Multicellularity Makes Cellular Differentiation Evolutionarily Stable

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Multicellularity and cellular differentiation, two traits shared by all developing organisms, have evolved independently in many taxa and are often found together in extant species[1]. Differentiation, which we define as a permanent and heritable change in gene expression, produces somatic cells from a totipotent germ line. Though somatic cells may divide indefinitely, they cannot reproduce the complete organism and are thus effectively sterile on long timescales. How has differentiation evolved, repeatedly, despite the fitness costs of producing non-reproductive cells? The absence of extant unicellular differentiating species, as well as the persistence of undifferentiated multicellular groups among the volvocine algae[2] and cyanobacteria[3], have fueled speculation that multicellularity must arise before differentiation can evolve[4-7]. We propose that unicellular differentiating populations are intrinsically susceptible to invasion by non-differentiating mutants ("cheats"), whose spread eventually drives differentiating lineages extinct. To directly compare organisms which differ only in the presence or absence of these traits, we engineered both multicellularity and cellular differentiation in budding yeast, including such essential features as irreversible conversion, reproductive division of labor, and clonal multicellularity. We find that non-differentiating mutants overtake unicellular populations

but are outcompeted effectively by multicellular differentiating strains, suggesting that multicellularity evolved before differentiation.

Prevailing opinion holds that in single-celled species, the growth advantages attainable through differentiation would be too small to warrant the resulting loss of reproductive potential[6-8]. Whereas the soma can contribute structure and motility to multicellular organisms, somatic cells of a unicellular species can benefit the germ line only by secreting products into a shared extracellular milieu. Nevertheless, nutrient exchange between members of microbial consortia demonstrates the potential for cooperation between cell types in the absence of physical adhesion[9,10]. We therefore propose that unicellular differentiation could offer fitness benefits in a pure population, but remains rare because it is not an evolutionarily stable strategy[11]. For example, mutants that do not differentiate ("cheats") can take advantage of somatic cell products in the shared media without paying the reproductive costs of differentiation, thus increasing in frequency until the predominant phenotype reverts to non-differentiating. We further posit that if multicellularity results from cells of a single lineage failing to disperse (rather than the aggregation of cells from different lineages), differentiating populations may outcompete cheats: although cheats initially arise in a group with somatic cells, their descendants will eventually be confined to their own multicellular groups composed entirely of cheats and thus cannot benefit from the local accumulation of somatic cell products[12].

To test these hypotheses, we designed strains of the budding yeast *Saccharomyces cerevisiae* that differentiate, are multicellular, or both: one strain is a multicellular, differentiating organism and the other two represent both possible intermediates in its evolution from non-differentiating, unicellular ancestors (Figure 1a). Experimental tests with living organisms avoid the potential pitfall of biologically-unrealistic parameter regimes in

mathematical models of evolution, and using engineered strains which differ from one another at only a few, well-defined loci ensures that no other variables confound the direct comparison of fitness and evolutionary stability.

By our definition of differentiation, all differentiating species share a fundamental division of labor between a germ line, whose descendants can give rise to all cell types, and a soma with restricted developmental potential. We mimicked this arrangement by engineering fast-growing "germ" cells which can give rise to slower dividing, differentiated "somatic" cells that secrete invertase (Suc2), an enzyme that digests sucrose (which this yeast strain cannot take up directly) into the monosaccharides glucose and fructose, which any cell in the shared medium can then import[13,14] (Figure 1b). These somatic cells thus perform a digestive function, ensuring the availability of monosaccharides which serve as the sole carbon source during growth in sucrose minimal media. In nature, differentiation is typically effected through multiply-redundant gene regulatory networks that stabilize cell fate[15]: to simplify our system, we instead made differentiation permanent and heritable by forcing the expression of somatic cell-specific genes to depend on the excision of genes needed for rapid cell proliferation (Figure 1c).

Germ and somatic cells must be present at a suitable ratio for fast culture growth in sucrose media: germ cells have the higher maximum growth rate, but monosaccharides become limiting when somatic cells are rare. We predicted that the ratio between cell types would reach a steady-state value reflecting the balance between unidirectional conversion of germ cells into somatic cells and the restricted division of somatic cells. We designed tunable differentiation and division rates to allow us to regulate the ratio between cell types and thus control the growth rate of the culture as a whole.

