Experimental Evolution of Multicellular Complexity in *Saccharomyces cerevisiae*

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The origin and evolution of multicellularity was directly investigated using experimental evolution. Using settling selection, multicellularity evolved quickly and repeatedly from a common unicellular ancestor, Baker's yeast. The transition occurred by persistent adhesion of daughter cells following cell replication. The resulting multicellular individuals had a morphology reminiscent of snowflakes, with many characteristics of extant multicellular species, including cell–cell attachment, a single-cell bottleneck, and juvenile and adult life history stages. Cellular division of labor by apoptosis evolved in large snowflake clusters, ameliorating the effects of a trade-off between snowflake settling and growth rate. Continued settling selection led to additional adaptation, such as a more hydrodynamic cluster shape. The majority of the developmental changes that evolved after the transition to multicellularity were contingent on this transition and even on the specific mode of cluster formation. The origin and subsequent evolution of multicellular complexity in snowflake yeast can be directly attributed to natural selection.

Keywords: multicellularity, yeast, adaptation, macroevolution, evo-devo

Multicellular organisms are incredibly diverse, varying greatly in their morphology, life histories, and developmental modes. They can be found in virtually all aquatic and terrestrial habitats, including environments with extreme temperatures and pressures. Multicellular organisms play such an integral role in Earth's ecology that it can be hard to imagine what life would be like in their absence. Let us perform a simple thought experiment: Imagine a biome bustling with life, such as a tropical forest. Now remove the multicellular land plants—no trees, shrubs, herbs, or mosses. From the air where the trees used to stand would fall a rain of insects (there are over 450,000 species of beetles, alone, on Earth; Stork 1988) punctuated by the heavy thud of a few vertebrates. Get rid of these animals. What is left? Multicellular fungi, such as mushrooms and lichens. Remove these too. This empty landscape of bare soil dotted by microscopic photosynthetic bacteria and algae is representative of terrestrial habitats in the absence of multicellular organisms.

The evolution of multicellularity (box 1) radically changed Earth's ecology, because multicellular organization opened up previously inaccessible avenues for adaptation, which resulted in the exploitation (and creation) of many new niches (Butterfield 2007). Using cells as modular building blocks, multicellular organisms were capable of evolving to be both larger and more complex than their single-cell ancestors were (Bonner 2004). Crucial for the evolution of complexity is division of labor among cells (Buss 1987, Maynard Smith and Szathmáry 1995, McShea 2002, Michod 2007). Cells that split tasks can realize increases in efficiency (similar to the efficiency gained by task specialization during the Industrial Revolution; Smith 1776), and differentiation reduces the impact of functional trade-offs that may limit the total number of tasks that a single cell can perform (Rodríguez-Caso 2013).

In contrast, unicellular organisms are strongly limited in their modes of division of labor. The subcellular division of labor through organelles (e.g., mitochondria or chloroplasts) constrains organismal complexity and size through issues of functional scaling, transport, and organellar regulatory control (West et al. 2002). Although some unicellular species show a very simple division of labor among cells (e.g., differentiation by rhizobia into nitrogen-fixing bacteriods and nonfixing reproductive cells; Oono et al. 2010), most unicellular microbes tend to divide labor in time, not across space. Spatially explicit multicellular division of labor has resulted in the generation of far more complex phenotypes than have unicellular modes of division of labor.

Because of its obvious importance, most investigations into the evolutionary origins of multicellularity have been focused on the evolution of cellular differentiation (Shapiro 1998, Kirk 2005, Rokas 2008, Willensdorfer 2009, Yu et al.

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Box 1. Defining *multicellularity*.

When does a cluster of cells become a multicellular individual? This is, at its core, a philosophical question based on notions of biological individuality and organismality (Godfrey-Smith 2009, Queller and Strassmann 2009, Clarke and Okasha 2013). Definitions of *individuality* that make sense for highly derived organisms (e.g., plants and animals) are often challenging to apply to organisms with unusual life histories (Herron et al. 2013) or taxa early in this evolutionary transition. In this article, we use a simple and trait-agnostic evolutionary definition: Clusters of cells become a *multicellular individual* when whole clusters are capable of evolving as Darwinian individuals (Godfrey-Smith 2009). Specifically, the clusters must be capable of reproducing (otherwise, they are an evolutionary dead end; Libby and Rainy 2013), the clusters must vary from one another, this variation must be heritable, and this variation must affect fitness. One simple way to determine whether all of these conditions are met is by testing directly for multicellular adaptation.

2010, Schirrmeister et al. 2011, Strassmann et al. 2011). However, recent comparative evidence suggests that less-obvious multicellular traits may have been just as important. In the evolution of the volvocines, a group of green algae that includes both uni- and multicellular species, phylogenetic reconstruction suggests that the first steps in their multicellular evolution did not involve cellular differentiation (Herron et al. 2009). Rather, the first key changes are considered to be the retention of daughter cells in an extracellular matrix, a switch from environmental to genetic control of replicative timing, and the evolution of incomplete cytokinesis. Only after these initial changes did cellular differentiation begin to evolve. These results and others (e.g., colony formation in choanoflagellates; Richter and King 2013) indicate that factors besides cellular differentiation are crucial for the evolutionary origin of multicellularity.

Experimental evolution of multicellularity in *Saccharomyces cerevisiae*

Although understanding the astounding diversity and abundance of multicellular life is the focus of much biological research, the foundational basis for multicellular diversity (its origin from unicellular ancestors) remains obscure. Direct experimentation (in contrast to comparative or theoretical approaches) would be ideal to determine the causative factors in the origins of multicellularity. Such experiments would identify the environmental conditions promoting multicellularity and the genetic changes involved. The importance of selection, drift, mutation, and sex could be determined, as could the repeatability of specific evolutionary changes. Focusing on the first steps would avoid the obscuring complexity of subsequent evolution. Unfortunately, opportunities for such direct experimentation are limited. Although multicellularity has evolved repeatedly in different eukaryotic lineages (Grosberg and Strathmann 2007), the most recent of these transitions, in brown (Brown and Sorhannus 2010) and volvocine algae (Herron et al. 2009), occurred approximately 200 million years ago, which makes direct investigation of their origins impossible. Moreover, prior work suggests that the crucial early steps may have taken millions of years (e.g., Herron et al. 2009).

