

De novo evolution of macroscopic multicellularity

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

G. Ozan Bozdag^{1,†,*}, Seyed Alireza Zamani-Dahaj^{2,3,*}, Thomas C. Day³, Penelope C. Kahn^{1,4}, Anthony J. Burnett¹, Dung T. Lac¹, Kai Tong^{1,2}, Peter L. Conlin¹, Aishwarya H. Balwani⁵, Eva L. Dyer⁵, Peter J. Yunker^{2,†} and William C. Ratcliff^{1,†}

¹School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

²Interdisciplinary Graduate Program in Quantitative Biosciences, Georgia Institute of Technology, Atlanta, GA, USA

³School of Physics, Georgia Institute of Technology, Atlanta, GA, USA

⁴Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada

⁵School of Electrical & Computer Engineering, Georgia Institute of Technology, Atlanta, GA, USA

*These authors contributed equally to this work.

†Corresponding authors: ozan.bozdag@gmail.com, ratcliff@gatech.edu, peter.yunker@gatech.edu

27 **Abstract**

28 While early multicellular lineages necessarily started out as relatively simple groups of cells, little
29 is known about how they became Darwinian entities capable of open-ended multicellular
30 adaptation^{1,2}. To explore this, we initiated the Multicellularity Long Term Evolution Experiment
31 (MuLTEE), selecting for larger group size in the snowflake yeast (*Saccharomyces cerevisiae*)
32 model system. Given the historical importance of oxygen limitation³, our ongoing experiment
33 consists of three metabolic treatments⁴: anaerobic, obligately aerobic, and mixotrophic yeast. After
34 600 rounds of selection, snowflake yeast in the anaerobic treatment evolved to be macroscopic,
35 becoming $\sim 2 \cdot 10^4$ times larger (\sim mm scale) and $\sim 10^4$ -fold more biophysically tough, while
36 retaining a clonal multicellular life cycle. They accomplished this through sustained biophysical
37 adaptation, evolving increasingly elongate cells that initially reduced the strain of cellular packing,
38 then facilitated branch entanglements that enabled groups of cells to stay together even after many
39 cellular bonds fracture. In contrast, snowflake yeast competing for low oxygen remained
40 microscopic, evolving to be just ~ 6 -fold larger, underscoring the critical role of oxygen levels in
41 the evolution of multicellular size. Taken together, this work provides unique insight into an
42 ongoing evolutionary transition in individuality, showing how simple groups of cells overcome
43 fundamental biophysical limitations via gradual, yet sustained, multicellular adaptation.

44 **Introduction**

45 Organismal size plays a fundamental role in the evolution of multicellularity. The evolution
46 of larger size allows organisms to gain protection from the external environment⁵ and explore
47 novel niches⁶, while creating opportunities for the evolution of cellular differentiation⁷⁻¹¹.
48 Increases in organismal size have also been hypothesized to play a key role in the evolution of
49 trade-off breaking multicellular innovations, as large size creates an evolutionary incentive to solve
50 challenges of nutrient and oxygen transportation that are otherwise inescapable consequences of
51 diffusion limitations^{12,13}. However, little is known about how nascent multicellular organisms,
52 consisting of small groups of undifferentiated cells, evolve to form biomechanically tough,
53 macroscopic multicellular bodies, and whether selection for size itself can drive sustained
54 multicellular adaptation³.

55 The evolution of macroscopic size presents a fundamental challenge to nascent
56 multicellular organisms, requiring the evolution of biophysical solutions to evolutionarily-novel
57 stresses that act over previously-unseen multicellular size scales¹⁴⁻¹⁸. While prior work with yeast
58 and algae have shown that novel multicellularity is relatively easy to evolve *in vitro*, these
59 organisms remain microscopic, typically growing to a maximum size of tens to hundreds of cells¹⁹⁻
60 ²³. Extant macroscopic multicellular organisms have solved the above challenges through
61 developmental innovation, evolving mechanisms that either reduce the accumulation of
62 biophysical strain or increase multicellular toughness²⁴⁻²⁶. However, in nascent multicellular
63 organisms that have not yet evolved coordinated morphogenesis, we do not know how, or even
64 whether, simple groups of cells can evolve the increased biophysical toughness required for the
65 evolution of macroscopic size.

66 Here we examine the interplay between biological, biophysical, and environmental drivers
67 of macroscopic multicellularity using long-term experimental evolution. We subject snowflake
68 yeast²¹, a model of undifferentiated multicellularity, to 600 rounds (~3,000 generations) of daily
69 selection for increased size. Furthermore, because oxygen is thought to have played a key role in
70 the evolution of macroscopic multicellularity, we evolved snowflake yeast with either anaerobic,
71 mixotrophic, or obligately aerobic metabolism. All five of our replicate anaerobic populations
72 evolved macroscopic size, while all aerobic and mixotrophic populations remained microscopic
73 throughout the experiment, supporting the hypothesis that growth under low concentrations of
74 oxygen constraint the evolution of large multicellular size⁴. Macroscopic size convergently
75 evolved through two key changes in all five replicate populations. First, snowflake yeast increased
76 the length of their constituent cells, which delays organismal fracture caused by packing-induced
77 strain¹⁸. Next, they evolved to entangle branches of connected cells such that breaking a single
78 cell-cell bond no longer causes multicellular fracture, evolving to become $\sim 2 \cdot 10^5$ times larger,
79 forming millimeter-scale groups of clonal cells. Together these adaptations increased the
80 toughness of individual clusters by more than 10^4 -fold, transforming the initial snowflake yeast
81 ancestor, which was weaker than gelatin, to an organism with the strength and toughness of wood.
82 Fitness assays, sequencing, and synthetic strain constructions reveal that macroscopic
83 multicellularity evolved via selection acting on group size, an emergent multicellular trait of
84 mutations directly affecting cellular morphology.

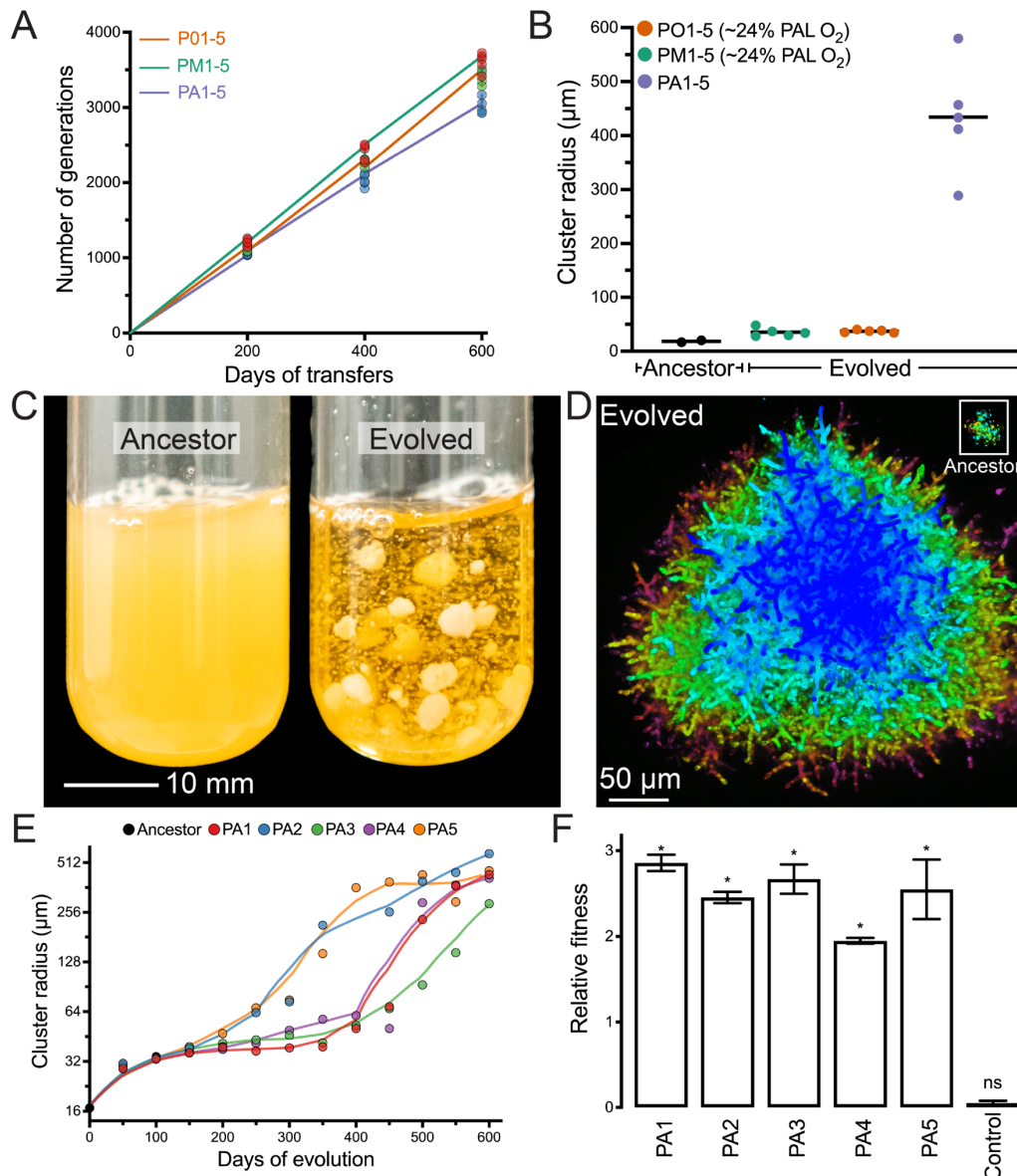
85 **Results**

86 In 2018 we initiated the Multicellularity Long Term Evolution Experiment (MuLTEE), named
87 after the pioneering Long Term Evolution Experiment with *E. coli* initiated by Rich Lenski²⁷. This
88 central goal of this project, which we intend to run over decadal time scales, is to observe open-

89 ended multicellular adaptation in a nascent multicellular organism. We began the MuLTEE by
90 engineering a unicellular isolate of *S. cerevisiae* strain Y55 to grow with the snowflake phenotype
91 by deleting the *ACE2* open reading frame, ensuring that each replicate population had the same
92 initial mechanism of group formation²⁸. To examine the effect of oxygen on the evolution of size,
93 we initiated three treatments in an otherwise isogenic ancestor: anaerobic growth (generated by
94 selecting for a spontaneous petite mutant incapable of respiration), mixotrophy (cultured with
95 glucose as the primary carbon source), and obligately aerobic growth (cultured with glycerol as
96 the primary carbon source)⁴. We refer to the five replicate populations of anaerobic, mixotrophic,
97 and obligately aerobic populations as PA, PM, and PO 1-5, respectively. We maintained strong
98 directional selection favoring larger cluster size throughout the experiment by selecting for
99 increasingly rapid sedimentation prior to transfer to fresh media (see Methods for details). We
100 evolved these 15 populations over 600 rounds of growth and settling selection (~3,000 generations,
101 Fig. 1A).

102 All five populations of anaerobic snowflake yeast evolved macroscopic size, with
103 individual clusters visible to the naked eye (Fig. 1B-D, Supplementary Movie 1). In contrast,
104 snowflake yeast capable of metabolizing oxygen remained microscopic (Fig. 1A & Extended Data
105 Fig. 1), a result consistent with recent work showing that competition for scarce oxygen imposes
106 a powerful constraint on the evolution of large multicellular size⁴. Here, we focus on the evolution
107 of macroscopic size in the five replicate anaerobic populations. Yeast in this treatment increased
108 their mean cluster radius from 16 μm to 434 μm , a $\sim 2 \cdot 10^4$ -fold increase in volume (Fig. 1E, $p <$
109 0.0001; $F_{5, 13321} = 2100$, Dunnett's test in one-way ANOVA). This corresponds to an estimated
110 increase from ~ 100 cells per cluster to $\sim 450,000$ (comparing average cluster volumes, accounting
111 for changes in mean cell volume and cellular packing density within clusters).

112 The largest clusters of 600-day evolved macroscopic snowflake yeast are over a millimeter
113 in diameter (Fig. 1C), which is comparable to the size of an adult *Drosophila*²⁹. Much like their
114 microscopic snowflake yeast ancestor^{18,28}, macroscopic snowflake yeast possess a life cycle in
115 which groups of cells both grow in size and reproduce, generating multicellular propagules, over
116 the course of the ~24 h culture period (Extended Data Fig. 2). This analysis establishes that group
117 size is heritable, and our time series data (Fig. 1E) show every replicate population evolved to
118 form larger clusters at each 200-day sampling interval, strongly suggesting that larger size is an
119 adaptive trait evolving in response to settling selection. To test this hypothesis, we performed a
120 fitness assay competing the microscopic ancestor against each t600 PA1-5 population, under our
121 standard selective conditions of growth and settling selection. The 600-day evolved macroscopic
122 snowflake yeast were far more fit than their ancestor (mean daily selection rate constant = 2.5, Fig.
123 1F), increasing from a mean starting frequency of 52% to 99.9% over just three days.



