Aneuploidy underlies a multicellular phenotypic switch

Zhihao Tan[a,b,1], Michelle Hays[a,1], Gareth A. Cromie[a], Eric W. Jeffery[a], Adrian C. Scott[a], Vida Ahyong[c], Amy Sirr[a], Alexander Skupin[a,d], and Aimée M. Dudley[a,b,2]

[a]Institute for Systems Biology, Seattle, WA 98109; [b]Molecular and Cellular Biology Program, University of Washington, Seattle, WA 98105; [c]Tetrad Program, University of California, San Francisco, CA 94122; and [d]Luxembourg Center for Systems Biomedicine, University of Luxembourg, L-4362 Esch-sur-Alzette, Luxembourg

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Although microorganisms are traditionally used to investigate unicellular processes, the yeast *Saccharomyces cerevisiae* has the ability to form colonies with highly complex, multicellular structures. Colonies with the "fluffy" morphology have properties reminiscent of bacterial biofilms and are easily distinguished from the "smooth" colonies typically formed by laboratory strains. We have identified strains that are able to reversibly toggle between the fluffy and smooth colony-forming states. Using a combination of flow cytometry and high-throughput restriction-site associated DNA tag sequencing, we show that this switch is correlated with a change in chromosomal copy number. Furthermore, the gain of a single chromosome is sufficient to switch a strain from the fluffy to the smooth state, and its subsequent loss to revert the strain back to the fluffy state. Because copy number imbalance of six of the 16 *S. cerevisiae* chromosomes and even a single gene can modulate the switch, our results support the hypothesis that the state switch is produced by dosage-sensitive genes, rather than a general response to altered DNA content. These findings add a complex, multicellular phenotype to the list of molecular and cellular traits known to be altered by aneuploidy and suggest that chromosome missegregation can provide a quick, heritable, and reversible mechanism by which organisms can toggle between phenotypes.

colony morphology | copy number variation | phenotypic switching | bet-hedging

Phenotypic switching allows cells or organisms to reversibly toggle between multiple, heritable states (1–3). These phenotypic states can confer distinct properties to cells that may be advantageous in some environments, e.g., increased resistance to heat (4), faster proliferation rates in the presence of excess nutrients (5), or altered interaction with host immune systems (6). Although some phenotypic switches can be induced by a change in environment (7), others may arise stochastically, possibly as a bet-hedging strategy that increases the probability that a subpopulation of genetically isogenic cells survive a wider range of environmental perturbations (4, 8, 9).

The ability to switch phenotypes could prove especially useful for microorganisms that are unable to physically relocate to an environment of choice and need to weather environmental fluctuations. Indeed, microbes are known to adopt multiple phenotypic states, and though the nature of these states can vary widely, they are all characterized by rates of switching too high to be that of spontaneous mutation (2, 3). Several interesting examples have been discovered in opportunistic fungal pathogens. Perhaps the best characterized state switch in fungi is the white-opaque switch of the *Candida albicans* (10), which is regulated by transcriptional feedback loops (11) and is associated with differential virulence (12), host colonization (13) and mating competency (14). *C. albicans* can also differentiate a subpopulation of "persister" cells that, like their bacterial counterparts, exhibit increased resistance to antimicrobial drugs (15). *Candida glabrata* exhibits at least two spontaneous and reversible phenotypic switches: a "core switching system" and an "irregular wrinkle switching system" (16) which exhibit differential tissue colonization and clearing rates in vivo (17). *Cryptococcus neoformans* switches between smooth, mucoid, pseudohyphal, and wrinkled colonies: state changes that are associated with biochemically distinct capsular polysaccharides (18), changes in karyotype (18), and differential fungal burden in infected host organs (19). Because these different states may exhibit differential virulence, cell surface properties, or resistance to host immune defenses (2, 3), understanding the mechanisms underlying phenotypic switching could prove essential for the development of effective therapeutics against microbial pathogens.

Some strains of *S. cerevisiae* also exhibit a striking phenotypic switch. Although most laboratory strains produce relatively unstructured "smooth" colonies, some natural isolates of *S. cerevisiae* produce colonies with complex multicellular features, such as folds, crevices, and channels that form as the colony grows on solid media (20, 21). These "fluffy" colonies possess properties similar to those of microbial biofilms, including the secretion and maintenance of an extracellular matrix (21–23), localized expression of drug efflux pumps (23), increased adherence (24), and the use of cell–cell communication (25). Fluffy colony formation requires the function and coordination of numerous pathways that underlie the trait, and the deletion of key factors produces a smooth colony phenotype (26–28). Fluffy colonies may benefit from both rapid acquisition of larger territories as well as an increased capacity for nutrient and water transport (21). Interestingly, some fluffy strains can switch to the smooth morphology at a rate significantly higher than that expected for spontaneous mutations (21, 29).