We have embarked on an alternative experimental program, using experimental evolution to study new multicellular linages in the laboratory (see supplemental video S1 for a summary of the project). These experiments complement phylogenetic and molecular studies and provide tests of extant hypotheses and suggest routes for further investigative directions. In particular, our approach will provide insight into three long-standing questions on the origins of multicellular complexity. First, natural selection acts on variation in fitness and not directly on complexity. How and why does evolution sometimes result in greater complexity if it is not directly beneficial? Second, differentiated cells forgo opportunities for independent reproduction to benefit the multicellular individual as a whole. How can natural selection drive the evolution of the loss of individual cellular reproductive capability, given its direct negative consequences? Finally, the growth, structure, and organization of multicellular individuals involve developmental processes associated with the number of cells, their location within an individual, and their size and shape. How readily does developmental complexity arise, given its essential, central role in multicellularity?

Experimental overview

Our initial selection experiment was composed of 10 replicate populations of *Saccharomyces cerevisiae* strain Y55. This is a relatively wild yeast strain that has not been heavily lab adapted in the way that widely used genetic workhorses (e.g., S288c or W303) have. We propagated these populations for 60 days, with daily settling selection (approximately 500 generations). Each population was founded with the same single ancestral outbred diploid genotype, and the populations were maintained asexually throughout the selection experiment. The genetic variation that developed during the experiment was therefore the result of *de novo* mutation. After 24 hours of growth in a shaking incubator, each population was subjected to settling selection. During the first 7 days of the experiment, the entire population was allowed to settle on the bench for 45 minutes, then the lower 100 microliters (μL) of yeast was transferred to fresh medium. To increase the efficiency of this settling step, from day 7 to day 60, we replaced the bench-top settling with a light centrifuge step:

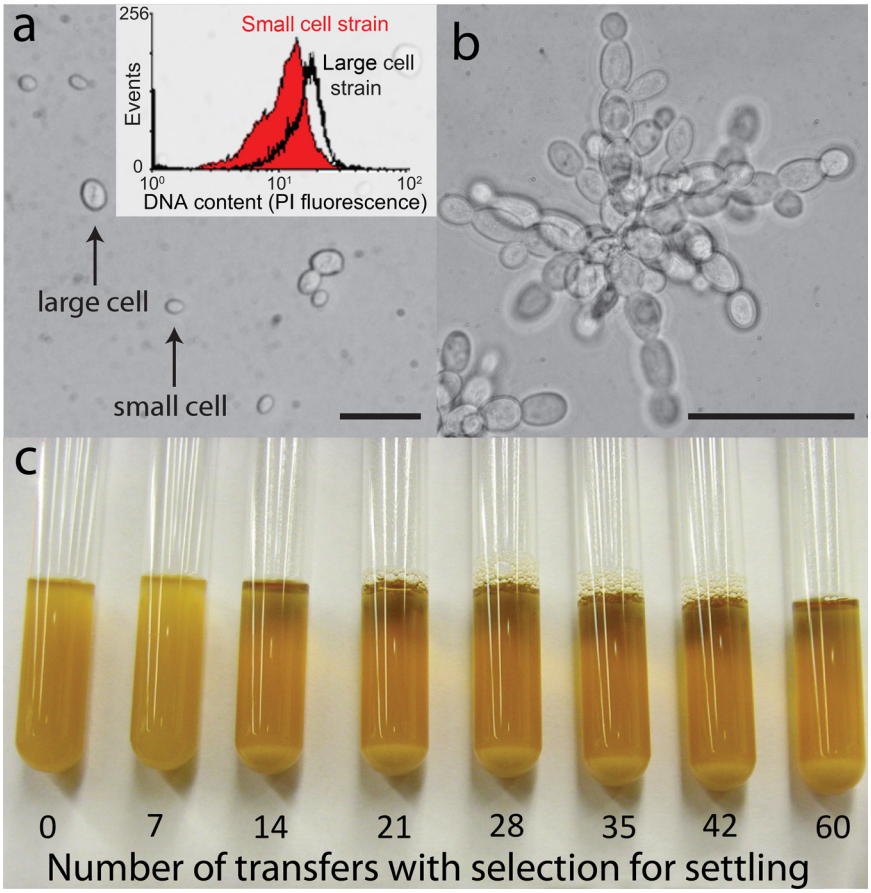

Figure 1. Responses to settling selection. (a) Three out of 10 populations evolved large cell size prior to the origin of snowflake yeast. Large-cell yeast contained an average of 1.8 times as much DNA as the ancestral small-cell morph (a, inset). The inset shows the results from a flow cytometric analysis of single cells (with the cell count on the y-axis and per cell DNA content, as measured by propidium iodide staining, on the x-axis). (b) Snowflake yeast evolved in all 10 replicate populations within 60 transfers, driving the unicellular strains to extinction. (c) Snowflake yeast clusters settle far faster than their unicellular ancestors do. Snowflake yeast invaded this population between 7 and 14 transfers (note the difference in settling at this time). The populations shown here have been settling on the bench for 15 minutes. The scale bars represent 25 micrometers. Photograph: William C. Ratcliff and Michael Travisano.

1.5 milliliters (mL) from each population was centrifuged at 100 gravitational force units for 10 seconds. After settling selection, the populations were incubated with shaking for 24 hours, at which point the populations reached roughly 10^9 cells per mL and were again subjected to settling selection and transfer to fresh medium. Because clusters of cells settle more rapidly than do single cells (they have a lower surface area to volume ratio than single cells do and, therefore, experience more downward force from gravity relative to friction caused by surface interactions with the growth medium), we expected these conditions to select for clusters of cells.

In these experiments, we employed techniques from both natural and artificial selection approaches. We did not screen populations for individual variants, as would be performed in artificial selection, but, instead, imposed a complex selective environment across 10 populations. Thus, beneficial genetic variation was systematically enriched by natural selection. The imposition of a form of truncation selection (only those individuals in the bottom 100 µL of the sample were transferred to fresh medium) is reminiscent of artificial selection. Even so, individual cells were not directly assayed, nor was the precise cause of reaching the bottom of the tube. In fact, nonsnowflake yeast (characterized by a larger but still unicellular growth form) were initially favored in three populations, highlighting the fact that our selection protocol was not a direct screen for phenotype, as is typically the case with artificial selection approaches.

