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Application of a Tunable Promoter System to Introduce Alien Damage Suppressor Protein to Single-cell Yeast Aging Studies

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Hetian Su

Committee in charge:

Professor Nan Hao, Chair
Professor Jeff Hasty
Professor Lorraine Pillus

2022

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The thesis of Hetian Su is approved, and it is acceptable in quality and form for publication on microfilm and electronically

University of California San Diego

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ABSTRACT OF THE THESIS

Application of a Tunable Promoter System to Introduce Alien Damage Suppressor Protein to Single-cell Yeast Aging Studies

by

Hetian Su

Master of Science in Biology

University of California San Diego, 2022

Professor Nan Hao, Chair

Yeast as a well-studied model organism has also been a major subject of genetic modifications. Various engineering has been performed on yeast gene circuits to not only rewire the gene transcriptional regulation network, but also gain control over certain gene expression via extracellular signals. This thesis demonstrates the application of exogenous signal-induced promoters to study the aging process of yeast cells. By performing single-cell aging analysis

based on microfluidics and fluorescent microcopy with yeast cells that contain tunable promoter-controlled tardigrade unique damage suppressor (Dsup), I examined how the inducible promoter system could be used to study the dynamics and effects of an alien damage suppressor in the yeast aging process. The experiments showed promising capacities of an inducible promoter system to control gene expression of the alien gene in yeast cells. Dsup protein affected the aging phenotypes of yeast cells and its effects were independent of the protein's dynamics.

Introduction

Tunable promoter systems can be used to switch on and off the expression of genes and control the level of gene expressions. Unlike direct gene knockout or overexpression that could potentially impair cell functions in the first place, tunable promoters enable more precise temporal control, so that experimental perturbations of gene expression can be induced only when needed and at certain stages of a model organism's life. Exogenously controlled promoters, whose activities are correlated with concentration of chemicals or intensity of other signals like light, have been developed in mammalian models (1, 2, 3) and constantly optimized (4, 5). The application of tunable promoter systems in biology can be considered two-fold. One is harnessing its temporal control to obtain more detailed knowledge about the function of a gene during a given stage of a cell's life or under certain conditions, the other is utilizing it to engineer genetic circuits, such as switches or oscillators, that can change a cell's phenotype or turn cells into therapeutics (6).

In the research of aging, the application of the first aspect of tunable promoters has had a long history (7). Up to now, from single-cell yeast models to multicellular mammalian models, the more traditional engineered bacterial inducible promoters and the more recent CRSPR-derived methods have led to mechanistic insights of cellular aging (8, 9,10,11,12,13). However, despite ample knowledge that has been accumulated over years of research, there is still little application of the second aspect of tunable promoter systems that turns knowledge into engineering approaches that manipulate a model's aging phenotype. In this study, tunable promoters are introduced into yeast cells to test the possibility of using it to control an alien protective factor to shape the aging process.

Tardigrade unique damage suppressor (Dsup) protein was identified to contribute to tardigrade's high resistance to DNA damage by oxidation and radiation (15). It was also demonstrated to be functional in and provide protection against radiation for human cells in vitro (16). Since reactive oxygen species (ROS) are important aging-related factors and have both positive and negative roles depending on concentration (17,18,19), perhaps Dsup could help contain their detrimental roles when they accumulate. Additionally, previous study found that 2 mutually inhibiting genes, Sir2 and Hap4, underlie yeast cells' aging trajectories to either age with low Sir2 and nucleolar decline (Mode 1) or low Hap4 and mitochondrial decline (Mode 2) (14). When yeast cells age, the balance of the mutual inhibition will break, and one of the two genes will overwhelmingly repress the expression of the other. The result is an overt accumulation of aging-related factor/damage otherwise suppressed by the silenced gene. Mitochondria is a center of ROS production (18) and its functional decline in Mode 2 cells will lead to increasing ROS production (23, 24), which then leads to oxidative stress and cytosolic chromatin formation by the ROS-JNK retrograde signaling pathway from mitochondria to the nucleus (31). Dsup, which protects against ROS, might mitigate both effects. On the other hand, in Mode 1 cells the expression of the chromatin stability keeping gene Sir2 is suppressed by repressors such as upstream activating factor (UAF) complex of rRNA gene (32). Dsup may or may not help block these factors. However, due to its unique and alien nature, yeast cells might lack the mechanisms to efficiently regulate Dsup level and related pathways that are also unique to tardigrades. A direct expression of a Dsup gene in yeast cells would probably be not effective. Therefore, a tunable promoter system is used to gain better control over the dynamics of Dsup gene expression. I hypothesize that tunable promoters can allow reliable observations of Dsup's

effects on yeast cellular aging, via maintaining a fine-tuned level of Dsup gene expression or inducing pulses of expressions.

