Using adaptive laboratory evolution of multicellular snowflake clusters in yeast *Saccharomyces cerevisiae* to study reversibility of evolutionary processes.

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Abstract

The question of chance vs. determinism in dictating evolutionary trajectories has been a broad question of interest in the last few decades. This question has not been addressed in the context of reverse evolution. By reverse evolution, we mean a scenario where selection is reversed. In this work, we use evolution of multicellularity in *S. cerevisiae* as a model to answer this question. When selected for fast-settling variants, multicellularity rapidly evolves in the organism. On reversing selection, unicellularity evolves from the multicellular clusters. However, the dynamic trajectories of the two processes are completely different. The molecular determinants dictating the two adaptive processes are also distinct from each other. In this context, evolution is not reversed dynamically or at a molecular level. The phenotypic reversal, however, is driven by epistatic interactions in the genome. How epistatic interactions evolve in a genome and shape evolutionary trajectories remains largely unknown.

Keywords

Multicellular yeast clusters, reverse evolution, epistasis.

Introduction

Reversibility, in an evolutionary context, is often seen as return to ancestral state (Teotónio et al., 2002, MULLER, 1939, Durrant, 1954). Historically, investigation of reversibility of evolutionary processes has been an important theme (Darwin, 1859, Dollo, 1893, Durrant, 1954, Lewontin, 1966).

In microbial systems, this question has been addressed in a number of experimental studies, and has led to different answers (Crill et al., 2000, Teotónio & Rose, 2000). In these experiments, a population is shifted from one environment to another, and adapted for a certain number of generations. The evolved population is then shifted back to the original environment, and its adaptation studied. In this context, reversibility is studied as the ability/dynamics of a population to re-adapt in an environment, when shifted from another (Teotónio & Rose, 2000). Towards this effect, one of the most well-studied systems has been evolution of sensitive bacteria in a population of resistant bacteria, when a stressor (antibiotic/phage) is removed from the environment (Lenski, 1988a, Lenski, 1988b, Duncan et al., 2011). In the context of microbial systems, sequencing makes it possible to trace the reversibility of evolution. In bacteria, when antibiotic resistance was removed, the original genotype was not restored (Larsson, 1992, Schrag et al., 1997). On the other hand, evolution experiments with virus have demonstrated that phenotypic reversals can map to precise nucleotide reversals (Bull et al., 1997, Crill et al., 2000). Enzymes too have been shown to recover activity, albeit via different mutational paths (Kaltenbach et al., 2015). The broad lesson from these experiments is that the longer the time spent in the novel environment, the more difficult the likelihood of reverse evolution becomes.

However, reversibility in another context, has been largely unexplored. What happens to the evolutionary fate of a population, when the selection pressure is reversed? In this context, to study reversibility of evolution, we need systems where an exact and opposite selection pressure is applied on a system. Such a choice of a system is not trivial. For instance, is absence of an antibiotic the reverse of evolution in presence of (how much?) antibiotic? Or, is evolution in presence of glucose the reverse of evolution in glycerol? To answer this question, we study evolution (and reverse) of multicellularity in yeast, using settling (or lack of) as a selection force.

The evolutionary transition from single cells to multicellular organisms was one of the major transitions in the history of life on Earth (King, 2004) (West et al., 2015). Eukaryotic multicellularity has evolved independently at least 25 times in separate lineages, leading to the evolution of complex multicellular organisms such as animals, plants, seaweed, and multicellular fungi (Grosberg & Strathmann, 2007, Rokas, 2008, Wolpert & Szathmáry, 2002). However, understanding the evolution of complex multicellular organisms from unicellular organisms is challenging, because the first steps in this process occurred in the deep past (Grosberg & Strathmann, 2007, Koschwanez et al., 2011b). It has been suggested that a combination of environmental, ecological, and genetic factors are associated with this transition to

multicellularity(Rokas, 2008). Sequencing the genomes of several independently evolved pairs of multicellular and unicellular ancestors enable identification of the genetic basis of *de novo* origin of multicellularity from unicellular forms (Koonin et al., 2004, Chothia et al., 2003). Transitions to multicellularity are typically associated with increases in the numbers of genes involved in three important processes such as cell differentiation (eg., Homeobox-*Hox* genes) (Ferrier & Holland, 2001), cell-cell communication (e.g., Wingless type-Wnt genes and receptor tyrosine kinases genes) (Kusserow et al., 2005, King & Carroll, 2001), and adhesion (e.g., cadherins and integrins) (Abedin & King, 2008, Nichols et al., 2006, Rokas, 2008).

The key step involved in the evolution of multicellularity likely was the evolution of simple, undifferentiated cell clusters. The multilevel selection hypothesis suggests that evolutionary transition from single-celled forms to complex multicellular groups occurs in two different modes. In the first mode genetically distinct single cell ancestors come together to form clusters (or collectives). For example, multicellularity in dictyostelid cellular slime molds and myxobacteria has evolved as a result of aggregation (Rokas, 2008, Ratcliff et al., 2013). In the second mode, the descendants of a single cell stay stuck together due to incomplete cell separation (mother-daughter cell adhesion), and then selection shifts to the collective level (i.e. selection of multicellular-level) (Michod, 1997, Bonner, 1998, Wolpert & Szathmáry, 2002). For example, multicellularity in Volvocine green algae likely evolved due to incomplete cell separation. This second mode of incomplete cell separation seems to be involved in the origins of multicellular individuals (Okasha, 2005). The clustering of single cells into a multicellular clump of undifferentiated cells was likely a necessary precursor for subsequent innovations such as division of labor and germ-soma separation (Koschwanez et al., 2011b). This multicellular cluster form is selected only when the fitness of an average cell in a clump exceeds the fitness of an average solitary cell that is not part of a clump. The conditions required for multicellular-level adaptation are stringent. First, collectives must be capable of reproducing. Second, collectives must have the properties required for Darwinian evolution, namely they must vary in their collective level traits, this variation must be heritable, and these collective level traits must affect fitness (Ratcliff et al., 2015). Finally, internal conflicts must be minimized.

A transition to cluster form confers a likely selective advantage over unicellularity in ecological niches in the context of effective protection from the changes/stresses in the environment (Bonner, 1998, Rokas, 2008). Several examples of this phenomenon exist, including protection from phagocytosis (Boraas et al., 1998), predators (Kessin et al., 1996), environmental stressors (ethanol and antibiotics) (Smukalla et al., 2008) and uptake of harmful compounds (Váchová et al., 2009). Clustering may be beneficial for more efficient exchange of external resources between component cells of a cluster by reducing interactions with non-cooperative individuals (or cheaters) (i.e. increased efficiency of cooperative feeding) (Koschwanez et al., 2011a). It has also been suggested that cluster levels selection is beneficial for the use of growth-promoting secretions as well as exclusion of cheaters (Koschwanez et al., 2011b). This division of labor can allow

multicellular organisms to perform tasks more efficiently, and can facilitate the evolution of complex traits (like differentiated tissues) that provide novel multicellular-level functionality (2014).

Mathematical models have demonstrated key steps in the transition from unicellular form to multicellular clusters (Pfeiffer & Bonhoeffer, 2003, Ispolatov et al., 2012, Willensdorfer, 2009, Amado et al., 2017). Experimental progress has been limited by a lack of suitable model systems. Taxonomic groups that contain both multicellular and unicellular species serve as good model system for testing specific hypotheses about the origin of multicellularity (Herron & Michod, 2008, Carr et al., 2008, Fairclough et al., 2010). Nevertheless, microbes provide a good model to investigate ecological and evolutionary questions, in this context. These organisms have short generation times and are easily maintained under controlled environmental conditions. All these properties allow for high reproducibility and high comparative power (Travisano, 2009). Several past studies have examined the evolution of multicellular snowflake clusters from unicellular yeast *S. cerevisiae* (Ratcliff et al., 2012, Ratcliff et al., 2013, Koschwanez et al., 2011a, Rebolleda-Gomez et al., 2012, Driscoll & Travisano, 2017, Jacobeen et al., 2018, Rebolleda-Gomez & Travisano, 2018, Kuzdzal-Fick et al., 2019, Herron et al., 2019). The studies have demonstrated that, under selection, the transition from unicellular to multicellularity is relatively quick (of the order of a few dozen generations).

In this study, we first evolved yeast multicellular clusters from unicellular yeast *S. cerevisiae* using settling as a selection force. The cluster is then reverse evolved. Would reversal of selection lead the population to re-trace its path on the sequence space to the ancestral phenotype? Or, would availability of a large number of genetic solutions, in response to a precisely opposite environmental selection, dictate that the reverse path is largely unpredictable? How do epistatic interactions in the genome play a role in dictating this dynamics? We show that tThe multicellular clusters perform better than the ancestor in presence of various stresses. Thereafter, we reverse evolve the clusters, using slow settling as the selection pressure. Our results show that while at the phenotypic level the kinetics of evolution of unicellularity does not trace the path that was taken to reach unicellularity to multicellularity. The genetic solutions that help accomplish this change are also distinct. Thus, reverse evolution in this context is dictated by epistatic interactions.