Results

Bidirectional Toggling Between Phenotypic States. While studying colony morphology in the haploid progeny of a cross between a Japanese sake strain and an Ethiopian white tecc strain (*SI Appendix*, Table S1), we observed a high frequency (10^{-3}) of colonies that switched from a fluffy to a smooth morphology. The smooth state of these isolates was clonally heritable and stable through multiple cell divisions, as evidenced by the formation of smooth colonies from single cells isolated from 4-d-old smooth colonies (Fig. 1*A*). However, when grown for longer periods of time (10–16 d), "blebs" appeared on the colony surface of otherwise smooth colonies (Fig. 1*B*). We hypothesized that these patches of cells had adopted characteristics distinct from the neighboring cells, and indeed, a portion of single cells isolated from these patches had regained the ability to form fluffy colonies

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Data deposition: Raw restriction-site associated DNA tag sequencing reads of strains for which ploidy was analyzed are deposited in the National Center for Biotechnology Information Sequence Read Archive, www.ncbi.nlm.nih.gov/sra/ (accession no. ERP002462).

[1]Z.T. and M.H. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: aimee.dudley@gmail.com.

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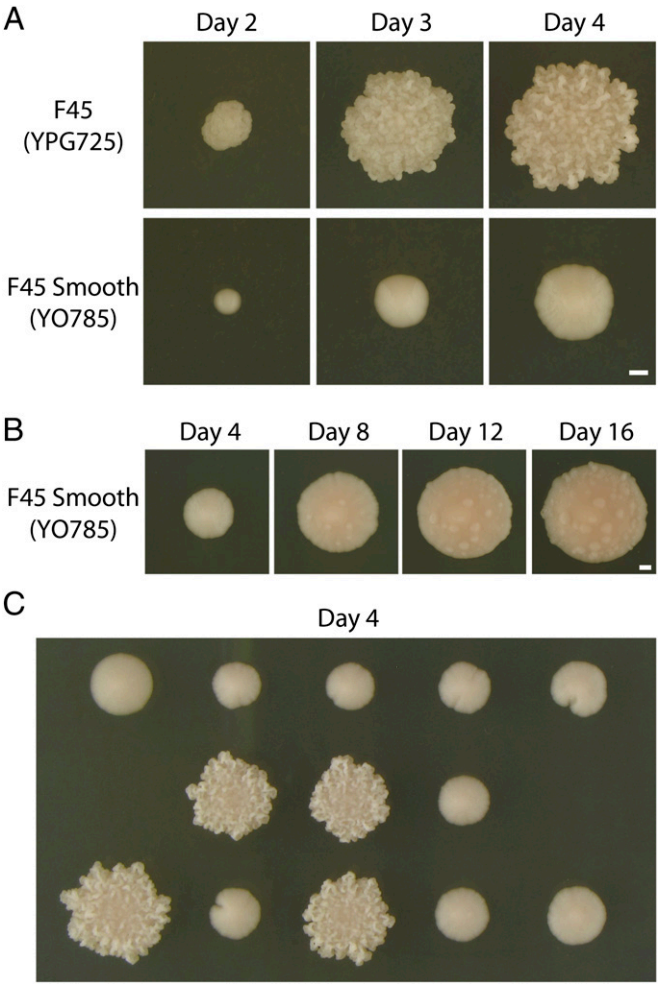

Fig. 1. Toggling between the fluffy and smooth states. (*A*) Morphology development of a colony growing from a single cell of the F45 fluffy strain and a smooth variant (F45 Smooth). (*B*) Development of "blebs" on the surface of a F45 Smooth colony. (*C*) Single cells isolated by micromanipulation from the "blebs" on day 16 yielded entirely fluffy or smooth colonies. Scale bars are 1 mm.

with shapes indistinguishable from that of the original strain (Fig. 1*C*). We therefore set out to determine the mechanism underlying this bidirectional switch.