Materials & Methods

Yeast strains construction

All yeast strains used in the experiments were derived from the BY4741 strain. Standard protocols for growth, maintenance, and transformation of yeast and E. coli cells were used. The strain referred as the WT strain contained a nuclear-anchored iRFP reporter and ac GFP reporter in the rDNA region. An engineered doxycycline-inducible TetO-rtTA3 system (20,21,22) was fused with an N-terminus tagged Dsup, and this construct was introduced to the WT yeast strain by homologous recombination. The Dsup gene was purely synthesized based on reported gene sequence and with codon optimization for use in yeast cells.

Single-cell aging experiment using microfluidics devices

Microfluidics devices were designed and prepared as previously described (14, 25). Each microfluidics device contained 4 individual channels in which individual cells could be loaded and trapped into separate traps. Prepared devices were vacuumed for 20mins and placed on an inverted microscopy in a 30°C chamber. Inlets and outlets of devices were sealed with input medium before loading the cells. The input media contained no cells and were connected to the inlets of the devices via plastic tubes and needles. The outlets of the devices were connected to needles and plastics tubes. The outlet tubes were first fixed to the microscope table and then slowly moved into bleach-containing tubed held on the ground. All tubes used

in the experiments were ensured to be filled with medium or ddH2O and containing no bubbles before connection to the devices. Cells were grown to saturation in 1.5ml low-fluorescent SD medium and to OD 0.7 in 10ml of the same type of medium, and then loaded into the channels of the devices. During cell loading, inlet tubes were disconnected from the devices and sealed with knotted tubes, so that cell containing medium could be connected to the inlets, also via plastic tubes and needles. In all experiments conducted in this study, 2 device channels were used to perform one experimental condition. In the experiments where pulses of inducer chemicals were given to the cells, the inlets of the devices were connected to electronic valves that could switch between 2 different input mediums – SD medium with no chemical and SD medium with the corresponding concentrations of chemicals. A MATLAB program was used to control the switching times of each valve.

Time-lapse microscopy and image analysis

The experiments were performed with 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). Phase and fluorescence images of cells were taken every 15mins for 90 hours. The exposure and intensity of light sources for each channel were set as the following: Phase 50ms, mCherry (mRuby) 50ms 10%, iRFP 300ms 15%. The start and death time point, the time points of buddings, and the death mode for the mother cell in each trap was recorded by hand. Then replicative lifespan and cell cycle analysis were performed using a custom python script. All images were preprocessed using

ImageJ. Image alignment, cell tracking, and fluorescence data analysis were then performed with a set of custom MATLAB scripts. The death mode of each cell was determined primarily based on the aging phenotype and assisted by nuclear iRFP intensity. Mother cells that produced elongated daughter cells and had high iRFP intensity during the last 4 generations were categorized as Mode 1. Mother cells that produced small, round daughter cells and had low iRFP intensity during the last 4 generations were categorized as Mode 2. In yeast strains with Dsup, some cells that produced small, round daughters but had high iRFP intensity were also categorized as Mode 2.

Results

Doxycycline-regulated Dsup show near-binary induction but slow degradation in yeast cells.

A yeast strain was constructed with Dsup controlled by a doxycycline-inducible promoter system and mRuby tagged to its N-terminus. The yeast strain was grown in media with different concentrations of doxycycline to identify optimal concentrations that did not affect cell growth rate. It was found that at high doxycycline concentrations, Dsup would significantly impair cell growth, and 500nM was the concentration with acceptable growth rate (Figure 1. A). Therefore, a population-level Dsup induction time trace was performed with 500nM doxycycline (Figure 1. B). It can be seen that despite a high variance among the cells, during the 8 hours of induction, the median Dsup level increased significantly in an exponential manner and seemed able to continue increasing. At the same time, the control group made of the same strain under 0nM doxycycline showed near zero Dsup expression over time,

indicating a minimal leaking in the promoter system. Overall, the dox-inducible system could be used to control the expression of Dsup in yeast cells. However, the induction seemed to be so strong that a medium dox concentration never saturated the system, but a high concentration would produce overwhelming Dsup that negatively impacted cell growth. This turned out to be a problem in studying the yeast aging process and could be resolved by the dynamic nature of tunable promoter systems.