Materials and Methodology

Yeast strain and growth medium composition

Yeast *Saccharomyces cerevisiae* diploid SC644 (*MATa MEL1ade1 ile trp1-HIII ura3-52*) strain was used for the generation of multicellular snowflake clusters. Yeast populations were grown in test tubes (25 x 150 mm) containing liquid YPD (1% yeast extract, 2% peptone and 2% glucose) medium at 30°C. Unless stated otherwise, all cultures were grown in an orbital shaker at a speed of 250 rpm for 24 h.

Selection for multicellularity

Multicellular yeast clusters were generated as described by Ratcliff et al 2012 (Ratcliff et al., 2012). A single isogenic yeast colony was inoculated into 10 mL of YPD medium and incubated at 30°C for 24 h. The resulting culture was then used as a pre-inoculum for the initiation of the selection for multicellularity. Two different selection pressures were used to evolve multicellularity. In addition, a line with no selection pressure for multicellularity was also evolved as a control. In the first set, after growth for 24 h (30°C, 250 rpm), cells were allowed to settle at room temperature for 45 min. Thereafter, the bottom 100 µL from the culture tube was transferred into a tube containing fresh YPD and allowed to grow for the next 24 h. The process was then repeated for a period of six weeks. In the second set, an aliquot (1 mL) of overnight grown yeast culture was transferred to a 1.5 mL Eppendorf tube and then centrifuged at a speed of 100xg for 15 sec. The bottom 100 µL was transferred to a tube containing fresh YPD media and allowed to grow (30°C, 250 rpm) for 24 h. A daily transfer of bottom few microliters of yeast cells after centrifugation at low speed for up to 60 transfers resulted in the generation of multicellular yeast snowflake clusters. As a control, an independent line was evolved in YPD, from which, 100 µL cultures was transferred into fresh YPD after growth for 24 h (30°C, 24 h). In this control, the transfer was immediate without allowing the cells to settle down, either by gravity or by centrifugation. Six independent lines of each of the two selection pressure environments and the control were studied in this work. All lines were propagated for a period of 60 days. Glycerol freezer stocks were made for every fifth transfer in 30% glycerol and stored at -80°C for future use.

Selection for Unicellularity

The frozen glycerol stocks of multicellular clusters obtained after 60 transfers were streaked onto YPD plates and incubated at 30°C for 48 h. Cells from a single colony were spread on glass slide and a cover slip, and then studied under a microscope (give details of what kind of microscope) for the presence of

multicellular clusters. The multicellular clusters were then inoculated into fresh YPD medium and incubated at 30°C for 24 h with shaking. These cultures were used as ancestors for the reverse selection of unicellular phenotype from the multicellular cluster phenotype. As described above, an aliquot (1 mL) of overnight grown yeast culture was transferred to centrifuge tubes and centrifuged at a speed of 100xg for 15 sec. In this experiment, the top 100 µL was transferred into tubes containing fresh YDP medium every 24 h. Glycerol stocks were made for every tenth transfer in 30% glycerol and stored at -80°C for further use. The same process was followed to select for unicellularity for all the lines, under each selection pressure.

Characterization of phenotype

Each of the overnight grown (24 h) yeast population was serially diluted 10 fold with deionized water and spotted onto YPD plates. These plates were then incubated at 20°C, 30°C and 37°C for 48 h, at which point images of the plates were taken (Driscoll & Travisano, 2017).

Measurement of Growth curve

Overnight yeast cultures were adjusted to get an initial OD_{600} nm to 0.01. Then different concentrations of ethanol (0, 2.5%, 5%, and 10%) were added to the cultures and the cell growth was checked by measuring the OD at 600 nm using a spectrophotometer. OD_{600} values were determined for every 1h over a 30 h period.

Determination of percentage of yeast clusters

Overnight grown yeast clusters were diluted 1:10 in PBS. A volume of 10 µL was placed on a glass slide covered with coverslip. The number of cells and clusters present in 10 different fields of view was counted manually using a microscope (Labomed Binocular) and the percentage of yeast clusters present was counted.

Measurement of yeast Cluster size

Yeast populations were grown in 10 mL of fresh YPD media shaken at 250 rpm for 24 h. These overnight cultures were transferred to fresh YPD medium and grown until exponential phase. About 100 µL of exponentially growing cells were washed thrice with PBS and analyzed for FSC-A of each cells/cluster

using flow cytometer. About 20,000 clusters per population were analyzed and the average of the FSC-A at the indicated tome of transfer time was calculated (Tan et al., 2019).

Settling assay

Strains were inoculated from colonies into 10 mL YPD liquid cultures and grown overnight at 30°C. Each culture tube was vigorously vortexed and placed on a lab bench at room temperature to allow for settling. Images of each population during the settling were taken at different time intervals (0, 15, 30, and 60 min) (Hope & Dunham, 2014).

Stress resistance assay

Each of the yeast populations was grown at 30°C with 250 rpm shaking for 20 h. Cells were serially diluted to initial OD_{600} such as 3, 1, 0.3 and 0.1 and 4 µL was spotted onto YPD plates containing different stressors (acetic acid, H_2O_2, menadione, tBOOH). Plates were incubated at 30°C for 2 days and photos were taken.

In order to test the effect of cold and heat stress, a set of potted plates were also incubated at 20°C and 37°C, respectively. A single plate was incubated at 30°C as a control. Images of the plates were taken at different days of incubation.

Sequencing of ACE2 gene

Genomic DNA was isolated from each of the six replicate populations in the two settling selection regimens as described in Burst and Grabs' method (Harju et al., 2004). The purity of DNA was checked by Agarose gel electrophoresis and though spectrophotometer. PCR amplification target gene Activator of CUP1 Expression (ACE2) was done in a thermo cycler with cycling conditions: initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing temperature at 64°C for 30 s, extension at 72°C and final extension at 72°C for 10 min for a total of 30 cycles. Forward and reverse primers used for amplification were given in the following table. Following PCR, about 60 µL of amplified product was run on the gel. Then the PCR product on the gel was excised, purified using Thermo Scientific GeneJET Gel Extraction and used for sequencing.

Chemicals

Yeast extract and Peptone were procured from Himedia, Mumbai, India. Glucose was procured from Sigma Aldrich, USA. All the chemicals used in this study were of highest analytical grade.

Results and discussion

Evolution of multicellularity on serial propagation under centrifugal selection

Evolution of complex multicellular life began from the emergence of a life cycle involving the formation of cell clusters (Gao et al., 2019). In this study, we used adaptive laboratory evolution approach to evolve yeast multicellular snowflake clusters under centrifugal selection. Centrifugal settling selection pressure was chosen because it is an easy method to select for larger cluster size (Ratcliff et al., 2012). Moreover, it is also a selection pressure, which enables reversal of the selection pressure. Figure 1 shows the representative images of yeast multicellular cluster populations during centrifugal selection. By 60 transfers of gravitational selection, we note complex multicellular clusters in replicate populations (although cluster sizes vary across different lines). The dynamics of the change in phenomenology between the different independent lines is remarkably consistent with each other in four of the six lines; but not the other two. Such parallelism in evolutionary experiments has been reported in the past, and explaining it remains a major question (Bertels et al., 2019, Woods et al., 2006, Bailey et al., 2017). It has been suggested that even under extremely controlled environmental growth conditions in an incubator, the evolution of multicellular clusters generates spatial structure with increasing localized interactions and creates new environmental gradients (Koschwanez et al., 2011b). It is also suggested that chance and environmental changes play a key role in the evolution of multicellular clusters and these factors might account for the high degree of cluster complexity in yeast populations (Rebolleda-Gomez et al., 2012, Ratcliff et al., 2012).

Yeast unicellular ancestor and multicellular yeast colonies show different morphology

A key step in the evolution of multicellularity was a transition from single celled to multicellular forms, which necessitated the formation of simple cellular clusters(Kirk, 2005, Pfeiffer & Bonhoeffer, 2003). When streaked on YPD agar plates, the evolved lines exhibit an altered colony morphology. These plates were incubated at 30°C until we see single colonies and the images of the single colonies were taken. As shown in Figure 2, yeast ancestor and multicellular cluster colonies exhibited a distinct surface morphology. Yeast multicellular colonies showed rugose (rough) colony morphology (image is shown for one of the multicellular replicate populations), whereas unicellular ancestor showed a smooth morphology. This is a result of the cluster structure dictating the colony structure in the evolved lines.