The results and approaches described here were reported in Ratcliff and colleagues (2012, 2013) and Rebolleda-Gomez and colleagues (2012). Please refer to those papers for experimental methods, statistical analysis, and detailed results.

Faster settling and the appearance of multicellular phenotypes

We initially observed two different modes of adaptation in response to the settling selection. In some of the replicate populations, the initial response was the evolution of large cells, roughly twice the size of those of the ancestral genotype (figure 1a). These large cells contained nearly twice as much DNA as did the unicellular strains (figure 1, inset), which suggests that their large size is due to chromosomal duplication (*aneuploidy*). However, this was not general and only occurred in 3 of the 10 replicates. In other replicates, the appearance of cell clusters was the first dramatic response to settling selection (figure 1b). The individual clusters were similar in appearance to snowflakes and were vastly superior in settling to the unicellular ancestors (figure 1c). Snowflake yeast clusters were the dominant phenotype in all of the replicates within 60 days, but the timing of their appearance varied substantially among the replicate populations, ranging from 7 to 60 days.

The large amount of variation in the rate at which snowflake yeast evolved is likely due to two factors: the stochastic nature of mutations and clonal interference. Because each of our populations initially lacked standing genetic variation,

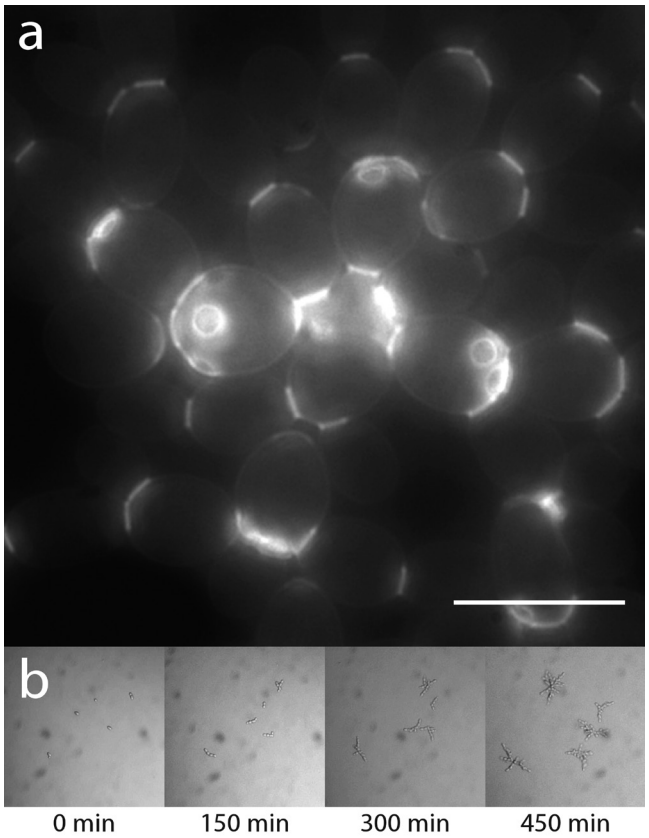

Figure 2. Snowflake yeast growth form. (a) Yeast cell walls were stained with the fluorescent dye calcofluor white. All connections between the cells occur at the bright bud scars, where daughter cells are connected to their parent cell. (b) A snowflake yeast cluster was digested to unicells with the enzyme lyticase and β-glucuronidase. These were then grown and tracked with time-lapse microscopy. Single cells regenerate clusters through mother–daughter cell adhesion, not through floclike aggregation. The scale bar represents 10 micrometers. Abbreviation: min, minutes. Micrographs: William C. Ratcliff and Michael Travisano.

each population had to independently acquire the mutations for the snowflake growth form *de novo*. The ability for natural selection to act on mutations conferring the snowflake phenotype can be impeded by competition from different beneficial mutations in other asexual linages (this process is known as *clonal interference*; Kao and Sherlock 2008). For example, a mutation increasing the growth rate of a unicellular lineage may initially outcompete a lineage that forms small snowflakes, which may lead to the loss of this otherwise beneficial mutation from the population.

Cell clusters formed by the aggregation of adhesive cells (a process known as *flocculation*) are a well-described life history trait in yeast (Jin and Speers 2000, Claro et al. 2007). These clusters, known as *flocs*, are formed when yeast cells produce adhesive glycoproteins in their cell walls and then physically come into contact (Veelders et al. 2010). These cell surface factors generally attach poorly to most surfaces but form relatively strong attachments to similar proteins. Depending on the expression of cell adhesion factors among cells, very large cell clusters can arise. During fermentation, cell clusters are a mode for yeast settling and are observed in bottom fermentations (Zhao and Bai 2009).

The snowflake clusters that evolved in our system are not flocs but, rather, formed by a failure of daughter cells to separate after mitosis. The physical structure of snowflake yeast clusters indicate mother–daughter adhesion: The clusters have approximate spherical symmetry, with a single point of cell attachment (to their mother cell) toward the center of the clusters and multiple attachments (to their daughter cells) facing outward. This is a modification of typical yeast cell replication, which occurs through asymmetric division: A small daughter cell buds out of its mother cell at a bud site. In most yeasts, the mother and daughter cells separate, but this does not occur in snowflake yeast. The bud sites can be stained, and they are readily observed in snowflake clusters as sites of cell attachment (figure 2a). The cell wall connecting mother and daughter cells can be enzymatically digested, resulting in free-floating individual cells. When allowed to grow, these individual cells form new snowflake clusters through mother–daughter cell adhesion, not floclike aggregation (figure 2b).

Competition among snowflake yeast clusters

We found substantial genetic variation in snowflake cluster size and morphology among (Ratcliff et al. 2012) and within (Rebolleda-Gomez et al. 2012) the replicate selected populations. This variation provided the basis for natural selection and an opportunity to experimentally investigate the evolution of a key multicellular trait: cluster size. The original selection scheme strongly favored increased settling rates. Pairs of cells fall faster than single cells do, and cell clusters settle faster still. However, there is a potential cost to increasing cluster size. Although cells at the periphery of a snowflake have unimpeded access to resources, the interior cells do not, which suggests a trade-off between settling and growth rates, which we observed in the first snowflake isolates to evolve in five separate lineages (figure 3). This trade-off is important, because the yeast experiences strong selection for growth during the 24 hours of shaking incubation, in addition to settling. On the basis of this trade-off, we hypothesized that altering the selection regime by varying the strength of settling selection would result in the evolution of different cluster sizes.

