loaded population, confirming that the device loading does not select for a subpopulation of cells. A potential caveat of microfluidic devices for aging studies is that the limited size of cell traps might affect the lifespan. However, given that lifespans measured using our device are comparable to those from classical microdissection studies (Fig. S1C), the effect of the trap size on lifespan, if any, should be modest.

To construct the microfluidic device, designs were first drawn in AutoCAD (Autodesk Inc.). Modeling of fluid flow in the device using COMSOL Multiphysics aided in design (COMSOL Inc.). Two chrome quartz glass masks (HTA Photomask), one for each layer of the device, were used to pattern SU-8 negative epoxy photoresist (MicroChem Corp.) onto clean silicon wafers (University Wafer Inc.). SU-8 2005 was used to build the first layer (cell trapping region) and was spun at 3250 rpm while SU-8 2015 was used to build the second layer (main channels), spun at 2000 rpm. These spin parameters yield approximately 4.3 µm and 20 µm tall features for the cell trapping and main channel layers respectively, as measured by a Dektak 150 surface profiler (Veeco Instruments Inc.). The mask for the second layer was aligned to the first layer of the wafer using an EVG620 mask aligner (EV Group Inc.). Once patterning of the SU-8 was complete, the wafer was exposed to 40 µl of trichloro(1H, 1H, 2H, 2H-perfluoro-octyl)silane (Sigma-Aldrich Co.) in a vacuum chamber for 7 minutes. After this, poly-dimethylsiloxane (PDMS, Sylgard 184, silicone elastomer kit with base and curing agent, Dow Corning Corp.) molds of the features on the wafer were made, cleaned and bonded to glass coverslips for experiments as described in (7).

Setting up a microfluidics experiment
Each microfluidic device was checked carefully before use to ensure no dust or broken features were present. Before setting up a microfluidics experiment, the device was vacuumed for 20 min. After vacuum, all of the inlets of the device were covered with 0.075% Tween 20 (Sigma-Aldrich Co.) for 5 min. The microfluidic device was placed on the stage of an inverted microscope with a 30°C incubator system. Three media ports were connected to plastic tubing, which connected to 60 ml syringes with fresh SC medium containing 0.04% Tween-20. The height of all three medium syringes is about 20 inches above the stage. The waste port of the microfluidic device was connected to plastic tubing, which was set to stage height. Yeast cells were inoculated into 2 ml of synthetic complete medium (SC, 2% dextrose) and cultured overnight at 30°C. 2 µl of saturated culture was diluted into 20 ml of fresh SC medium and grown at 30°C overnight until it reached OD600n~ 1.0. For loading, cells were diluted by 10-fold and transferred into a 60 ml syringe (Luer-Lok Tip, BD) connected to plastic tubing (TYGON, ID 0.020 IN, OD 0.060 IN, wall 0.020 IN). To load cells, the connected syringe on the middle port was replaced with a syringe filled with yeast culture. The height of the cell-loading syringe is also about 20 inches above the stage. The flow of medium in the device was
maintained by gravity and drove cells into traps. Most traps were filled with cells within 2-5 minutes, after which cell loading tubing was replaced with media supply tubing and syringe as above. Then the height of all medium tubing and the waste tubing were adjusted to make the height difference around 60 inches. Waste medium was collected in a 50 ml tube to measure flow rate, which was about 2.5 ml/day.

For experiments with media switching, left and right medium ports were connected to syringes with fresh SC medium containing 0.04% Tween-20 and the middle medium port was connected to a syringe containing medium with NA or NAM and 0.1 µg/ml Atto 655 cy5 dye (Sigma 93711). The waste port of the microfluidic device was connected to plastic tubing set to a position about 20 inches below the stage. Before cell loading, the heights of syringes need to be calibrated for media switching. Syringes connected to the left and right medium ports were moved to the same height as the microscope stage and the syringe containing NA or NAM was moved to about 33 inches above the stage. To further calibrate the positions, the junction of all three media channels was imaged under the cy5 channel and the positions of syringes were adjusted to ensure the cell trapping region was fully filled with the NA or NAM medium. The position of all syringes were marked and used for NA or NAM during the experiment. To switch from the NA or NAM medium to SC medium, the left and right syringes were moved to about 33 inches above the stage and the middle syringes was moved to the same height as the stage. The positions of all syringes were further calibrated under the cy5 channel to ensure only SC medium went into the cell trapping region and then marked the positions of all syringes for the condition of no NA or NAM in the cell culture chamber. Cell loading was as for experiments without media switching. After cell loading, all syringes were moved to previously marked positions. The flow rate is about 2.5 ml/day.