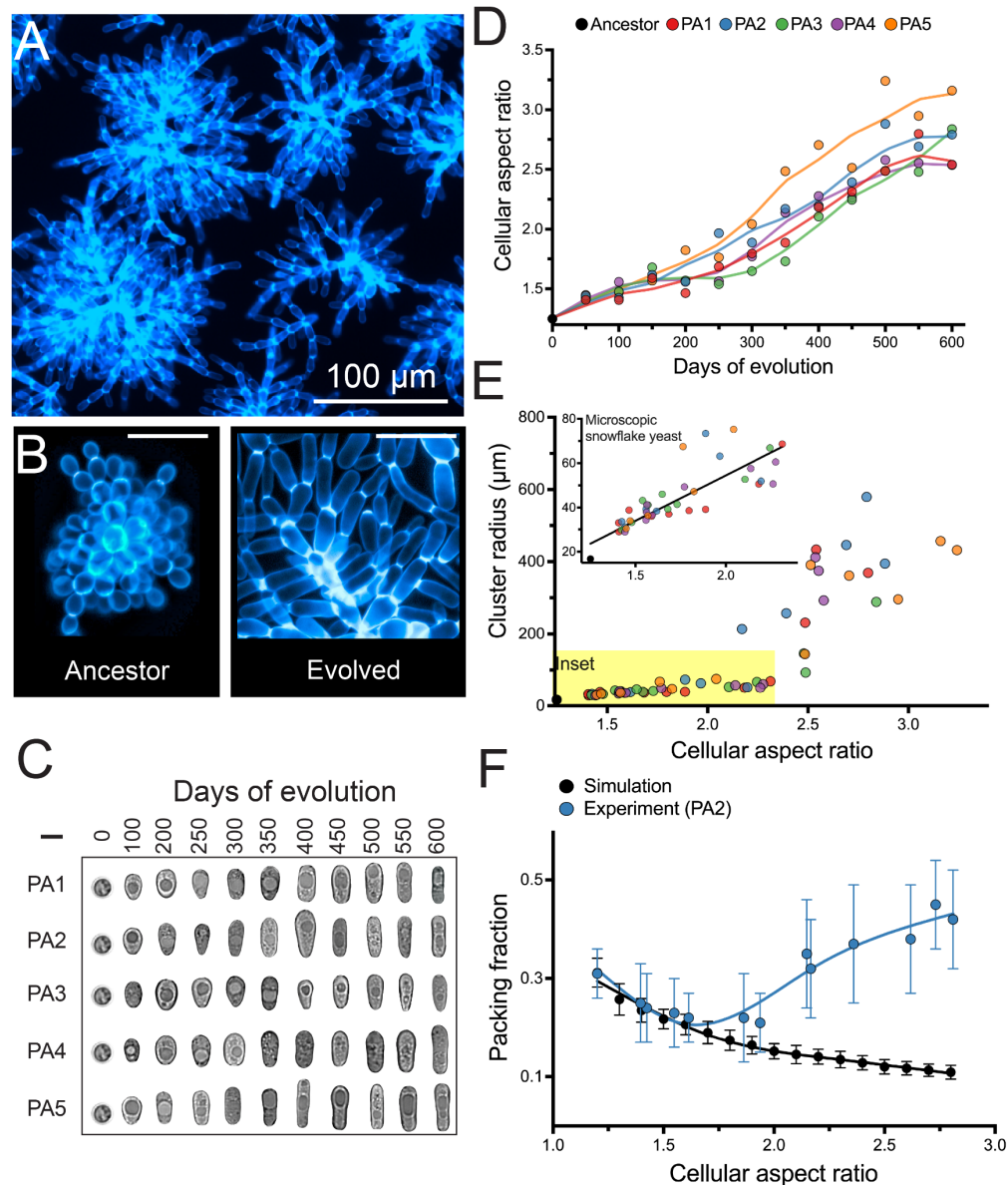
124
125

126 **Figure 1. Evolution of macroscopic multicellularity in five replicate snowflake yeast populations.** (A)
127 We selected for larger size over 600 daily transfers, which represents ~3,000 generations. (B) Only the
128 anaerobic populations (PA1-5) evolved macroscopic size over this time. (C) Individual snowflake yeast
129 clusters from t600 are visible to the naked eye. (D) Representative clusters of the same two genotypes
130 (ancestor in the upper right corner) shown under the same magnification (color represents depth in the z
131 plane). (E) Temporal dynamics of size evolution in the anaerobic treatment (PA). (F) Macroscopic
132 snowflake yeast were considerably more fit (calculated as a per-day selection rate constant $(2.5)^{30}$) than
133 their microscopic ancestor ($n=3$, error bars represent the SEM, asterisks denote significance at the 0.05
134 level). In A and E, the data points show the biomass-weighted mean radius (see Methods for details). See
135 Extended Data Fig. 1 for additional data on the evolution of cluster size in oxygen-using populations (PM
136 and PO) and Extended Data Fig. 3 for cluster size distributions for the 600-day anaerobic populations (PA1-
137 5). Lines in (E) are Lowess smoothing curves intended to aid the eye.

138 As with their ancestor, macroscopic snowflake yeast grow via incomplete mother daughter
139 cellular separation, forming a branched, tree-like structure (Fig. 2A&B). When compressed,
140 macroscopic clusters fracture into small modules that resemble microscopic snowflake yeast in
141 terms of branching morphology (Fig. 2 A&B). Their cellular morphology, however, changed
142 markedly. Throughout the experiment, snowflake yeast cells evolve to be more elongate across all
143 five replicate populations, increasing in average aspect ratio (ratio of length to width) from ~1.2
144 to ~2.7 (Fig. 2C&D; $F_{5, 1993} = 206.2, p < 0.0001$, Dunnett's test after one-way ANOVA; Extended
145 Data Fig. 4). Even in macroscopic snowflake clusters, cell size and shape did not depend on
146 location in the cluster (*i.e.*, interior or exterior; Extended Data Fig. 5). Initially, cluster size was a
147 roughly linear function of cellular aspect ratio (Fig. 2E inset), but this relationship changes once
148 they evolve macroscopic size (Fig. 2E).

149 Prior work has shown that the evolution of more elongate cells increases the size to which
150 microscopic snowflake yeast grow by decreasing the density of cellular packing (*i.e.*, their packing
151 fraction) in the cluster interior, which reduces cell-cell collisions that drive multicellular
152 fracture^{18,31}. To establish a null expectation for the effect of cell aspect ratio on cluster packing
153 fraction, we simulated the growth of individual clusters from a single cell using an experimentally-
154 validated model (see methods for details)¹⁸. In these simple 3D simulations, cellular packing
155 fraction decreased monotonically with increasing cellular aspect ratio (Fig. 2F). We then examined
156 this relationship over the course of our long-term experiment in replicate population two (PA2),
157 which was one of the first lineages to evolve macroscopic size. As predicted by our simulation,
158 cellular elongation decreased the packing fraction of microscopic multicellular groups— but only
159 initially, from aspect ratio ~1.2-2.0. Beyond this, clusters with more elongate cells actually became
160 more densely packed, and experimentally-measured packing fraction became increasingly

161 divergent from model predictions (Fig. 2F). This divergence suggests that this lineage evolved a
 162 novel biophysical mechanism for increased multicellular toughness, capable of withstanding
 163 growth to macroscopic size and a high cellular packing fraction.



164

165 **Figure 2. Evolution of novel cell morphology.** (A) When compressed, macroscopic snowflake yeast
 166 fracture into modules, retaining the same underlying branched growth form of their microscopic ancestor
 167 as seen in (B) (scale bars are 20 μm). Cell walls are stained with calcofluor-white in (A) and (B). (C) and
 168 (D) show the parallel evolution of elongated cell shape, resulting in an increase in average aspect ratio from
 169 ~ 1.2 to ~ 2.7 (for each point in D, 453 cells were measured on average. The t0 ancestor is the same for PA1-
 170 5. Scale bar in C is 5 μm). An expanded version of (C) is shown in Extended Data Fig. 6. (E) Early in their

171 evolution (aspect ratio 1-2.3), cluster size (weighted mean radius) is an approximately linear function of
172 cellular aspect ratio (inset; $p < 0.0001$, $y = 41.1x - 27.8$, $r^2 = 0.72$). This relationship does not hold beyond
173 aspect ratio ~ 2.5 . (F) A biophysical model of snowflake yeast predicts that increasing cellular aspect ratio
174 should decrease cellular packing fraction (black points). We see a close correspondence with these
175 predictions for low aspect ratios, but our experimental data diverges from model predictions for aspect
176 ratios beyond 2. Each datapoint in (F) reports the mean of 15 snowflake yeast clusters or 25 replicate
177 simulations, \pm one standard deviation.

178 The simplest way that snowflake yeast could evolve to become macroscopic is to become
179 adhesive, forming large aggregates of many separate snowflake yeast clusters. Indeed, aggregation
180 is a common mechanism of group formation in yeast (i.e., via flocculation³²), and this would
181 explain the modular structure of macroscopic snowflake yeast (Fig. 2A). To determine if clusters
182 of macroscopic yeast form via aggregation, or if they develop as a single clonal lineage, we labeled
183 a single-strain isolate (taken from PA2 after 600 days of selection), with either GFP or RFP. If
184 adhesive aggregation were responsible for their large size, we would expect to see chimeric groups
185 composed of both red and green-fluorescent sub-clusters³². After five rounds of co-culture,
186 however, all multicellular clusters ($n=70$; Extended Data Fig. 7) remained monoclonal. This is
187 unlikely to occur with aggregation. If we conservatively assume each macroscopic snowflake yeast
188 cluster we measured was the result of just a single fusion event, occurring with equal probability
189 between two groups of red and green cells, then the binomial probability of finding no chimeric
190 groups in our sample would be 10^{-6} . Floc-like aggregation thus does not explain the evolution of
191 macroscopic size in snowflake yeast.

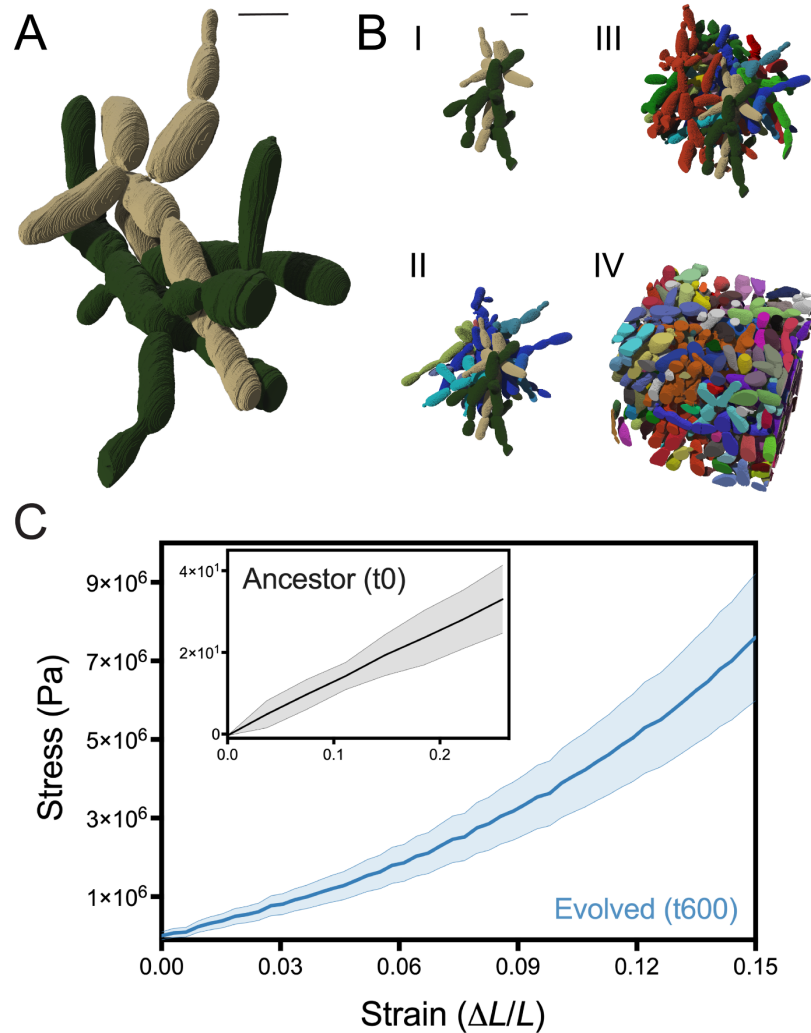
192 To examine how changes in the topology of macroscopic snowflake yeast may underlie
193 their increased size, we imaged clusters via Serial Block Face Scanning Electron Microscopy
194 (SBF-SEM). This technique enables us to image the interior of macroscopic clusters that are
195 difficult to resolve with light-based microscopy, allowing us to map their internal architecture with
196 nanometer precision³³. Within macroscopic clusters, separate branches contact, intercalate, and

197 even wrap around each other (Fig. 3A). As these clusters are densely packed, moving one
198 component would require moving many other components as well. Further, we found that
199 individual macroscopic snowflake yeast were not composed of a single topologically-connected
200 component, like their ancestors. Instead, they contained disconnected branches of cells, suggesting
201 that the cluster remained intact even when cell-cell connections were broken (Fig. 3A). Based on
202 these observations, we hypothesized that branches of macroscopic clusters are entangled, in a
203 manner reminiscent of physical gels³⁴ and entangled granular materials³⁵. Entanglement would
204 provide a mechanism for branches of cells to remain in the same, densely packed group even after
205 cell-cell bonds break.

206 Following prior work in entangled chains and knotted strings^{35,36}, we used our SBF-SEM
207 dataset to quantify branch entanglement in macroscopic snowflake yeast by analyzing chain
208 topology and geometry. Specifically, we constructed the convex hull of each connected component
209 within a sub-volume, which denotes the smallest convex polyhedron containing this component
210 (see Extended Data Fig. 8A). If a cell from one connected component overlaps with the convex
211 hull of a second, then the two can be considered entangled. By percolating entanglement among
212 adjacent connected components throughout the sub-volume, we can measure the extent to which
213 the cluster's biomass is mutually entangled (Fig. 3B, Extended Data Fig. 8B). For entanglement
214 to underlie macroscopic size, the largest entangled component (consisting of many entangled
215 pieces) must be able to resist mechanical stress, meaning that there must be an entangled
216 component that spans the vast majority of the cluster³⁷. In analyses of 10 randomly selected sub-
217 volumes from different macroscopic snowflake yeast clusters from population PA2 t600
218 macroscopic yeast, we found that the largest entangled component contains 93% +/- 2% of all

219 connected components. This observation supports the hypothesis that entanglement between cell
220 branches can prevent cluster fracture in the event that a cell-cell bond fractures.

221 As a further test, we investigated the mechanics of macroscopic snowflake yeast. Entangled
222 materials are known to exhibit two key mechanical signatures: strain stiffening and high material
223 toughness^{35,38}. Strain stiffening describes the fact that, when compressed, the effective stiffness of
224 entangled chains increases with increased strain. By efficiently distributing stress across
225 constituent bonds, entangled materials can withstand stress orders-of-magnitude greater than their
226 non-entangled counterparts^{38,39}. As the microscopic ancestor is presumably not entangled, it should
227 not exhibit strain-stiffening behavior or possess high toughness. We measured the mechanical
228 stress response of 10 macroscopic snowflake yeast clusters under uniaxial compression using a
229 macroscopic mechanical tester (Zwick Roell Universal Testing Machine). We repeated the same
230 experiment for 10 ancestral microscopic snowflake yeast clusters using an atomic force
231 microscope (AFM Workshop LS-AFM). The stress-strain plot for the microscopic ancestor is
232 linear ($r^2 = 0.97 \pm 0.02$, average and standard deviation of the regression for 10 samples, Fig. 3C
233 inset), clusters fracture at stress as low as 240 Pa and have toughness as low as 8.9 J/m^3 ¹⁸. By
234 contrast, macroscopic snowflake yeast clusters have a convex stress-strain curve (Fig. 3C), can
235 support stresses at least as large as $\sim 7 \text{ MPa}$ without failing, and have toughness greater than 0.6
236 MJ/m^3 . Thus, entanglement both enables separate branches within macroscopic snowflake yeast
237 to stay together and allows them to endure the large stresses necessary for growth to macroscopic
238 size.



239

240 **Figure 3. Branch entanglement underlies the evolution of macroscopic size.** (A) Shown are two
241 entangled components (green and tan), obtained via SBF-SEM imaging. (B) Branch entanglement is
242 pervasive in macroscopic snowflake yeast. Starting with the two-component sub-volume in (A), we
243 percolated entanglement by adding on adjacent entangled components in four steps (I-IV). Scale bars on A
244 and B are $5 \mu\text{m}$. (C) Stress vs. strain plot for macroscopic snowflake yeast (PA2, t600) clusters in blue and
245 the ancestor in grey (ancestor shown again in inset with a rescaled y axis). Macroscopic snowflake yeast
246 experience strain stiffening, a hallmark of entangled systems, while the ancestor's stress-strain plot is linear,
247 which is expected for non-entangled systems. The shaded area shows one standard deviation based on 10
248 repeated measurements for each.

249 To rule out alternative hypotheses, we made additional measurements on macroscopic
250 snowflake yeast (PA2 t600) and their microscopic ancestor. First, we measured the stiffness of
251 individual cells to determine if they were becoming tougher. We did not detect a change between
252 than ancestor and t600 macroscopic strain (0.018 and 0.019 N/m for the ancestor and evolved,
253 respectively, Extended Data Fig. 9a). To determine if, in the absence of macroscopic entangled
254 structures, individual PA2 t600 cells still show strain stiffening behavior, we crushed macroscopic
255 clusters into smaller, microscopic branches before compressing them. These small groups
256 displayed a linear stress-strain curve like their unevolved microscopic ancestor. In the absence of
257 their macroscopic phenotype, the cells of the PA2 t600 yeast do not behave like an entangled
258 material (Extended Data Fig. 9B).