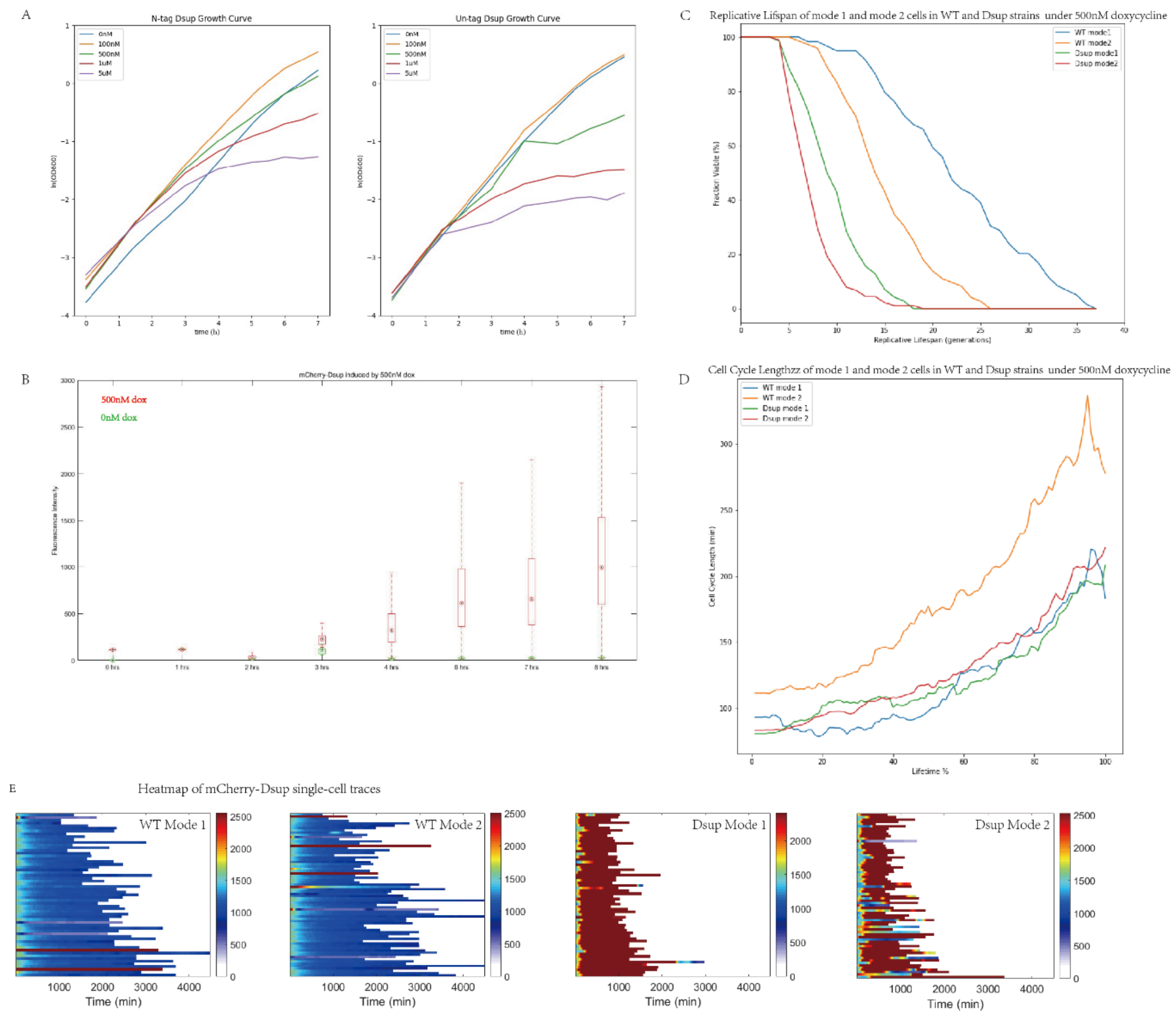

Figure 1: characterization of yeast cells' growth and aging phenotype with Dsup induced by 500nM doxycycline. (A) Dsup constructs with mCherry tagged to the N-terminus or the C-terminus were tested for growth rates by OD time trace. N-terminus tagged constructs show better growth and 500nM or lower doxycycline concentrations show minimal decrease in growth rate. (B) Dsup fluorescence time trace under 500nM (red) vs 0nM (green) doxycycline induction. (C) Replicative lifespan of WT strain and Dsup strain under 500nM (continued) doxycycline, modes separated. (D) Average cell cycle lengths time trace during aging of WT and Dsup strains under 500nM doxycycline, modes separated. (E) Heatmaps of Dsup fluorescence levels, a trace of an individual cell in each row, during aging of WT and Dsup strains, modes separated.