To test this hypothesis, we performed a divergent selection experiment. Nine new populations were initiated from one of the replicate populations from the original selection experiment. We chose a 30-day population in which the snowflake phenotype was first detected after 7 days. These nine new populations were maintained under the same conditions as those in the original experiment, except for the amount of time to settle: Three replicate populations were each allowed to settle on the bench for 5, 15, or 25 minutes before we

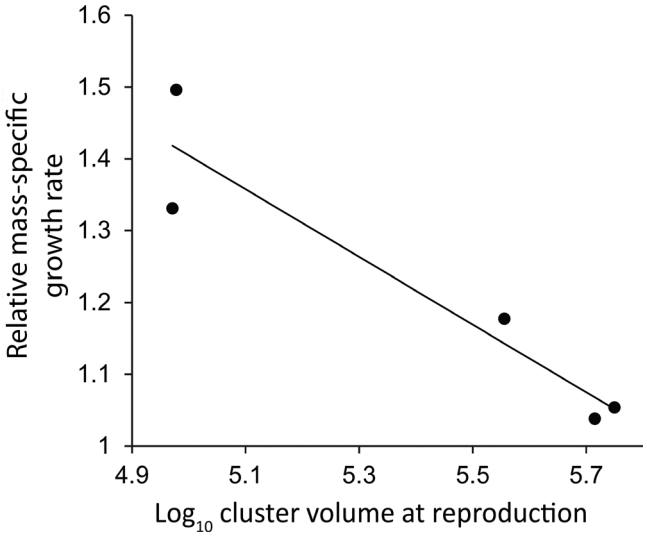

Figure 3. The cost of a larger cluster size. Larger clusters do not grow as rapidly (for an equivalent amount of biomass) as smaller clusters do. Here, we have plotted the relationship between the relative mass-specific growth rate (as a proportion of the hourly increase in biomass) and cluster volume at reproduction (in cubic micrometers). The slower growth of the large clusters is probably due to diffusion limitation of interior cells, which reduces their access to growth-limiting resources (e.g., oxygen, glucose).

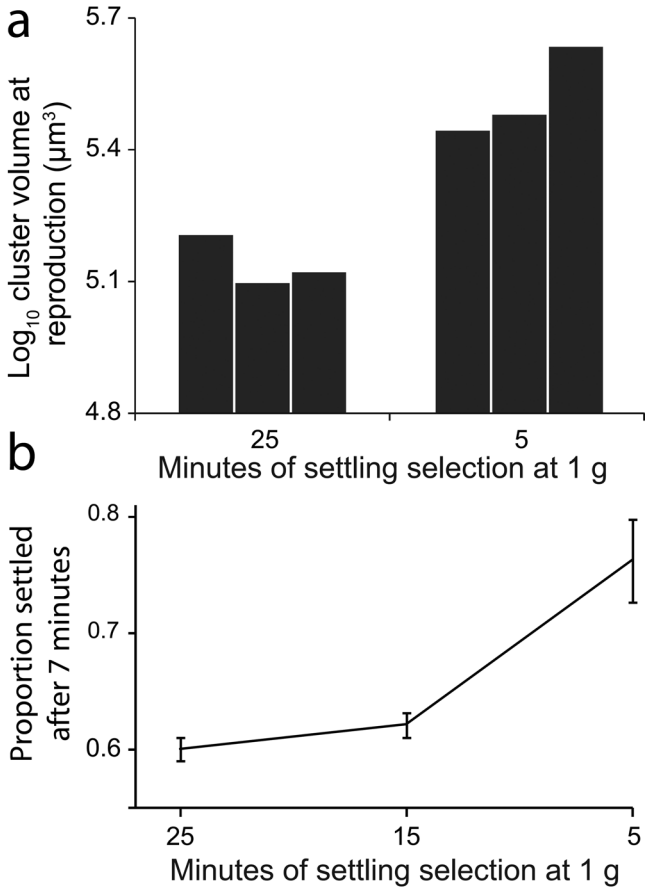

Figure 4. Whole clusters respond to selection. A single population was replicated and subject to divergent selection. Three replicates were transferred with 5, 15, or 25 minutes of settling at 1 gravitational force unit (g) prior to transfer. Shorter settling times impose stronger selection for fast settling. (a) After 35 transfers, snowflake yeast from the 5-minute line evolved to delay reproduction until the cluster was 2.4 times as large as those given 25 minutes to settle. Each bar represents an isolate randomly selected from an independent replicate population. (b) The larger yeast in the 5-minute treatment settles more rapidly. Abbreviation: μm^3, cubic micrometers.

transferred the lower 100 μL. The evolutionary responses to selection were rapid and dramatic. Within just 35 days, the snowflake yeast exposed to the strongest settling selection (5 minutes) evolved to form clusters more than twice as large as those in the other two treatments (figure 4a); these larger clusters settled more than 20% faster (figure 4b). Although strong selection for faster settling (5 minutes) resulted in a twofold increase in cluster size from the ancestor, weak settling selection (25 minutes) actually resulted in the evolution of reduced cluster size. These results demonstrate that the trade-off observed with the first isolates was not simply a temporary constraint that would be quickly overcome but is rather a persistent factor that can substantially affect subsequent evolution.

More important, these results show how complexity can appear and evolve through natural selection. We selected yeast that settled rapidly through liquid but did not directly select for any particular mode of evolutionary adaptation. We observed two initial responses to selection: increased size of individual cells and snowflake clusters of cells. Although both can result in faster settling rates, larger cells experience well-known negative fitness consequences associated with a diffusion of resources and components within a cell, which are increasingly costly as the cells increase in size (Lane and Martin 2010). In the three populations in which large-size unicellular strains evolved, they were outcompeted by snowflake yeast. A simple genetically determined phenotypic change—daughter cell adhesion to mother cells—was the superior evolutionary strategy. The responses to the divergent selective regimes show that, once body size became an important mode through which settling speed evolved, subsequent evolution occurred through changes in body size, a composite trait of the multiple cells constituting a multicellular individual.