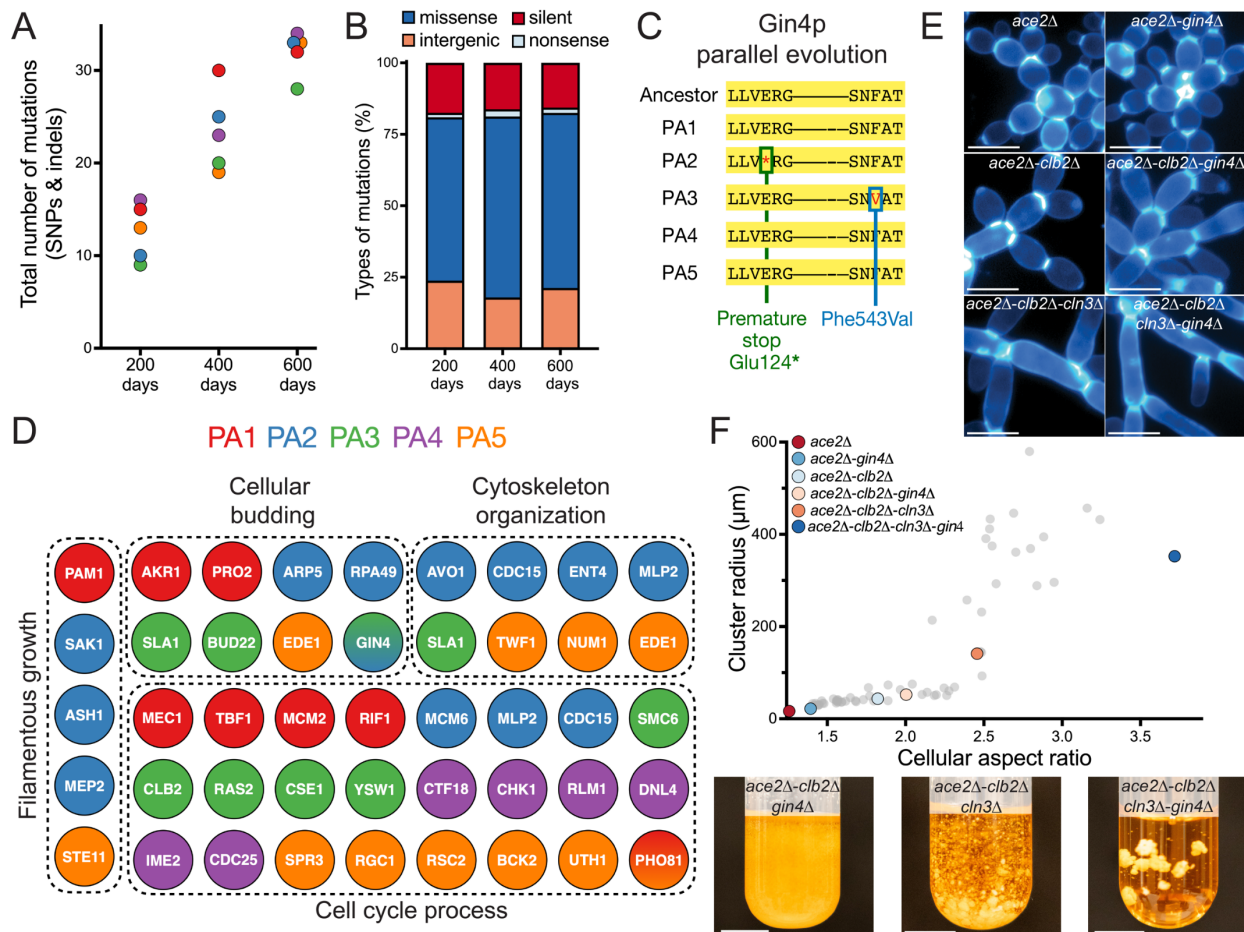
259 Finally, we performed a positive control: we created persistently entangled groups
260 experimentally. As described in Extended Data 7, growth in well-mixed liquid media prevents the
261 formation of chimeric groups through entanglement. Yet if entanglement is critical for
262 multicellular toughness, allowing fractured branches to remain in the same group, then chimeric
263 clusters held together only by entanglement should be possible to grow under the right
264 environmental conditions. We allowed GFP and RFP-tagged versions of PA2 t600 to grow at high
265 density on solid media for 48 h, then cultured these yeast in liquid media for two rounds of growth
266 and settling selection. These yeast readily formed and maintained chimeric groups. Specifically,
267 30% (31/101) of the clusters of the macroscopic genotype were still chimeric, with visibly
268 entangled branches of green and red yeast (Extended Data Fig. 10). In contrast, only 1/110 clusters
269 of the ancestral genotype were chimeric when cultured under the same conditions (0.9%, $p < 0.001$,
270 $t = 6.59$, $df = 209$, two-tailed t -test). Taken together, this experiment shows that entanglement
271 allows evolved snowflake yeast to remain intact, even when constituent branches lack continuous

272 mother-daughter cellular bonds (*i.e.*, red and green branches are not attached to each other by
273 permanent bonds).

274 To uncover the genomic basis of cell-level changes underlying multicellular adaptation,
275 we sequenced the genomes of a single strain from each of the five populations (PA1-PA5) that
276 independently evolved macroscopic multicellularity after 600 transfers (Fig. 4A&B). Over ~3,000
277 generations, snowflake yeast in our anaerobic treatment evolved dramatically more elongate cells
278 (Fig. 2C&D), which plays a central role in the evolution of increased cluster size (Fig. 2E) and
279 biophysical toughness (Figs. 2F & 3). Gene Ontology (GO) terms associated with cell length were
280 significantly enriched, namely genes of the cell cycle⁴⁰ (29 / 123 mutations, $p = 0.02$) and
281 filamentous growth (7 / 123 mutations). In addition, we found 11 nonsynonymous mutations in
282 genes with known roles in cellular budding (Fig. 4D), which includes eight genes that have
283 previously been shown to increase the size of the bud neck (*AKR1*, *ARP5*, *CLB2*, *GIN4*, *PRO2*,
284 *RPA49*, *RSC2*, *PHO81*)^{41,42}. Mutations arose in two of these genes in different populations (*i.e.*,
285 *PHO81* in populations PA1 and PA5, and *GIN4* in populations PA2 and PA3, Fig. 4C), indicating
286 parallel evolution. Larger bud scars should increase the amount of cell wall connecting cells,
287 increasing the strength of the bond and toughness of the group. In our ancestral strain Y55, *gin4Δ*
288 cells formed bud necks that had a 1.7-fold larger cross-sectional area ($t=2.8$, $df = 8$, $p < 0.01$).
289 Consistent with this, we found that PA2-t600 macroscopic snowflake yeast evolved to form bud
290 necks that had a 2.4-fold larger cross-sectional area ($t = 5.3$, $df = 24$, $p < 0.001$), and bud scars that
291 were 5.8x larger 3D volume ($t = 7.3$, $df = 24$, $p < 0.001$) than their microscopic ancestor (Extended
292 Data Fig. 11).

293 As a final proof of principle, we set out to show that cellular elongation, aided by increased
294 cell-cell bond strength, is sufficient to underpin the origin of macroscopic size in snowflake yeast.

295 Starting with the microscopic *ace2Δ* ancestor, we deleted the cyclins *CLN3* and *CLB2* in order to
296 artificially increase cellular aspect ratio, and deleted *GIN4* to increase bud scar size, and thus
297 strength. *CLN3*, while not present in evolved isolates, has a large, well-understood phenotypic
298 effect on cell shape. Deleting *CLN3* and *CLB2* increased cellular aspect ratio (AR) by 21% and
299 45% respectively in single mutants, and 95% in the double mutant, with *GIN4* deletion further
300 increasing the aspect ratio of each genotype in addition to its effects on bud scars (Fig. 4E and F).
301 Our results mirror those from our evolution experiment: strains with AR < 2.5 were clearly
302 microscopic, with increasing AR resulting in a gradual increase in group size. At AR ~2.5, *ace2Δ*
303 *clb2Δ cln3Δ* yeast were at the threshold of macroscopic size, but still quite a bit smaller than our
304 t600 isolates. The *ace2Δ clb2Δ cln3Δ gin4Δ* mutant, with an AR of 3.7, formed well-developed
305 macroscopic clusters (Fig. 4F; experimentally-evolved strains described in Fig. 1E are shown in
306 gray to facilitate direct comparison).



307

308 **Figure 4. Whole-genome sequencing reveals the dynamics of molecular evolution and the genetic**

309 **basis of cell-level and cluster-level changes.** (A) and (B) show the number and types of mutations in

310 evolved single strains from each population. (C) *GIN4*, a kinase whose loss of function increases bud neck

311 size, is mutated in two independent populations. (D) Macroscopic snowflake yeast were enriched in

312 mutations affecting cell cycle progression, cytoskeleton, and filamentous growth. In addition, we saw

313 mutations affecting budding (*i.e.*, the location of buds on the cell surface, and bud neck size). These

314 constituted 11 non-synonymous mutations out of the 123 total mutations in genotypes isolated from PA1-

315 PA5 after 600 days of evolution. (E) Representative images of cells from strains used to re-engineer

316 macroscopic size. Scale bars are 10 μ m. (F) Engineered strains recapitulated the evolutionary trajectory

317 established over 600 rounds of selection. With cellular aspect ratio below \sim 2.5, snowflake yeast remained

318 microscopic, greatly increasing in size beyond this threshold. Scale bars are 10 mm.

319 Discussion

320 In this paper, we show that snowflake yeast, a model system of undifferentiated multicellularity,
321 were capable of sustained multicellular adaptation, evolving macroscopic size over 600 days of
322 experimental evolution. Macroscopic snowflake yeast are readily visible to the naked eye,
323 containing hundreds of thousands of clonal cells. They achieved this remarkable increase in size
324 by evolving highly elongate cells that become entangled within the cluster interior. This critical
325 innovation allows multicellular groups to remain physically attached even when individual cellular
326 connections are severed, increasing cluster toughness by more than 10,000-fold. As a material,
327 snowflake yeast evolve from being ~100-fold weaker than gelatin⁴³, to having the strength and
328 toughness of wood⁴⁴. Sequencing revealed an enrichment in mutations affecting the cell cycle and
329 budding - traits that increase cell length and the amount of cell wall material at the point of
330 attachment. Engineered strains with mutations increasing cell length and bud scar size
331 recapitulated our evolutionary progression from microscopic to macroscopic size.

332 In our system, novel multicellular traits arise as an emergent property of changes in cell-
333 level traits. Two cell-level innovations appear to have played a key role in the evolution of
334 macroscopic size: more elongate cells and larger bud scars. Increased cell length initially reduces
335 the strain generated from cellular packing, which is the primary manner in which size increased
336 early in the experiment, and may underlie entanglement by facilitating cellular intercalation.
337 Larger bud scars increase the amount of shared cell wall connecting cells, which all else equal
338 should increase multicellular toughness by strengthening cell-cell bonds³¹. While the evolution of
339 larger, tougher multicellular groups necessarily has underlying cell-level causation, these group
340 and cell-level traits are distinct and non-commensurable (*i.e.*, group size and toughness cannot be
341 measured at the single-cell level). This demonstrates that snowflake yeast are evolving under

342 MLS2, a shift in evolutionary dynamics that is critical for the transition multicellular individuality,
343 as it allows groups as a whole, not just their constituent members, to gain adaptations^{45,46}.

344 Entanglement is a common mechanism through which filamentous materials can solidify.
345 It can operate on nearly any length scale, ranging from nanoscale polymers⁴⁷ and nanofibers⁴⁸, to
346 macroscopic staples⁴⁹, and beaded chains⁵⁰. Relatively little is known about the role of
347 entanglement in the materials properties of macroscopic biological structures, though recent work
348 has shown that California Blackworm collectives are entangled, and can vary their degree of
349 entanglement to solidify and melt their groups in response to environmental change⁵¹⁻⁵³.
350 Macroscopic multicellularity has evolved repeatedly in fungi⁵⁴, and while to our knowledge no
351 prior work has formally examined whether the cells of fungal fruiting bodies and lichen thalli are
352 physically entangled, they are generally composed of densely-packed, overlapping hyphae,
353 strongly suggesting entanglement⁵⁵⁻⁵⁷. The prevalence of entanglement in superficially different
354 systems is likely due to its simplicity and efficacy; if pairs of constituents are easily entangled,
355 large mutually-entangled clusters readily form, greatly increasing the strength and toughness of
356 the material. While further work will be required to test this hypothesis, the relative ease with
357 which multicellular fungi form entangled structures may have facilitated the highly convergent
358 evolution of macroscopic multicellularity within this clade⁵⁴, allowing different fungal lineages to
359 independently evolve robust multicellular structures.

360 Our results depend on the fact that snowflake yeast grow as topologically-structured groups
361 with permanent cellular bonds, and we would not necessarily expect similar biophysical exaptation
362 in organisms with alternative means of group formation. These features, however, make it well
363 suited as a model system for the lineages that have ultimately evolved complex multicellularity.
364 Of the five lineages that independently evolved complex multicellularity (fungi, animals, plants,

365 red algae, and brown algae), all but animals possess permanent cell-cell bonds, and early
366 multicellular lineages in each are thought to have started out as simple, topologically-structured
367 networks⁵⁸. While animals do not currently have permanent cell-cell bonds, little is known about
368 their ancestral mode of cellular adhesion. Indeed, their closest living relatives, the
369 choanoflagellates, can form topologically structured multicellular groups with permanent cell-cell
370 bonds⁵⁹⁻⁶¹, suggesting that early animals may have possessed a similar mode of growth.

371 Despite 600 rounds of selection for increased size, our mixotrophic and obligately aerobic
372 lineages remained microscopic (Fig. 1C & Extended Data Fig. 1), increasing their radius by less
373 than two-fold. Extending prior work examining the role of oxygen diffusion in the evolution
374 multicellular size⁴, these results highlight the importance of environmentally-dependent tradeoffs
375 on the evolution of multicellularity. Oxygen can serve as a resource, allowing increased cellular
376 growth by increasing ATP yields from metabolism⁶² and allows growth on non-fermentable carbon
377 sources⁶³. For simple, diffusion-limited organisms like snowflake yeast, low concentrations of
378 oxygen create a cost to large size by reducing the proportion of cells in the group that have access
379 to it- a cost which our anaerobic populations did not face.

380 During the evolutionary transition to multicellularity, groups of cells must become
381 Darwinian entities capable of adaptation^{64,65}. This requires that they reproduce, and have heritable
382 variation in traits that affect fitness⁶⁶. For groups of cells to become more than simply the sum of
383 their parts, adaptation must take place in multicellular traits that are distinct from those of their
384 constituent cells (*i.e.*, they must evolve under MLS2)^{45,46}. Until now, it has not been clear if simple
385 groups of cells are capable of making this transition, or whether they first require innovations that
386 allow for the heritable expression of novel multicellular traits^{67,68}. Using long-term experimental
387 evolution, we show even simple groups of cells, initially differing from their unicellular ancestor

388 by a single mutation, have an innate capacity for open-ended multicellular adaptation. In response
389 to selection on group size, a broadly important trait for simple multicellular organisms⁶⁹, snowflake
390 yeast evolved to form radically larger and tougher multicellular groups by leveraging the emergent
391 biophysical properties of altered cellular morphology. These results demonstrate how selection on
392 group size can drive sustained multicellular adaptation and biophysical innovation, and highlight
393 the surprising ease with which evolutionary transitions in Darwinian individuality can occur.