We note that Tween-20 is a non-ionic surfactant capable of coating the hydrophobic PDMS surface of microfluidic devices, thereby reducing protein interactions and cell friction on the PDMS (8, 9). We have used Tween-20 or Tween-80 at very low concentrations in all the microfluidic experiments for more than 10 years on a variety of organisms including budding yeast (10-16) without any adverse effects on cells. We have found that Tween-20 aids in preventing clogs and air bubbles from forming and therefore helps with the robustness and reproducibility of microfluidics experiments (7). We have observed no difference in the cell doubling times in media with or without Tween-20 and have obtained lifespan measurements using our microfluidics setup that are comparable to values in the literature (for WT and mutants; Fig. S1C), confirming that Tween-20 has no significant effect on lifespan or physiology.

**Time-lapse microscopy**

Time-lapse microscopy experiments were performed using a Nikon Ti-E inverted fluorescence microscope with Perfect Focus, coupled with an EMCCD camera (Andor iXon X3 DU897). The light source is a spectra X LED system. Images were taken using a CFI plan Apochromat Lambda DM 60X oil immersion objective (NA 1.40 WD 0.13MM). During experiments, the microfluidic device was taped to a customized device holder inserted onto the motorized stage (with Encoders). In all experiments, the microscope was programmed to acquire images for each fluorescence channel every 15 min for a total of 80 hours or more. The exposure and intensity setting for each channel were set as follows: Phase 50 ms, GFP 10 ms at 10% lamp intensity with an EM Gain of 50, mCherry 50 ms at 10% lamp intensity with an EM Gain of 200, and iRFP 300
ms at 15% lamp intensity with an EM Gain of 300. The EM Gain settings are within the linear range. We confirmed that this fluorescence imaging setting did not affect lifespan (Fig. S1D).

**Quantification of single-cell traces**

Fluorescence images were processed with a custom MATLAB code. Background of images from each channel was subtracted. Cell nuclei were identified by thresholding the iRFP images. Each image was evenly divided into 6 parts, each containing a single cell trap. The position of the dent in the cell trap was labeled. In each trap, the positions of all nuclei of each single cell were labeled. Mother cells are identified by comparing the positions of the dent and the positions of nuclei. Since the fluorescence reporter of rDNA silencing is evenly distributed inside the cell, nuclei of mother cells were further dilated to generate a mask to quantify the intensities of fluorescence reporters. The mean intensity value of the top 50% pixels of fluorescence reporter is used as the intensity of the rDNA silencing reporter. All single-cell time traces were normalized by the mean reporter intensity of WT cells’ 1st cell cycle and smoothed with local regression using weighted linear least squares and a 2nd degree polynomial model. The normalized and smoothed data were used for plotting of trajectories and density maps.

We also tested segmenting the whole cell using phase images and quantified the mean fluorescence intensities of the whole cell. The resulting time traces were similar to those obtained using nuclei segmentation and quantification as described above. Because nuclei segmentation and quantification is more robust than whole cell segmentation and allows us to identify and analyze more cells automatically, we used the former method for all imaging analysis.

Cell divisions of each mother cell were manually identified and counted at the time that the nuclei separated between mother and daughter cells. Cells were categorized based on their aging phenotypes, characterized by the morphologies of later daughters they produced. Mothers continually producing elongated daughters at the last few generations were categorized as “cells aging with elongated daughters”, whereas mothers continually producing round daughters at the last few generations were categorized as “cells aging with rounded daughters.” A small fraction of cells show abnormal morphologies even at the very beginning of the experiment and have a lifespan shorter than 5 generations. Those cells were excluded from analysis.

To identify the peaks and troughs of silencing loss pulses for aging cells, we first took the time derivative of the fluorescence trajectory of each single cell and then digitalized the curve by setting positive derivatives as 1 and negative derivatives as -1. The time points with the derivative transition from -1 to 1 were identified as “troughs” and the time points with the derivative transition from 1 to -1 were identified as “peaks”. This identification process is illustrated in Fig. S4B.