Apoptosis and snowflake reproduction

Many multicellular organisms pass through a unicellular genetic bottleneck as they grow (Bonner 1974), such as the fertilized zygote in metazoans. This bottleneck aligns the genetic interests of cells in an individual (Buss 1987, Grosberg and Strathmann 1998, Folse 2011), because the

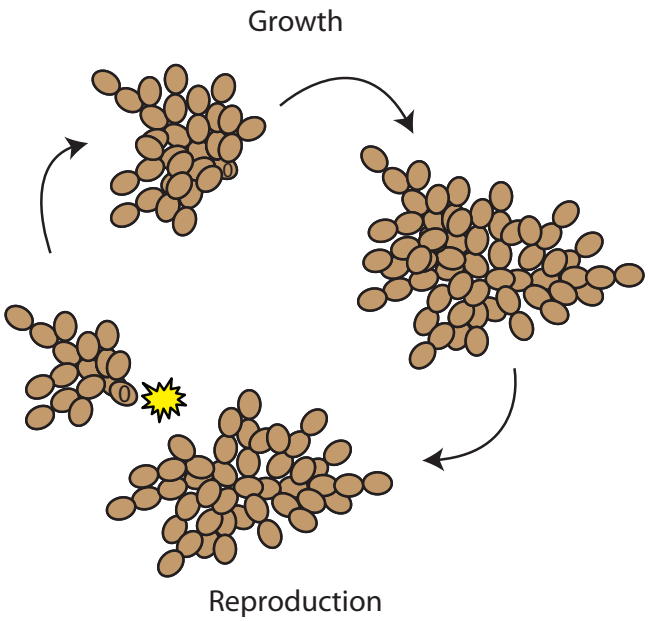

Figure 5. The snowflake yeast life cycle. Snowflake yeast clusters reproduce when the connection between two cells in a branch is severed. This also results in a single-cell bottleneck at the focal cell (labeled 0). As a result, all of the cells in the snowflake yeast cluster are clonal, which facilitates the evolution of traits that are costly to individual cells but beneficial to the cluster (e.g., apoptosis). Propagules grow through mitotic division, and when they reach their parents' size, they begin producing their own propagules.

cells are initially genetically identical (within-individual variation can arise by mutation, however). The high level of genetic relatedness of cells within the cluster facilitates cellular division of labor, which allows cells that forgo opportunities for independent reproduction to benefit indirectly, through the reproduction of the multicellular individual as a whole.

The snowflake yeast life cycle also includes a single-cell genetic (but not physiological) bottleneck. Snowflake clusters consist of a network of branches (figure 2), all of which lead back to a single focal cell. This branching pattern is a necessary consequence of daughter–mother cell adhesion, because connections between cells can be formed only by cellular replication. Cluster reproduction occurs when a branch within a cluster breaks (figure 5). The new offspring cluster separates with a new focal cell (labeled *0* in figure 5), the site at which the cluster broke away. The first snowflake clusters that evolved broke into two roughly symmetric clusters during reproduction, which is consistent with the mechanism of tension driving branch breaking. Individual cellular replication causes cells to press against one another, and the greatest tension occurs where the largest branches connect—the focal cell at the center of the cluster.

Symmetric snowflake cluster division is not necessarily beneficial, however. Large snowflakes grow slowly (figure 3), and symmetric division ensures that clusters are always fairly large and fairly slow growing. In contrast, clusters that instead reproduce asymmetrically—producing a larger number of smaller offspring—may partially compensate for the growth-rate cost of a larger cluster size. Clusters that start out small will initially grow at a much faster rate than their parents did and, if they are given sufficient time to grow large before selection, can realize a high rate of survival during settling selection. We observed that snowflakes at the end of the original selection experiment had evolved increasingly asymmetric reproduction, in contrast to the first snowflake clusters that evolved (figure 6, inset). We have partially uncovered the mechanism driving this asymmetric division; it is caused by an increased rate of apoptosis (figure 6).

In the unicellular ancestor, there was a very low occurrence of apoptosis (less than 0.5%), and the rates of apoptosis showed no relationship with cluster size in the first snowflake isolates, regardless of their size (figure 7a, dashed line), which indicates that the large cluster size did not affect the apoptosis rate. However, the rate of apoptosis was strongly correlated with snowflake size in the 60-transfer snowflake yeast (figure 7a, solid line), and the shift in correlation suggests that the apoptosis rates evolved in response to selection. To test the functional effects of increased rates of apoptosis, we chemically induced apoptosis in a low-apoptosis snowflake yeast strain, which resulted in a substantial increase in reproductive asymmetry. In fact, this brought its reproductive asymmetry in line with a 60-day high-apoptosis genotype isolated from the same population. We next examined this process at the cellular level by examining the location of dead cells in freshly produced propagules. On the basis of their overall frequency, we determined that the cell at the site of propagule separation (cells labeled *0* in figure 5) had a 6% chance of being dead by chance alone. However, we found that these cells were, in fact, dead 76% of the time. This means one of two things: Either dead cells increase the probability of branch fragmentation or branch fragmentation was causing the cells at the site of the break to die. To test the latter hypothesis, we manually fragmented clusters and found that branch breakage did not cause appreciable amounts of cell death (figure 7b). In combination with our results showing that higher rates of apoptosis reduced cluster size (figure 6, inset), this strongly suggests that dead cells act as weak links in the branches. Fragmentation caused by cell death has previously been described in filamentous microbes (Daft and Stewart 1973, Ning et al. 2002, Adamec et al. 2005).

Increased rates of apoptosis alone did not explain the differences in cluster size at reproduction. The lack of an initial correlation between cluster size and apoptosis rates (figure 7a, dashed line) indicates that the size differences among strains involved other factors, such as cell size, shape, or cell–cell attachment strength. Although apoptosis was highly correlated with cluster size after 60 transfers (figure 7a, solid