Dynamics of changes in yeast cluster size with serial propagation under centrifugal selection

Centrifugal selection and selection for fast settlers favors the evolution of larger cluster size (Ratcliff et al., 2012) and dramatic divergence in cluster size evolves rapidly during the transition from unicellularity to multicellularity (Rebolleda-Gomez et al., 2012). Therefore, in this study the average cluster size of yeast

replicate populations was quantified as mean value of forward scatter area following every ten transfers using a flow cytometer. In our experimental study, the size of the yeast clusters increased with concomitant increase in transfer time in most of the replicate populations (MC1-4 and MC6, but not MC5) (Figure 3). An important question in evolutionary biology is to what extent repeatability of evolution or parallel evolution occurs. Parallel evolution is defined as the repeated evolution of the similar phenotype or genotype in populations that are evolved independently (Bertels et al., 2019). Previous studies have documented the parallel evolution in *Escherichia coli* (Fong et al., 2005, Cooper et al., 2003). In this study, we have noticed that multicellularity has evolved in all six replicate populations. However, it is also clearly evident from the Figure 3 that all multicellular clusters did not evolve to the same size, instead showed diversity in their size (Figure 3A), when compared to other replicate populations after 60 rounds of centrifugal selection. Microbial evolution experiments have demonstrated that diversity may depend largely on environmental conditions (Rainey & Travisano, 1998) and ecological interactions between different genotypes (Kerr et al., 2002).

Once cluster growth meets volumetric constraints (*i.e* cells experience volume limitations in larger clusters that allow cell death of older cells, apoptosis), further growth results in cell–cell scission, producing a relatively small cluster (termed as propagule) in which the oldest cell in the cluster is the genetic bottleneck (Ratcliff et al., 2015). It is reported that the mechanism of cluster formation in yeast is through post-division adhesion, but not through aggregation (Ratcliff et al., 2012). This method of growth ensures high relatedness among component cells of a multicellular cluster, therefore reducing internal conflicts. A previous study has demonstrated that new clusters arise by producing unicellular or multicellular propagules. Clusters reproduce by fragmentation especially when tension among cells in the cluster exceeds the tensile strength of cellular adhesion (Ratcliff et al., 2012). These two different modes of propagule reproduction are common in plants, whereas propagules in animals reproduce from a single cell (Grosberg & Strathmann, 1998). Apoptosis plays a major role in the development and somatic maintenance in metazoans and is also observed in unicellular *S. cerevisiae* (Meier et al., 2000, Carmona-Gutierrez et al., 2010). At the same time, it is suggested that the existence of apoptotic cellular suicide in the unicellular ancestors of metazoans may be an important preadaptation, facilitating the evolution of complex multicellularity (Huettenbrenner et al., 2003). Apoptotic cells act as break points within yeast multicellular clusters, allowing production of a greater number of new propagules (Ratcliff et al., 2012, Pentz et al., 2016).

Expectedly, the multicellular yeast clusters settles faster with increasing cluster size. It is evident from the Figure 3B that, four of the multicellular replicate populations (R1-R4 and R6) started settling faster after 40 transfers. However, replicate populations R5 showed moderate settling even after 60 transfers. The first snow flake yeast clusters formed are small during early transfer times, therefore they settle slowly. However, settling rate increased rapidly with the evolution of large cluster size during later stages of transfer times.

Yeast multicellular snow flake clusters show slower growth compared to unicellular counterpart

Multicellularity is associated with multiple benefits such as reduced predation and division of labor between cells. However these benefits come with costs. In multicellular organisms body size can have profound effects on resource use. The efficient exchange of nutrients is one of the important basic factors affecting the fitness of multicellular organisms. Multicellular clusters showed lower growth rates relative to their unicellular counterparts because of limitations of space and resource acquisition (Rebolleda-Gomez et al., 2012). The geometric constraints such as low surface area to volume ratio and of spatial organization in multicellular clusters limit the extent of interactions with the nutrient resources in the environment, and subsequently affects the metabolism, growth rate and viability (Solari et al., 2006). It has been suggested that there may be a trade-off between individual cluster size and growth rate (Rebolleda-Gomez et al., 2012). Therefore in the present study we have performed kinetic assay to investigate whether cluster phenotype has any effect on the growth curve. As shown in Figure 4, all multicellular cluster populations are slow growers when compared to unicellular counterpart. It is well know that as per the size-complexity rule, bigger entities have a greater division of labor than smaller ones (Bonner, 2004). Therefore it can be suggested that more number of cells in a multicellular cluster could lead to the slower growth of the cluster phenotype than the unicellular counterpart.

Yeast multicellular cluster phenotype confers resistance against stresses.

Heat stress

Previous studies have reported that wild type yeast cluster strains were resistant to free/thaw treatment (Kuzdzal-Fick et al., 2019) and the stress resistance was measured using a cell counter. In contrast, in the present study, we used spot assay to investigate the sensitivity of yeast cluster phenotype to cold (20°C) and heat stress (37°C). As evident from the Figure 5A, multicellular yeast cluster lines (MC 1-6) were found to be resistant to heat stress (37°C) when compared to unicellular ancestor. Even after five days of incubation, there is no significant growth of ancestor at indicated cell densities viz at OD_{600} of 0.3 and 0.1. Eukaryotes respond to heat stress by activating mitogen-activated protein kinase (MAPK) pathways. In the yeast *Saccharomyces cerevisiae*, two MAPK pathways regulate the response to stress (Gustin et al., 1998, Kamada et al., 1995). The cell wall integrity pathway, containing the MAPK Mpk1, is activated by heat stress. Heat stress mediated activation of yeast cell wall integrity pathway might be the possible reason for the benefit of multicellular cluster phenotype when compared to unicellular phenotype (Winkler et al., 2002). Cold stress has no effect on the survival of both unicellular and multicellular cell lines.

Solvent stress.

It is well established that ethanol as a potent inhibitor of the growth of microorganisms and its toxic effect on yeast is mainly caused by damaging the mitochondrial DNA and inactivation of enzymes such as hexokinase and dehydrogenase (Augustin et al., 1965). Therefore we wanted to investigate how multicellularity affects stress resistance to ethanol when compared to unicellularity. From the Figure 5B, it is clear that except 3rd cell line, remaining yeast multicellular clusters (MC 1, 2, 4-6) show better tolerance to ethanol (2.5%) when compared to unicellular yeast cell lines. None of the cultures could tolerate higher concentrations of ethanol (5% and 10%) (data not shown).

Mutations in ACE2 transcription factor leads to evolution of the multicellular phenotype.

It has been reported that *de novo* origin of snowflake yeast clusters is mainly due to a mutations in activator of CUP1 expression (ACE2) gene (a transcription factor) (Oud et al., 2013, Ratcliff et al., 2015). These experiments demonstrate that a single genetic mutation not only can create a new level of biological organization, but can also facilitate the evolution of higher-level complexity (Ratcliff et al., 2015). All six lines from our experiments acquired mutations in the ACE2 gene. All lines had insertions or deletions in the coding region of the gene, implying that no functional copies of ACE2 were being produced in the cell. As a check of this, when the mutant versions of the ACE2 were shifted to the parent genotype, the multicellular phenotype was recovered. Thus, mutations in ACE2 is a key molecular determinant in dictating the evolution of the multicellular phenotype.

Does the centrifugal selection favor the reverse evolution from multicellular cluster to unicellularity?

Clustering of cells is likely also disadvantageous, because multicellular organisms require more resources and take longer to mature than unicellular organisms. Compared with their unicellular counterparts, clustering cells face locally increased cell densities resulting increased competition for local resources (Pfeiffer & Bonhoeffer, 2003). Further, their complexity makes them more vulnerable to disruption (2014). On other hand one of the evolutionary questions that have not attracted much attention is whether reverse evolution from multicellularity to unicellularity is possible or not. The HeLa cell line (Cervical cancer cells) presents an interesting example of the evolution of unicellularity from a human ancestor. HeLa cells are exceptionally adapted to life as a single-celled microbe (Skloot, 2010, 1991). However, lack of suitable environments where exact and opposite selection forces can be applied to an organism made it difficult to study the reversibility of multicellularity.

In the present study, we aimed at investigating the reverse evolution from multicellularity to unicellularity using experimental evolution, under the same centrifugal selection pressure. Growing the multicellular cells the same way, and after the same processing, the cells from the topmost layer of the liquid were transferred to the next tube. In this way, the selection pressure in the experiment was exactly the opposite of the one applied in the uni- to multicellular evolution. This reverse evolution experiment lead to several physiological changes.