During aging experiments, about 25% of mother cells escaped from chambers before their final death. The major reason for a mother cell to escape from the chamber is that when the mother produces a daughter cell toward the bottom of the chamber, depending on the bud position, occasionally the daughter cell will not go through the small opening at the bottom of the chamber. Instead, the daughter will stay in the chamber and push the mother out. We observed that the times that these cells escaped were randomly distributed throughout the experiments. We further
quantified the silencing time traces of these escaped cells. During their time in the device, all of the escaped cells showed intermittent silencing loss dynamics with no obvious difference to those presented in Fig. 2. The data from escaped cells have been excluded from further analysis or presentation in this work.

**Correlation analysis of single-cell data**
Correlation coefficients (R) for all the scatter plots were calculated using the MATLAB function: ‘corrcoef.’

To determine the time-dependent relationship between silencing loss in aging mother cells and the occurrence of elongated daughters, we calculated the cross-correlation between silencing reporter fluorescence trajectories of each mother cell (from Fig. 2A) and the morphology change trajectories of its daughters.

The daughter morphology was mapped as a binary variable that is set to “1” when the daughter is elongated and “0” when the daughter is rounded. Because daughter morphology traces are digitalized, we also digitized silencing reporter fluorescence trajectories of mother cells. To this end, we first took the time derivatives of a fluorescence trajectory, and then set positive derivatives as 1 (representing the silencing loss state) and negative derivatives as -1 (representing the silenced state).

For each pair of mother silencing loss trajectory, $M_{SL}(1, 2, \ldots n)$, and daughter morphology trace $i$, $D_{M}(1, 2, \ldots n)$, with n time points, we aligned them with a time shift $\Delta \tau$: $M_{SL(i)}(1, 2, \ldots n-\Delta \tau)$ and $D_{M(i)}(1+\Delta \tau, 2+\Delta \tau, \ldots n)$ for $\Delta \tau \geq 0$, or $M_{SL(i)}(1+|\Delta \tau|, 2+|\Delta \tau|, \ldots n)$ and $D_{M(i)}(1, 2, \ldots n-|\Delta \tau|)$ for $\Delta \tau < 0$. For each $\Delta \tau$, we concatenated all mother silencing loss trajectories into one long silencing loss trajectory and all daughter morphology trajectories into one long morphology trajectory. We then calculated the correlation coefficient between the concatenated pair of mother-daughter trajectories with the shift $\Delta \tau$ using Matlab function corrcoef.

To provide a control for this cross correlation analysis, we disrupted the original pairing between mother silencing loss and daughter morphology trajectories. Instead of pairing each mother silencing loss trajectory with its own daughter morphology trajectory, we randomly paired mother silencing loss trajectories with daughter morphology trajectories, and then performed the same cross correlation calculation for the concatenated mother trajectory and “randomly reshuffled” daughter trajectory (the dash line in Fig. S2).

The correlation analysis revealed a ~140 min time lag between silencing loss and daughter morphology trajectories, indicating a ~140 min time delay between the changes at the molecular level – the occurrence of silencing loss and the changes at the phenotypic level – the production of elongated daughters during the aging process.

**Development of the phenomenological model of cell aging**
We developed a phenomenological model that relates silencing and cell aging based on our experimental data. The model only considers the aging process with elongated daughters and postulates that each mother cell can be in one of two states during aging: state 0 – the silencing
state in which it produces normal daughters, and state 1 – the silencing loss state with elongated daughters, and a cell may only die from state 1 (Fig. 4A).

The transitions between the states are purely stochastic and are characterized by transition probabilities \( p_{01} \) and \( p_{10} \) that depend on the replicative age of the cell. All the experimental data are from Fig. 2A. Due to the phenomenological nature of the model, we only used the age-dependent phenotypic change (elongated daughter) data for model fitting. To deduce the transition probabilities from the single-cell data, we computed the fraction of all the cells at the state with normal daughters (state 0) of a given generation that switch to the state with elongated daughters (state 1) at the next cell cycle \( (f_{0\rightarrow 1}) \) and the fraction of the cells at the state with elongated daughters (state 1) that return to state 0 at the next cell cycle \( (f_{1\rightarrow 0}) \), as a function of the replicative age. As this data shows, the transition rate from state 0 to state 1 gradually increases with age, while the rate of the reverse process decreases and reaches zero after ~25 generations (Fig. 4B). Using linear regression of the data, we approximate the dependence of \( p_{01} \) and \( p_{10} \) on the replicative age \( n \) by linear functions, 

\[
p_{01}(n) = 0.016 \cdot n - 0.04 \quad \text{and} \quad p_{10}(n) = 0.27 - 0.01 \cdot n.
\]