Aneuploidy Is Sufficient for the Phenotypic Switch. Because transition to a prion state is an epigenetic phenomenon that can affect colony morphology (29), we tested for the effect of prions by passaging the original F45 fluffy strain on guanidine hydrochloride (*SI Appendix*, *SI Materials and Methods*), a method known to cure prions in *S. cerevisiae* (30), and then reassayed colony morphology on our assay medium (yeast extract/peptone/dextrose, YPD). The results (*SI Appendix*, Fig. S1) showed that the fluffy morphology of F45 was independent of the prion status, and therefore prions were unlikely to be the molecular mechanism underlying the switch in our strain background.

Because changes in DNA copy number can also occur reversibly and at relatively high frequencies, we next tested our strains for aneuploidy (chromosome numbers that deviate from multiples of the haploid chromosome count). Using a high-throughput restriction-site associated DNA tag sequencing (RAD-seq) strategy that directs genome sequencing to specific restriction sites (31) thereby sequencing the same ∼1% of each strain in a highly multiplexed fashion, we determined the karyotype of our strains based on ∼3,000 markers across the genome. Together with DNA content estimates determined by flow cytometry (*SI Appendix*, Fig. S2), RAD-seq showed that although the original fluffy strain (F45) was euploid, transition to the smooth state (F45 Smooth) was accompanied by the gain of an extra copy of chromosome XVI (Fig. 2*A*). Remarkably, the additional copy of chromosome XVI was lost when the strain transitioned back to the fluffy state (Fig. 2*A*, F45 "2nd gen"), exhibiting a morphology indistinguishable from that of the original strain.

To test whether this phenomenon was specific to F45, we chose a strain (YO880) from an independent genetic background that also harbored an extra copy of chromosome XVI. YO880 is a haploid strain derived from a cross between strains originating from "Evolution Canyon" in Israel and an oak tree in North America (*SI Appendix*, Table S1). Similar to F45, the version of YO880 containing an extra copy of chromosome XVI was smooth (Fig. 2*B*), but upon extended growth produced patches of fluffy colony producing cells. RAD-seq confirmed that these fluffy isolates had also lost the extra copy of chromosome XVI (Fig. 2*B*). Thus, in two independent genetic backgrounds, smooth and fluffy colony morphologies were correlated with chromosome XVI copy number.

To determine whether chromosome XVI disomy was sufficient for the phenotypic switch, we engineered the original strain (F45) to enrich for chromosome XVI disomes, independent of their phenotype (Fig. 3). This system (32) modifies the centromere of a target chromosome so that it can be temporarily inactivated (by exposure to galactose), thereby increasing the frequency of chromosome-specific nondisjunction (33). Galactose-induced nondisjunction events are selected using a marker that can exist in one of two states, an intact *HIS3* or an intact *URA3* (32). Only cells that have obtained two copies of the target chromosome can excise *HIS3* from one copy and maintain *URA3* on the other, allowing them to grow on the selective medium (SC–His–Ura). In our strain background, $\sim 10^3$ out of 10^7 galactose-induced cells produced His^+ Ura^+ colonies. Because colony morphology was difficult to score on the selective medium (*SI Appendix*, Fig. S3), we randomly chose 48 colonies and assayed their morphology on rich medium (YPD) (*SI Appendix*, Fig. S4). All of these colonies were smooth, and RAD-seq of a representative subset ($n = 22$) confirmed the presence of an additional copy of chromosome XVI (*SI Appendix*, Fig. S5). To test whether restoration of the euploid state was sufficient to switch colonies back to the fluffy morphology, we used minimal medium containing 5-fluoroorotic acid (5-FOA) to select for the loss of the *URA3*-containing copy of chromosome XVI (*SI Appendix*, Fig. S6). In agreement with the original phenotypic selection, RAD-seq confirmed that all randomly selected colonies with adequate sequence coverage ($n = 12$) were euploid and exhibited the fluffy colony morphology (Fig. 3 and *SI Appendix*, Fig. S5). Thus, the gain and loss of chromosome XVI is sufficient to reversibly toggle cells between the fluffy and smooth states.

Multiple Routes to the Same Phenotype. While analyzing additional fluffy to smooth isolates of F45, we were surprised to find that, in addition to chromosome XVI, several other aneuploidies altered the colony forming state of the strain. Disomies of chromosomes III, X, and XV displayed smooth colony morphologies, and chromosome V disomy displayed an intermediate phenotype (Fig. 4). As with chromosome XVI, the subsequent reversion of all four disomic strains back to the original fluffy morphology was accompanied by restoration of the euploid karyotype (*SI Appendix*, Fig. S7). Thus, the ability of different disomies to trigger the bidirectional toggle suggests that there are multiple, reversible routes to the same phenotypic state. We note that the screen also yielded a number of euploid smooth isolates (*SI Appendix*, *SI Text*), suggesting that additional mechanisms may exist.