Firstly, the average cell size in the clusters decreased rapidly and unicellular lines were evolved (Figure 6). As a result, the colony morphology was restored from rugose to smooth colony morphology during reverse evolution from multicellularity to unicellularity. As shown in the Figure, a transition from rough surfaced colony morphology to smooth surfaced colony morphology was noticed. It has been suggested that both genotypic and phenotypic factors can affect the colony morphology. Some cellular properties, such as cell adhesion, and budding pattern, have also been associated with morphological changes in *S. cerevisiae* (Gil de Prado et al., 2014). Second, the growth kinetics of the reverse evolved lines was restored closer to that of the ancestral strain (Figure 6B). Thus is likely due to the transport limitations being eliminated in the reverse evolved unicellular lines.

Reverse evolution is not due to mutations in ACE2 sequence.

The trajectory of the cluster size in the reverse evolution is shown in Figure 7. The line 1-4 were reverse evolved and the dynamics of the cluster size with the transfer number compared with the forward evolution experiment. As shown, the transition from multicellular to unicellular phenotype has a trajectory which is distinct from the original transition from unicellular to multicellular phenotype. To understand the molecular basis which trigger the reverse evolution, we sequenced the ACE2 region of the reverse evolved lines. However, none of the 4 lines had acquired a mutation in the ACE2 region, when compared to the corresponding sequence of the multicellular cluster that each line had started from. Thus, the evolutionary transition from the multicellular to the unicellular phenotype is brought about by mutations elsewhere on the chromosome, via epistatic interactions.

Conclusion

In this work, we report forward and reverse evolution between unicellularity and multicellularity in the yeast *S. cerevisiae*. In this context, selection of settling under gravity or centrifugation provides an environment where study of evolution, when selection is reversed can be studied. As has been reported previously, multicellularity readily evolves under the appropriate selection pressure. Of our six lines, four of them exhibit dynamics of cluster size increase quite close to each other. To that effect, the evolution of multicellularity is a repeatable and predictable process. Somewhat more surprisingly, all six lines acquire mutation at a single locus in the genome. This single set of mutations in the ACE2 gene is responsible for exhibition of the multicellularity trait. The mutations at this locus suggests that the selective advantage associated with an ACE2 mutation is very high. On reversal of the selection pressure, all four lines exhibit reversal of the multicellular phenotype. While the phenotype is completely reversed in these line, the trajectory of the change in the cluster size does not mimic the forward evolution experiment. Moreover, sequencing the ACE2 region in the reverse evolved lines tells us that the reversed-evolved lines do not have a functional ACE2 gene. This tells us that the molecular basis of the reverse evolution experiment is at another locus/loci on the genome. Studying this genetic determinant, and its possible epistatic/redundant interactions with ACE2 is the next step in this work.

Our results also show that although the selection pressure of gravity lead to evolution of multicellularity, the multicellular phenotype confers advantages in the face of several environmental stresses too. Thus, in the context of evolution of multicellularity during the evolution, any of these stresses could provide an avenue for evolution of multicellularity.

From our reverse evolution experiment, we note that the dynamic trajectory of reverse evolution is different from that of the forward one. In the context of reverse evolution, what is the genetics at the organismal level, which constrains this evolutionary process? (Travisano et al., 1995, Losos et al., 1998). If we assume that the strength of selection is reversed exactly in the reverse experiment, then what could lead to the difference in the trajectories? Several factors could be responsible for this. First, with the evolution of multicellularity, the size of the population in the culture media changes. Population size is an important determinant of the rate of adaptation (Neher et al., 2010). Second, mutation rates across the genome are known to be variable (Chattopadhyay et al., 2009). Hence, since in this case, the molecular determinants of the forward and reverse evolution are different, these might be subject to different mutation rates. Third, the number of adaptive mutations facilitating forward and reverse evolution might be different, and as a result, the time of transition from one state to the other may be different. What role, if any, does epistasis play in this process is also not known and can be a determinant in dictating the evolutionary trajectory of the cluster size (Phillips, 2008). Adaptation to an environment also leads to establishment to newer epistatic interactions in the genome, which is likely to mitigate the effect of previous adaptive changes (Gros et al., 2009, Anholt, 2020). Finally, how cluster size relates with the accumulation of mutations is not yet known. The relationship between these two variables in the forward and reverse evolution experiment need not be

the same. Determining the answers to these questions related to the forward and backward adaptive processes forms the basis of the future work in this direction.

Conflict of interest

Authors declare that there is no conflict of interest.

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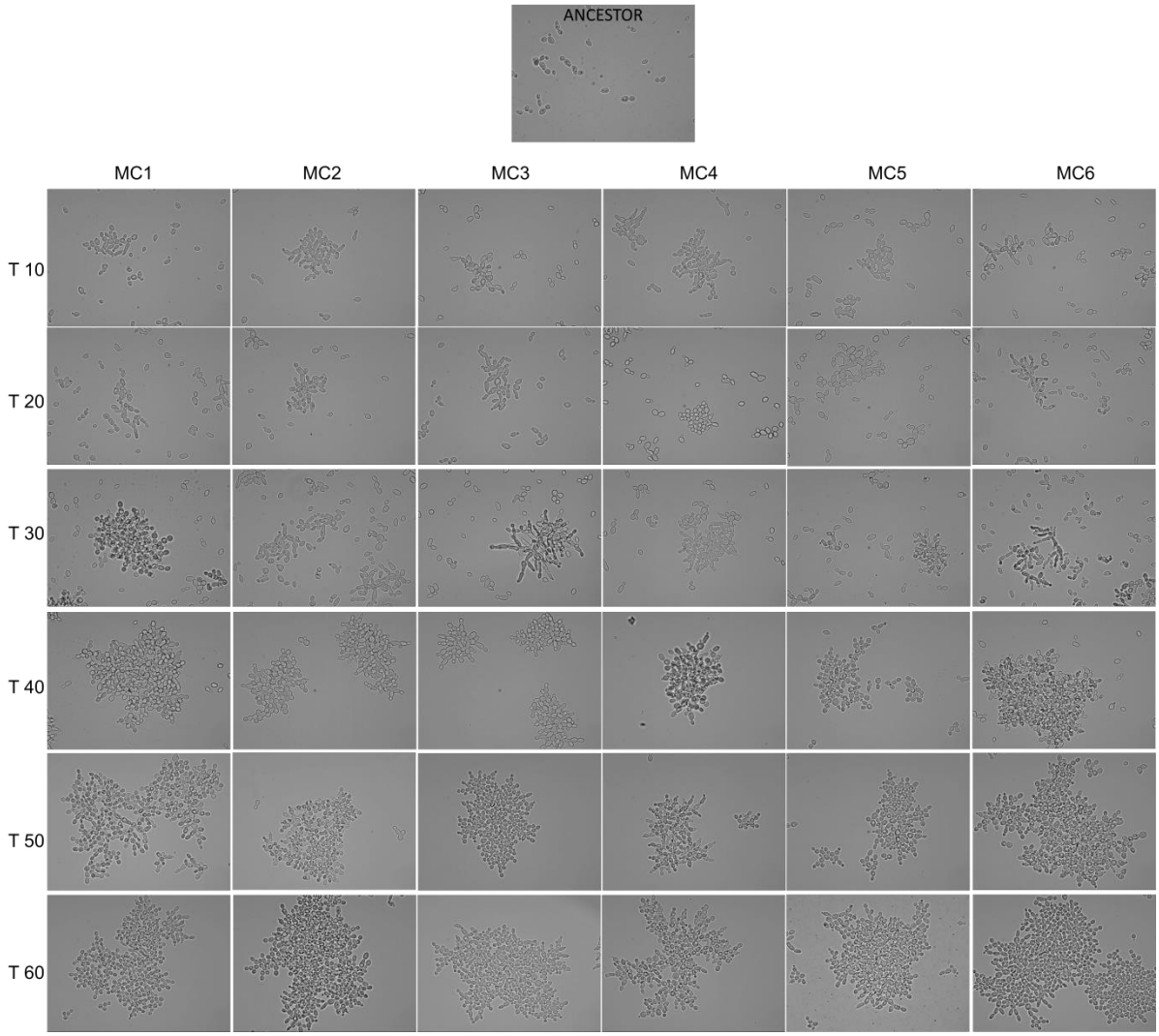


Figure 1. The representative images of yeast multicellular cluster populations during centrifugal selection. An aliquot of overnight grown yeast cultures (both ancestor and multicellular cluster populations (MC1-MC6) from different transfer times (T10-T60) were placed on a microscopic slide covered with a cover slip. Images were taken using a microscope under 40X magnification.