We also postulate that in the silencing loss state (state 1), a damage factor \( D \) accumulates continuously, and the probability of a cell to die is proportional to \( D \). To obtain the relationship between damage accumulation and cell death, we calculated the fraction \( f_{1\rightarrow D} \) of cells that died after \( N \) consecutive generations in state 1 to total number of cells that lived through \( N \) consecutive generations in state 1 (Fig 4C). Evidently, this fraction becomes higher with \( N \), however at large \( N>10 \) the data becomes very noisy since only small number of cells remain living. We use the data for \( N<10 \) to approximate the probability of transition to death from state 1 by a linear function of \( N \),

\[
p_{1D}(N) = 0.0297 \cdot N + 0.00025
\]

and used this expression for all \( N \). We assume that the damage \( D \) is proportional to the time the cell spent in state 1 and, once a cell transits back to the silencing state 0, \( D \) is reset to zero.

Thus, the phenomenological model depends on two transition rates \( p_{01} \) and \( p_{10} \) that are linear functions of the generation number \( n \), and the death rate \( p_{1D} \) that is a linear function of the number of consecutive generations in state 1. Time unit in simulations is one generation. Based on the experimental data we set one generation in the silencing state is 80 min, and in the silencing loss state, 110 min. We simulated this model stochastically and in each \textit{in silico} experiment we generated 79 cell state trajectories (same number as cells in experimental data in Fig. 2A). Using these data and averaging over 200 \textit{in silico} experiments, we computed the number of cells alive at replicative age \( n \) as a function of \( n \) (Fig. 4D, red line), the fraction of cells in state 1 as a function of their replicative age (Fig. 4E, red line), and the distribution of continuous generations in state 1 before death (Fig. 4F, red line). The error bars indicate standard deviations of the distribution for the corresponding results from multiple runs. All these simulation results match our experimental data very well. We also plotted the individual cell trajectories from one \textit{in silico} run ordered according to the replicative lifespan (Fig. 4G).

To simulate the effects of silencing perturbations, we increased \( p_{01} \) and decreased \( p_{10} \) by tenfold to generate an induced silencing loss, see Fig. 4H. To simulate the transient silencing loss
(Fig. 4I), we used the same high $p_{01}$ and low $p_{10}$ as in Fig. 4H at the beginning of simulation for 4 generations then switch them back to the $p_{01}$ and $p_{10}$ used in Fig. 4G. We have included the MATLAB code for simulation as Code S1.

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**Code S1.** The MATLAB code used to generate simulations in Fig. 4G-I.

**Figures S1 - 8**
References


**Fig. S1. Validation of the technologies - the microfluidic device and the silencing reporter.**

(A) Left: A representative confocal image showing bud scars stained with wheat germ agglutinin (WGA). Right: Bud scar distribution of cells at OD$_{600} \sim 1.0$. Cells with OD$_{600} \sim 1.0$ from the same culturing procedure (see SI: Setting up a microfluidics experiment) have been consistently used to load the device throughout our work.

(B) A boxplot showing the distributions of cell widths for cells from the culture population before loading (black) and cells loaded in the device (blue). Snapshot images were taken with cells from the culture population immediately before loading or with cells just loaded into the device, respectively. Cell widths for single cells were quantified using ImageJ. In the plot, the bottom and top of the box are first (the 25th percentile of the data, q1) and third quartiles (the 75th percentile of the data, q3); the band inside the box is the median; the whiskers cover the range between q1-1.5 x (q3-q1) and q3+1.5 x (q3-q1). These results confirm that the device loading does not select for a subpopulation of cells.

(C) Replicative lifespans measured using the microfluidic device. Lifespan curves have been shown for WT and $sgf73\Delta$ cells in SC medium supplemented with 2% glucose. In our device, deletion of $SGF73$ increases lifespan by 27% comparing to that of WT, consistent with the previous report (McCormick et al, *Cell Metabolism* 2015, Table S2; the $sgf73$ mutant increases lifespan by 25.8% in MATa strains).