394 **Methods**

395 **Long-term evolution experiment.** To generate our ancestral snowflake yeast for the MuLTEE,
396 we started with a unicellular diploid yeast strain (Y55). In this yeast, we replaced both copies of
397 the *ACE2* transcription factor using a *KANMX* resistance marker (*ace2::KANMX/ace2::KANMX*)
398 and obtained a snowflake yeast clone (see²⁸ for a detailed description of strains and growth
399 conditions, including measurements of oxygen concentrations in growth media). When grown in
400 YEPD media (1% yeast extract, 2% peptone, 2% dextrose), these yeast are mixotrophic, both
401 fermenting and respiring. When grown in YEPG media, which is the same as YEPD but with the
402 dextrose replaced by 2.5% glycerol, these yeast are incapable of fermentation and are obligately
403 aerobic. From this initial clone of *ace2Δ* snowflake yeast, we selected a randomly produced ‘petite’
404 (p⁻) mutant. Due to a large deletion in its mitochondrial DNA (identified via sequencing), this
405 snowflake yeast is unable to respire and is therefore metabolically ‘anaerobic,’ and was cultured
406 in YEPD.

407 We evolved five replicate populations of mixotrophic snowflake yeast (referred to as
408 populations PM1-PM5), obligately aerobic (PO1-PO5) and anaerobic (PA1-PA5) snowflake yeast
409 in 10 mL of culture media, growing them in 25 x 150 mm culture tubes for 24 hours at 30°C with
410 225 rpm shaking. We used settling selection to select for larger cluster size. Once per day, after
411 ~24 h of growth, we transferred 1.5 ml of culture into 1.5 mL Eppendorf tubes, let them settle on
412 the bench for 3 minutes, discarded the top 1.45 mL of the culture, and only transferred the bottom
413 50 µl of the pellet into a new 10 mL of culture media for the next round of growth and settling
414 selection. Once the anaerobic populations (PA1-PA5) had started to evolve visibly larger clusters
415 with all biomass settling to the bottom of the tube in under a minute, we decreased the length of
416 gravitational selection to 30 seconds, thus keeping them under directional selection for increased

417 size. The timing of this change corresponded to ~350 days for PA2 and PA5 and ~500 days for
418 PA1, PA3, and PA4. We used wide-bore filtered pipette tips (Thermo Scientific) for our daily
419 transfers. In total, we applied 600 rounds (days) of growth and settling selection. We archived a
420 frozen glycerol stock of each population at -80°C every 10-15 transfers.

421 **Measuring cluster size.** We developed a standard visualization protocol to be able to measure the
422 size of both microscopic and macroscopic snowflake yeast from each population over the 600-day
423 evolution experiment. To prepare yeast for imaging, we revived evolved frozen cultures for each
424 population in 50-day intervals (12 for each of the 15 replicate populations). We then inoculated
425 each sample into 10 mL fresh media and brought them to equilibrium over a five-day culture
426 process, performing daily settling selection prior to transfer to fresh media. After 5 transfers, we
427 pipetted a random 1mL subsample of each 24-hour culture, placing them in 1.5 ml Eppendorf
428 tubes. We added 0.5 ml of sterile water to each well of 12-well culture plates, then gently vortexed
429 each snowflake yeast sample and diluted them into the water (1,000-fold dilution for microscopic
430 populations, and 100-fold dilution for macroscopic populations). We shook each well plate gently
431 to disperse the yeast clusters evenly over the bottom of each well. We then imaged each well using
432 a 4X Nikon objective, capturing the cross-sectional area of clusters without disrupting their 3D
433 structure. Next, we used ImageJ-Fiji to calculate the cross-sectional area of each cluster,
434 converting pixels to microns by including a physical 100 μm scale bar in each image.

435 **Calculating weighted average cluster size.** The distribution of cluster size across various isolates
436 are not consistent- while microscopic populations are unimodal, while macroscopic populations
437 contain a substantial number of small groups that may only contain a few cells. Even when these
438 small groups constitute a trivial amount of the population's biomass, variation in their abundance
439 can have a large impact on sample statistics, like average cluster size. Because of their skewed size

440 distribution, mean size is an unreliable and often uninformative measure of the central tendency
441 of the cluster size distribution, and does not accurately describe how cells are distributed across
442 different cluster size classes. To account for this, we calculated the distribution of cellular biomass
443 over the range of cluster sizes, and found the mean of this biomass distribution (which is the same
444 as weighting mean cluster size by its biomass). This weighted mean cluster size represents the
445 expected size group any given cell will be in (see Extended Data Fig. 2 for a visual representation),
446 and is an accurate measure of changes in the distribution of cellular biomass across different cluster
447 sizes over evolutionary time. Rather than presenting the weighted mean group size as a volume,
448 we transformed these into an average (micron) radius to be consistent with the units that have
449 historically been used in the paleontological literature documenting the evolution of macroscopic
450 multicellular organisms.

451 **Assessing fitness.** We measured the relative fitness of the evolved macroscopic populations (PA1-
452 5) in competition against the ancestor in liquid culture under the same conditions as our evolution
453 experiment. To differentiate competing strains, we used an ancestral snowflake yeast strain
454 carrying a hemizygous red fluorescent protein (*ura3::dTOMATO/URA3*). Before coculturing these
455 strains, we first grew evolved populations and the ancestral strain in separate cultures overnight.
456 Then we mixed the two types in a 1.5 mL microcentrifuge tube to start the competition assay in
457 fresh 10 mL YEPD cultures. We examined the fitness of PA1-5 t600, as well as an
458 ancestor:ancestor control, with three replicate competitions per treatment. We grew these
459 competitions for 24 hours in 10 mL YEPD (conditions as described in the evolution experiment),
460 followed by 3 minutes of settling selection in 1.5 mL Eppendorf tubes. We then transferred the
461 bottom 50 μ l into a fresh culture tube for the next round of growth and settling, repeating the same
462 procedure for three rounds across the fitness assay. The evolved populations' initial frequency

463 ranged between 35-70%, and after three rounds of growth and settling selection, they reached a
464 range between 99.8-99.9%.

465 To calculate the relative fitness of the evolved populations against the common ancestor,
466 we calculated their selection rate constant, as described in ³⁰. To do so, we estimated the initial and
467 final cellular density of yeast by measuring the cross-sectional area of the evolved and ancestral
468 snowflake yeast clusters at the beginning and end of the fitness assay using a Nikon Eclipse Ti
469 inverted microscope at 100x magnification. Then we calculated the selection rate by dividing the
470 estimated density of the evolved populations at the end and beginning of the competition assay,
471 followed by subtracting the natural log of this value from that of the ancestral strain³⁰. Finally, by
472 coculturing the ancestral snowflake yeast strains with and without the hemizygous *dTOMATO*
473 constructs, we confirmed that the expression of this protein had no significant fitness cost, as
474 reported in Fig1F, right column (p=0.22, t=1.7, df=2, one sample t-test). Image analysis was
475 performed in ImageJ (v2.3.0).

476 **Aspect ratio data collection and analysis.** To measure the evolution of cellular aspect ratio in
477 populations PA1-PA5 over the 600-day evolution experiment, we first inoculated 61 samples (1
478 ancestor + 5 replicates x 12 time points, each separated by 50 days) and grew them overnight in
479 shaking incubation as described above. Following the same growth protocols as in our cluster size
480 measurements, we grew these samples for five consecutive days with settling selection. On the
481 final day, we transferred 100 μ l of each culture into tubes with fresh YEPD and incubated them
482 for 12 hours. Next, we stained samples in calcofluor-white by incubating them in the dark for 30
483 minutes (at a final concentration of 5 μ M) prior to imaging (40x objective, UV excitation of blue
484 fluorescent cell wall stain, imaged on a Nikon Ti-E). We measured the aspect ratio of individual

485 cells within snowflake yeast clusters on ImageJ-Fiji, analyzing an average of 453 cells per
486 population.

487 **Simple biophysical model examining packing fraction as a function of aspect ratio.** We
488 simulated the growth of snowflake yeast from a single cell. Cells were modeled as prolate
489 ellipsoids with one long (major) axis and two equal shorter (minor) axes. Clusters started as a
490 single cell and were grown for nine cellular generations. New cells first emerged from their
491 mother's distal pole; subsequent cells emerged with a polar angle of 45° and a random azimuthal
492 angle. If adding a new cell would cause too much overlap with existing branches, the new cell was
493 deleted and the mother cell lost its chance to reproduce that generation. We simulated the growth
494 of 50 clusters of each genotype, which varied in their cellular aspect ratio, defined as the ratio of
495 the major axis to minor axis length, ranging from 1.2-2.8. We then calculated each simulated
496 cluster's packing fraction by fitting a convex hull to the cluster, and measuring the ratio of the total
497 volume to the volume specifically occupied by cells. The MATLAB code to grow snowflake yeast
498 using this protocol is attached as Supplementary File 1.

499 **Testing aggregative vs. clonal development.** To determine if macroscopic snowflake yeast
500 aggregate or develop clonally (Extended Data Fig. 7), we isolated a single genotype from PA2,
501 t600 (strain GOB1413-600), and engineered it to constitutively express either green or red
502 fluorescent proteins. To do that, we amplified the *prTEF_GFP_NATMX* construct from a pFA6a-
503 eGFP plasmid and the *prTEF_dTOMATO_NATMX* construct from a pFA6a-tdTomato plasmid.
504 We then separately replaced the *URA3* open reading frame with *GFP* or *dTOMATO* constructs in
505 an isogenic single strain isolate following the LiAc transformation protocol⁷⁰. We selected
506 transformants on Nourseothricin Sulfate (Gold Biotechnology Inc., U.S.) YEPD plates and
507 confirmed green or red fluorescent protein activity of transformed macroscopic clusters by

508 visualizing them under a Nikon Eclipse Ti inverted microscope. To test whether they grow clonally
509 or aggregatively, we first inoculated *GFP* or *dTOMATO* expressing clones individually overnight.
510 We then mixed the two cultures in equal volume and diluted 100-fold into a 10 mL fresh culture.
511 We co-cultured this mixed population for five days, transferring 1% of the population to fresh
512 media every 24 h. Finally, we washed this culture in 1 mL sterile water and visualized 70 individual
513 clusters under both red and green fluorescent channels, allowing us to count the number of
514 snowflake yeast clusters that were green, red, or chimeric.

515 We examined the potential for entanglement alone to allow for persistent interactions among
516 disconnected components (Extended Data Fig. 10) by crushing GFP- and RFP-tagged macroscopic
517 snowflake yeast (PA2-t600) into smaller groups, and then growing a mixture of them on the surface
518 of agar plates for 48 hours, potentially allowing branches of adjacent genotypes to entangle through
519 growth. We then scraped these populations and grew them in 10 mL YEPD (yeast-extract, peptone,
520 dextrose) media with shaking at 250 rpm for two 24 h rounds of growth and settling selection. We
521 imaged the resulting clusters under widefield microscopy (Nikon Ti-E), taking pictures of
522 individual clusters under bright field, green, and red channels, collecting data for a random sample
523 of clusters (101 for the PA2-t600, and 110 for the ancestor control). We analyzed the images in
524 ImageJ-FIJI, and any clusters that contained both green and red cells were scored as chimeric. The
525 images shown in Extended Data Fig. 10 were taken on a Nikon AR1 confocal, allowing better
526 view of the 3D structure of chimeric intercalation.

527 **Specimen preparation for Serial Bulk Faced Scanning Electron Microscopy (SBF-SEM).** We
528 fixed snowflake yeast in 2% formaldehyde (fresh from paraformaldehyde (EMS)) containing 2
529 mM CaCl₂, incubating at 35°C for 5 minutes followed by 2-3 hours on ice. Next, we incubated
530 these yeast for an hour in a solution of 1.5% potassium ferrocyanide, 0.15M cacodylate buffer, 2

531 mM CaCl₂, and 2% aqueous osmium tetroxide. This last step was performed on ice and under
532 vacuum. Finally, we washed our yeast and incubated them in thiocarbohydrazide solution (10 g /
533 L double-distilled water) for 60 minutes at 60°C, followed by en bloc uranyl acetate and lead
534 aspartate staining^{71,72}.

535 **SBF-SEM.** We imaged fixed yeast on a Zeiss Sigma VP 3View. This system has Gatan 3View
536 SBF microtome installed inside a Gemini SEM column. For this work, yeast clusters that were
537 embedded in resin were typically imaged at 2.5 keV, using 50-100 nm cutting intervals, 50 nm
538 pixel size, beam dwell time of 0.5-1 µsec and a high vacuum chamber.

539 **SEM Image analysis.** Images were initially in.dm3 format, which we converted to .tiff using
540 GMS3 software. We then cleaned the images and passed them through a gaussian filter in Python.
541 Using the interactive learning and segmentation toolkit (ilastik), we segmented images into 3 parts:
542 live cells, dead cell debris, and background. We then imported segmented HDF5 files in Python.
543 First, we identified connected cells using the nearest neighbor algorithm to identify connected
544 cells. We call a set of connected cells inside a sub-volume a connected component. Then, using a
545 3D extension of the gift-wrapping algorithm, we extracted the convex hull of each connected
546 component.

547 **Visualization of SEM images.** After segmenting images as described above, we created a mesh
548 of individual cells by dilating binarized images. After creating the surface mesh of each individual
549 cell using the mesh tool in Mathematica 12, we imported whole sub-volumes in Rhino6. Then we
550 manually identified cell-to-cell connections and colored each connected component differently.

551 **Volume fraction data collection and analysis.** We measured the packing fraction (proportion of
552 the cluster volume that is cellular biomass) by measuring the number of cells within a cluster, their
553 size, and the volume of the cluster, following the protocol described in Zamani et al. (2021)⁷³.

554 **Mechanical testing.** To test the response of ancestral clusters to uniaxial compression we
555 submerged individual clusters under water, and then compressed them using a Puima Chiaro
556 nanoindenter (Optics11, 19.5 μ m spherical glass probe). For mechanical measurements of
557 macroscopic snowflake yeast, we used a Zwick Roell Universal Testing Machine (UTM) with 5
558 N probe. As above, individual clusters were extracted from the growth tube and placed on the
559 testing stage while submerged under water.

560 **Preparing glass slides with attached cells.** We coated glass slides with Concanavalin A to make
561 a sticky glass surface to which individual cells could adhere. We started by preparing a 10 mg/mL
562 solution of ConA dissolved in sterile DI water, which can be stored at -20C. This stock solution
563 was diluted 1:10, and then 200 μ L of diluted solution was pipetted onto a glass slide in a sterile
564 environment. The slide was allowed to incubate for 5 min at room temperature, then washed with
565 sterile DI water twice, then left to aspirate dry in the hood. Cell cultures were inoculated (100 μ L)
566 onto the glass surface and left to settle for 5 minutes.

