Hsp90 and the Evolution of Novel Cellular Differentiation

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Introduction

Cellular differentiation is a prerequisite for complex multicellular life, yet little is known about how it evolves *de novo*. Several mechanisms may explain how early multicellular organisms evolved to perform cell-specific roles. In this work, I explore the potential role of cellular aging in driving age-dependent differences that evolve to become co-opted for a novel role in cellular differentiation. Specifically, I am examining how, in the snowflake yeast model system of nascent multicellularity, the Hsp90 class chaperone proteins have evolved to act in a novel age-dependent manner, thus driving the emergence of adaptive differences in protein activity among cells.

Snowflake yeast initially arose through experimental evolution [1], but more recently we have been starting out our experiments simply by deleting the *ACE2* gene, which makes unicellular yeast grow as simple multicellular clusters. The loss of *ACE2* prevents daughter cells from separating from mother cells after budding [2]. The two strains used in my work are the ancestor strain of snowflake yeast (*ACE2* deletion) and a macroscopic strain experimentally evolved after 600 days of selection for larger size (this corresponds to about 3,000 generations of evolution). This evolved strain is ~19,000 times larger than the ancestor and has evolved novel biophysical mechanisms of group maintenance that give it

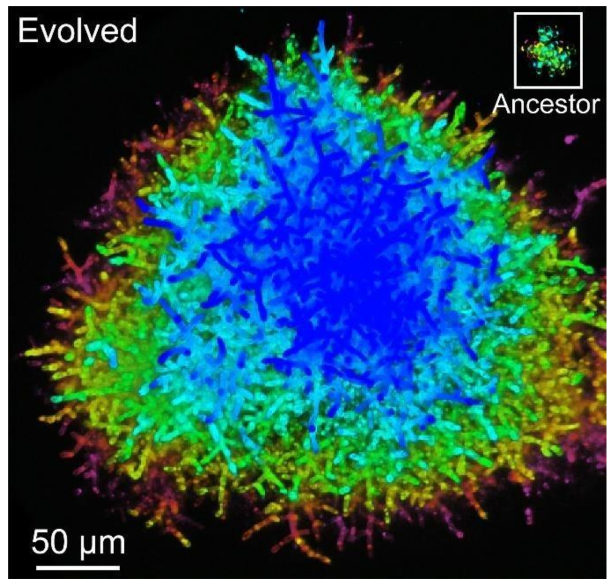

Figure 1 Comparative sizes between the ancestor snowflake yeast and the evolved macroscopic yeast strains.

new mechanical traits, one of which may be the formation of ‘side buds’, which facilitate the entanglement of cellular branches and make the clusters 10,000x biophysically tougher.

Hsp90 is a highly conserved class of chaperone proteins that is involved in diverse molecular processes. Due to aging, its expression may differ among cells, resulting in differential phenotypes in older versus younger cells. In this case, we hypothesize that Hsp90 is involved in the origin of the evolved side-budding trait, which is primarily a feature of older cells.

To examine the potential of Hsp90 in being co-opted for cellular differentiation, I identified Hsp90 activity across cells of all ages in the ancestor and evolved macroscopic yeast strains. Additionally, I observed differences both in Hsp90 activity amongst cells of all ages per strain and between the strains. To quantify this, I developed an image analysis pipeline that incorporates machine learning to automatically segment yeast cells and to measure cell traits including Hsp90 activity, aspect ratio, and angle of budding. This approach determines if evolved cells exhibit age-dependent Hsp90 activity more frequently than ancestral cells

This experimental evolution approach enables real-time observation of cells evolving more complex forms, giving us an unprecedented ability to observe the fine details of this transition. Furthermore, insights into the evolution of cellular differentiation are particularly important considering how ubiquitous this process is in most multicellular organisms. Finally, given the multiple origins of multicellularity, such knowledge is valuable in charting the emergence of life forms on earth.

Literature Review

The evolution of multicellularity is a foundational step in the evolution of complex life, having occurred 8-11 times in fungi alone [5]. A key factor in evolving complex multicellularity is the evolution of cellular differentiation, allowing cells within an organism to perform different tasks (i.e., division of labor). However, we know little about how cellular differentiation first arose in simple, undifferentiated clusters of cells. We aim to explore one possible mechanism for the evolution of cellular differentiation: the co-opting of age-dependent differences in gene expression (here using the chaperone protein Hsp90) between individual cells.

As the science of genetics and evolution advances, we are discovering that drastic genomic and regulatory novelties are not always necessary for major transitions in evolutionary history [6, 7]. In fact, much of the molecular machinery integral in the transition from unicellular life to multicellular life was in fact already present in unicellular ancestors [8].