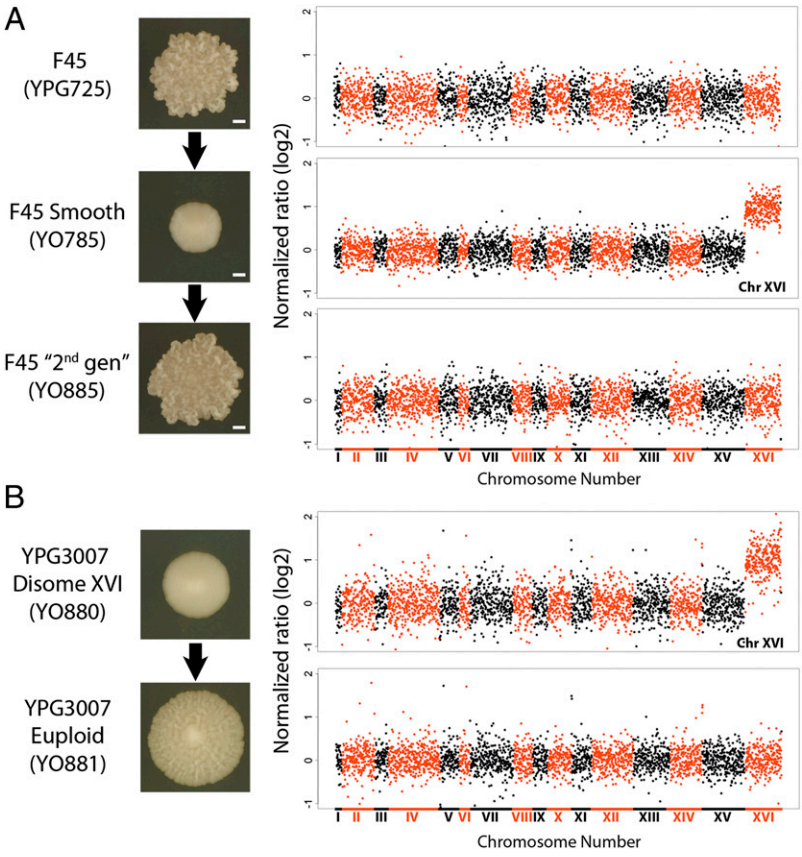

Fig. 2. Change in morphology affected by chromosome XVI copy number. (*A*) Phenotypic toggling of naturally derived strains. Plots of RAD-seq data show the presence of an extra chromosome XVI in F45 Smooth and its subsequent loss in the fluffy revertant (F45 "2nd Gen"). Chromosomes are alternatively colored black and red. (*B*) Colony morphology in YO880 (*SI Appendix*, Table S1), a strain unrelated to F45. The loss of an extra chromosome XVI also correlates with the gain in complex colony morphology in this strain background.

Switch Modulated by Gene-Specific Copy Number Variation. Our results are consistent with either of two models. First, because numerous biological processes are required to form a fluffy colony (26–28), these five chromosomes could each contain dosage sensitive genes necessary for the function or regulation of key processes. Alternatively, the fluffy state could be sensitive to some more general aspect of aneuploidy, such as the DNA content of the cell. To test whether any chromosomal aneuploidy could confer the smooth morphology, we introduced conditional centromere constructs onto the 11 remaining chromosomes (I, II, IV, VI, VII, VIII, IX, XI, XII, XIII, and XIV) and selected for each disomy (*SI Appendix*, Fig. S8 and Table S2). Of the 11 disomies selected, four were either too sick or unstable to assay (chromosome II, VI, XI, and XIII) and two could only be stably recovered in conjunction with additional aneuploidies (disome IV paired with VII and disome XII paired with XV) (*SI Appendix*, *SI Text*). This last set of strains containing multiple disomies suggested that genetic interactions may play a role in the formation of colony morphology. For example, whereas strains disomic for chromosome III were smooth, and strains disomic for chromosome IX were fluffy, strains disomic for both III and IX were smooth (Fig. 5). However, of the five chromosomes that could be stably recovered as individual disomies, only one (chromosome VII) yielded smooth colonies through day 4 of growth, whereas the remaining four disomies (chromosome I, VIII, IX, and XIV) were all fluffy at the same time point (Fig. 5). The fact that several disomies, spanning the continuum of chromosome sizes, failed to trigger the smooth transition strongly supports the hypothesis that the switch is due to specific gene effects rather than general DNA content changes.

