Supporting Information

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SI Materials and Methods

Gravitational Selection, Experiment 1. Ten replicate populations of initially genetically uniform dioloid *Saccharomyces cerevisiae* strain Y55 were grown in 10-mL aliquots of Yeast Peptone Dextrose (YPD; per liter: 10 g yeast extract, 20 g peptone, 20 g dextrose, pH 5.8) in 25- × 150-mm glass culture tubes at 30 °C, shaking at 250 × *g*. Every 24 h, the entire population was transferred to a 16- × 150-mm sterile glass tube and allowed to settle on the bench for 45 min, and the bottom 100 μL was transferred to the next 25-mm culture tube containing fresh rich media. After 7 d, the transfer step was made more efficient: 1.5 mL of each culture was removed and centrifuged at 100 × *g* for 10 s, and then the bottom 100 μL was transferred to the next 25-mm culture tube containing fresh media.

Gravitational Selection, Experiment 2. To examine the relative fitness of snowflake-phenotype yeast vs. unicellular yeast, we repeated experiment 1 (using 100 × *g* centrifugations to impose settling selection) on five replicate populations of the ancestral strain of Y55. In a parallel treatment, five replicate populations were transferred without gravitational settling. For both this and the previous experiment, a single, representative isolate was obtained from each replicate population after 60 transfers by picking and restreaking a single colony on YPD petri plates three times.

Relative Fitness of Unicellular vs. Multicellular Genotypes. Five snowflake-phenotype or unicellular single-strain isolates (experiment 2, above) were grown for 24 h in YPD and then diluted by a factor of 1/200 into 10 mL YPD (in 25- × 150-mm tubes) along with a common competitor: GFP-labeled unicellular Y55, also diluted by 1/200 from an overnight culture. After 24 h of growth, 100 μL from each tube was transferred to fresh media, either with or without selection imposed by centrifugation (100 × *g* for 10 s), and grown for another 24 h. The population size of both test and reference strains was determined at time 0 and after 48 h growth by imaging five predetermined fields of view in a hemocytometer for each replicate population. Image analysis in ImageJ was used to count the number of clusters with more than seven cells in brightfield illumination, and GFP-labeled unicells were counted by fluorescence microscopy. We determined the number of evolved unicellular cells by subtracting the number of GFP-marked cells from a count of the total number of unicells in the field of view. Malthusian growth parameters for each test strain relative to the common competitor were determined following the method of Lenski et al. (1). For this assay, we measured the fold increase in individuals (either multicellular clusters or individual cells for the snowflake and unicellular genotypes, respectively). Our a priori hypotheses were that clustering would increase settling rate, and thus fitness, when transferred with gravitational selection, but would decrease the rate at which nutrients and oxygen are absorbed by interior cells, resulting in reduced fitness in the absence of gravitational selection. Significance was thus tested with one-sided *t* tests.

Divergent Selection for Settling Rate. A single population of snowflake-phenotype yeast (from experiment 1, replicate population 1, 30 transfers) was put under divergent selection for settling rate. Cell culture was performed as above. Three replicate populations per treatment were exposed to strong, medium, or weak gravitational selection for settling rate by subculturing 1.5 mL into a microcentrifuge tube and allowing this to settle on the bench for 5, 15, or 25 min, respectively. As in the other experiments, the lower 100 μL was then transferred to fresh media. This was carried out for 35 daily transfers. Settling rate was measured by placing 1 mL of stationary-phase cells in a 1.5-mL centrifuge tube, allowing the yeast to settle at 1 × *g* for 7 min and then fractionating the upper 700 μL and lower 300 μL. These subsamples were pelleted and double-washed in deionized (DI) water, excess water was removed, and the pellet was air-dried at 50 °C for 2 d. Settling rate was determined by the percentage of total biomass in each fraction.