567 **AFM measurements.** Prior to measuring the properties of individual cells with the AFM, we
568 restored ACE2 functionality to increase the frequency of single cells available for mechanical
569 testing. To do this, we reinserted a single copy of the ancestral *ACE2* allele fused with the antibiotic
570 resistance gene *HYGNT1* into the genome of the PA ancestor and PA1 t600 isolate under the
571 control of its native promotor using the LiAc/SS-DNA/PEG method of yeast transformation⁶⁹.
572 Transformants were then plated on YEPD agar plates (1% yeast extract, 2% peptone, 2% dextrose,

573 1.5% agar) supplemented with 200 mg l⁻¹ of the antibiotic hygromycin B (Enzo Life Sciences).
574 All atomic force measurements used an atomic force microscope from Asylum Research that was
575 integrated with an inverted optical microscope (Nikon). For single-cell measurements, we used a
576 silicon nitride cantilever with a nominal stiffness of 0.06 N/m with an attached borosilicate glass
577 bead with diameter 2µm (Novascan Technologies). The cantilever was measured via thermal
578 analysis to have a stiffness of 0.0593 N/m. For cluster-level measurements, we used tipless,
579 aluminum coated cantilevers that have a rectangular shape (length 225 µm, width 40 µm) that have
580 a nominal stiffness of 30 N/m (AppNano). For measurements, either single cells or entire clusters
581 were visually aligned with the cantilever probe, which was then moved at a velocity of 1 µm/s to
582 compress the cell or cluster with increasing force.

583 **Chitin staining protocol.** We stained cells with calcofluor white via the following protocol. First,
584 we mixed 500µL of cell culture from the ancestor and PA2 t600 strains into the same tube. Then,
585 we sampled 150 µL (containing both the ancestor and t600 yeast clusters) from the mixed culture.
586 We removed the supernatant via an iterated process of centrifugation and pipetting media removal.
587 Then, we diluted 15 µL of 1 mg/mL calcofluor solution into 500 µL 1x Phosphate-buffered saline
588 solution (PBS) and mixed with the yeast pellet. We incubated the sample in darkness at room
589 temperature for 25 minutes, then we removed the calcofluor media via centrifugation and pipetting.
590 Finally, we added 200 µL 1x PBS on top of the pellet. 20 µL of this cell suspension was pipetted
591 onto a clean glass slide and covered with a coverslip for microscopy.

592 **Single Cell and Bud Scar Confocal microscopy.** We used a Nikon A1R confocal microscope
593 equipped with a 60x oil immersion objective to obtain z-stack images of individual cells stained
594 with calcofluor white. To track the location and size of bud scars, we wrote a MatLab script to
595 extract the brightest calcofluor signals, since the chitinous bud site region makes bud scars brighter

596 than the other portions of the cell wall. Brightness isosurfaces isolated the bud scars themselves,
597 and the brightness of each voxel contained within the isosurface was recorded to track the density
598 of chitin. Next, the isosurface points were rotated to the x-y plane by finding the principal axes of
599 the shape via principal component analysis. The rotated surface points were then used to calculate
600 the height and cross-sectional area of the bud scars.

601 **DNA extraction and genome sequencing.** To extract DNA for whole-genome sequencing, we
602 isolated clones from each of the evolved replicate populations of anaerobic yeast (*i.e.*, PA1-PA5)
603 and their common ancestor after 200, 400, and 600 days of evolution. To pick clonal isolates, we
604 diluted populations of snowflake yeast clusters in 1.5 mL tubes and plated them at a density of
605 100-200 colonies per plate. Next, we restreaked those initial single colonies onto fresh plates, thus
606 ensuring that each colony on a plate results from a single snowflake yeast cluster. Because
607 snowflake yeast grow clonally, we expected that these isolates would only represent a single clone
608 of cells, with no more variation than would be expected from any single cell isolate that grew into
609 a population, generating *de novo* mutation along the way (subsequent analysis of the genomes
610 confirmed this: we never saw evidence of >1 genotype present in any isolate). We inoculated these
611 16 samples in YEPD for 12 hours and extracted their genomic DNA using a commercially
612 available kit (Amresco, Inc. VWR USA). We measured the concentration of DNA with a Qubit
613 fluorometer (Thermo Fisher Scientific, Inc.). We prepared genomic DNA libraries for the 16
614 samples using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Inc).
615 We quantified the quality of the genomic DNA library using the Agilent 2100 Bioanalyzer system
616 located at the Genome Analysis Core Laboratories at Georgia Institute of Technology (Agilent
617 Technologies, Inc). Finally, whole genomes were sequenced using the HiSeq 2500 platform

618 (Illumina, Inc) by the Genome Analysis Core Center located in the Petit Institute, Georgia Tech.

619 As a result, we obtained paired 150 bp (R1 & R2) FASTQ reads from two lanes (L1 & L2).

620 **Bioinformatic analysis.** For our bioinformatic analysis, we used the bash command-line interface
621 on a Linux platform. To identify *de novo* mutations (single nucleotide changes, or 'SNPs,' and
622 small insertion/deletions, or 'indels') in the ancestral and evolved genomes, we first filtered out
623 low-quality reads using a sliding window approach on Trimmomatic (v0.39). We aligned reads to
624 the yeast reference genome (S288C, SGD) using an algorithm in the BWA software package (*i.e.*,
625 BWA-MEM)⁷⁴. Next, we used the genome analysis toolkit (GATK) to obtain and manipulate .bam
626 files⁷⁵. Duplicate reads were marked using the Picard - Tools (MarkSuplicates v2.18.3). We called
627 SNPs using two different tools, *i.e.*, GATK4 HaplotypeCaller (v4.0.3.0) and FreeBayes
628 (v1.2.0)^{75,76}. We validated SNP calls by comparing results obtained by two independent tools. For
629 indels, we used the output from HaplotypeCaller. To filter variants according to their quality/depth
630 scores and generate an overview of the variant calling step's statistical outcome, we used
631 VCFTOOLS (v0.1.16)⁷⁷. Finally, after manually checking each variant call by visualizing SAM
632 files and VCF files on Integrative Genomics Viewer (IGV)⁷⁸, we extracted *de novo* variants by
633 making a pairwise comparison of each VCF file of the evolved genomes against the VCF file of
634 the ancestral genome by using bcftools-isec (v1.10)⁷⁹. Lastly, we annotated evolved mutations
635 using SnpEff (v4.3T)⁸⁰.

636 To search for gene ontology (GO) term enrichment for *de novo* mutations, we generated a
637 combined list of synonymous and nonsynonymous mutations within gene coding regions. We then
638 searched for enriched gene ontology terms using GO Term Finder and GO Slim Mapper on the
639 yeast genome database⁸¹.

640 **Genetically engineering macroscopic snowflake yeast.** To genetically engineer snowflake yeast
641 strains with cell lengthening (*CLB2* and *CLN3*) and bud-scar strengthening (*GIN4*) mutations that
642 are shown in Fig. 4, we used homozygous unicellular (GOB76) and multicellular strains (GOB8).
643 To engineer each gene deletion, we first amplified hygromycin, nourseothricin, and G418
644 resistance marker cassettes from plasmids and individually transformed them into yeast cells (see
645 Supplementary Table 1 for genotypes, plasmids, and primers). Next, we induced sporulation of
646 individual heterozygous mutant strains (2% KAc), dissected tetrads, and obtained homozygous
647 deletions through auto-diploidization. By subsequent rounds of sporulation and inter-tetrad mating
648 on appropriate multi-drug plates, we generated double, triple, and quadruple mutant strains (Fig.
649 4 E&F). Finally, we quantified the cellular aspect ratio and cluster size (cross-sectional area) of
650 these mutants by imaging clusters under a Nikon Eclipse Ti inverted microscope, using the same
651 methods as previously described.

652 **Life-cycle experiment (Extended Data Fig. 2).** To characterize the life cycle of the ancestral
653 (microscopic) and evolved (macroscopic, PA2-600) snowflake yeast, we inoculated both strains
654 starting from frozen glycerol stocks. Then, we grew them under the conditions of the evolution
655 experiment for five rounds of growth and settling selection. Applying a five-day growth and
656 settling selection brings cultures to an equilibrium, reflecting the physiology and size distribution
657 observed during the evolution experiment. On the final day, we sampled from the growing cultures
658 at 0, 3, 6, 12, and 24 hours and measured the number, size, and volume of cultures using the same
659 methods described under "Measuring cluster size."

660 References

- 661 1 Godfrey-Smith, P. *Darwinian populations and natural selection*. (Oxford University
662 Press, 2009).
- 663 2 Bourke, A. F. *Principles of social evolution*. (2011).
- 664 3 Knoll, A. H. The multiple origins of complex multicellularity. *Annual Review of Earth and
665 Planetary Sciences* **39**, 217-239 (2011).
- 666 4 Bozdag, G. O., Libby, E., Pineau, R., Reinhard, C. T. & Ratcliff, W. C. Oxygen
667 suppression of macroscopic multicellularity. *Nature Communications* **12**, 1-10 (2021).
- 668 5 Smukalla, S. *et al.* FLO1 is a variable green beard gene that drives biofilm-like
669 cooperation in budding yeast. *Cell* **135**, 726-737 (2008).
- 670 6 Bonner, J. T. *The evolution of complexity by means of natural selection*. (Princeton
671 University Press, 1988).
- 672 7 Bell, G. & Mooers, A. O. Size and complexity among multicellular organisms. *Biological
673 Journal of the Linnean Society* **60**, 345-363 (1997).
- 674 8 Bonner, J. T. Perspective: the size-complexity rule. *Evolution* **58**, 1883-1890 (2004).
- 675 9 Willensdorfer, M. Organism size promotes the evolution of specialized cells in
676 multicellular digital organisms. *Journal of evolutionary biology* **21**, 104-110 (2008).
- 677 10 Solari, C. A., Kessler, J. O. & Goldstein, R. E. A general allometric and life-history model
678 for cellular differentiation in the transition to multicellularity. *The American Naturalist* **181**,
679 369-380 (2013).
- 680 11 Fisher, R., Shik, J. & Boomsma, J. The evolution of multicellular complexity: the role of
681 relatedness and environmental constraints. *Proceedings of the Royal Society B* **287**,
682 20192963 (2020).
- 683 12 Knoll, A. H. & Hewitt, D. Phylogenetic, functional and geological perspectives on
684 complex multicellularity. *The major transitions in evolution revisited*, 251-270 (2011).
- 685 13 Bonner, J. T. *Why size matters: from bacteria to blue whales*. (Princeton University
686 Press, 2011).
- 687 14 Boudaoud, A. An introduction to the mechanics of morphogenesis for plant biologists.
688 *Trends in plant science* **15**, 353-360 (2010).
- 689 15 Höhn, S., Honerkamp-Smith, A. R., Haas, P. A., Trong, P. K. & Goldstein, R. E.
690 Dynamics of a Volvox embryo turning itself inside out. *Physical review letters* **114**,
691 178101 (2015).
- 692 16 Bi, D., Yang, X., Marchetti, M. C. & Manning, M. L. Motility-driven glass and jamming
693 transitions in biological tissues. *Physical Review X* **6**, 021011 (2016).
- 694 17 Delarue, M. *et al.* Self-driven jamming in growing microbial populations. *Nature physics*
695 **12**, 762-766 (2016).
- 696 18 Jacobsen, S. *et al.* Cellular packing, mechanical stress and the evolution of
697 multicellularity. *Nature physics* **14**, 286-290 (2018).
- 698 19 Boraas, M. E., Seale, D. B. & Boxhorn, J. E. Phagotrophy by a flagellate selects for
699 colonial prey: a possible origin of multicellularity. *Evolutionary Ecology* **12**, 153-164
700 (1998).
- 701 20 H. Koschwanez, J., R. Foster, K. & W. Murray, A. Sucrose utilization in budding yeast as
702 a model for the origin of undifferentiated multicellularity. *PLoS biology* **9**, e1001122
703 (2011).
- 704 21 Ratcliff, W. C., Denison, R. F., Borrello, M. & Travisano, M. Experimental evolution of
705 multicellularity. *Proceedings of the National Academy of Sciences* **109**, 1595-1600
706 (2012).
- 707 22 Ratcliff, W. C. *et al.* Experimental evolution of an alternating uni-and multicellular life
708 cycle in *Chlamydomonas reinhardtii*. *Nature communications* **4**, 1-7 (2013).

- 709 23 Herron, M. D. *et al.* De novo origins of multicellularity in response to predation. *Scientific*
710 *reports* **9**, 1-9 (2019).
- 711 24 Westbrook, J. W. *et al.* What makes a leaf tough? Patterns of correlated evolution
712 between leaf toughness traits and demographic rates among 197 shade-tolerant woody
713 species in a neotropical forest. *The American Naturalist* **177**, 800-811 (2011).
- 714 25 Veres, S. P. & Lee, J. M. Designed to fail: a novel mode of collagen fibril disruption and
715 its relevance to tissue toughness. *Biophysical journal* **102**, 2876-2884 (2012).
- 716 26 Prakash, V. N., Bull, M. S. & Prakash, M. Motility-induced fracture reveals a ductile-to-
717 brittle crossover in a simple animal's epithelia. *Nature Physics* **17**, 504-511 (2021).
- 718 27 Grant, N. A., Maddamsetti, R. & Lenski, R. E. Maintenance of metabolic plasticity
719 despite relaxed selection in a long-term evolution experiment with *Escherichia coli*. *The*
720 *American Naturalist* **198**, 93-112 (2021).
- 721 28 Ratcliff, W. C., Fankhauser, J. D., Rogers, D. W., Greig, D. & Travisano, M. Origins of
722 multicellular evolvability in snowflake yeast. *Nature communications* **6**, 1-9 (2015).
- 723 29 Heinrich, E. C., Farzin, M., Klok, C. J. & Harrison, J. F. The effect of developmental
724 stage on the sensitivity of cell and body size to hypoxia in *Drosophila melanogaster*.
725 *Journal of Experimental Biology* **214**, 1419-1427 (2011).
- 726 30 Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-term experimental
727 evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *The*
728 *American Naturalist* **138**, 1315-1341 (1991).
- 729 31 Jacobsen, S. *et al.* Geometry, packing, and evolutionary paths to increased multicellular
730 size. *Physical Review E* **97**, 050401 (2018).
- 731 32 Pentz, J. T. *et al.* Ecological advantages and evolutionary limitations of aggregative
732 multicellular development. *Current Biology* **30**, 4155-4164. e4156 (2020).
- 733 33 Denk, W. & Horstmann, H. Serial block-face scanning electron microscopy to reconstruct
734 three-dimensional tissue nanostructure. *PLoS Biol* **2**, e329 (2004).
- 735 34 Edwards, C. E., Mai, D. J., Tang, S. & Olsen, B. D. Molecular anisotropy and
736 rearrangement as mechanisms of toughness and extensibility in entangled physical gels.
737 *Physical Review Materials* **4**, 015602 (2020).
- 738 35 Brown, E., Nasto, A., Athanassiadis, A. G. & Jaeger, H. M. Strain stiffening in random
739 packings of entangled granular chains. *Physical review letters* **108**, 108302 (2012).
- 740 36 Raymer, D. M. & Smith, D. E. Spontaneous knotting of an agitated string. *Proceedings of*
741 *the National Academy of Sciences* **104**, 16432-16437 (2007).
- 742 37 Wilhelm, J. & Frey, E. Elasticity of stiff polymer networks. *Physical review letters* **91**,
743 108103 (2003).
- 744 38 Kim, J., Zhang, G., Shi, M. & Suo, Z. Fracture, fatigue, and friction of polymers in which
745 entanglements greatly outnumber cross-links. *Science* **374**, 212-216 (2021).
- 746 39 Tauber, J., Rovigatti, L., Dussi, S. & Van Der Gucht, J. Sharing the load: Stress
747 redistribution governs fracture of polymer double networks. *Macromolecules* **54**, 8563-
748 8574 (2021).
- 749 40 Sheu, Y.-J., Barral, Y. & Snyder, M. Polarized growth controls cell shape and bipolar bud
750 site selection in *Saccharomyces cerevisiae*. *Molecular and cellular biology* **20**, 5235-
751 5247 (2000).
- 752 41 Watanabe, M., Watanabe, D., Nogami, S., Morishita, S. & Ohya, Y. Comprehensive and
753 quantitative analysis of yeast deletion mutants defective in apical and isotropic bud
754 growth. *Current genetics* **55**, 365-380 (2009).
- 755 42 Sopko, R. *et al.* Mapping pathways and phenotypes by systematic gene overexpression.
756 *Molecular cell* **21**, 319-330 (2006).
- 757 43 Lee, D. H., Tamura, A., Arisaka, Y., Seo, J.-H. & Yui, N. Mechanically reinforced gelatin
758 hydrogels by introducing slidable supramolecular cross-linkers. *Polymers* **11**, 1787
759 (2019).