Both features depend on a single, genetically-engineered locus (Figure 1c). In germ cells, this locus expresses the fluorescent protein mCherry and a gene that accelerates cell division, the cycloheximide resistant (*cyh2*^{r}) allele of the ribosomal protein L28 (ref. 16); in somatic cells, the locus expresses a different fluorescent protein (mCitrine) and the invertase Suc2. The germ line form is converted to the somatic form by a version of Cre recombinase engineered by Lindstrom et al.[17] to be active only in the presence of β-estradiol. Adding β-estradiol to a growing culture induced conversion of germ to somatic cells, apparent as the onset of mCitrine expression and slow loss of mCherry fluorescence by dilution (Figure 1d, Extended Data Figure 1, Supplementary Video 1). Conversion rates ranged from undetectable levels ($< 10^{-3}$ conversions per cell per generation) to approximately 0.3 conversions per cell per generation as the β-estradiol concentration increased (Figure 1e, Extended Data Figure 2a). Expression of the codominant, cycloheximide-sensitive wild-type allele of *CYH2* from its native locus permitted continued growth following *cyh2*^{r} excision, but at a reduced rate which depended on cycloheximide concentration. The growth rate deficit of somatic cells ranged from undetectable (< 1%) to nearly 30% as the cycloheximide concentration increased (Figure 1f, Extended Data Figure 2b).

The combination of irreversible differentiation and restricted somatic cell division caused cultures to approach a steady-state ratio between the two cell types over time (Figure 2a). The steady-state fraction of somatic cells increased with conversion rate and decreased with somatic cell growth disadvantage, as expected (Figure 2b). The ratio between cell types could be tuned over four orders of magnitude through appropriate choices of cycloheximide and β-estradiol concentrations (Figure 2b).

To investigate the ability of invertase secretion from somatic cells to support germ cell proliferation, we determined how the culture's growth rate on sucrose depended on the ratio between cell types. In the presence of cycloheximide, cultures containing both cell types at an intermediate ratio grew more quickly in sucrose media than cultures of either cell type alone (Figure 2c), confirming that somatic cells benefit their germ line through invertase secretion.

Clonal multicellularity arises through the maintenance of contact between daughter cells following cytokinesis[18]. In budding yeast, clonal multicellularity can be produced by mutations that disrupt degradation of the septum, a specialized part of the cell wall that connects mother and daughter cells after their cytoplasms have been separated by cytokinesis[19]. Deletion of *CTS1*, a chitinase gene required for septum degradation, causes formation of "clumps" (groups of daughter cells attached through persistent septa) that typically contain 4-30 cells during growth in well-mixed liquid medium (ref. 20, Figure 3a). In the presence of β-estradiol, differentiating strains that lack *CTS1* (*Δcts1*) produced clumps that frequently contained both germ and somatic cells, as evaluated by fluorescence microscopy (Figure 3a) and flow cytometry (Figure 3bc). Combining our gene excision-based differentiation system with *CTS1* deletion thus allowed us to produce strains exhibiting all life strategies needed to compare the evolutionary stability of unicellular and multicellular differentiation.

Somatic cells in unicellular strains can only benefit germ cells by secreting useful products into a shared medium; non-differentiating cheats (e.g., Cre^- germ cells) and germ cells in well-mixed media have equal access to somatic cell products, but cheats do not pay the reproductive toll of differentiation. We therefore predicted that cheats would enjoy a fitness advantage over germ cells, allowing them to invade unicellular, differentiating populations (Figure 4a, top). In multicellular species, however, significant local accumulation of somatic cell

products (in our experiment, monosaccharides) within multicellular groups can give differentiating lineages an advantage over cheats as long as the benefit of better nutrition overcomes the cost of producing slower-replicating, somatic cells[21]. In clonally multicellular species, such as our *Δcts1* strain, novel cheats arising by mutation will eventually be segregated into cheat-only groups by cell division and group fragmentation. We hypothesized that cheats would then experience reduced access to somatic cell products, potentially negating their growth advantage over germ cells (Figure 4a, bottom).