Cluster-Level Life-History Analysis. Individual clusters were inoculated into 0.5-μL droplets of YPD and placed on the bottom of an eight-well Lab-Tek II chambered coverglass slide. Ten microliters of water was placed in each corner of the chamber, and clear tape was placed over the chamber top to keep the microdroplet from drying out. These clusters were then imaged on an Olympus IX70 inverted microscope. Using the 10× objective, brightfield illumination (set extremely low to avoid overheating the yeast), and a 1-s acquisition time, images were captured every minute with a SPOT 4MP camera in overnight time courses. Cluster size at reproduction was determined by manually outlining the perimeter of the reproducing cluster (one frame before a propagule was produced) in ImageJ and then measuring the outlined area. Propagule size was also determined by manual annotation.

Trade-Off Between Growth and Settling Rates. We chose 5/10 of the replicate populations from our first experiment that captured the range of cluster sizes evolved after 60 transfers. A representative genotype was isolated from each replicate (single colony selection, repeated three times serially) for the first time point at which we detected snowflake yeast and from 60 transfers. For the settling rate assay, five replicates of each genotype were conditioned by 24 h growth in 10 mL YPD, transferred to fresh media with selection for settling (100 × *g* for 10 s), and incubated at 30 °C for 24 h. Settling rate was determined as described in the divergent selection experiment above. Growth rate was determined by growing five replicates of each genotype for 24 h, diluting 1:100 without gravitational selection into 10 mL of fresh YPD (thereby transferring similar biomass), and then allowing yeast to grow for 12 h. From these actively growing cells, 100 μL was removed and added to 900 μL fresh YPD in 15-mL centrifuge tubes. These cells were grown for 4 h at 30 °C, and then the fold increase in biomass was determined by pellet washing and drying as previously described.

Quantification of Apoptosis. Apoptosis was measured by dihydrorhodamine 123 (DHR) staining of reactive oxygen species (2–4). Following the procedure of Madeo et al. (3), we stained cells with 1:100 of DHR stock solution (2.5 mg/mL in ethanol) for 2 h in the dark; cells were double-washed in sterile DI water and imaged microscopically. Before staining, yeast were conditioned by 24 h growth in YPD, followed by a 1:100 dilution (without gravitational selection) into fresh YPD where they were incubated for 12 h. Clusters were flattened into two dimensions by placing 5 μL of cell suspension between a standard slide and a 22- × 22-mm coverslip. Sample drying was minimized by sealing coverslip edges with clear nail polish. The frequency of apoptotic cells was measured for three independent replicates of each genotype on five fields of view per replicate. For each field of view, total cluster area was measured with brightfield microscopy, and DHR-stained cell area was measured by fluorescence microscopy for the same cells. In each case, background pixels were removed by thresholding; threshold values were kept as consistent as possible with small adjustments made for minor variation in background intensity.

Fluorescence Overlay. Fluorescence overlay was done in ImageJ with the "merge channels" command. The background brightfield, phase contrast, or differential interference contrast image was set to gray, propidium iodide (PI) to red, and DHR to green. To make fluorescence more visible in merged images, PI and DHR brightness was increased. Brightness was increased identically for all samples of a particular fluorophore in each experiment.

Experimental Induction of Apoptosis. Apoptosis was induced in snowflake yeast from replicate 1 at 14 transfers, a strain with wild-type levels of apoptosis. Following the proceedure of Ludovico et al. (5), we incubated stationary-phase snowflake yeast in YPD supplented with 40 mM acetate at pH 3.0 for 4 h and then washed cells centrifugally and resuspended them in standard YPD.

Dead Cell Involvement in Propagule Production. To determine if dead cells frequently serve as a break point for propagule production, we examined 17 randomly selected propagules that contained at least one dead cell. Propagules were obtained by diluting stationary-phase replicate 1, 60 transfer snowflake yeast 1:100 into fresh YPD and culturing for 4 h. Propagules were distinguished from parental clusters by size. For each, the center cell (the site of propagule separation) was determined as described in Fig. S3. Viability of this center cell was determined by PI staining. Statistical significance was assessed with a binomial probability test. The overall frequency of dead cells (live/dead cells were counted manually for all clusters) was used as our null expectation that the center cell would be dead.