- 760 44 Gerhards, C. *Effects of type of testing equipment and specimen size on toughness of*
761 *wood*. Vol. 97 (Forest Products Laboratory, 1968).
- 762 45 Okasha, S. Multilevel selection and the major transitions in evolution. *Philosophy of*
763 *science* **72**, 1013-1025 (2005).
- 764 46 Rainey, P. B. & Kerr, B. Cheats as first propagules: a new hypothesis for the evolution of
765 individuality during the transition from single cells to multicellularity. *BioEssays* **32**, 872-
766 880 (2010).
- 767 47 Graessley, W. W. in *The entanglement concept in polymer rheology* 1-179 (Springer,
768 1974).
- 769 48 Chen, W., Yu, H., Li, Q., Liu, Y. & Li, J. Ultralight and highly flexible aerogels with long
770 cellulose I nanofibers. *Soft matter* **7**, 10360-10368 (2011).
- 771 49 Gravish, N., Franklin, S. V., Hu, D. L. & Goldman, D. I. Entangled granular media.
772 *Physical review letters* **108**, 208001 (2012).
- 773 50 Zou, L.-N., Cheng, X., Rivers, M. L., Jaeger, H. M. & Nagel, S. R. The packing of
774 granular polymer chains. *Science* **326**, 408-410 (2009).
- 775 51 Savoie, W., Tuazon, H., Bhamla, M. S. & Goldman, D. I. Amorphous Entangled Active
776 Matter. *arXiv preprint arXiv:2207.03665* (2022).
- 777 52 Ozkan-Aydin, Y., Goldman, D. I. & Bhamla, M. S. Collective dynamics in entangled
778 worm and robot blobs. *Proceedings of the National Academy of Sciences* **118**,
779 e2010542118 (2021).
- 780 53 Patil, V. P. *et al.* Ultrafast reversible self-assembly of living tangled matter. *arXiv preprint*
781 *arXiv:2210.03384* (2022).
- 782 54 Nagy, L. G. in *The Evolution of Multicellularity* 279-300 (CRC Press, 2022).
- 783 55 Moore, D. *Fungal biology in the origin and emergence of life*. (Cambridge University
784 Press, 2013).
- 785 56 García-Segovia, P., Andrés-Bello, A. & Martínez-Monzó, J. Rehydration of air-dried
786 Shiitake mushroom (*Lentinus edodes*) caps: Comparison of conventional and vacuum
787 water immersion processes. *LWT-Food Science and Technology* **44**, 480-488 (2011).
- 788 57 Roth, R., Wagner, R. & Goodenough, U. Lichen 3. Outer layers. *Algal Research* **56**,
789 102332 (2021).
- 790 58 Yanni, D. *et al.* Topological constraints in early multicellularity favor reproductive division
791 of labor. *Elife* **9**, e54348 (2020).
- 792 59 Larson, B. T. *et al.* Biophysical principles of choanoflagellate self-organization.
793 *Proceedings of the National Academy of Sciences* **117**, 1303-1311 (2020).
- 794 60 Brunet, T. *et al.* Light-regulated collective contractility in a multicellular choanoflagellate.
795 *Science* **366**, 326-334 (2019).
- 796 61 Dayel, M. J. *et al.* Cell differentiation and morphogenesis in the colony-forming
797 choanoflagellate *Salpingoeca rosetta*. *Developmental biology* **357**, 73-82 (2011).
- 798 62 Hinkle, P. C., Kumar, M. A., Resetar, A. & Harris, D. L. Mechanistic stoichiometry of
799 mitochondrial oxidative phosphorylation. *Biochemistry* **30**, 3576-3582 (1991).
- 800 63 Raymond, J. & Segrè, D. The effect of oxygen on biochemical networks and the
801 evolution of complex life. *Science* **311**, 1764-1767 (2006).
- 802 64 Szathmáry, E. & Smith, J. M. The major evolutionary transitions. *Nature* **374**, 227-232
803 (1995).
- 804 65 Buss, L. W. *The evolution of individuality*. Vol. 796 (Princeton University Press, 2014).
- 805 66 Lewontin, R. C. The units of selection. *Annual review of ecology and systematics*, 1-18
806 (1970).
- 807 67 Michod, R. E. in *The Evolution of Multicellularity* 25-52 (CRC Press, 2022).
- 808 68 Libby, E. & Rainey, P. B. A conceptual framework for the evolutionary origins of
809 multicellularity. *Physical biology* **10**, 035001 (2013).

- 810 69 Tong, K., Bozdag, G. O. & Ratcliff, W. C. Selective drivers of simple multicellularity.
811 *Current Opinion in Microbiology* **67**, 102141 (2022).
- 812 70 Gietz, R. D. & Schiestl, R. H. High-efficiency yeast transformation using the LiAc/SS
813 carrier DNA/PEG method. *Nature protocols* **2**, 31-34 (2007).
- 814 71 Deerinck, T. J. *et al.* High-performance serial block-face SEM of nonconductive
815 biological samples enabled by focal gas injection-based charge compensation. *Journal*
816 *of microscopy* **270**, 142-149 (2018).
- 817 72 Ngo, H. T. & Yin, C. S. *Luteimonas terrae* sp. nov., isolated from rhizosphere soil of
818 *Radix ophiopogonis*. *International journal of systematic and evolutionary microbiology*
819 **66**, 1920-1925 (2016).
- 820 73 Zamani-Dahaj, S. A. *et al.* Spontaneous emergence of multicellular heritability. *bioRxiv*
821 (2021).
- 822 74 Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM.
823 *arXiv preprint arXiv:1303.3997* (2013).
- 824 75 McKenna, A. *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing
825 next-generation DNA sequencing data. *Genome research* **20**, 1297-1303 (2010).
- 826 76 Garrison, E. & Marth, G. Haplotype-based variant detection from short-read sequencing.
827 *arXiv preprint arXiv:1207.3907* (2012).
- 828 77 Danecek, P. *et al.* The variant call format and VCFtools. *Bioinformatics* **27**, 2156-2158
829 (2011).
- 830 78 Thorvaldsdóttir, H., Robinson, J. T. & Mesirov, J. P. Integrative Genomics Viewer (IGV):
831 high-performance genomics data visualization and exploration. *Briefings in*
832 *bioinformatics* **14**, 178-192 (2013).
- 833 79 Danecek, P. *et al.* Twelve years of SAMtools and BCFtools. *Gigascience* **10**, giab008
834 (2021).
- 835 80 Cingolani, P. *et al.* A program for annotating and predicting the effects of single
836 nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster*
837 strain w1118; iso-2; iso-3. *Fly* **6**, 80-92 (2012).
- 838 81 Cherry, J. M. *et al.* *Saccharomyces* Genome Database: the genomics resource of
839 budding yeast. *Nucleic acids research* **40**, D700-D705 (2012).
- 840 82 Day, T. C. *et al.* Cellular organization in lab-evolved and extant multicellular species
841 obeys a maximum entropy law. *Elife* **11**, e72707 (2022).
- 842

843 **Acknowledgements.** We thank Jennifer T. Pentz for teaching us Illumina library preparation,
844 Shweta Biliya at the High Throughput DNA Sequencing Core at Georgia Tech for sequencing the
845 genomes of evolved strains, and Kingsley A. Boateng at Core Facilities at the Carl R. Woese
846 Institute for Genomic Biology for the SEM imaging. Siyi Cao helped us with microscopy during
847 the early stages of this project. We thank Christian Orlic, László Nagy, and all members of the
848 Ratcliff group for insightful comments on the manuscript. This work was supported by NIH grants
849 R35-GM138030-01 to W.C.R. and R35-GM138354-02 to P.J.Y., and a Packard Fellowship for
850 Science and Engineering to W.C.R.

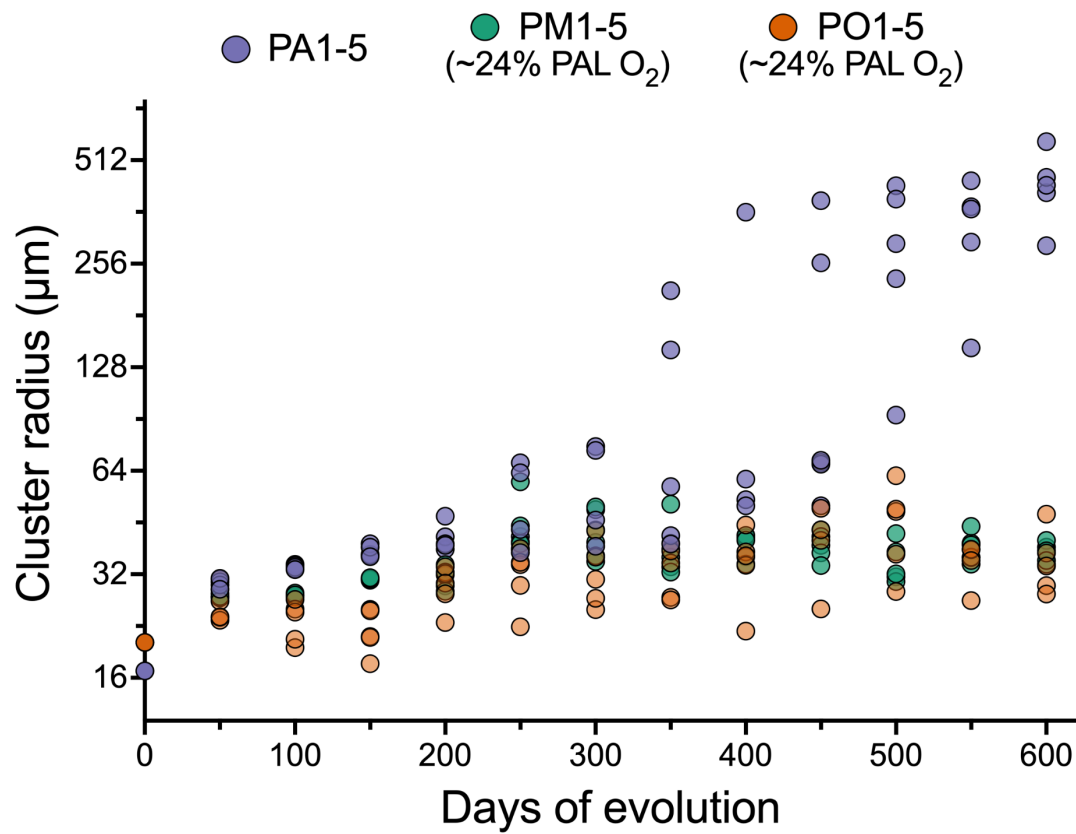
851 **Author Contributions.** G.O.B., A.Z., P.J.Y, and W.C.R. conceived of the project. G.O.B. and
852 W.C.R. designed the MuLTEE, and G.O.B. performed the evolution experiment. G.O.B., A.Z.,
853 P.K., and T.C.D. designed and collected data. A.Z. generated SBF-SEM images. A.Z., T.C.D., and
854 P.J.Y performed the yeast biophysical simulations. E.L.D. and A.H.B. assisted G.O.B. and A.Z.
855 with image analysis. A.J.B. genetically engineered large snowflake yeast, K.T. performed life-
856 cycle experiments, D.T.L. measured the number of generations, and P.L.C. performed unicellular
857 reversion experiments. G.O.B., A.Z., T.C.D., W.C.R., and P.Y. analyzed the data. G.O.B. made
858 the figures. G.O.B., W.C.R., and P.J.Y. wrote the first draft of the paper, and all authors contributed
859 to the revision.

860 **Competing interests.** The authors have no competing interests to declare.

861 **Data and code availability.** All source data and code are available at
862 https://github.com/ozanbozdog/de_novo_evolution_of_macroscopic_multicellularity.

863 Microscopy images are available upon request.

864 **Extended Data Figures**

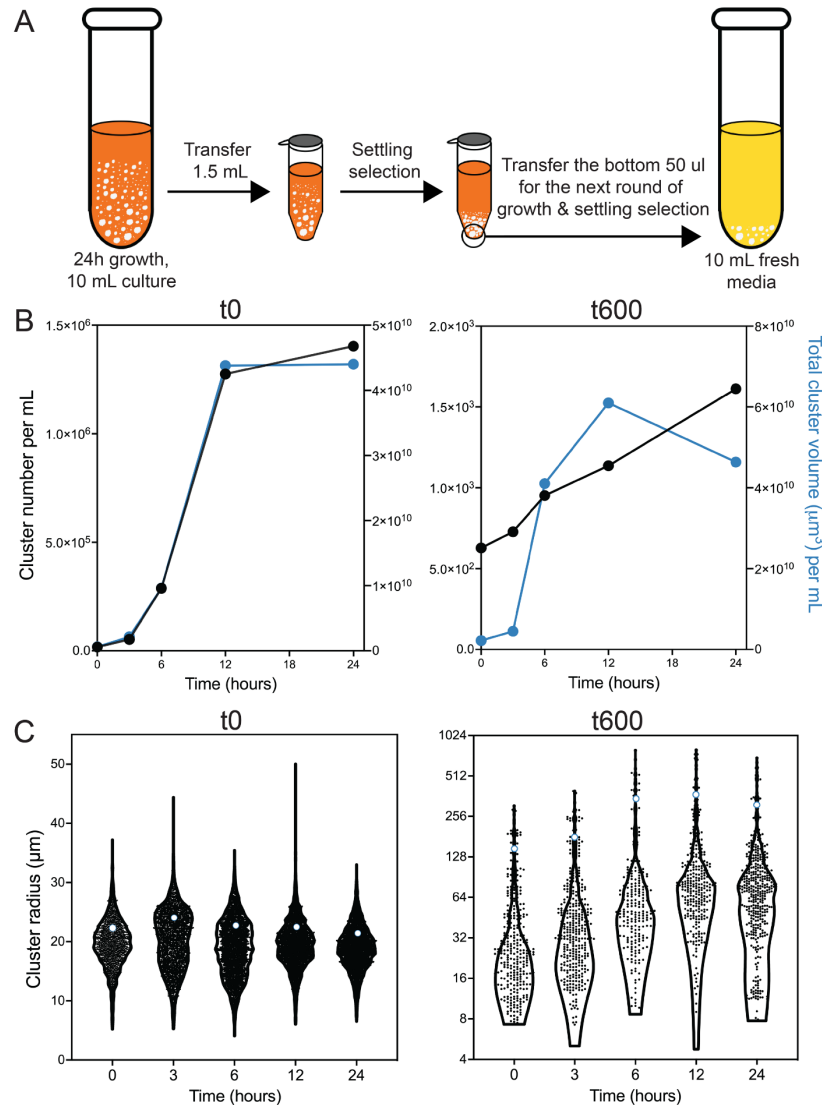


865

866 **Extended Data Figure 1.** Temporal dynamics of size evolution in each population and treatment