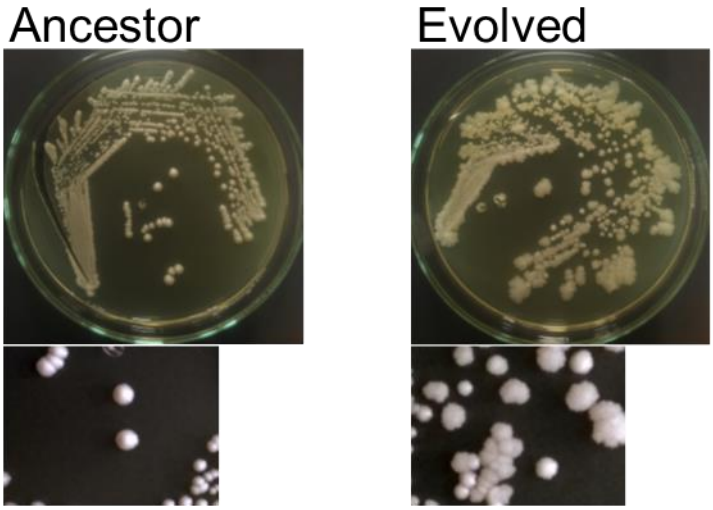
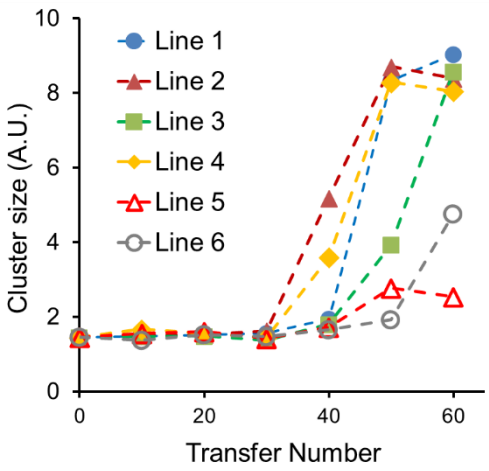


Figure 2. Smooth and rugose (rough) morphologies of yeast unicellular and multicellular colonies, respectively. Overnight grown yeast cultures (both unicellular ancestor and multicellular cluster populations) were streaked on to YPD agar plates. Plates were allowed to incubate at 30°C until single colonies appear and images of the plates were taken.

A



B

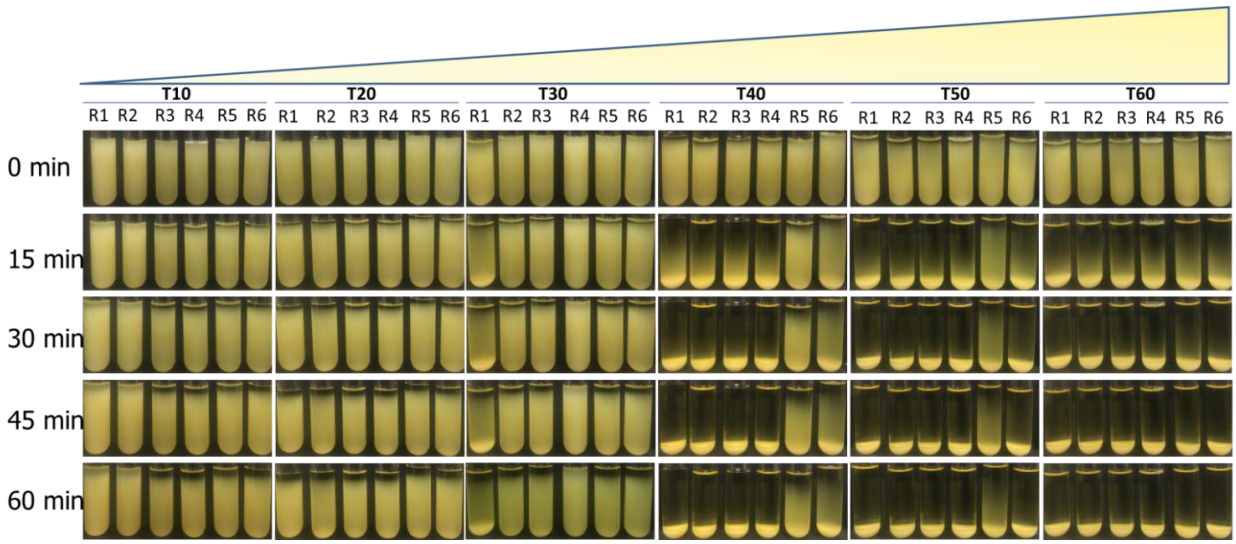
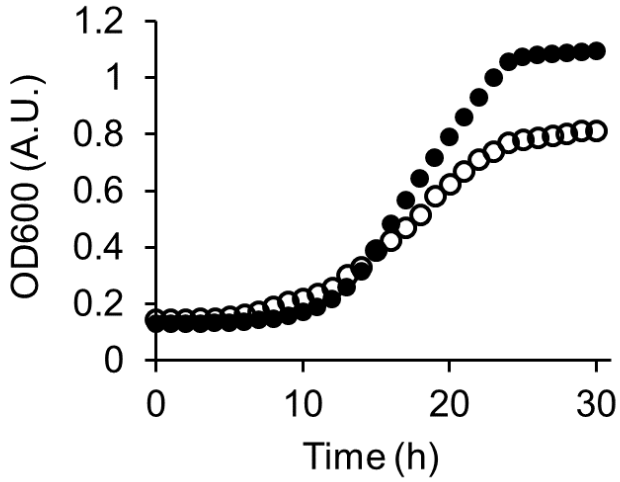


Figure 3. (A) Dynamics of changes in yeast cluster size after 60 rounds of centrifugal selection. Exponentially growing yeast multicellular cluster populations. Cells were washed with PBS and analyzed for FSC-A of each cells/cluster using flow cytometer. About 20,000 clusters per population were analyzed and the average of the FSC-A at the indicated time of transfer was calculated**.** Standard error of the mean in the cluster size variation in each line was less than 0.65%. **(B)** Settling of multicellular cell lines.



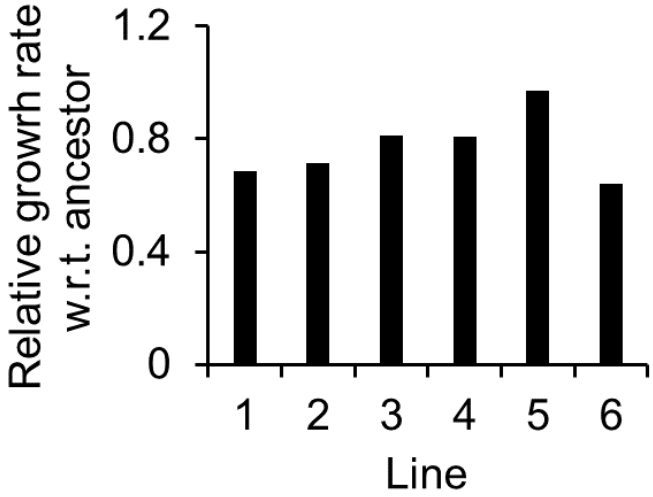
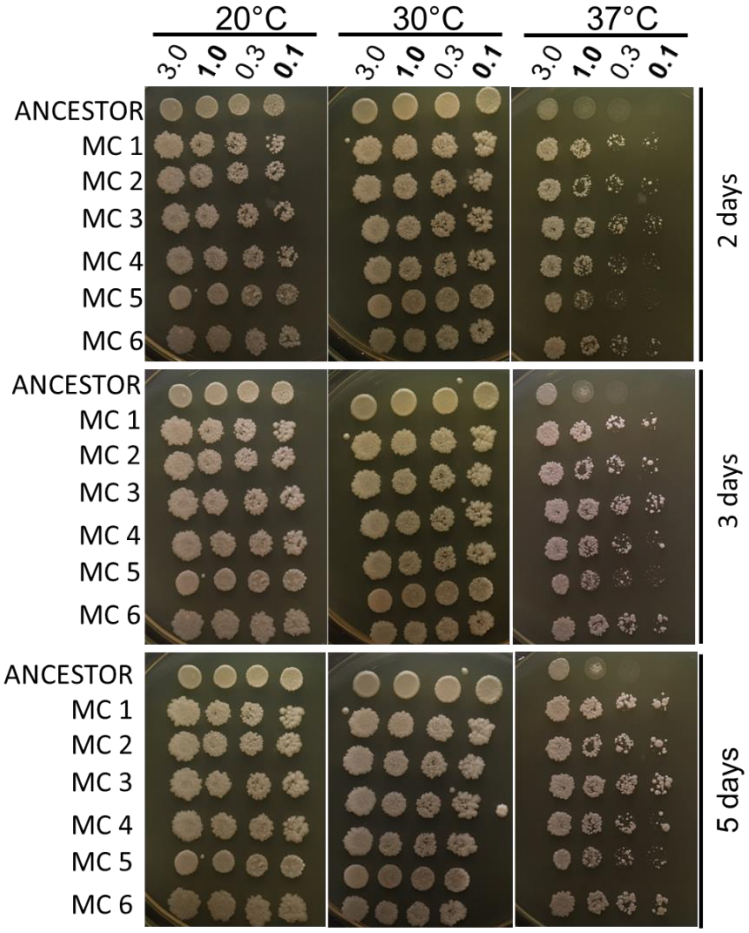


Figure 4. Growth curves of yeast cultures. Yeast Yeast multicellular cluster populations (A-F represents six replicate multicellular cluster populations MC1-MC6, respectively) show slow growth compared to unicellular ancestor phenotype. Overnight yeast clusters and ancestor lines were diluted to an initial OD_{600} of 0.1 and the optical density was measured for every 1 h. The data represents average of six independent repeats. The standard deviation of the data is less than 3% for all six lines.