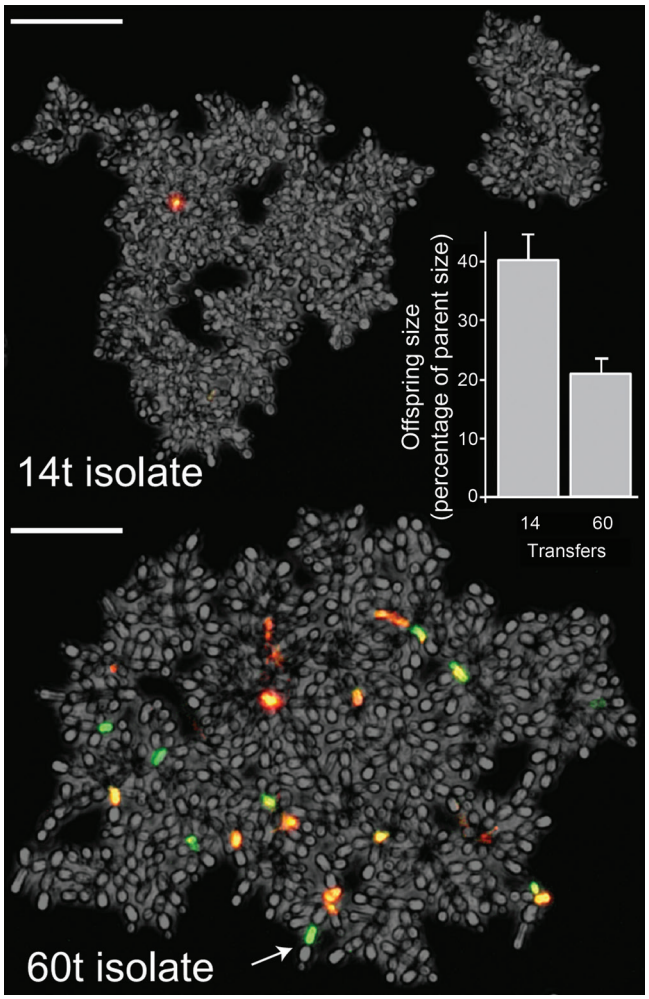

Figure 6. The evolution of elevated apoptosis. Snowflake yeast from the 14-transfer (14t) isolate show little apoptosis (labeled green with the reactive oxygen species dye dihydrodhodamine 123); a single dead cell is visible (labeled red with the DNA stain propidium iodide). By 60 transfers, however, high rates of apoptosis have evolved. The 60-transfer (60t) strain also produced substantially smaller propagules than its 14t ancestors did (inset). Apoptosis is not restricted to old cells; even young cells near the periphery can undergo apoptosis (the arrow). The scale bar represents 100 micrometers.

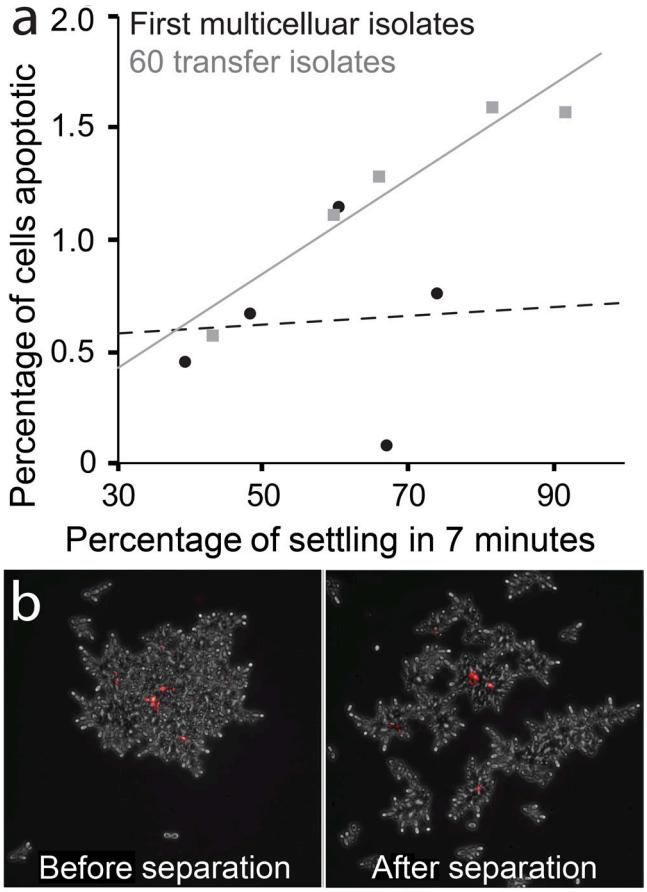

Figure 7. High rates of apoptosis evolve only in large-body snowflake yeast. (a) Apoptosis frequency was not correlated with cluster size in the first snowflake yeast to evolve in five separate populations (the dashed line). After 60 transfers, however, cluster size and apoptosis were highly correlated, which suggests that the higher rates of apoptosis evolved in the context of a large cluster size. (b) Propagules have a strong tendency to have a dead cell at the site of branch fragmentation. To determine whether this causes cell separation or whether cell separation causes cell death, we manually fragmented clusters in the presence of dead-cell stain propidium iodide. No cells were killed by cluster fragmentation. The high frequency of dead cells at the site of fragmentation suggests that cell death through apoptosis creates weak links in the cluster, which allows it to produce a greater number of faster-growing, smaller propagules.

line), we doubt that it has been fully optimized by natural selection. We are currently investigating the molecular mechanisms of increased rates of apoptosis, and, although we have not yet determined a specific mechanism, we can exclude two potential routes. The apoptotic cells cannot be the oldest cells in the cluster, because cells at the snowflake periphery (the very youngest cells) also undergo apoptosis (figure 6b, arrow). Nor can the apoptotic cells be those experiencing the most severe nutrient limitation in the interior of the clusters, because apoptosis appears throughout the clusters. Moreover, dead cells in the center of a snowflake, which is where the oldest and the most nutrient-deprived cells are, are unlikely to be adaptive, because they would generate largely symmetric offspring.

The evolution of development

Understanding how multicellular form originates in development and evolution is one of the central aims of biology. Multicellularity provides the central avenue for the

emergence of form during development and morphological innovation during evolution. This is because morphological form and function are altered by the number of cells within an individual, their spatial relationship, and their individual characteristics (e.g., shape and differentiation). However, the apparent simplicity by which cellular changes affect morphology belies the underlying complexity of the developmental mechanisms affecting cell number, organization, and differentiation. Changes in developmental evolution can be evaluated by a consideration of the evolutionary and functional context. Specifically, research examining the origin of development should focus on the following questions: How important is the evolutionary order of developmental traits? What functional dependencies are involved for specific developmental changes? Do developmental changes alter interactions among cells, and do these changes increase fitness by modifying the multicellular phenotype? Are these fitness benefits context dependent (e.g., would they accrue under other cellular contexts)?