Inducible promoter-driven Dsup had minimal effects on yeast replicative lifespan but reduced cell cycle lengths in mode 2 cells.

The first single-cell aging experiment was performed with WT yeast strains and the

dox-regulated Dsup strain, both captured within a microfluidics device and kept in medium with 500nM doxycycline. The Dsup strain showed significantly reduced replicative lifespan as compared to the WT strain (Figure 1. C). Dsup production quickly reached very high levels in nearly all cells and likely impaired cellular functions (Figure 1. E). At the same time, cell cycle lengths of mode 2 cells in the Dsup strain seemed to be reduced and closely resembled those of mode 1 cells (Figure 1. D). However, it was possible that this effect on cell cycle was due to quick death of the Dsup strain. Either because yeast cells lacked the machinery to efficiently degrade Dsup proteins or because Dsup production was overwhelming, the degradation rate and dilution rate seemed to never match the production rate of Dsup under 500nM doxycycline.

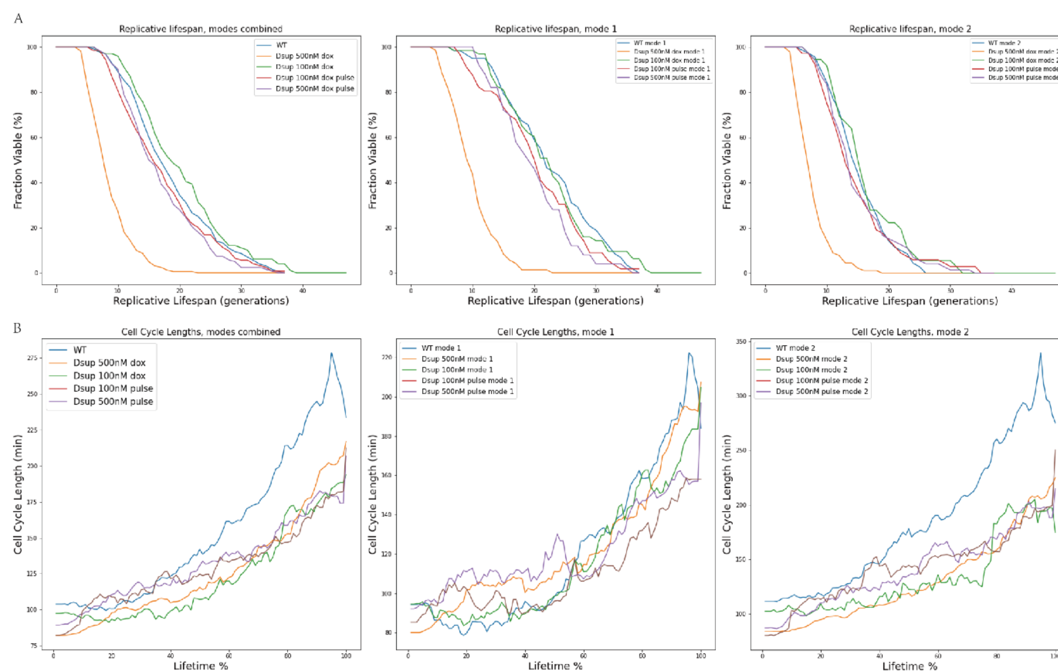

Figure 2: Multiple patterns of doxycycline input had minimal effect on the replicative lifespan but significantly reduced cell cycle lengths in mode 2 cells. (A) The replicative lifespans of WT, Dsup with 500nM dox, Dsup with 100nM dox, Dsup with pulses of 100nM dox, Dsup with one pulse of 500nM dox. Modes taken together and viewed separately. (B) The cell cycle lengths time traces of cells under these conditions. Modes taken together and viewed separately.