(A)



(B)

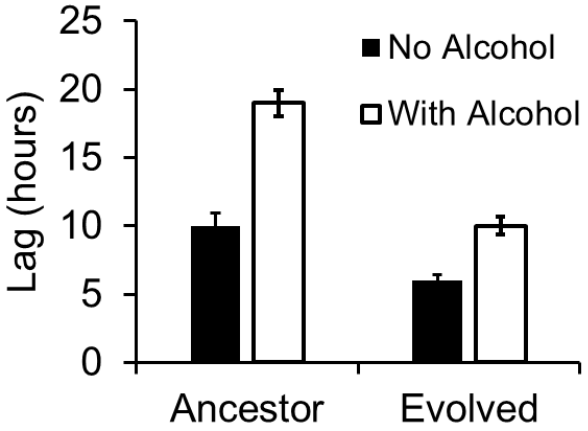


Figure 5. (A) Yeast multicellular cluster phenotype confers heat stress resistance. Overnight yeast clusters and ancestor lines were diluted to OD_{600} of 3, 1, 0.3 and 0.1 and spotted on to YPD plates. Plates were incubated at indicated temperatures and images were taken at different days. MC=multicellular cluster

population. **(B) Effect of ethanol on the growth curves of yeast cultures.** Yeast Yeast multicellular cluster populations (A-F represents six replicate multicellular cluster populations MC1-MC6, respectively) shows resistance to ethanol stress compared to unicellular ancestor phenotype. Overnight yeast clusters and ancestor lines were diluted to an initial OD_{600} of 0.1 and exposed to ethanol (2.5 percent). Then the optical density was measured for every 1 h.

(A)

(B)

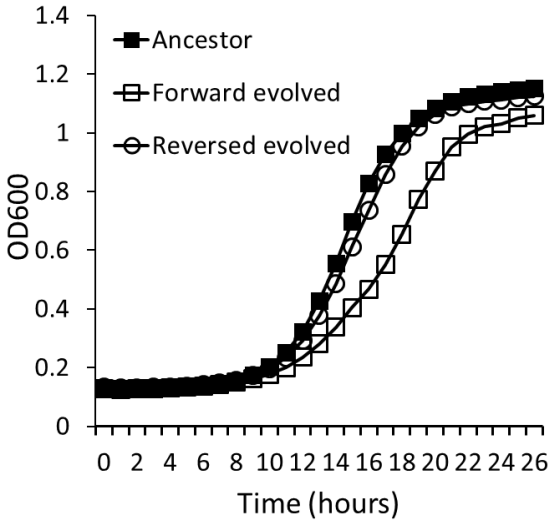


Figure 6. (A) Reversion of rough colony morphology to smooth colony morphology during reverse evolution from multicellularity to unicellularity (one of the six lines is shown)**.** Overnight grown yeast cultures (both multicellular cluster population and population evolved from it during reverse evolution) were streaked on to YPD agar plates. Plates were allowed to incubate at 30°C until single colonies appear and images of the plates were taken. **(B)** Growth kinetics of ancestor, forward-, and reverse-evolved line. Data for one of the six lines is shown. The data is average for six independent experiments. The standard deviation for the data is less than 3% of all data points.

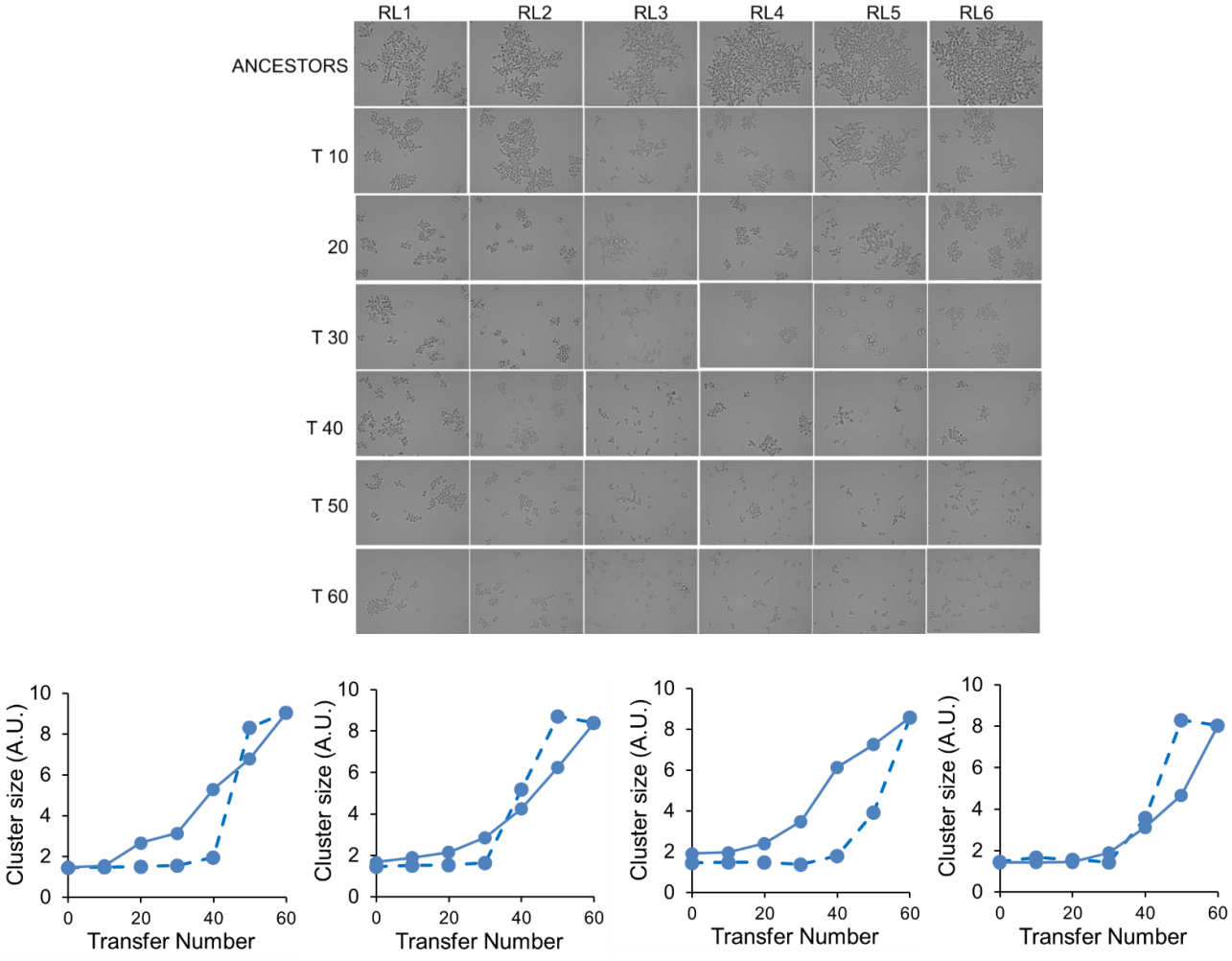


Figure 7. **(Top)** The representative images of yeast populations during reverse evolution from multicellularity to unicellularity. An aliquot of overnight grown yeast cultures (including multicellular ancestor cluster populations) from different transfer times (T10-T60) were placed on a microscopic slide covered with a cover slip. Images were taken using a microscope under 40X magnification. **(Bottom)** The kinetic trajectory of cluster size during forward (dashed lines) and reverse-evolution (solid lines).

References

Abedin, M. & King, N. 2008. The premetazoan ancestry of cadherins. *Science (New York, N.Y.)* **319**: 946-948.

Amado, A., Batista, C. & Campos, P. R. A. 2017. A mechanistic model for the evolution of multicellularity. *bioRxiv*: 115832.