867 group. Data points show the weighted average radius of cluster size for the whole population (see

868 Methods for details).



869

870

871

872

873

874

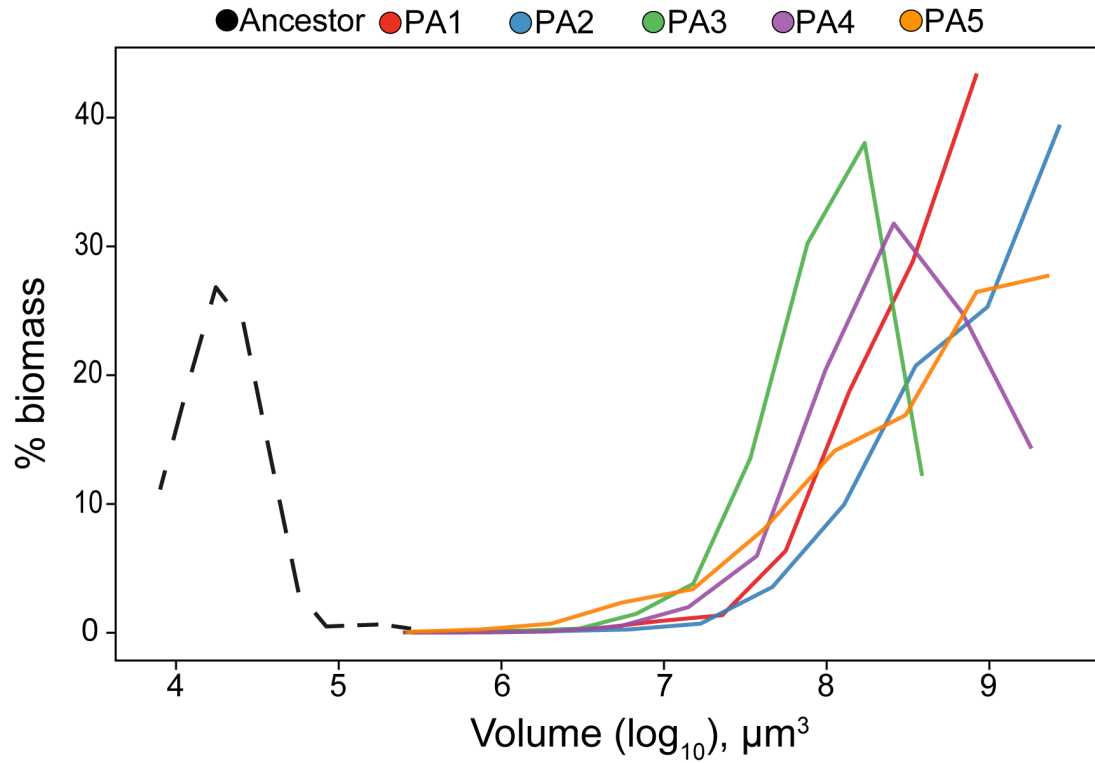
875

876

877

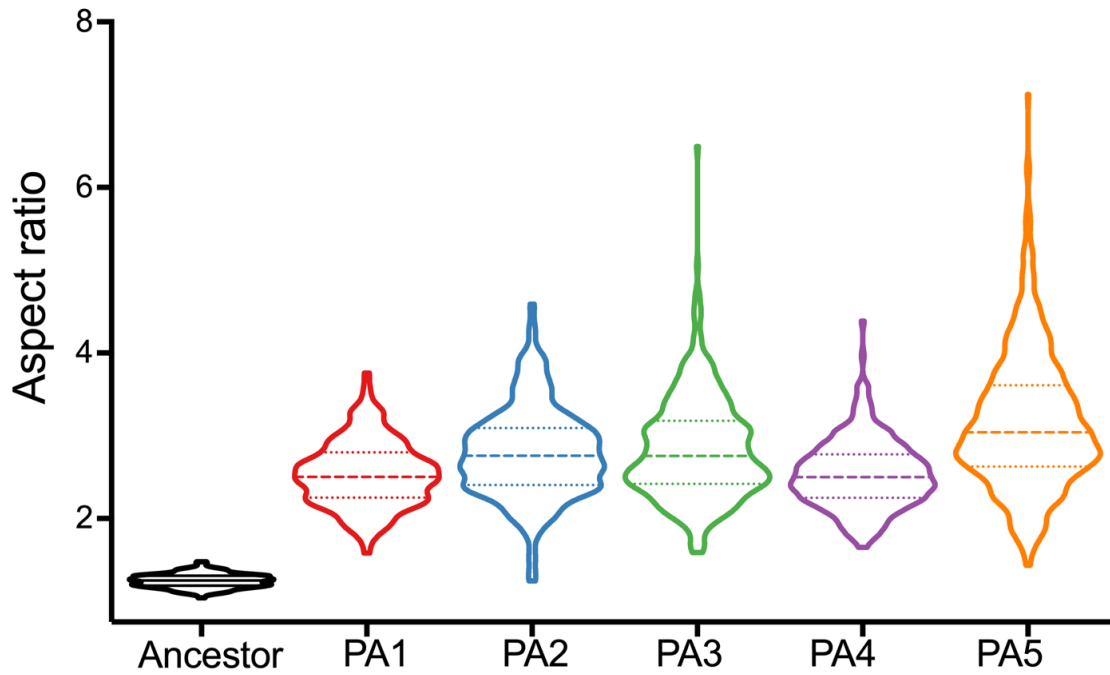
Extended Data Figure 2. Characterizing the life-cycle of the ancestral (microscopic) and evolved (macroscopic) snowflake yeast. A) During the \sim 24-hour growth cycle, snowflake yeast compete for growth and reproduction in 10 mL of YPED (250 RPM at 30°C). At the end of the growth phase, we select for larger group size via settling selection. While there is a theoretical maximum survival rate of 15% (that is, if all of the cells survived settling selection), we only transfer the bottom 50 μ L of pellet biomass regardless of how many cells settle, creating an arms race that favors the fastest groups within the population. Our measurements of the number of cellular generations per day in Figure 1A suggests about 3%

878 of the cells survive from one day to the next on average. B) Both the microscopic (ancestral)
879 and macroscopic (t600) snowflake yeast clusters have a life cycle, reproducing during the
880 growth phase. C) Consistent with entanglement producing tough groups, macroscopic
881 snowflake yeast release mostly microscopic propagules, possibly from branch tips at the
882 exterior of the group, where the opportunity for entanglement is minimal. Despite the
883 presence of many small propagules, most of the biomass in the population is contained
884 within macroscopic clusters. The open circles represent the biomass-weighted mean size,
885 which is the average sized group the mean cell finds itself in.



886

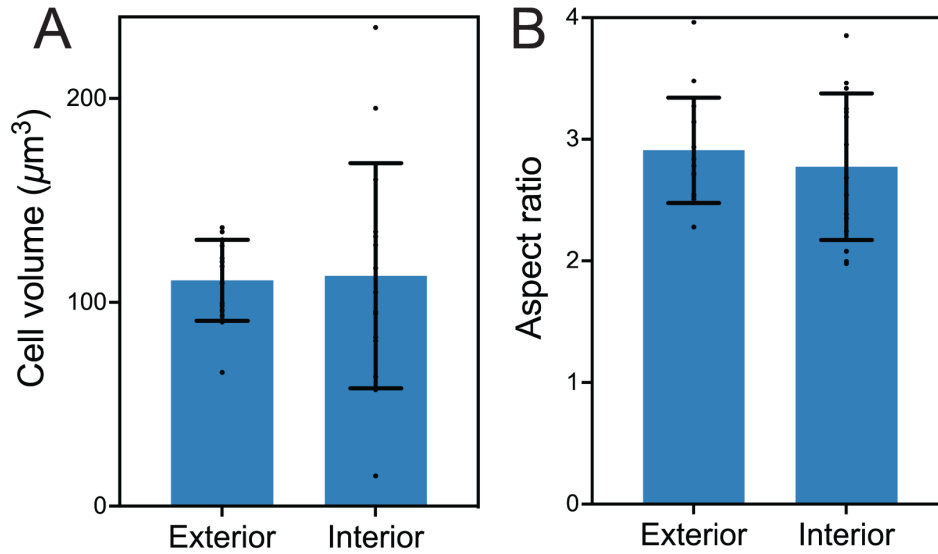
887 **Extended Data Figure 3.** Biomass distribution as a function of group size for the ancestral
888 snowflake yeast (dotted line) and 600 day evolved populations of PA1-PA5. The ‘weighted mean
889 size’ used in Figures 1, 2 and 4 is the mean of the biomass distribution.



890

891 **Extended Data Figure 4.** Distribution of aspect ratios for ancestral and 600-day evolved

892 populations of anaerobic snowflake yeast.



893

894 **Extended Data Figure 5.** Cell shape is not substantially affected by location within macroscopic

895 yeast. (A) and (B) show cell volume and cell shape (aspect ratio) measured for 10 cells from the

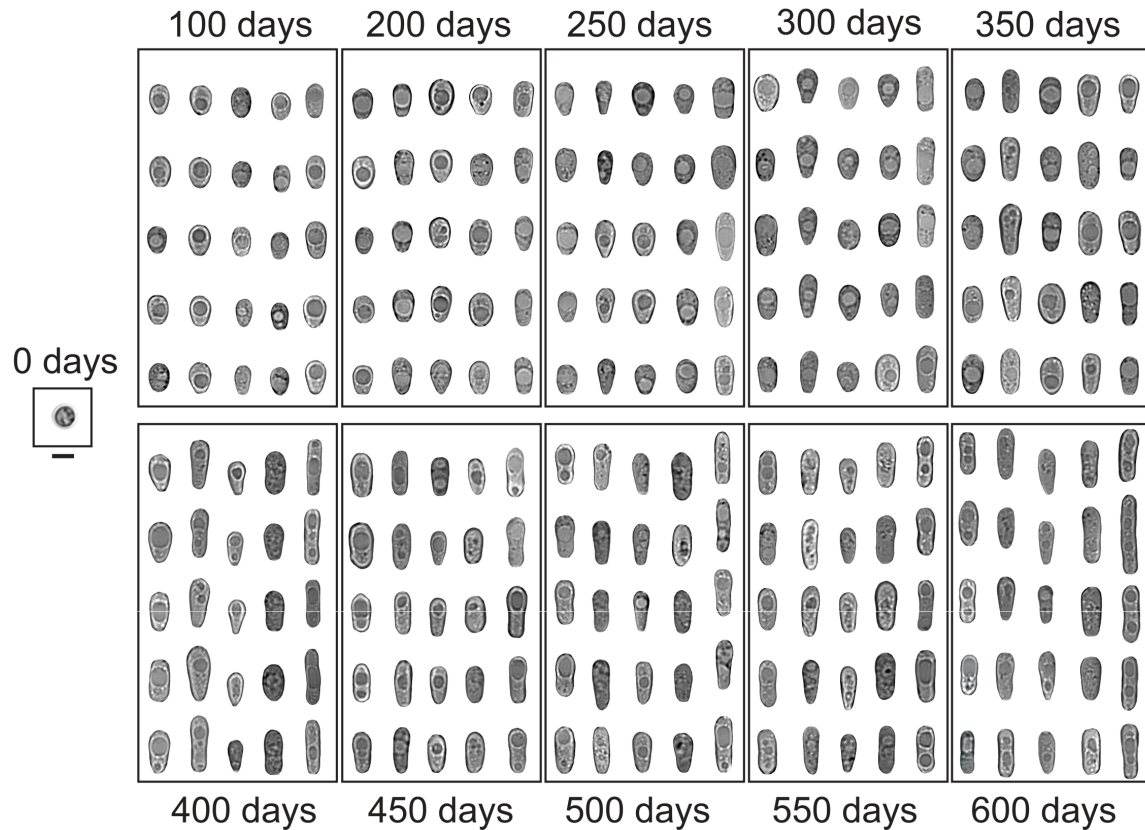
896 interior of a macroscopic cluster and 10 cells from the exterior of a cluster (measured in t600

897 macroscopic clusters). Average cell volume for exterior and interior are $110.8 \mu\text{m}^3$ and $113.1 \mu\text{m}^3$

898 ($p=0.88$, $t=0.15$ $df=17.55$, Welch's t -test), and average cell shape for exterior and interior are 2.9

899 and 2.8 ($p=0.51$, $t=0.68$, $df=14$, Welch's t -test). Individual measurements are marked as points,

900 the mean and one standard deviation are indicated by the bar plot.