Chaperone proteins, which assist in folding other proteins, are an essential part of this machinery. The heat shock protein family Hsp90 performs different roles in unicellular and multicellular organisms, with its multicellular analogs contributing to neurodegenerative diseases and cancers [9]. Thus, it is worth investigating how Hsp90 could have been coopted to serve different purposes.

Hsp90 may be essential to understanding the evolution of multicellularity because previous research has indicated a strong association between Hsp90 and the stress response. It may serve as a 'stress sensor' in yeast, allowing for phenotypic diversity when environmental conditions change [10, 11]. The function of Hsp90 is then capable of linking environmental changes with evolution [11].

Currently, a large amount of research has been done on aging in *S. cerevisiae*. Consequently, our knowledge on characteristics of aging in this species is robust. One of the reasons why yeasts are ideal for aging studies is because individual yeast cells have a finite lifespan, and their ages can easily be determined. Two models of aging budding yeast are in use: chronological aging and replicative aging. Chronological aging sets the age zero of the cell at the time point that it finishes its birthing division. Using this method, its age is essentially the amount of time it has been alive since the first division. Replicative aging, unlike chronological aging, is far easier to determine for large quantities of cells. Replicative age is determined simply by the number of bud scars a single yeast cell has, which is the same as the number of divisions the cell has gone through [12].

Much of the literature on budding yeast aging has centered on the asymmetric replicative aging. Yeast replication is referred to as 'asymmetric' relative to age because yeast cell division is asymmetrical, resulting in unequal segregation of age-related factors between the mother and daughter. Thus, a mother cell will bud a daughter cell whose age begins at zero, while the mother cell's age will not revert to zero, despite the daughter budding off from the mother cell. Age-dependent phenotypes are thus observed between mother and daughter cells. Henderson and Gottschling (2008) claim that other mother cells will produce daughter cells with relatively shorter lifespans compared to those of younger mother cells because of senescence factors that affect the older mother cells [13]. Higuchi-Sanabria *et al*. (2014) explored similar themes of asymmetrical aging, but unlike Henderson and Gottschling, they do not assert that daughter cells of older mother cells exhibit shorter lifespan. Interestingly, they do summarize some of the cellular consequences of asymmetric aging. They expand on molecular differences between a mother cell and daughter cell that cause asymmetric aging.

One of these differences is the asymmetric apportioning of aging determinants, like damaged aggregates of proteins, between the mother and daughter cells. Further, there is asymmetrical inheritance of mitochondria, vacuole, and the endoplasmic reticulum [14].

These differences in cells of different ages may have implications for cellular differentiation.

Interestingly, differentiation in cells based on age has already been observed in yeast populations [15], which exists as subpopulations with different traits that possibly affect longevity. Post-division cells within colonies also have higher fitness, even when further away from the agar nutrient source. However, this study only classified 'age' into two categories: currently budding off from a mother cell and nondividing (elder). There remains much to learn about how differentiation correlates with aging. In my research, I aim to bridge this gap by observing differences in Hsp90 expression levels, which affect many aspects of the cell, across cells of all replicative ages.

To begin studying the origins of complex multicellularity and cellular differentiation, a simple multicellular study organism is required. In their 2012 paper, Ratcliff *et al.* unveiled "snowflake yeast"—a model of simple multicellularity [1, 2]. The lack of *ACE2* allows daughter cells to remain attached to mother cells after budding, thus creating a network of attached cells. As a result, the *ACE2* mutant yeast exist in a network of simple multicellularity. Additionally, due to the fast life cycles of *S. cerevisiae*, snowflake yeast undergoes five generations per day making it an ideal organism for laboratory evolution experiments.

Next, if we want to correlate aging with differentiation, we must be able to observe Hsp90 activity. As a class of chaperone proteins, Hsp90 is associated with many transcription factors and other proteins that it folds. Because of this, the activity of Hsp90 can be quantified by observing its downstream transcription factors in addition to tagging Hsp90 proteins themselves. Hsf1 is one such transcription factor. It is both regulated by and regulates Hsp90 activity, making it a good candidate for tracking Hsp90 activity in our research [8].

Additional proteins that can be used to approximate Hsp90 activity are found in the network of transcription factors that affect chromatin accessibility. These include Abf1 and Cbf1 [16]. Our team is currently using these transcription factors as a proxy for Hsp90 levels in our image analysis.

Considering the scope and content of previous research on *S. cerevisiae* and cellular aging, it is interesting to further consider how proteins, like the Hsp90 class of chaperones, can mediate age-dependent differences. By studying how the expression of downstream Hsp90 transcription factors differ between cells of different age groups, a correlation in expression may be determined.