(D) Replicative lifespans measured in the device with or without fluorescent imaging. For experiments without fluorescent imaging, only phase images (50 ms exposure) were taken every 15 mins. For experiments with fluorescent imaging, setting and acquisition frequency have been provided in SI: Time-lapse microscopy. For experiments without fluorescent imaging, only phase images (50 ms exposure) were taken every 15 mins.

(E) Validation of the reporter. Left: Representative images of WT or $sir2\Delta$ cells with the fluorescence reporter gene inserted at $URA3$, or NTS1 at the rDNA. Right: Average fluorescence intensities quantified from different strains in the left panel and normalized to the reporter level at $URA3$ in WT cells.
To determine the time-dependent relationship between silencing loss in aging mother cells and the occurrence of elongated daughters, we calculated the cross-correlation between silencing reporter fluorescence trajectories of each mother cell (from Fig. 2A) and the morphology change trajectories of its daughters.

The daughter morphology was mapped as a binary variable that is set to “1” when the daughter is elongated and “0” when the daughter is rounded. Because daughter morphology traces are digitalized, we also digitized silencing reporter fluorescence trajectories of mother cells. To this end, we first took the time derivatives of a fluorescence trajectory, and then set positive derivative as 1 (representing the silencing loss state) and negative derivative as -1 (representing the silenced state).

For each pair of mother silencing loss trajectory, MSL (1, 2, … n), and daughter morphology trace i, DM (1, 2, … n), with n time points, we aligned them with a time shift Δτ : MSL(i) (1, 2, … n-Δτ) and DM(i) (1+Δτ, 2+Δτ, … n) for Δτ ≥ 0, or MSL(i) (1+|Δτ|, 2+|Δτ|, … n) and DM(i) (1, 2, … n-|Δτ|) for Δτ < 0. For each Δτ, we concatenated all mother silencing loss trajectories into one long silencing loss trajectory and all daughter morphology trajectories into one long morphology trajectory. We then calculated the correlation coefficient between the concatenated pair of mother-daughter trajectories with the shift Δτ using Matlab function corrcoef.

To provide a control for this cross correlation analysis, we disrupted the original pairing between mother silencing loss and daughter morphology trajectories. Instead of pairing each mother silencing loss trajectory with its own daughter morphology trajectory, we randomly paired mother silencing loss trajectories with daughter morphology trajectories, and then performed the same cross correlation calculation for the concatenated mother trajectory and “randomly reshuffled” daughter trajectory (the dashed line).

The correlation analysis revealed a ~140 min time lag between silencing loss and daughter morphology trajectories, indicating a time delay between the changes at the molecular level – the occurrence of silencing loss and the changes at the phenotypic level – the production of elongated daughters during the aging process.
Fig. S3. Validation of the silencing reporter dynamics during aging. (A) Dynamic patterns of reporter fluorescence are not caused by age-associated global effects on gene expression. Left: A schematic illustrating the dual-color strain, in which mCherry under the TDH3 promoter is inserted at the NTS1 region at rDNA and GFP under the same promoter is inserted at URA3. Middle: Representative single-cell time traces of the dual-color cells throughout their lifespans. Each plot shows the time traces of NTS1-mCherry and URA3-GFP in a single cell. Vertical dashed lines represent division times of the cell, in which the distance between two adjacent dashed lines indicates the cell cycle duration. The number of dashed lines in each trace represents the cell’s lifespan. Reporter fluorescence is normalized to the baseline level. Right: Single-cell density map trajectories for rDNA-mCherry divided by URA3-GFP in the same cells (cells aging with elongated daughters). Because the expression of URA3-GFP reporter shows little change during aging, the density map trajectories of rDNA-mCherry, normalized by URA3-GFP in the same cells, show similar patterns to those observed in Fig. 2A. (B) Dynamic patterns of silencing loss are not specific to the NTS1 region of rDNA. Left: A schematic illustrating the strain in which mCherry under the TDH3 promoter is inserted at the NTS2 region at rDNA. Right: Single-cell density map trajectories for NTS2-mCherry during aging. Silencing loss at the NTS2 region shows similar patterns to those observed for the NTS1 region. (C) Representative single-cell time traces for loss of silencing, recombination, or ERC of the reporter gene. Top: Loss of silencing features a gradual fluorescence increase. Middle: Recombination features a fast 2-fold fluorescence change in one cell division and at the same time the daughter cell from that cell division stably loses the reporter gene. Bottom: ERC features a drastic exponential fluorescence increase until detection saturation.