To better test the effects of Dsup and verify the validity of the observed change in cell cycle lengths, attempts were made to control the expression level of Dsup low enough by using

consistent 100nM doxycycline, pulses of 100nM doxycycline (6hr on - 10hr off x 4), and one pulse of 500nM doxycycline (4hr on x 1). It can be seen that these manipulations did not significantly change yeast cells' replicative lifespans regardless of modes, suggesting that a controlled low level of Dsup has neither positive nor negative effects on the lifespan (Figure 2.A). However, in all cases, the cell cycle time traces of mode 2 cells were reduced (Figure 2.B). The result was valid given that the replicative lifespans were unperturbed. It seemed that Dsup protein's effect on yeast cell cycle is decoupled from that on lifespan.

In the Dsup strain iRFP was tagged to Nhp1α as a reporter of heme concentration (14). Higher iRFP levels suggested presence of heme molecules and healthy mitochondria, which were characteristic of mode 1 cells, and the opposite was characteristic of mode 2 cells. Judging from the single-cell iRFP traces, mode 2 cells in all Dsup samples showed higher iRFP than mode 2 cells of the WT strain, and iRFP intensities in many Dsup+ mode 2 cells became closer to those of mode 1 cells in every sample (Figure 3.A). Dsup seemed to push the iRFP pattern of mode 2 cells to those of mode 1 cells, but ultimately the mode should not have changed in these cases since the replicative lifespans of mode 2 cells were consistent across control and experiment groups. At the same time, there seemed to be a cutoff at 1000min in WT mode 1 cells, after which most of them showed high iRFP levels (Figure 3.A). However, in all Dsup strains the cutoff seemed to be postponed to around 1500min (Figure 3.A), suggesting that Dsup protein might have impaired mitochondria in mode 1 cells.