Selfing Snowflake Yeast. To determine if apoptosis and cluster size are genetically independent traits, we selfed a large-cluster-forming, high-apoptosis strain. This strain evolved in the 5-min settling treatment of the divergent selection experiment. To induce sex, we streaked cells out onto sporulation agar (per liter: 20 g potasium acetate, 2.2 g yeast extract, 870 mg synthetic complete amino acid mix, 0.5 g glucose) and incubated the cells at 30 °C for 4 d. Individual spores were obtained by enzymatically digesting tetrads in 1,000 units of lyticase and 2% β-glucuronidase/arylsulfatase (Roche) for 1 h at 28 °C and then by vortexing tetrads with 50% (vol/vol) 0.1-mm glass beads for 60 s. Complete digestion of tetrads was confirmed by microscopy. Individual spores were plated on YPD agar, and one isolate was obtained from one colony by three rounds of streaking and single-colony isolation. Selfing a single spore was possible because the ancestral strain Y55 switches mating types. All isolates obtained were diploid and are presumed homozygous at all loci, excepting the *MAT* loci.

1. Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-term experimental evolution in escherichia coli. I. Adaptation and divergence during 2,000 generations. *Am Nat* 138:1315–1341.
2. Rodriguez-Menocal L, D'Urso G (2004) Programmed cell death in fission yeast. *FEM Yeast Res* 5(2):111–117.
3. Madeo F, et al. (1999) Oxygen stress: A regulator of apoptosis in yeast. *J Cell Biol* 145: 757–767.
4. Carmona-Gutierrez D, et al. (2010) Apoptosis in yeast: Triggers, pathways, subroutines. *Cell Death Differ* 17:763–773.
5. Ludovico P, Sousa MJ, Silva MT, Leão C, Côrte-Real M (2001) Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. *Microbiology* 147:2409–2415.

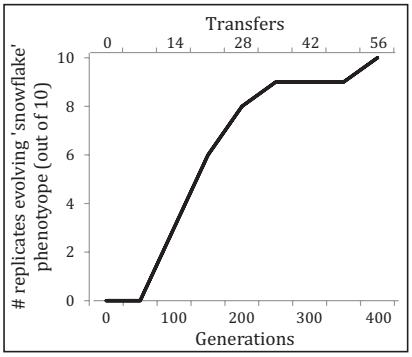


Fig. S1. Rapid evolution of snowflake-phenotype yeast. Snowflake phenotypes arose rapidly in response to strong selection for rapid settling. Plotted is the number of replicates with detectable snowflake phenotype (determined by plating on YPD agar and isolation of nonsmooth colony morphs and confirmed by microscopy) as a function of the number of transfers (or estimated generations) in our first 10-replicate selection experiment.

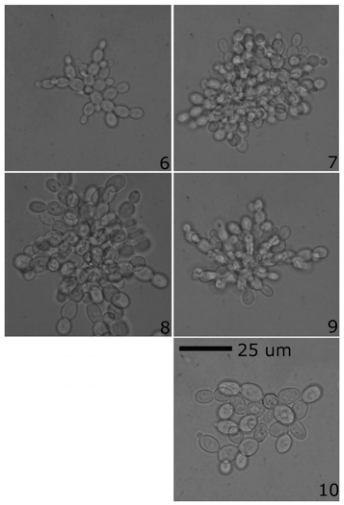


Fig. S2. Representative snowflake-phenotype yeast from replicate populations 6–10, isolated after 60 transfers (see Fig. 1 for replicate populations 1–5).