Anholt, R. R. H. 2020. Evolution of epistatic networks and the genetic basis of innate behaviors. *Trends Genet* **36**: 24-29.

Augustin, H. W., Kopperschläger, G., Steffen, H. & Hofmann, E. 1965. Hexokinase as limiting factor of anaerobic glucose consumption of saccharomyces carlsbergensis ncyc74. *Biochimica et biophysica acta* **110**: 437-439.

Bailey, S. F., Blanquart, F., Bataillon, T. & Kassen, R. 2017. What drives parallel evolution?: How population size and mutational variation contribute to repeated evolution. *Bioessays* **39**: 1-9.

Bertels, F., Leemann, C., Metzner, K. J. & Regoes, R. R. 2019. Parallel evolution of hiv-1 in a long-term experiment. *Molecular Biology and Evolution* **36**: 2400-2414.

Bonner, J. T. 1998. The origins of multicellularity. *Integrative Biology: Issues, News, and Reviews* **1**: 27-36.

Bonner, J. T. 2004. Perspective: The size-complexity rule. *Evolution; international journal of organic evolution* **58**: 1883-1890.

Boraas, M. E., Seale, D. B. & Boxhorn, J. E. 1998. Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. *Evolutionary Ecology* **12**: 153-164.

Bull, J. J., Badgett, M. R., Wichman, H. A., Huelsenbeck, J. P., Hillis, D. M., Gulati, A., Ho, C. & Molineux, I. J. 1997. Exceptional convergent evolution in a virus. *Genetics* **147**: 1497-507.

Carmona-Gutierrez, D., Eisenberg, T., Büttner, S., Meisinger, C., Kroemer, G. & Madeo, F. 2010. Apoptosis in yeast: Triggers, pathways, subroutines. *Cell death and differentiation* **17**: 763-773.

Carr, M., Leadbeater, B. S. C., Hassan, R., Nelson, M. & Baldauf, S. L. 2008. Molecular phylogeny of choanoflagellates, the sister group to metazoa. *Proceedings of the National Academy of Sciences* **105**: 16641-16646.

Chattopadhyay, S., Weissman, S. J., Minin, V. N., Russo, T. A., Dykhuizen, D. E. & Sokurenko, E. V. 2009. High frequency of hotspot mutations in core genes of escherichia coli due to short-term positive selection. *Proc Natl Acad Sci U S A* **106**: 12412-7.

Chothia, C., Gough, J., Vogel, C. & Teichmann, S. A. 2003. Evolution of the protein repertoire. *Science (New York, N.Y.)* **300**: 1701-1703.

Cooper, T. F., Rozen, D. E. & Lenski, R. E. 2003. Parallel changes in gene expression after 20,000 generations of evolution in escherichiacoli. *Proc Natl Acad Sci U S A* **100**: 1072-7.

Crill, W. D., Wichman, H. A. & Bull, J. J. 2000. Evolutionary reversals during viral adaptation to alternating hosts. *Genetics* **154**: 27-37.

Darwin, C. 1859. *On the origin of species by means of natural selection, or preservation of favoured races in the struggle for life*. London : John Murray, 1859.

Dollo, L. 1893. The laws of evolution. *BULLETIN DE LA SOCIÉTÉ BELGE DE GÉOLOGIE* **VII**: 164-166.

Driscoll, W. W. & Travisano, M. 2017. Synergistic cooperation promotes multicellular performance and unicellular free-rider persistence. *Nat Commun* **8**: 15707.

Duncan, A. B., Fellous, S. & Kaltz, O. 2011. Reverse evolution: Selection against costly resistance in disease-free microcosm populations of paramecium caudatum. **65**: 3462-3474.

Durrant, S. D. 1954. Simpson, george gaylord. The major features of evolution. Columbia university press, new york city, new york. Xx plus 434 pp., 52 figs., 28 tables. 1953. Price $7.50. *Journal of Mammalogy* **35**: 600-601.

Fairclough, S. R., Dayel, M. J. & King, N. 2010. Multicellular development in a choanoflagellate. *Current Biology* **20**: R875-R876.

559 Ferrier, D. E. & Holland, P. W. 2001. Ancient origin of the hox gene cluster. *Nature reviews. Genetics* **2**: 33-
560 38.
561 Fong, S. S., Joyce, A. R. & Palsson, B. 2005. Parallel adaptive evolution cultures of escherichia coli lead to
562 convergent growth phenotypes with different gene expression states. *Genome Res* **15**: 1365-72.
563 Gao, Y., Traulsen, A. & Pichugin, Y. 2019. Interacting cells driving the evolution of multicellular life cycles.
564 *PLoS computational biology* **15**: e1006987-e1006987.
565 Gil de Prado, E., Rivas, E.-M., de Silóniz, M.-I., Diezma, B., Barreiro, P. & Peinado, J. M. 2014. Quantitative
566 analysis of morphological changes in yeast colonies growing on solid medium: The eccentricity
567 and fourier indices. *Yeast* **31**: 431-440.
568 Gros, P. A., Le Nagard, H. & Tenaillon, O. 2009. The evolution of epistasis and its links with genetic
569 robustness, complexity and drift in a phenotypic model of adaptation. *Genetics* **182**: 277-93.
570 Grosberg, R. K. & Strathmann, R. R. 1998. One cell, two cell, red cell, blue cell: The persistence of a
571 unicellular stage in multicellular life histories. *Trends in ecology & evolution* **13**: 112-116.
572 Grosberg, R. K. & Strathmann, R. R. 2007. The evolution of multicellularity: A minor major transition?
573 *Annual Review of Ecology, Evolution, and Systematics* **38**: 621-654.
574 Gustin, M. C., Albertyn, J., Alexander, M. & Davenport, K. 1998. Map kinase pathways in the yeast
575 saccharomyces cerevisiae. *Microbiol Mol Biol Rev* **62**: 1264-300.
576 Harju, S., Fedosyuk, H. & Peterson, K. R. 2004. Rapid isolation of yeast genomic DNA: Bust n' grab. *BMC*
577 *Biotechnology* **4**: 8.
578 Herron, M. D., Borin, J. M., Boswell, J. C., Walker, J., Chen, I. C. K., Knox, C. A., Boyd, M., Rosenzweig, F. &
579 Ratcliff, W. C. 2019. De novo origins of multicellularity in response to predation. *Scientific Reports*
580 **9**: 2328.
581 Herron, M. D. & Michod, R. E. 2008. Evolution of complexity in the volvocine algae: Transitions in
582 individuality through darwin's eye. *Evolution* **62**: 436-451.
583 Hope, E. A. & Dunham, M. J. 2014. Ploidy-regulated variation in biofilm-related phenotypes in natural
584 isolates of saccharomyces cerevisiae. *G3 (Bethesda)* **4**: 1773-86.
585 Huettenbrenner, S., Maier, S., Leisser, C., Polgar, D., Strasser, S., Grusch, M. & Krupitza, G. 2003. The
586 evolution of cell death programs as prerequisites of multicellularity. *Mutation research* **543**: 235-
587 249.
588 Ispolatov, I., Ackermann, M. & Doebeli, M. 2012. Division of labour and the evolution of multicellularity.
589 *Proceedings. Biological sciences* **279**: 1768-1776.
590 Jacobeen, S., Graba, E. C., Brandys, C. G., Day, T. C., Ratcliff, W. C. & Yunker, P. J. 2018. Geometry, packing,
591 and evolutionary paths to increased multicellular size. *Phys Rev E* **97**: 050401.
592 Kaltenbach, M., Jackson, C. J., Campbell, E. C., Hollfelder, F. & Tokuriki, N. 2015. Reverse evolution leads
593 to genotypic incompatibility despite functional and active site convergence. *Elife* **4**.
594 Kamada, Y., Jung, U. S., Piotrowski, J. & Levin, D. E. 1995. The protein kinase c-activated map kinase
595 pathway of saccharomyces cerevisiae mediates a novel aspect of the heat shock response. *Genes*
596 *Dev* **9**: 1559-71.
597 Kerr, B., Riley, M. A., Feldman, M. W. & Bohannan, B. J. M. 2002. Local dispersal promotes biodiversity in
598 a real-life game of rock-paper-scissors. *Nature* **418**: 171-174.
599 Kessin, R. H., Gundersen, G. G., Zaydfudim, V. & Grimson, M. 1996. How cellular slime molds evade
600 nematodes. *Proceedings of the National Academy of Sciences* **93**: 4857-4861.
601 King, N. 2004. The unicellular ancestry of animal development. *Developmental cell* **7**: 313-325.
602 King, N. & Carroll, S. B. 2001. A receptor tyrosine kinase from choanoflagellates: Molecular insights into
603 early animal evolution. *Proceedings of the National Academy of Sciences* **98**: 15032-15037.
604 Kirk, D. L. 2005. A twelve-step program for evolving multicellularity and a division of labor. *BioEssays :*
605 *news and reviews in molecular, cellular and developmental biology* **27**: 299-310.