Materials and Methods

We tagged *HSC82* (an Hsp90 class chaperone) and several transcription factors regulated by Hsp90 using green fluorescent protein (GFP). We tagged these proteins in snowflake yeast of several strains, all various days evolved under settling selection, which selects for large size in multicellular clusters, from the ancestor. One difficulty of studying Hsp90 activity is that not all of its proteins can be directly measured. To get around this, we use protein stains that target the transcription factors regulated by Hsp90 chaperones, such as Cbf1 and Abf1. The idea is that by tracking the activity of several transcription factors regulated by Hsp90, we can get a good idea of how Hsp90 expression as a whole varies with age. One Hsp90 protein, *HSC82*, can be directly detected, and was used in our first round of analysis. Essentially, *HSC82* and Abf1 activity were tagged in order to approximate Hsp90 activity.

To gain insight as to how Hsp90 and cellular aging can facilitate cellular differentiation, I began with the images of snowflake yeast clusters and measured several factors, including Hsp90 activity, cell age, area, aspect ratio, the angle of budding, and the location of budding sites using ImageJ software. The values for each of these traits were then plotted against each other to ascertain any trends, with a special focus on Hsp90 activity and age.

We performed the ImageJ measurement portion using a pipeline coded in the ImageJ macro language. This pipeline performs several operations, such as background subtraction, in addition to measuring the attributes listed above. All the attributes with the exception of age, which I initially input measurements for manually, are easily measured by the software. A large portion of my work this semester focuses on finding a way to automate the cell age measurement process. When measured manually, the age of an individual yeast cell is determined by counting its bud scars (excluding the "birth scar" obtained when the cell originally budded from its mother cell). However, since each image usually has 200–300 cells, and I would have 4–8 images per batch, manually measuring age became very tedious. Therefore, we decided to work on developing an algorithm for automated measurement. Our first method was approximating age via measuring the Calcofluor fluorescence of the bud scars in each cell, with higher florescent values meaning older age. Recently, we began to take the age approximation into a machine-learning direction. We are currently compiling a training set of individual cell images with their manually measured ages in order to train a machine learning algorithm to recognize and count the bud scars in a given cell.

Our output from the ImageJ pipeline, with all the various measurements, was then analyzed using Python in Jupyter Notebook. Each measured variable was plotted in appropriate graph types against every other variable, with special attention paid to changes based on age and Hsp90 activity.

Results

The results of our image analysis indicate several trends that varied with age and with evolved strain. The ancestor strain had more Hsp90 (specifically *HSC82*) than the evolved strains. The ancestor additionally had little variance of Abf1 with age, and the evolved 400 and 600 days had lower *HSC82* as age increased (Fig. 1).