To test this prediction, we introduced cheats into unicellular or multicellular differentiating cultures and investigated their fate. We mixed differentiating cultures expressing a third fluorescent protein, Cerulean, with cheats that lack this third color and cannot differentiate because they lack the recombinase whose action gives rise to somatic cells (Cre^-). We monitored the relative frequency of the two strains over a series of growth and dilution cycles. In sucrose media, cheats invaded unicellular, differentiating populations but were outcompeted in multicellular, differentiating populations (Figure 4b). The growth advantage of multicellular, differentiating strains was nullified in monosaccharide-containing media, where somatic cells should confer no fitness advantage to clumps (Figure 4b). Moreover, the growth advantage of differentiating strains depended strongly on the conversion rate (β-estradiol concentration): higher conversion rates were advantageous only in the multicellular differentiating case (Figure 4b), where they increased the fraction of somatic cells overall as well as the fraction of clumps containing at least one somatic cell (Figure 3c). In unicellular cultures grown on sucrose, or in cultures grown on glucose (unicellular or multicellular), increasing the conversion rate increased the growth advantage of cheats by reducing the growth rate of the germ cell population (Figure 4b).

Our results show that unicellular, differentiating strains are evolutionarily unstable to invasion by non-differentiating cheats. This finding is unlikely to depend on the specific molecular mechanisms that produce differentiation or allow somatic cells to assist germ cells: any form of differentiation in a single-celled species would require that the two cell types exchange resources through a shared medium. From the spontaneous mutation rate in budding yeast ($\approx 3 \times 10^{-10}$ per base pair per cell division[22]) and the size of the recombinase gene, we estimate mutations that inactivate our engineered recombination system would occur in about 1 out of every 10^7 cell divisions; this is likely an underestimate of the frequency of inactivating mutations in natural differentiation, which typically requires more loci and thus presents a larger target for mutation[15]. Because this frequency is high relative to typical microbial population sizes, cheats would thus likely arise and sweep to fixation shortly after the appearance of unicellular differentiation, explaining the absence of extant species with this life strategy. The short persistence time of unicellular differentiating species makes them an unlikely intermediate in the evolution of development relative to undifferentiated multicellular species, which have persisted for hundreds of millions to billions of years in some clades[1-3]. We cannot, however, rule out the possibility that the transient existence of a unicellular differentiating species might suffice for the secondary evolution of multicellularity, which has been observed experimentally in small populations on the timescale of weeks[23,24]. In any case, the differentiating phenotype cannot be stably maintained against cheats until clonal multicellularity evolves.

Our study demonstrates that synthetic biology can directly test hypotheses about evolutionary transitions, complementing retrospective inference based on comparing existing species, experimental evolution, mathematical modeling, and simulation. We note that other major evolutionary transitions, including the appearance of body plans (spatially-ordered

arrangements of cell types) and life cycles (temporal sequences of growth and dispersion), could be studied through experimental evolution or further engineering of the strains described above. Furthermore, our differentiating strain provides an experimentally-tractable version of a common simplifying assumption in population genetics: *cyh2*^{r} excision is a form of irreversible mutation that always produces the same fitness disadvantage[25]. Thus engineered organisms, including those we have developed, can permit robust experimental testing of a wide variety of outstanding hypotheses in evolutionary biology.

Figure Legends

Figure 1: Synthetic yeast model for cellular differentiation.

(a) Alternative evolutionary trajectories for the evolution of development. Multicellularity and cellular differentiation have separate biological bases and thus probably evolved sequentially. In some clades, the persistence of likely evolutionary intermediates suggests that multicellularity arose first (solid arrows); no examples of evolution through a unicellular differentiating intermediate (dashed arrows) are known.

(b) Schematic of germ-soma division of labor model engineered in differentiating strains.

(c) Cell type-defining locus in the differentiating strains. Each pair of cell type-specific proteins (mCherry and Cyh2^r for germ cells, mCitrine and Suc2 for somatic cells) is initially expressed as a single polypeptide with a ubiquitin linker (red triangles). Cellular deubiquitinating enzymes cleave this linker post-translationally at its C-terminus[26], allowing the two resulting peptides to localize and function independently: for example, Suc2 enters the secretory pathway while mCitrine remains in the cytoplasm. A transcriptional terminator (Term) blocks expression of the somatic cell proteins in germ cells. Cre recombinase-mediated gene excision between loxP sites removed the terminator as well as the germ cell-specific genes.

(d) The unicellular differentiating strain (yMEW192) during growth in yeast extract-peptone-dextrose media (YPD) before (top) or five hours after addition of 1 μM β-estradiol (bottom).