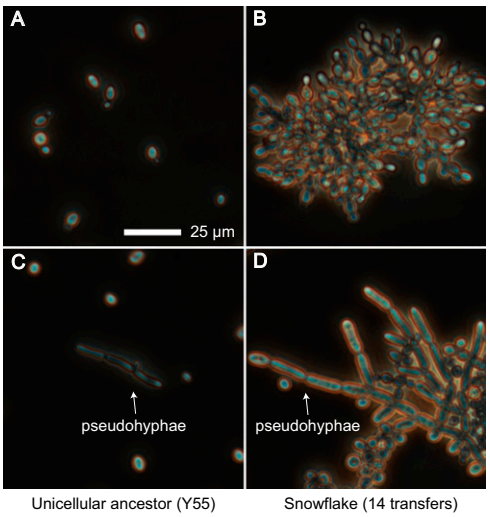


Fig. S3. Clustering occurs independently of pseudohyphal growth. *S. cerevisiae* has previously been shown to form clusters through pseudohyphal growth (1). Pseudohyphal cells are characterized by cellular elongation and are expressed on solid media in response to nitrogen starvation (2). (*A* and *B*) When grown in liquid YPD overnight, both the ancestral unicellular strain and snowflake-phenotype yeast exhibit normal, oval-cell morphology. To determine if pseudohyphae can be induced in the unicellular ancestor and snowflake yeast from replicate population 1, 14 transfers, we starved yeast by culturing them on solid YPD media for 5 d. Pseudohyphae were readily observed in both strains (*C* and *D*). We conclude that the snowflake phenotype is not the result of a mutation that made previously inducible pseudohyphal cell morphology constitutive.

1. Van de Velde S, Thevelein JM (2008) Cyclic AMP-protein kinase A and Snf1 signaling mechanisms underlie the superior potency of sucrose for induction of filamentation in Saccharomyces cerevisiae. *Eukaryot Cell* 7:286–293.
2. Gimeno CJ, Ljungdahl PO, Styles CA, Fink GR (1992) Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: Regulation by starvation and RAS. *Cell* 68:1077–1090.

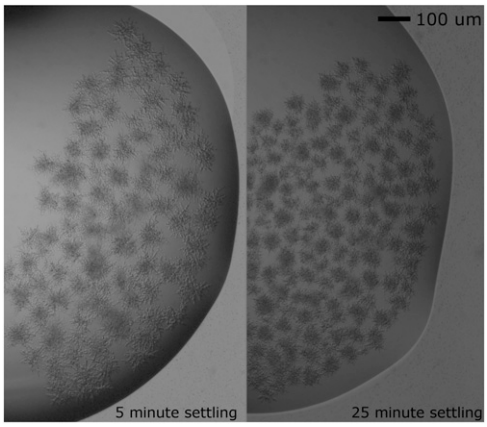


Fig. S4. Size of the multicellular cluster is approximately determinate. A single cluster was inoculated into a 0.5-μL droplet of YPD and grown for 16 h; shown are the resulting populations. The similar size distribution of the resulting offspring clusters (within each population), despite generational differences, demonstrates that the size of the multicellular cluster is approximately determinate. Cells in large clusters do not stop dividing. Instead, ongoing cell growth results in propagule production. (*Left*) A randomly selected isolate from the 5-min settling treatment. (*Right*) A randomly selected isolate from the 25-min settling treatment from the divergent selection experiment (Fig. 3).

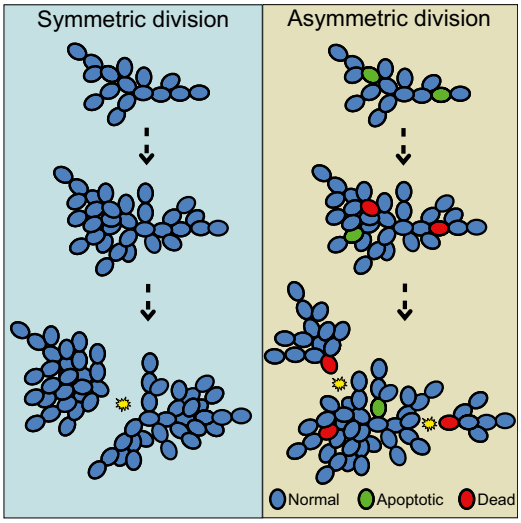


Fig. S5. Proposed role of apoptosis in reducing propagule size. Cell death through apoptosis generates "weak links" between chains of cells in a cluster. As cells within the cluster divide, they grow into one another, putting a strain on the connection between cells. Dead cells break more easily, resulting in earlier cell separation and the production of smaller propagules.