**Fig. S3**
**Fig. S4. Additional quantification of silencing loss dynamics in single cells.**

(A) A boxplot showing the distributions of (blue) Intermittent Phase length and (red) Sustained Phase length in single cells. Single-cell data are from Fig. 2A. In the plot, the bottom and top of the box are first (the 25th percentile of the data, q1) and third quartiles (the 75th percentile of the data, q3); the band inside the box is the median; the white circle is the mean; the whiskers cover the range between q1-1.5 x (q3-q1) and q3+1.5 x (q3-q1).

(B) A schematic illustrates the identification of peaks (orange circles) and troughs (black squares) within Intermittent Phase in a representative single-cell time trace (green curve). The grey trace is the stepwise derivative trajectory of the time trace. The identification procedure are described in Supplemental Information - Quantification of single-cell traces.

(C) A scatter plot showing the relationship between the baseline shift values of troughs and their sequential positions in time traces. For each time trace, the fluorescence value of each trough is subtracted with the value of the 1st trough to calculate the silencing baseline shift during aging of this cell. The baseline shift value of each trough versus its sequential position (1st, 2nd, 3rd …) in a single-cell time trace was plotted. If the baseline level increases in the majority of cells, we would expect to see a general trend with positive baseline shifts increasing with the trough positions. As shown in the panel, we observed little relationship between baseline shift and trough positions, with a correlation coefficient of 0.08. Therefore, we conclude that sporadic waves of silencing loss show modest effects on the basal silencing level during aging.

(D) A scatter plot showing the relationship between the number of early silencing loss pulses and final lifespan at the single-cell level. Each circle represents a single cell. Correlation coefficient (R) has been calculated and shown in the plot.

(E) Sporadic transient waves of silencing loss have little contribution to cell death. Left: A scatter plot showing the relationship between the summed duration of sporadic transient silencing loss (summed t1 in Fig. 3b for each cell) and the duration of Sustained Phase preceding cell death (t2 in Fig. 3B for each cell) at the single-cell level. Single-cell data are from Fig. 2A. Each circle represents a single cell. Correlation coefficient (R) has been calculated and shown. Right: A scatter plot showing the relationship between total number of sporadic transient waves of silencing loss and the duration of Sustained Phase at the single-cell level. If transient pulses of silencing loss can contribute to cell death, additively to sustained silencing loss, cells with more transient pulses would exhibit shorter Sustained Phase of silencing loss before cell death. In fact, the summed duration (or number) of transient pulses of silencing loss show very poor correlation with the length of Sustained Phase, suggesting little contribution to cell death.
Fig. S5. Dynamics of silencing loss in \textit{sgf73}\textDelta. (A) Representative single-cell time traces of silencing loss in \textit{sgf73}\textDelta. Vertical dashed line represents each division time of the cell, in which the distance between two adjacent dashed lines indicates the cell cycle length. Reporter fluorescence is normalized to the baseline level. (B) A scatter plot showing the relationship between the number of early silencing loss pulses and final lifespan at the single-cell level for WT (black circles) and \textit{sgf73}\textDelta (red squares). Each symbol represents a single cell.
Fig. S6. A constant treatment of 0.5 mM NA shortens the lifespan. Lifespan curves have been shown for WT cells with (red solid curve) or without (black solid curve) 0.5 mM NA treatment. The NA concentration used in Fig. 5D (2.5 mM) is lower than that used in most previous studies (5 mM)(Anderson et al, Nature, 2003; Bitterman et al, JBC, 2002). Here, the dosage of NA has been further reduced by 5 times to 0.5 mM, which is the lowest dose that can be found in the literature (Mei et al, PLoS Biol, 2015). A constant treatment of this very low dose of NA also resulted in a significantly shortened lifespan.
Fig. S7. The effects of NAM and NA treatments on lifespan are mediated through Sir2. Lifespan curves have been shown for WT cells with 5 mM NAM (red solid curve) or 2.5 mM NA (blue solid curve) treatments, or in normal growth medium (black solid curve), as well as sir2Δ cells with 5 mM NAM (red dashed curve) or 2.5 mM NA (blue dashed curve) treatments, or in normal growth medium (black dashed curve).
Fig. S8. The ‘inferno’ version of all the heatmaps shown in the main figures. The ‘inferno’ color map is used as a perceptually uniform sequential colormap to aid in interpretation of the intensity information even when printed or viewed in grayscale.