(e) Conversion rates estimated by flow cytometry during growth in YPD containing β-estradiol. Error bars represent 95% confidence intervals of the mean, determined using data obtained from three biological replicates.

(f) The relative growth disadvantage of somatic cells relative to germ cells was measured by competition assay during growth in YPD + cycloheximide. Error bars represent 95% confidence intervals of the mean, determined using data obtained from three biological replicates.

Figure 2: Stable maintenance of both cell types facilitates growth in sucrose.

(a) Representative timecourses of the fraction of somatic cells in cultures of the unicellular differentiating strain (yMEW192 and yMEW192 convertant) initiated at various cell type ratios and passaged in YPD containing 10 nM β-estradiol and 600 nM cycloheximide.

(b) The steady-state fraction of somatic cells was determined as in (a) for various cycloheximide and β-estradiol concentrations. Error bars represent two standard deviations, calculated using data obtained from three biological replicates.

(c) Dependence of growth rate on fraction of somatic cells in cultures of the unicellular differentiating strain growing in 0.5% sucrose minimal media. Error bars represent 95% confidence intervals of the mean, determined using data obtained from three biological replicates.

Figure 3: *Δcts1* strains form multicellular clumps containing both cell types.

(a) Representative image of clump size and cell type composition in a well-mixed liquid culture of the*Δcts1* multicellular differentiating strain (yMEW208) at a steady-state cell type ratio in sucrose minimal media + 10 nM β-estradiol + 150 nM cycloheximide.

(b) Representative distribution of clump mCherry and mCitrine fluorescence for the multicellular differentiating strain (yMEW208) at a steady-state cell type ratio in sucrose minimal media + 10 nM β-estradiol + 150 nM cycloheximide. Gating of clumps by cell type composition (lower sector: germ cells only; middle sector: both cell types; top sector: somatic cells only) is shown in red.

(c) The fraction of clumps containing one or both cell types was determined as in (b) for cultures of the multicellular differentiating strain (yMEW208) at a steady-state cell type ratio in sucrose minimal media + 150 nM cycloheximide at the indicated β-estradiol concentrations. Error bars represent 95% confidence intervals of the mean, determined with data obtained from six biological replicates.

Figure 4: Multicellular differentiating strains resist invasion by Cre$^-$ cheats.

(a) Hypothesized evolutionary outcomes for novel non-differentiating mutants (red) in unicellular (top) and multicellular (bottom) populations.

(b) Growth advantages of differentiating strains (unicellular: yMEW192, multicellular: yMEW208) relative to cheaters (unicellular: yMEW193, multicellular: yMEW209) in minimal media containing 150 nM cycloheximide. Error bars represent 95% confidence intervals of mean growth advantages, determined using data obtained from three biological replicates. Growth advantages of the multicellular strain growing in sucrose minimal medium at 1 nM, 3 nM, and 10 nM β-estradiol are significantly greater than zero ($p < 10^{-3}$, one-tailed t-test); growth advantages of the unicellular strain growing in sucrose are significantly less than zero at all β-estradiol concentrations ($p < 10^{-6}$, one-tailed t-test).

Methods

Conversion timecourses and conversion rate assays

Conversion rate timecourses were performed by adding β-estradiol at the indicated concentration to cultures of pure germ cells (yMEW192) which were pre-grown in log phase in YPD. Cultures were maintained in log phase for additional growth while aliquots were collected for analysis by flow cytometry. At the indicated time point, cultures were washed twice with phosphate-buffered saline to remove β-estradiol and resuspended in YPD for additional growth before a final flow cytometry analysis.

For conversion rate determinations, β-estradiol was added to pure cultures of germ cells (yMEW192) pre-grown in log phase in YPD (Extended Data Figure 2a). Pure cultures of germ cells were maintained in parallel to permit calculation of the number of germ cell generations elapsed through cell density measurement with a Coulter counter (Beckman Coulter, Danvers, MA). Aliquots collected at indicated time points were washed twice in phosphate-buffered saline solution to remove β-estradiol, then resuspended in YPD for continued growth, allowing recently-converted somatic cells to dilute out remaining mCherry so that cell types could be unambiguously distinguished by flow cytometry. Linear regression of the log fraction of cells which were germ cells vs. the number of germ cell generations elapsed was used to determine a 95% confidence interval of the conversion rate for each experiment using the fit() and confint() functions in Matlab (Mathworks, Natick, MA). The conversion rates reported in Figure 1e correspond to the weighted arithmetic mean and corresponding variance calculated with data obtained from three or more independent experiments.