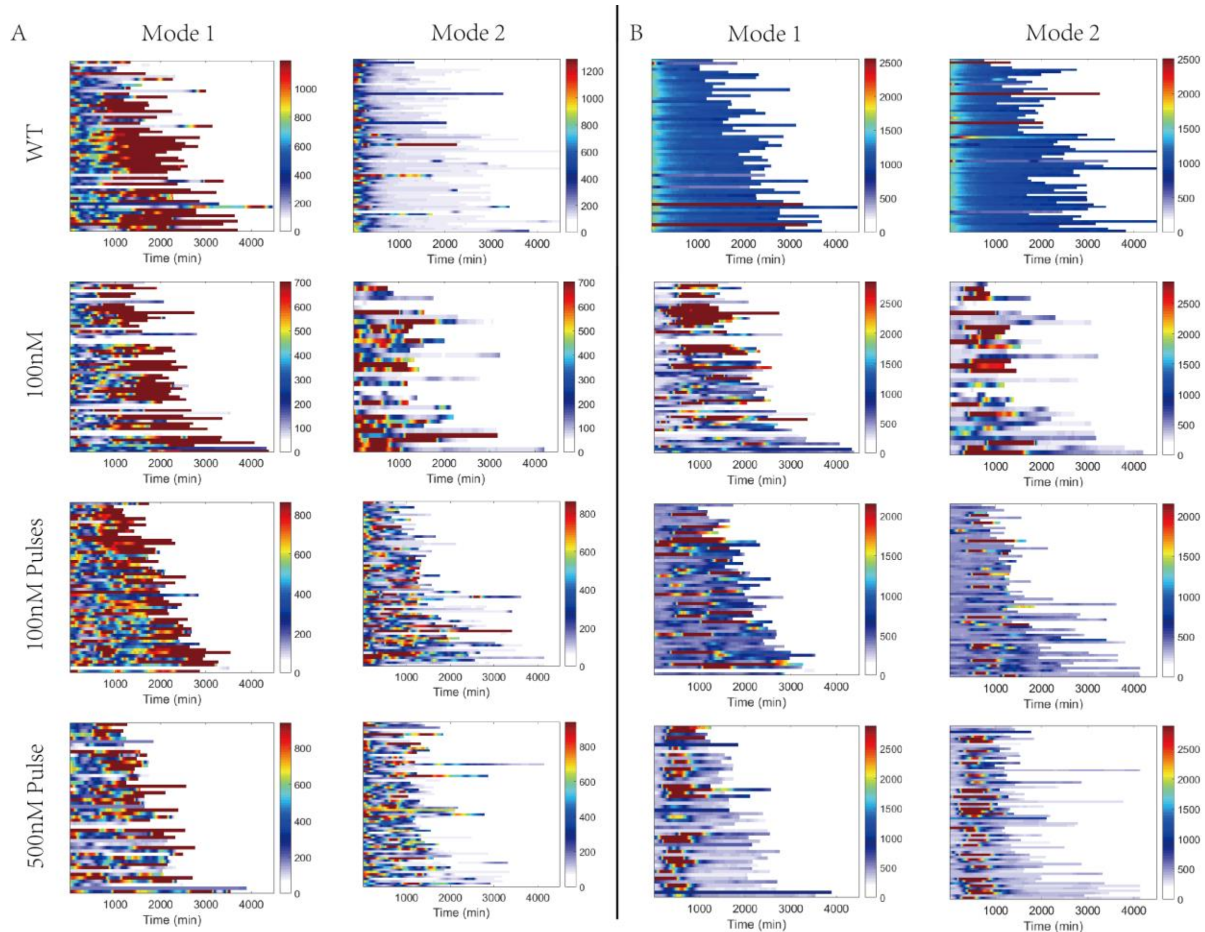

Figure 3: Single cell traces of nuclear iRFP (A) and mRuby-Dsup (B) under WT (continued) and the conditions that did not impair lifespan. Each row in the heatmaps is a time trace of the fluorescence in a single cell. All plots are death modes viewed separately.

Cell Age Affects Induction of the dox-inducible promoter

From the single-cell mRuby traces, it can be seen that the Dsup level patterns were identical between mode 1 cells and mode 2 cells in samples treated with 500nM dox pulse (Figure 3.B), which was expected as there was only one pulse at the beginning. On the other hand, in relatively long-lived cells treated with consistent and multi-pulses of 100nM dox, the patterns of Dsup levels in mode 2 cells were different from those in mode 1 cells (Figure 3.B). In both cases, Dsup production in many long-lived mode 2 cells was silenced during later phases of time. However, under the same induction conditions, many mode 1 cells had

consistently active production of Dsup. This indicated that the induction of the promoter system could be in an age and mode-dependent manner.

Discussion

By inducing Dsup gene expression with multiple patterns of doxycycline input and measuring the aging phenotypes of yeast cells, this study found that a strong induction impaired cell growth and lifespan, while weaker inductions reduced the cell cycle lengths of mode 2 cells during aging regardless of the input pattern. Also, weak Dsup expression seemed to improve the health of mitochondria in mode 2 cells but impair that in mode 1 cells. Both cell cycle and nuclear iRFP reporter seemed to suggest that Dsup protein drew mode 1 and mode 2 cells closer, but such effects were decoupled from replicative lifespans, which were unperturbed. Since cell cycle arrests when cells remove damages, the lengths can be considered as an indicator of stress accumulation (27, 28, 29, 30), Dsup protein might have significantly relieved stress during aging in mode 2 cells, or perhaps it triggered bypass of certain damage checking mechanism. The impacts of increased ROS production by mitochondrial decline in mode 2 cells (23, 24) might have been mitigated by Dsup, since protection against oxidation is a known function of it (15). If the damages were indeed better controlled, why was the replicative lifespan eventually not prolonged? Perhaps there are always non-damage related factors that increase mortality rate during aging, or the damages that can be limited by Dsup are not the key, or primary, aging-related damage factors. After all, these data support the idea that tunable promoter systems are highly helpful for introducing an alien factor into yeast cells.

The data collected in this study regarding Dsup protein's effects on yeast cells are truly

preliminary. To better answer the above questions, more knowledge about Dsup function and more advanced construction of inducible promoter systems are likely needed. It was not until recently that the structure of Dsup protein was computationally solved (26), and perhaps the knowledge about Dsup's function and related machinery in tardigrades are not complete in the first place. So far, we only know its role of protecting nucleosomes from radiation and oxidative particles (15,16). It is possible the apparent improvement of mode 2 cells' health was the result of Dsup protecting against ROS or influencing the expression of Hap4 gene. After all, more knowledge about the functions of Dsup would be helpful for further understanding the observations in this study.

Besides, in tardigrades Dsup gene expression is believed to be triggered only in extreme conditions, such was when tardigrades dehydrate (15). However, in this study Dsup gene expression was induced constantly or by pulses. Judging from the mRuby traces, induction by pulses of 100nM dox triggered wave-like Dsup production only in few cells. Therefore, the function of Dsup is likely not being used optimally. A new promoter construction that let Dsup expression be triggered by stress would be needed. Also, since Dsup showed slow degradation in yeast cells, limiting both high induction and formation of pulses of production, a design for targeted degradation would be needed. Such new constructions might help reveal effects of Dsup on single-cell aging otherwise hidden by the current design.

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