606 Koonin, E. V., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Krylov, D. M., Makarova, K. S., Mazumder, R.,
607 Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Rogozin, I. B., Smirnov, S., Sorokin, A. V., Sverdlov,
608 A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J. & Natale, D. A. 2004. A comprehensive evolutionary
609 classification of proteins encoded in complete eukaryotic genomes. *Genome biology* **5**: R7-R7.
610 Koschwanez, J. H., Foster, K. R. & Murray, A. W. 2011a. Sucrose utilization in budding yeast as a model for
611 the origin of undifferentiated multicellularity. *PLoS Biol* **9**: e1001122.
612 Koschwanez, J. H., Foster, K. R. & Murray, A. W. 2011b. Sucrose utilization in budding yeast as a model for
613 the origin of undifferentiated multicellularity. *PLoS biology* **9**: e1001122-e1001122.
614 Kusserow, A., Pang, K., Sturm, C., Hrouda, M., Lentfer, J., Schmidt, H. A., Technau, U., von Haeseler, A.,
615 Hobmayer, B., Martindale, M. Q. & Holstein, T. W. 2005. Unexpected complexity of the wnt gene
616 family in a sea anemone. *Nature* **433**: 156-160.
617 Kuzdzal-Fick, J. J., Chen, L. & Balázsi, G. 2019. Disadvantages and benefits of evolved unicellularity versus
618 multicellularity in budding yeast. *Ecology and evolution* **9**: 8509-8523.
619 Larsson, P. O. 1992. Cost-effectiveness of misoprostol prevention. *Lakartidningen* **89**: 3657.
620 Lenski, R. E. 1988a. Experimental studies of pleiotropy and epistasis in escherichia coli. I. Variation in
621 competitive fitness among mutants resistant to virus t4.
622 Lenski, R. E. 1988b. Experimental studies of pleiotropy and epistasis in escherichia coli. Ii. Compensation
623 for maladaptive effects associated with resistance to virus t4.
624 Lewontin, R. C. 1966. Is nature probable or capricious? *BioScience* **16**: 25-27.
625 Losos, J. B., Jackman, T. R., Larson, A., Queiroz, K. & Rodriguez-Schettino, L. 1998. Contingency and
626 determinism in replicated adaptive radiations of island lizards. *Science* **279**: 2115-8.
627 Meier, P., Finch, A. & Evan, G. 2000. Apoptosis in development. *Nature* **407**: 796-801.
628 Michod, R. E. 1997. Cooperation and conflict in the evolution of individuality. I. Multilevel selection of the
629 organism. *The American Naturalist* **149**: 607-645.
630 MULLER, H. J. 1939. Reversibility in evolution considered from the standpoint of genetics1. **14**: 261-280.
631 Neher, R. A., Shraiman, B. I. & Fisher, D. S. 2010. Rate of adaptation in large sexual populations. *Genetics*
632 **184**: 467-81.
633 Nichols, S. A., Dirks, W., Pearse, J. S. & King, N. 2006. Early evolution of animal cell signaling and adhesion
634 genes. *Proceedings of the National Academy of Sciences of the United States of America* **103**:
635 12451-12456.
636 Okasha, S. 2005. Multilevel selection and the major transitions in evolution. *Philosophy of Science* **72**:
637 1013-1025.
638 Oud, B., Guadalupe-Medina, V., Nijkamp, J. F., de Ridder, D., Pronk, J. T., van Maris, A. J. & Daran, J. M.
639 2013. Genome duplication and mutations in ace2 cause multicellular, fast-sedimenting
640 phenotypes in evolved saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **110**: E4223-31.
641 Pentz, J. T., Taylor, B. P. & Ratcliff, W. C. 2016. Apoptosis in snowflake yeast: Novel trait, or side effect of
642 toxic waste? *J R Soc Interface* **13**.
643 Pfeiffer, T. & Bonhoeffer, S. 2003. An evolutionary scenario for the transition to undifferentiated
644 multicellularity. *Proceedings of the National Academy of Sciences* **100**: 1095-1098.
645 Phillips, P. C. 2008. Epistasis--the essential role of gene interactions in the structure and evolution of
646 genetic systems. *Nat Rev Genet* **9**: 855-67.
647 Rainey, P. B. & Travisano, M. 1998. Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69-
648 72.
649 Ratcliff, W. C., Denison, R. F., Borrello, M. & Travisano, M. 2012. Experimental evolution of multicellularity.
650 *Proc Natl Acad Sci U S A* **109**: 1595-600.
651 Ratcliff, W. C., Fankhauser, J. D., Rogers, D. W., Greig, D. & Travisano, M. 2015. Origins of multicellular
652 evolvability in snowflake yeast. *Nat Commun* **6**: 6102.

Ratcliff, W. C., Pentz, J. T. & Travisano, M. 2013. Tempo and mode of multicellular adaptation in experimentally evolved saccharomyces cerevisiae. *Evolution* **67**: 1573-1581.

Rebolleda-Gomez, M., Ratcliff, W. & Travisano, M. 2012. *Adaptation and divergence during experimental evolution of multicellular saccharomyces cerevisiae*.

Rebolleda-Gomez, M. & Travisano, M. 2018. The cost of being big: Local competition, importance of dispersal, and experimental evolution of reversal to unicellularity. *Am Nat* **192**: 731-744.

Rokas, A. 2008. The origins of multicellularity and the early history of the genetic toolkit for animal development. *Annual review of genetics* **42**: 235-251.

Schrag, S. J., Perrot, V. & Levin, B. R. 1997. Adaptation to the fitness costs of antibiotic resistance in escherichia coli. *Proc Biol Sci* **264**: 1287-91.

Skloot, R. 2010. *The immortal life of henrietta lacks*. Crown Publishers.

Smukalla, S., Caldara, M., Pochet, N., Beauvais, A., Guadagnini, S., Yan, C., Vinces, M. D., Jansen, A., Prevost, M. C., Latgé, J.-P., Fink, G. R., Foster, K. R. & Verstrepen, K. J. 2008. Flo1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell* **135**: 726-737.

Solari, C. A., Ganguly, S., Kessler, J. O., Michod, R. E. & Goldstein, R. E. 2006. Multicellularity and the functional interdependence of motility and molecular transport. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 1353-1358.

Tan, J., He, Q., Pentz, J. T., Peng, C., Yang, X., Tsai, M. H., Chen, Y., Ratcliff, W. C. & Jiang, L. 2019. Copper oxide nanoparticles promote the evolution of multicellularity in yeast. *Nanotoxicology*: 1-9.

Teotónio, H., Matos, M. & Rose, M. R. 2002. Reverse evolution of fitness in drosophila melanogaster. **15**: 608-617.

Teotónio, H. & Rose, M. R. 2000. Variation in the reversibility of evolution. *Nature* **408**: 463-466.

Travisano, M. (2009) Long-term experimental evolution and adaptive radiation. In: *Experimental evolution: Concepts, methods, and applications of selection experiments.* pp. 111-133.

Travisano, M., Mongold, J. A., Bennett, A. F. & Lenski, R. E. 1995. Experimental tests of the roles of adaptation, chance, and history in evolution. *Science* **267**: 87-90.

Váchová, L., Chernyavskiy, O., Strachotová, D., Bianchini, P., Burdíková, Z., Ferčíková, I., Kubínová, L. & Palková, Z. 2009. Architecture of developing multicellular yeast colony: Spatio-temporal expression of ato1p ammonium exporter. *Environmental Microbiology* **11**: 1866-1877.

West, S. A., Fisher, R. M., Gardner, A. & Kiers, E. T. 2015. Major evolutionary transitions in individuality. *Proceedings of the National Academy of Sciences* **112**: 10112-10119.

Willensdorfer, M. 2009. On the evolution of differentiated multicellularity. *Evolution* **63**: 306-323.

Winkler, A., Arkind, C., Mattison, C. P., Burkholder, A., Knoche, K. & Ota, I. 2002. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryotic cell* **1**: 163-173.

Wolpert, L. & Szathmáry, E. 2002. Multicellularity: Evolution and the egg. *Nature* **420**: 745-745.

Woods, R., Schneider, D., Winkworth, C. L., Riley, M. A. & Lenski, R. E. 2006. Tests of parallel molecular evolution in a long-term experiment with escherichia coli. *Proceedings of the National Academy of Sciences* **103**: 9107-9112.