Fitness assays for relative growth difference between cell types

The growth rate difference between cell types was determined using a fitness assay protocol described previously[27]. Briefly, pure cultures of germ (yMEW192) and somatic (yMEW192 convertant) cells pre-grown in log phase in YPD containing cycloheximide were combined and maintained in log phase through multiple growth and dilution cycles in like media (Extended Data Figure 2b). Aliquots taken at each dilution step were used to determine the fraction of each cell type by flow cytometry. Pure cultures of germ (yMEW192) cells were maintained in log phase in parallel to determine the number of germ cell generations elapsed through cell density measurements with a Beckman Coulter counter. The 95% confidence interval for the slope of the linear regression line of log(somatic cells/germ cells) vs. the number of germ cell generations elapsed was determined as described above. The growth rate differences reported in Figure 1f represent the weighted arithmetic means and corresponding variances of the slopes calculated in three independent experiments.

Steady-state ratio assays

Pure cultures of germ cells (yMEW192) and somatic cells (yMEW192) were mixed to initiate cultures from a range of cell type ratios. Cultures were washed with phosphate-buffered saline and resuspended in YPD containing β-estradiol and cycloheximide at the indicated concentrations. Aliquots were taken at the indicated time points to determine the number of culture doublings since initiation (determined from Coulter counter measurements of culture density) and the fraction of somatic ($mCitrine^{+}$ $mCherry^{-}$) cells in the culture. Cultures typically converged on a steady-state ratio after 30-40 culture doublings.

Growth rate assays in sucrose minimal media

Cultures of converting strains at their steady-state ratios were washed twice with phosphate-buffered saline and resuspended at approximately 10^5 cells/mL in minimal media

containing 0.5% sucrose and the concentrations of cycloheximide and β-estradiol in which they had been growing previously. Pure cultures of the Cre$^-$ strain yMEW193 and the yMEW192 convertant were also used to represent pure cultures of germ and somatic cells of the differentiating strain, respectively. After an acclimation period of approximately ten hours, culture density measurements were taken regularly with a Coulter counter and a 95% confidence interval of the slope determined by linear regression of log(culture density) vs. time. The reported growth rates (Figure 2c) were obtained from the weighted arithmetic means of slopes and their corresponding variances estimated from three or more independent experiments.

Determination of the distribution of cell type composition in clumps

Cultures of *Δcts1* strains growing in minimal media containing 0.5% sucrose and the indicated concentrations of cycloheximide and β-estradiol were analyzed by flow cytometry. Clumps in these cultures typically ranged in size from 4-30 clumps; forward and side scatter gating could therefore not be used to eliminate events consisting of two or more clumps. Instead, cultures were diluted and vortexed thoroughly prior to flow cytometry to reduce the probability of multiple clumps being counted as a single event. The efficacy of this strategy was checked by combining pure cultures of germ and somatic cells in the absence of β-estradiol: the fraction of mCherry+ mCitrine+ events in the mixed culture was less than 1% (data not shown). Clumps containing only germ cells had negligible fluorescence in the mCitrine channel and thus formed a distinguishable population; likewise, clumps containing only somatic cells had negligible fluorescence in the mCherry channel. Clumps with non-negligible fluorescence in both channels were considered to contain both cell types.

Competition assays

In competition assays, Cerulean$^+$ differentiating strains (unicellular: yMEW192; multicellular: yMEW208) were mixed with Cerulean$^-$ Cre$^-$ reference strains (unicellular: yMEW193; multicellular: yMEW209) at a 1:1 ratio and passaged through five cycles of growth and dilution in indicated media. Aliquots taken at each dilution step were analyzed by flow cytometry to determine the ratio between Cerulean$^+$ and Cerulean$^-$ events (cells or clumps). The 95% confidence interval of the slope of the linear regression line of log(Cerulean$^+$ events/Cerulean$^-$ events) vs. the number of culture doublings elapsed was determined as described above. The growth advantages of the differentiating strain reported in Figure 4b represent the weighted arithmetic means and corresponding variances of the slopes calculated in three or more independent experiments.

Please see the Supplementary Methods for descriptions of plasmid and strain construction, imaging, media, and growth conditions.

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