In the snowflake yeast, the evolutionary transition to multicellularity had multiple developmental effects that altered the context in which the cells interact. Mother–daughter cell adhesion following cell replication results in the branching pattern of cell connections within snowflakes, as was described above. As cells replicate within a snowflake cluster, tension across individual cells increases until the branch breaks, which produces a propagule cluster. Given the new multicellular context of selection, the size of a cluster at reproduction is a multicellular life history trait with substantial fitness consequences. Mutations that affect the size of a cluster (e.g., by increasing the strength of cell–cell adhesion) must be viewed in light of the multicellular context of selection. The snowflake phenotype is an excellent example of how a single phenotypic change—mother–daughter cellular adhesion after division—can dramatically change the context in which cells interact, with profound consequences for the subsequent evolution of development.

The evolution of increased apoptosis is a highly context-dependent developmental change. The cluster-level benefits associated with apoptosis necessarily depend on the particular mode of branching multicellularity found in snowflake yeast. Increased apoptosis should not provide a fitness benefit to single-cell yeast or even to flocculant yeast. The conditions under which organismal (e.g., a unicellular yeast cell) suicide is beneficial are limited (Nedelcu et al. 2011). Because of the multiple connection points among cells in a floc cluster, apoptosis of a single cell should not release a propagule. Moreover, altruistic suicide should not be evolutionarily favored in floc clusters, because clusters may be formed by unrelated cells (Smukalla et al. 2008). In snowflake yeast, the benefits of apoptosis are directed toward living clonemates that possess the genes for elevated apoptosis; in floc clusters, the benefits of apoptosis may be gained by cheats that do not possess the genes for apoptosis.

Additional examples of developmental evolution were obtained from an extension of the divergent selection experiment (Ratcliff et al. 2013). At the completion of the divergent selection, one 5-minute settling selection population was used to initiate three new populations in which the strength of selection was further increased; we allowed only 1.25 minutes of settling on the bench prior to transfer. We continued this selection for an additional 182 days. We then examined the settling speed over the full 227 days of selection and measured three key multicellular traits: the number of cells per cluster, cell mass, and cluster shape. During this time, the mean settling speed increased from 296 to 428 micrometers per second, but the rate of increase declined exponentially.

Multicellular adaptation occurred in three stages (figure 8). The number of cells per cluster increased at every time point measured, from 42 at 7 days to 114 at 227 days. This change is context dependent on multicellularity; it could not occur in the unicellular ancestor. It is not dependent on the mode of multicellularity, however, because floc clusters with more cells would settle faster, as well. Between 28 and 65 days, cell mass increased by more than twofold, increasing cluster size and settling speed by modifying not the number of units (cells) but their nature. This trait is totally context independent: Increased cell mass would increase settling rates regardless of whether the cells occur in a unicellular or multicellular context. Finally, between days 65 and 227, the clusters evolved to settle faster not simply by getting larger but also by forming a more hydrodynamic, spherical shape. This trait is not derived from a simple change to the underlying units but, instead, is a fully emergent trait of the group as a whole. This change is highly context dependent, because it necessarily involves a multicellular organization and probably depends on particular modes of multicellular development.

In the relatively short evolutionary history of snowflake yeast, we observed changes in the typical mechanisms affecting morphology: the number of cells, their shape, and their spatial organization. The majority of these changes are context dependent and could occur or be beneficial only in a multicellular context. This is consistent with the importance of evolutionary order in the generation of biological complexity. This context dependence is highlighted by two developmental changes: regulation of propagule size through apoptosis and the evolution of more hydrodynamic clusters. Neither of these traits could have evolved in the absence of the multicellular life history exhibited by snowflake yeast or the shift to cluster-level selection. Furthermore, both are cluster-level traits: Their benefits accrue at the multicellular level and cannot readily be decomposed into direct individual-cell-level benefits.

Conclusions

Under the appropriate selective conditions, multicellularity rapidly evolves in *S. cerevisiae*. Although larger unicellular yeast settles faster than the ancestral strain, snowflake yeast clusters are superior to both. Snowflake yeast evolved by a simple change in the ancestral mode of cell replication:

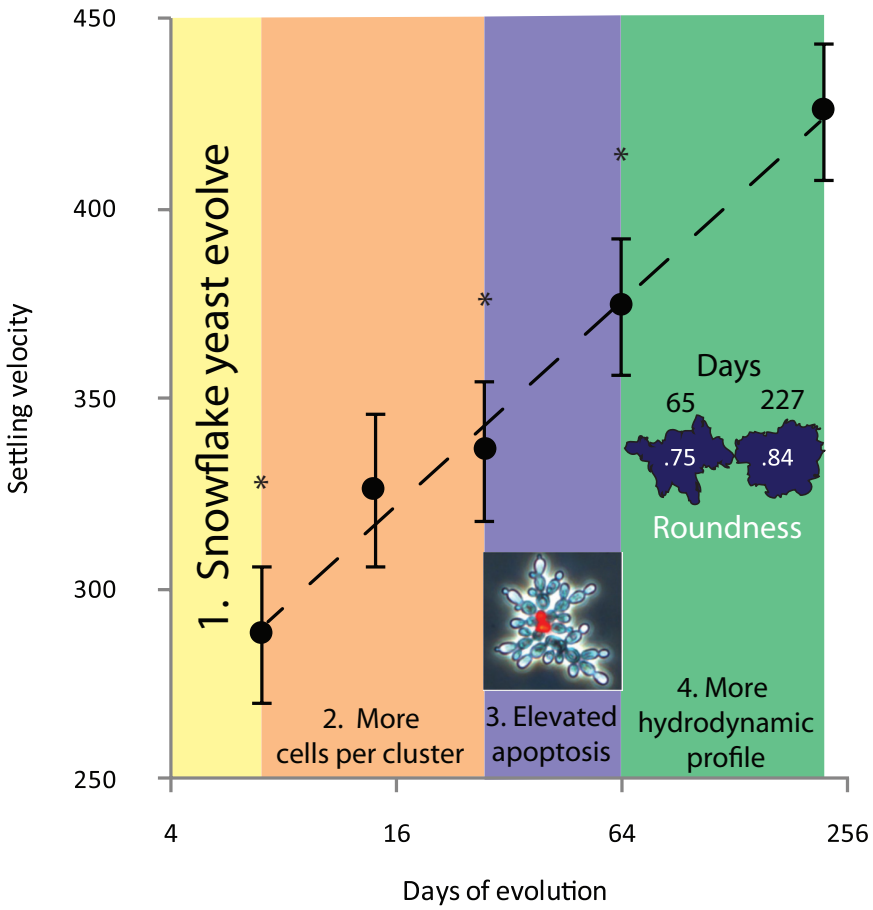

Figure 8. The tempo and mode of multicellular adaptation. Snowflake yeast evolved to settle faster over 227 days of evolution, but the rate of increase declined logarithmically. Shown here are four key developmental milestones under a regime of periodically increasing settling selection (changes to the selection scheme are marked with asterisks; see the text for details). Snowflake yeast arose after 7 days of settling selection and first evolved faster settling simply by forming larger clusters. Subsequently, snowflake yeast evolved higher rates of apoptosis between days 28 and 65 and more spherical clusters between days 65 and 227.