901

902

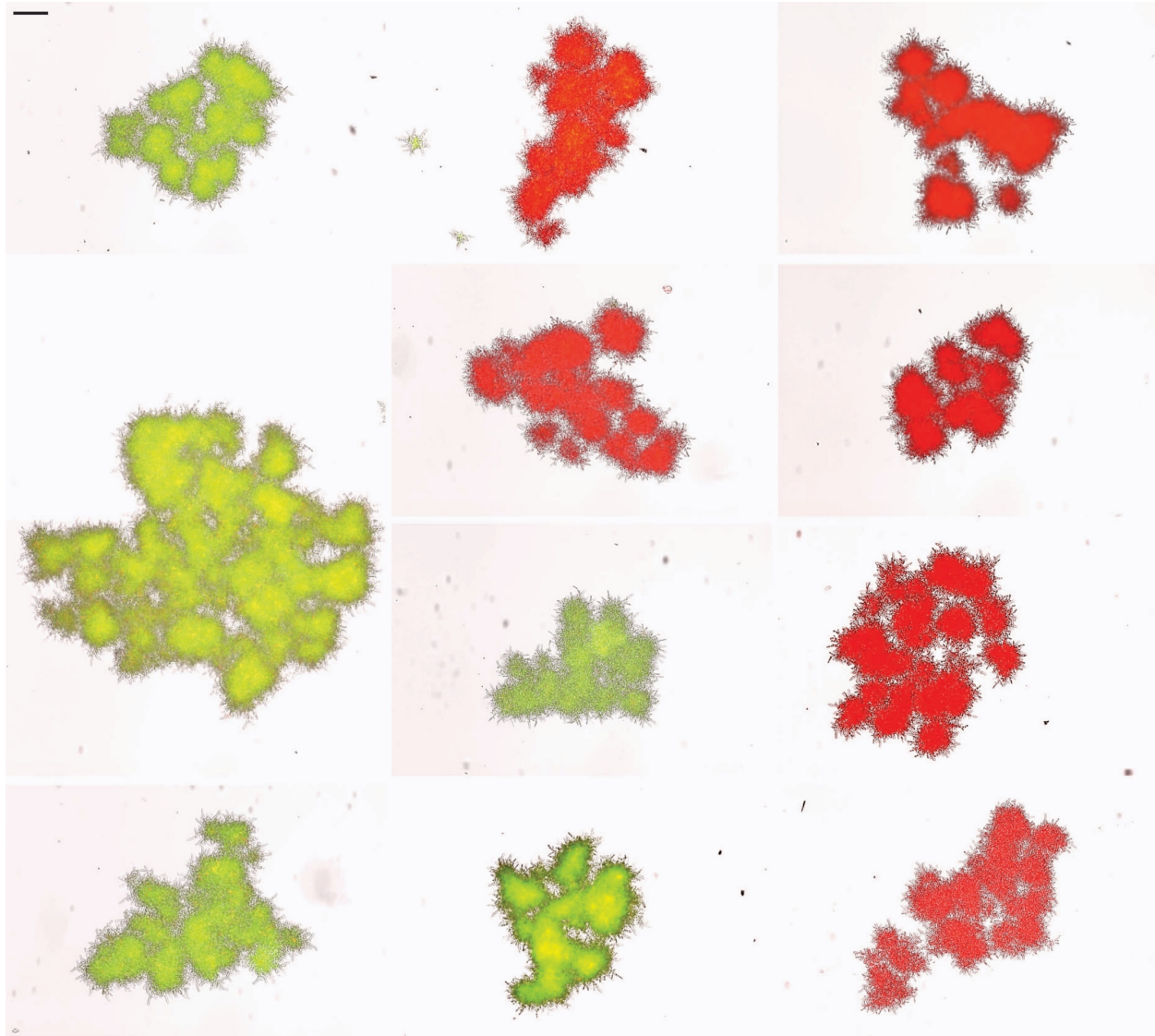
903

904

905

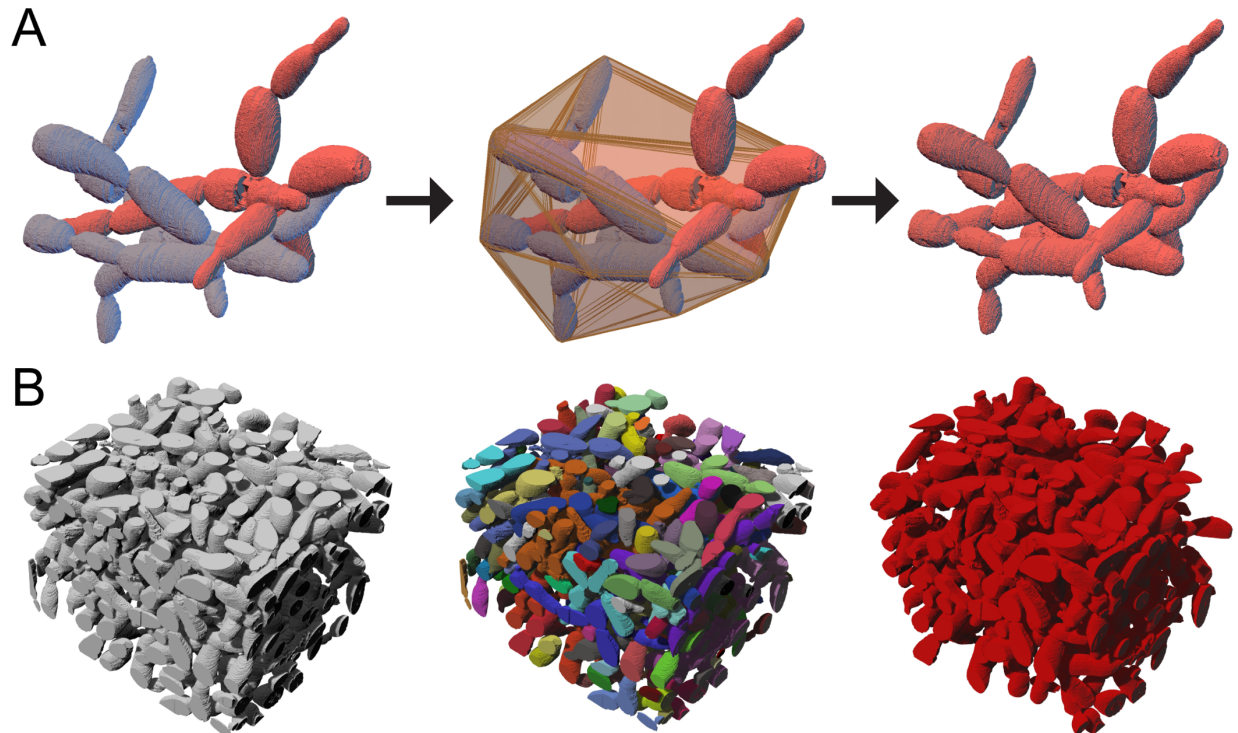
906

Extended Data Figure 6. Parallel evolution of elongated cell shape across all five replicates of each PA population. For each evolutionary time point and population, five different cells are shown (organized vertically from left to right: PA1 on the further left and PA5 on the further right in each box). Scale bar is 5 μm (under the ancestral cell). This is a more detailed version of the plot shown in Figure 2c.



907

908 **Extended Data Figure 7.** Macroscopic snowflake yeast are monoclonal, growing via permanent
909 mother-daughter cellular bonds, not aggregation. We co-cultured GFP and RFP-tagged genotypes
910 of a macroscopic single strain isolate (PA2, strain ID: GOB1413-600) for 5 days, then imaged 70
911 clusters on a Nikon Ti-E. Shown are a composite of 11 individual clusters, which all remain
912 entirely green or red. Individual clusters were compressed with a coverslip for imaging, resulting
913 in their fragmentation into multiple modules. Scale bar (top-left) is 100 μm .



914

915 **Extended Data Figure 8.** Quantifying entanglement via analysis of the topology and geometry of

916 a snowflake yeast cluster. (A) We measured entanglement of individual components by fitting a

917 convex hull around each component, and determining whether the other component overlaps with

918 the space bounded by this convex hull. Here we just show the convex hull for the blue component,

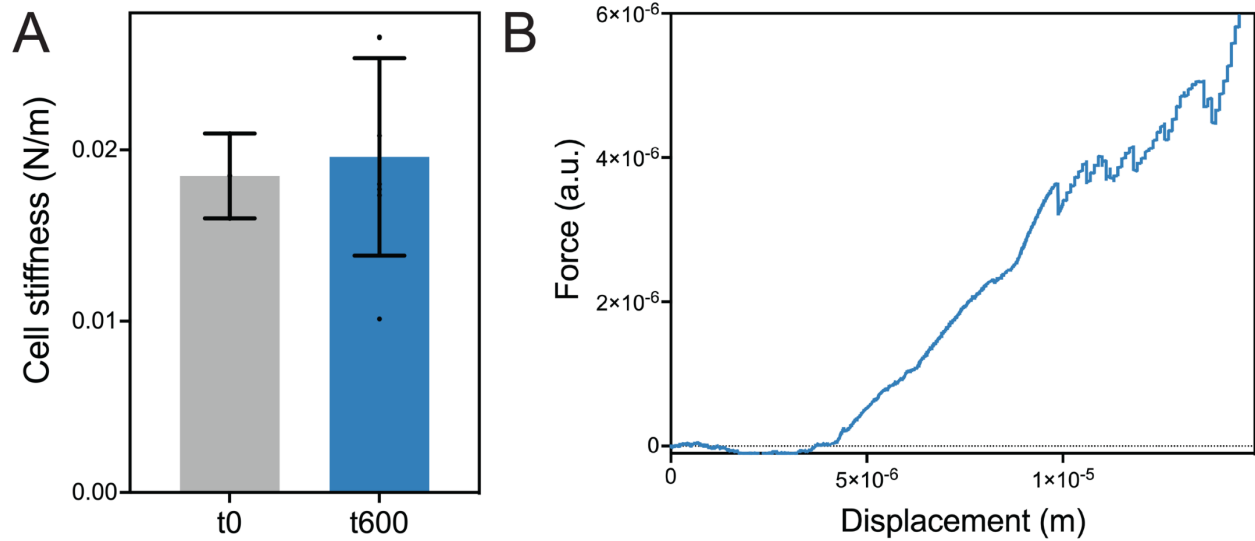
919 which overlaps with the red component. These components are thus part of the same entangled

920 component. (B) Using this approach, we identified the components within a sub-volume of a

921 macroscopic snowflake yeast, and used a percolation analysis to examine the fraction of the

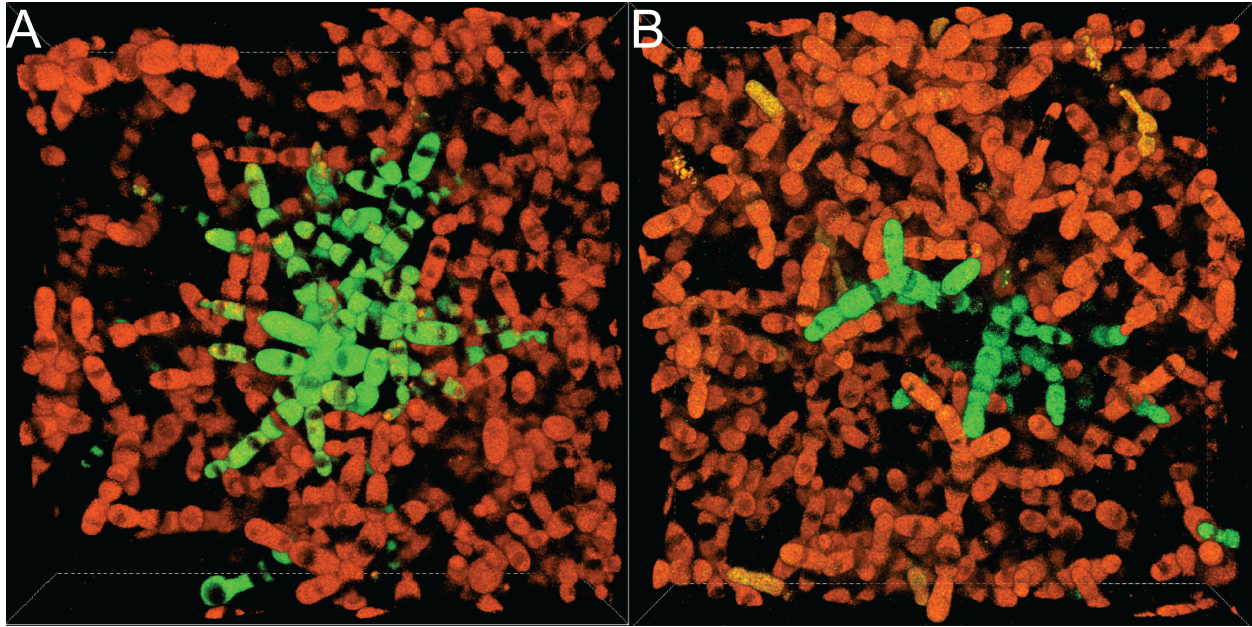
922 biomass that is part of the same entangled component (colored in red).

923



924

925 **Extended Data Figure 9.** (A) Individual cells do not change their stiffness over 600 rounds of
926 selection (average cell stiffness for the ancestor and t600 isolates are 0.019 and 0.020, respectively.
927 $p=0.77$, $t=0.31$, $df=8$, Welch's unequal variances t -test). Single-cell stiffness values measured from
928 atomic force microscopy (AFM) of individual cells. Error bars are one standard deviation. (B)
929 Macroscopic snowflake yeast fractured into small modules prior to compression do not show strain
930 stiffening behavior. Shown here is an AFM trajectory of cantilever deflection vs displacement for
931 one t600 cluster that has been crushed into small, unentangled pieces.

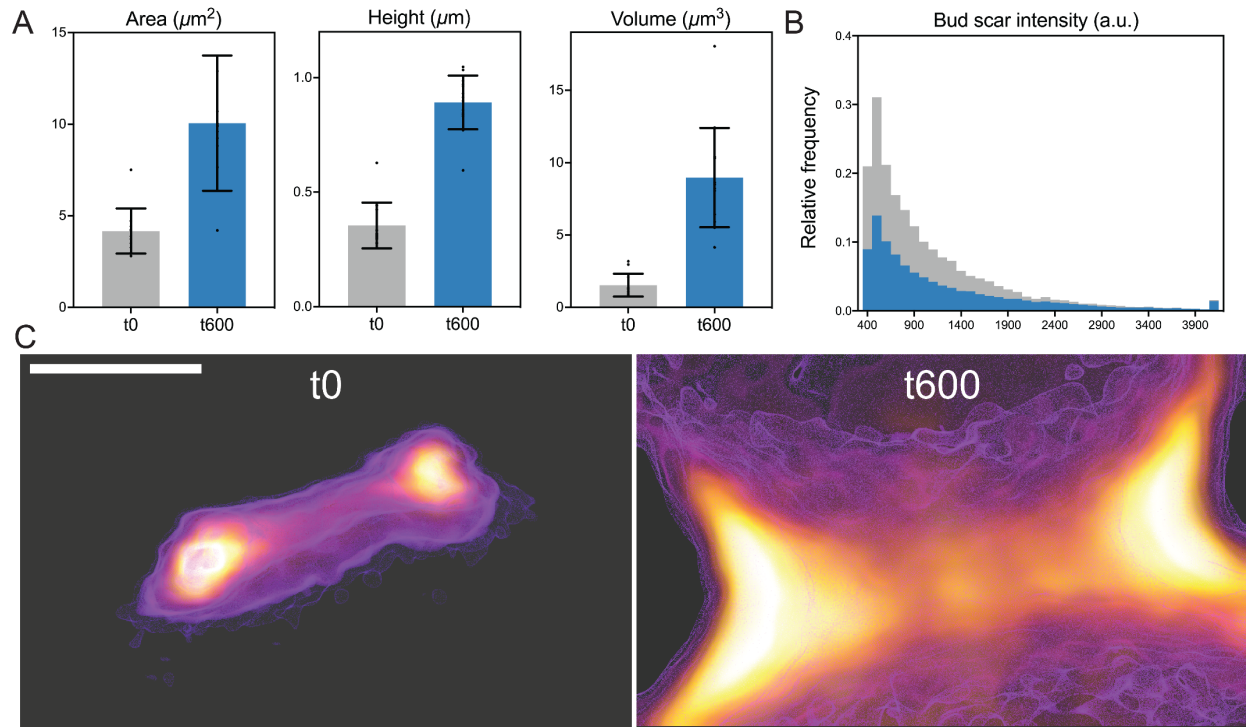


932

933 **Extended Data Figure 10.** Representative confocal images show chimeric clusters that are formed

934 after growth in liquid culture followed by entanglement on agar plates. Each frame is 139.64 x

935 139.64 x 34.50 μm in X, Y, and Z axes, respectively.



936

937 **Extended Data Figure 11.** Dimensions of bud scars connecting cells in microscopic, ancestral

938 (t0, gray) and macroscopic, evolved snowflake yeast clusters (PA2 t600, blue). Macroscopic t600

939 yeast had 2.4x larger bud scar cross-sectional area (A; $t=5.3$, $df=24$, $p<0.001$), 2.8x greater bud

940 scar height (B; $t=12.5$, $df=24$, $p<0.001$), resulting in bud scars with 5.8-fold greater volume (C;

941 $t=7.3$, $df=24$, $p<0.001$) than the microscopic ancestor. Error bars are one standard deviation. (B)

942 Histogram of pixel intensities for bud scars stained with chitin stain calcofluor white, isolated from

943 ancestor (t0, microscopic) and t600 (macroscopic) bud scars. The t600 strain has a 27% higher

944 mean fluorescence intensity, suggesting that they may have evolved moderately chitin density in

945 the bud scar. (C) The size differences in bud scars is readily visible. Shown are the side view of

946 buds from the ancestor (left) and t600 evolved (right), imaged at the same microscope settings.

947 The scale bar is 0.5 μm .

948 **Supplementary Movie 1.** Comparison of the ancestor and a population of macroscopic snowflake
949 yeast (PA2-t600, on the right).

950

951 **Supplementary Table 1.** A list of primers, plasmids, and strains used in the study.

952

953 **Supplementary File 1.** Code for the simple 3D biophysical simulation. These simulations,
954 described at a high level in the methods section of this paper, were adapted from previous work by
955 Jacobsen 2018 and Day 2022^{31,82}. The code is self-contained and commented. Please reach out to
956 Thomas Day or Peter Yunker with questions.