To test whether this phenotypic toggle is in fact modulated by the copy number variation of specific genes, we transformed the original F45 (fluffy) strain with a set of low copy plasmids containing portions of chromosome XVI (34). This screen identified a plasmid containing seven full-length genes that was able to confer the smooth state (*SI Appendix*, Fig. S9*A*). We subsequently determined that this effect was due to the increased copy number of a single gene *DIG1* (Fig. 6*B* and *SI Appendix*, *SI Text*). *DIG1* encodes a known repressor of the MAPK pathway (35), and our results are consistent with a model in which overexpression of *DIG1* leads to the down-regulation of the fluffy trait (26). However, the intermediate phenotype obtained by restoring *DIG1* copy number in the context of the XVI disomy (*SI Appendix*, Fig. S9*B*) suggests that additional genes on chromosome XVI may contribute to the phenotypic switch.

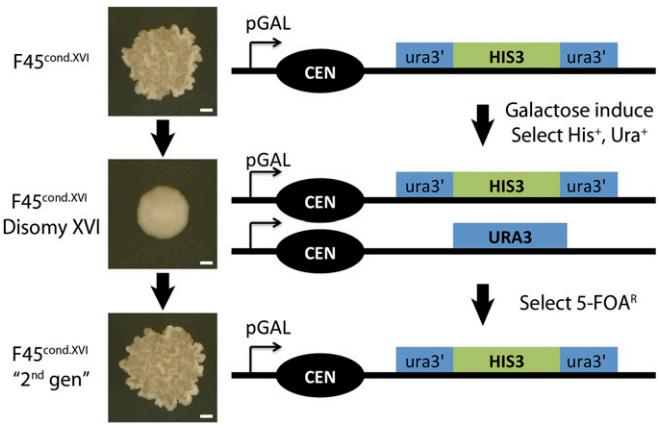

Fig. 3. Phenotypic toggling of the strain containing a conditional centromere on chromosome XVI ($F45^{cond.XVI}$). The protocol used to select chromosome gain and loss is depicted (right). Scale bar is 1 mm. Images were taken on day 4 of colony growth.

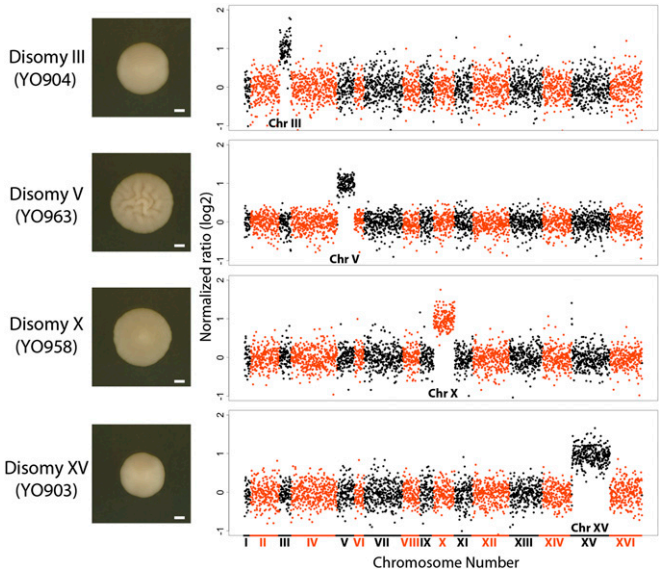

Fig. 4. Multiple disomies induce a similar phenotype. Colony morphology of the different disomic yeast strains obtained by phenotypic screening. Plots show the presence of the extra chromosomes identified by RAD-seq. Scale bar is 1 mm. Images were taken on day 4 of colony growth.

Growth Advantages of the Two States. Phenotypic switching suggests that the states may exhibit distinct fitness landscapes under different environmental conditions. Because the organization of cells into the fluffy colony structure is a solid media phenomenon and because previous work has shown that fluffy colonies contain more cells than smooth colonies (36), we chose to compare the growth characteristics of smooth and fluffy colony forming strains in both liquid and solid media (*SI Appendix*, *SI Materials and Methods*). We first compared the original fluffy euploid (F45) and the smooth XVI disome (F45 Smooth). On solid medium, the fluffy strain had a substantial growth advantage, as measured by the maximal growth rate achieved and number of cells produced by day 4 of growth (Fig. 6*C*). Although these results are consistent with previous comparisons of cell number in fluffy and smooth colonies (36), they could also merely reflect decreased growth rates resulting from aneuploidy (37). Interestingly, in liquid medium the smooth, aneuploid strain showed only a modest decrease in growth rate and even a statistically significant increase in cell yield at saturation (Fig. 6*E*). These results suggest that the smooth state has advantages for growth in liquid media even in the presence of the presumed growth disadvantage conferred by aneuploidy.