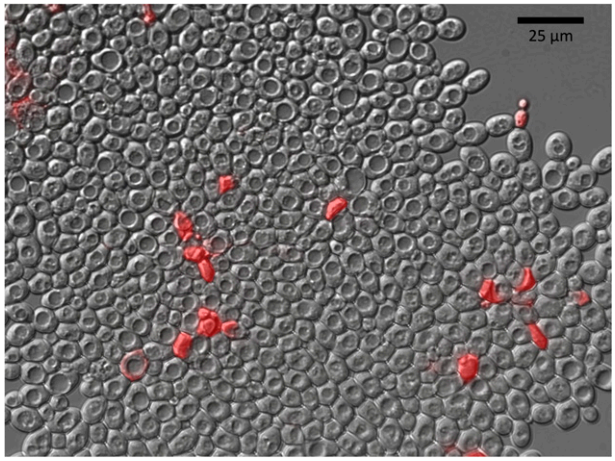


Fig. S6. Dead cells exhibit aberrant morphology. Snowflake-phenotype yeast (drawn from replicate population 1, day 60, of our first evolution experiment) were grown for 24 h in YPD, stained with propodium iodide (PI), flattened between a coverslip and slide, and imaged on a Nikon E800 microscope. Shown is a differential interference contrast background image with PI fluorescence overlay (42% opacity).

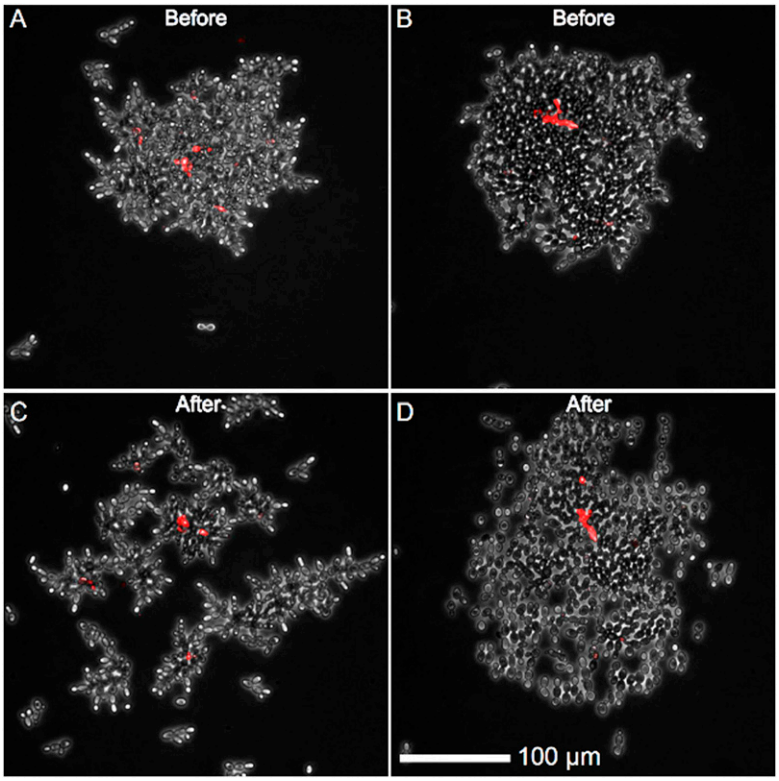
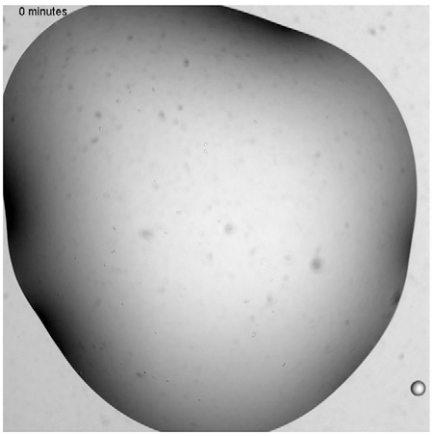
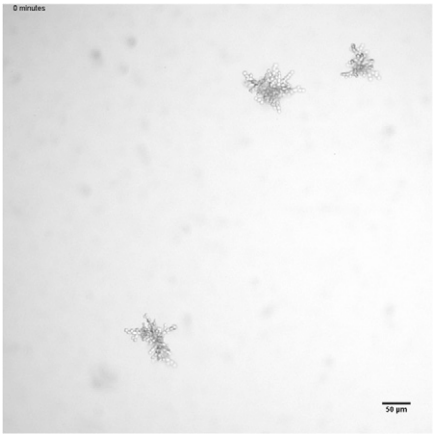