Daughter cells remain attached to their parent. Snowflake yeast possess several traits characteristic of familiar multicellular organisms, such as among-cell clonality, multicellular development, juvenile and adult life history stages, and cellular specialization. Many of these characteristics are direct consequences of mother–daughter cellular adhesion. These experiments demonstrate that natural selection can readily drive the evolution of complexity, particularly when a single change (e.g., cell–cell adhesion) has multiple phenotypic consequences.

Snowflake yeast reproduction occurs through branch fragmentation. As a result, every propagule undergoes a unicellular genetic bottleneck (but not a physiological one, because propagules are multicellular), and the clusters are almost always genetically uniform. The high genetic relatedness of cells within a cluster favors the evolution of cellular differentiation, because individual cells can benefit from the success of the snowflake cluster as a whole. Although apoptosis is highly detrimental to the fitness of individual cells, increased rates of apoptosis in large snowflakes evolves because of the reproductive benefits that dead cells confer to clonemates within the cluster. These experiments demonstrate that natural selection may strip cells of their evolutionary autonomy and thereby transition them from whole organisms to a part of an evolving higher-level (i.e., multicellular) organism.

The evolution of developmental complexity remains a topic of intense interest in biology, because small developmental changes can have profound effects on form and function (Gerhart and Kirschner 2007). A long-standing question is the tempo of developmental evolution (Peterson et al. 2005). Do such changes evolve gradually, through many small steps, or quickly, through a few large steps? Although the terminology has changed over the last 150 years, this question has been hotly debated since Darwin's (1861) publication of the *Origin of Species*. Our results suggest that the dichotomous division between gradual and punctuated change has obscured the evolutionary processes. We found that a physiologically simple evolutionary change—mother–daughter cell adhesion—had profound consequences for both short- and long-term phenotypic change. The snowflake yeast growth form, with its emergent multicellular life cycle (e.g., juvenile and adult stages), is dramatically different from the ancestral unicellular life history. Moreover, the shift to among-cluster selection led to the evolution of elevated programmed cell death and more hydrodynamic clusters. These traits could not have evolved without the multicellular context for evolution.

Looking forward, there are several important directions for investigation. The most obvious is a molecular genetic decomposition of the basis for multicellularity. Among replicate populations, are the same mutations, genes, and pathways necessarily involved in the transition to multicellularity? How dependent is this process on the specific mutations that give rise to snowflake yeast? We are particularly interested in determining the extent to which the transition to multicellularity leads to the evolution of new regulatory structures rather than the coopting of preexisting unicellular processes.

One mode for evolutionary conservation involves the preexisting phenotypic plasticity of unicellular ancestors (Gavrilets 2010). *Plastic* traits are those that are under regulatory or environmental control, and unicellular microbes are masters of altering their phenotype, depending on environmental conditions. In a nascent multicellular organism, the local environment of individual cells (their context) varies with the density and spatial organization of the neighboring cells, which exposes cells to diverse environmental conditions. Preexisting plastic responses to those differing conditions in the unicellular ancestor may result in location-specific gene expression (e.g., anaerobic fermentation by cells in the oxygen-poor cluster center). This among-cell variation provides the raw material on which natural selection acts during the origin of cellular specialization.

Other evolutionary modes for a cellular division of labor are possible, again building on the changing cellular context inherent in a multicellular individual and the changing context of a multicellular individual in its selective environment. One particularly intriguing possibility is an increase in additive genetic variation following a multicellular transition. Neutral genetic variants that have no selective consequences in a unicellular ancestor may have substantial fitness consequences in a multicellular context. This suggests that mixis of preexisting genetic variation through a sexual phase in the multicellular life cycle might substantially enhance the rates of adaptation and might increase developmental complexity. We are currently examining this hypothesis experimentally.

The evolutionary importance of multicellular complexity on the origin and maintenance of ecological complexity is widely recognized. Many instances of adaptive radiation in multicellular species implicitly or explicitly involve changes in development, such as the differences in bill size in Darwin's finches (Abzhanov et al. 2006), changes in body armor in stickleback fish (McKinnon and Rundle 2002), and mouth shape in cichlids (Albertson et al. 2003). However, the causal linkage between multicellular and ecological complexity remains obscure, and many basic questions remain open. For example, how does ecological complexity scale with increases in multicellular complexity, and what are the relevant factors? To what extent do ecological responses to multicellular complexity feed back and affect the subsequent evolution of multicellular complexity and on what timescale? The snowflake yeast model system allows for direct experimental investigation of these questions.

Experimental evolution can be used to directly investigate the origins of multicellular complexity. Multicellular snowflake yeast readily and rapidly evolved in replicate populations of initially unicellular *S. cerevisiae* under settling selection. The evolutionary transition was accomplished by persistent attachment of mother and daughter cells following cellular replication. Snowflake yeast clusters exhibit a branched network structure and pass through a unicellular genetic bottleneck each time they reproduce, which generates a high degree of within-cluster genetic uniformity. Snowflake yeast possess an emergent multicellular life history: Propagules are effectively juvenile and require a period of growth before they are capable of producing their own clusters. These multicellular traits affect fitness, and selection was able to act on cluster-level traits that have no single-cell analogue. The high degree of genetic uniformity within a snowflake cluster promotes cellular division of labor, which was observed as increased rates of apoptosis in large snowflakes. The majority of the developmental changes that evolved after the transition to multicellularity were context specific, contingent on both the evolution of multicellular clusters and cluster formation through mother–daughter cell adhesion. The origin and subsequent evolution of multicellular complexity in snowflake yeast can be directly attributed to natural selection.

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Supplemental material

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