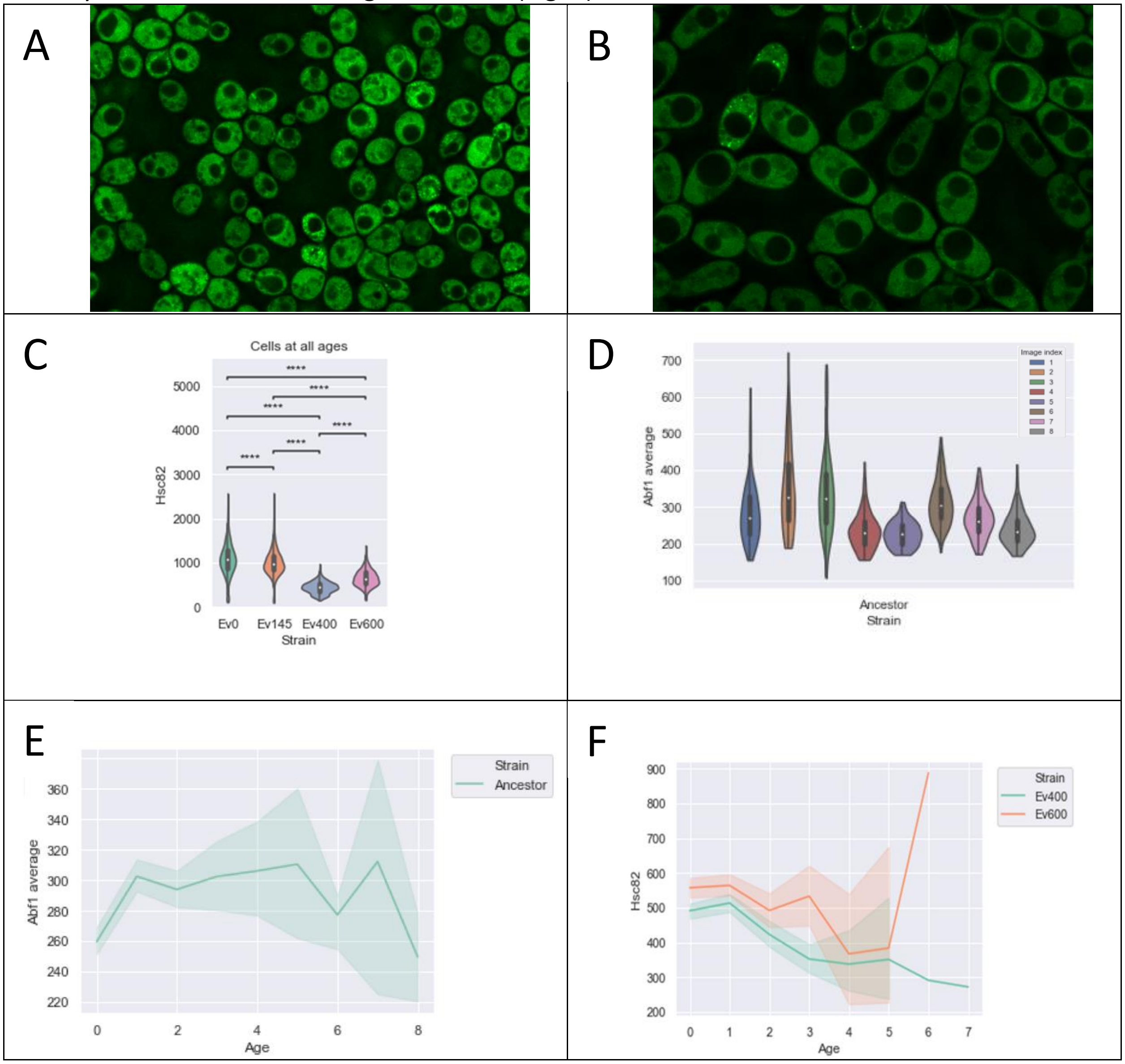

Fig. 1. Hsp90 image analysis results. *(A)* The ancestor strain with GFP tagged *HSC82*. *(B)* The 600 days evolved strain with GFP tagged *HSC82*. Images were edited without affecting the relative intensity of GFP; the lower intensity in the 600 days compared to the ancestor strain is likely due to lower *HSC82* activity. *(C)* Evolved strains at four different time points were analyzed. The ancestor (Ev0) was GFP tagged for *HSC82* along with the 145, 400, and 600 days evolved strains. Up until 400 days, more evolved strains have lower *HSC82*, but at 600 days the activity rises again. Differences are statistically significant. *(D)* A separate image set was analyzed with GFP tagged Abf1, a transcription factor regulated by Hsp90. Eight images were analyzed. This violin plot shows the variation of Abf1 levels across images, despite them being taken in one session. *(E)* Abf1 (type of Hsp90) GFP average pixel values remain steady across cells of all ages in the ancestor strain. *(F)* In the evolved 600 strain, *HSC82* (and thus Hsp90) average pixel values decrease in images of cells as the age of the cells increase. Ages 6 and 7 have very few cells due to the relative scarcity of old cells, so average *HSC82* activity is not statistically relevant.

Discussion

From our results we have tentative evidence that Hsp90 activity decreases with age in more evolved cells. However, one shortcoming of this work was that we only have results for approximating Hsp90 in the ancestor strain by tracking Abf1, and Hsp90 in the evolved 400 and 600 days by tracking *HSC82*. Thus, though Abf1 is a transcription factor regulated by Hsp90 proteins and though *HSC82* is a paralog of Hsp90 protein, the lack of variance in Abf1 across ages in the ancestor and the decrease of *HSC82* as age increases in the evolved 400 and 600 days cannot be definitive evidence that Hsp90 decreases with age in more evolved cells. This is because *HSC82* and Abf1 activity may not be accurate on their own in approximating Hsp90 levels. Therefore, it is necessary to further examine the activity of each protein in both the ancestor and evolved strains more exhaustively. Nevertheless, the result that Hsc82 activity decreased with age in evolved strains supports our hypothesis: age-related expression of *HSC82* can be a precursor to cellular differentiation in snowflake yeast.

We were able to analyze *HSC82* activity in cells of the ancestor, 145 days evolved, 400 days evolved, and 600 days evolved. *HSC82* activity was lower in the 145 days than in the ancestor, and lower in the 400 days than in the 145 days. However, the 600 days evolved strain had higher *HSC82* activity than the 400 days evolved strain, and this trend was verified in two other rounds of analysis with new images of new batches of cells. We expected Hsp90 (and thus *HSC82*) activity to decrease in the more evolved strains. While both 400 days and 600 days evolved had lower *HSC82* activity than the ancestor, it is unexpected that the 600 days evolved has more *HSC82* activity than the 400 days evolved.

Future work will further unearth the role of age-related Hsp90 expression in mediating cellular differentiation. A more robust set of data with *HSC82*, Cbf1, Abf1, and additional Hsp90-mediated transcription factors will be necessary.

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