Fig. S7. Cell separation does not cause cell death. To determine if cell separation, the process that produces propagules, causes cell death, we manually fragmented snowflake yeast from replicate population 1, 60 transfers. Yeast were grown for 24 h under standard culture conditions, diluted 1:10 into water, and stained with propidium iodide for 5 min. Five microliters of cells and staining solution was placed on a slide and covered with a 22- × 22-mm coverslip. Clusters were imaged immediately, and then the coverslip was moved in a small circular motion to break the cluster apart. After 5 min, fragmented clusters were reimaged. Regardless of whether snowflake yeast were broken into smaller clusters (*A* and *C*) or into mostly pairs of cells (*B* and *D*), no cell death was induced.



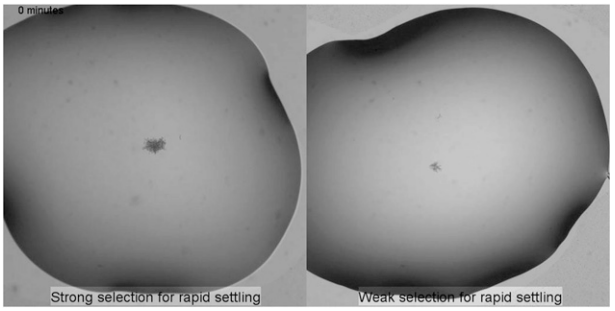
Movie S1. Single cells of snowflake-phenotype yeast regenerate new snowflake-phenotype clusters. Snowflake-phenotype clusters (drawn from replicate population 1, day 60, of our first evolution experiment) were broken down into unicells by digestion with lyticase (500 units/mL in H_2O, 45-min digestion at 22 °C, vigorous vortexing for 5 min). These cells were then diluted 300-fold and grown in 0.5 μL YPD. Time-lapse microscopy was performed at 100× magnification, with images taken every minute for 760 min. Note that these are all "juveniles"; no multicellular propagule production by any clusters was observed during the experiment.

Movie S1



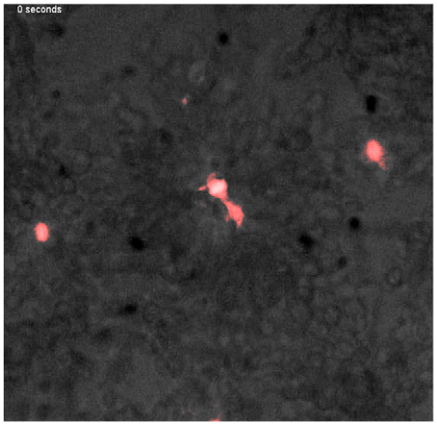
Movie S2. Growth of snowflake-phenotype yeast. Snowflake-phenotype clusters (same genotype as in Movie S1) were diluted by 300-fold and grown in 0.5 μL YPD. Time-lapse microscopy was performed at 100× magnification, with images taken every minute for 500 min.

Movie S2



Movie S3. Time-lapse microscopy of derived rapid settling (*Left*) and slow settling (*Right*) genotypes isolated from 5- and 25-min settling regimes, respectively. Cultures were grown for 24 h, diluted 300-fold, and grown in 0.5 μL YPD. Time-lapse microscopy was performed with images taken every minute for 600 min.

Movie S3



Movie S4. Cluster fragmentation occurring between a pair of dead cells. This video captures production of a propagule by fragmentation. Cell separation occurs at the connection between two cells that stain with propidium iodide (PI). Snowflake yeast (replicate population 1, 60 transfers) were grown in 100 μL of YPD with 1% PI stock solution in eight-well Lab-Tek II chambered coverglass slides. Snowflake yeast were illuminated with low-intensity halogen light and PI excited by green light (dimmed with a 50% opacity neutral density filter); images were captured every 5 s at 400× magnification. To compose the movie, individual frames (RGB Imaging) were decomposed into their component colors; the red component was then thresholded to remove cells that were not emitting PI fluorescence. The original images were converted to 8 bit, and the thresholded images containing PI fluorescence data were entered as the red channel using the "merge channels" command in ImageJ.

Movie S4