Because the strain overexpressing *DIG1* exhibits a smooth colony morphology (Fig. 6*B*) without the burden of an additional chromosome, the smooth state is genetically separable from the aneuploid state. We compared the growth characteristics of F45 transformed with either a low copy plasmid containing *DIG1* or an empty vector. In these experiments the fluffy (vector alone) strain also exhibited a growth advantage over the smooth (*DIG1*) strain on solid media (Fig. 6*D*), whereas in liquid medium the *DIG1*-mediated smooth strain exhibited both a higher maximal growth rate and cell yield (Fig. 6*F*). Thus, when the cost of aneuploidy is removed, the smooth state shows clear growth advantages in liquid.

Discussion

The phenotypic changes brought about by aneuploidy can arise from a general increase in DNA content, dosage changes in specific gene products, or a combination of both factors. In *S. cerevisiae* some traits, such as decreased growth rates and G1 cell cycle delays, appear to be general effects of aneuploidy (37), whereas others, such as altered drug resistance and protein abundance, have been linked to the duplication of specific chromosomes or genes (38). Our results add a complex, multicellular phenotype to the set of traits known to be regulated by aneuploidy. Cells in fluffy colonies organize to form intricate systems of hollow channels that increase the distances between cells, allowing for more rapid colonization of surfaces and greater acquisition of resources (21). At the same time, they perform metabolically intensive processes, such as the production of extracellular matrix, that while aiding the protection of the colony, require significant nutrient and energy expenditure (21). Switching to the smooth state when such protection is not needed has been proposed as an energy conservation strategy (39). Our results suggest that the copy number imbalance of specific chromosomes is one means by which cells can affect this state change, presumably by altering the dosage of a subset of genes required for the regulation or execution of these biological processes.

The surprisingly large number of chromosomes able to modulate the phenotypic toggle between fluffy and smooth may be

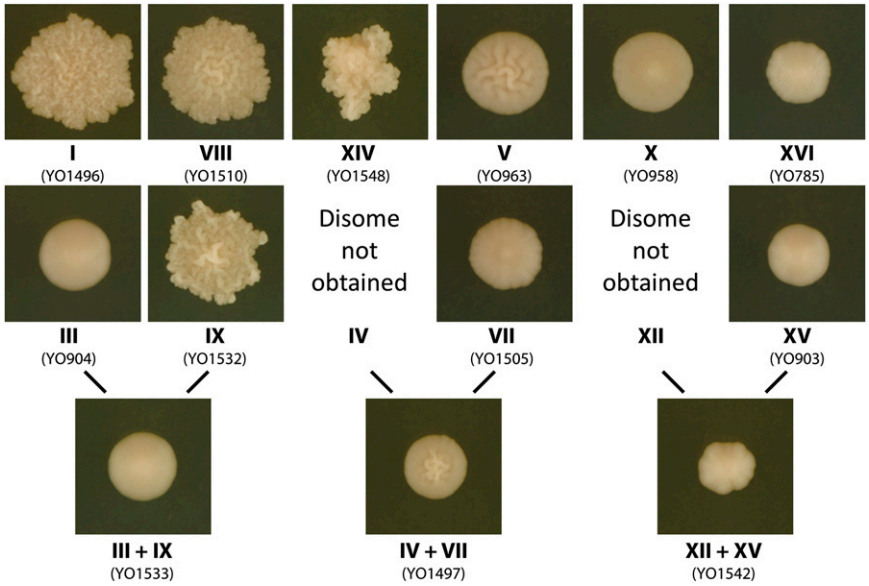

Fig. 5. Colony morphology for disomic strains. Morphologies of representative examples of each karyotype are shown on YPD agar. All strains in this figure were rekaryotyped following micromanipulation. Only stable, reproducible karyotypes are depicted. See *SI Appendix*, *SI Text* details on sick or unstable disomies (II, VI, XI, and XIII). Images were taken on day 4 of colony growth.

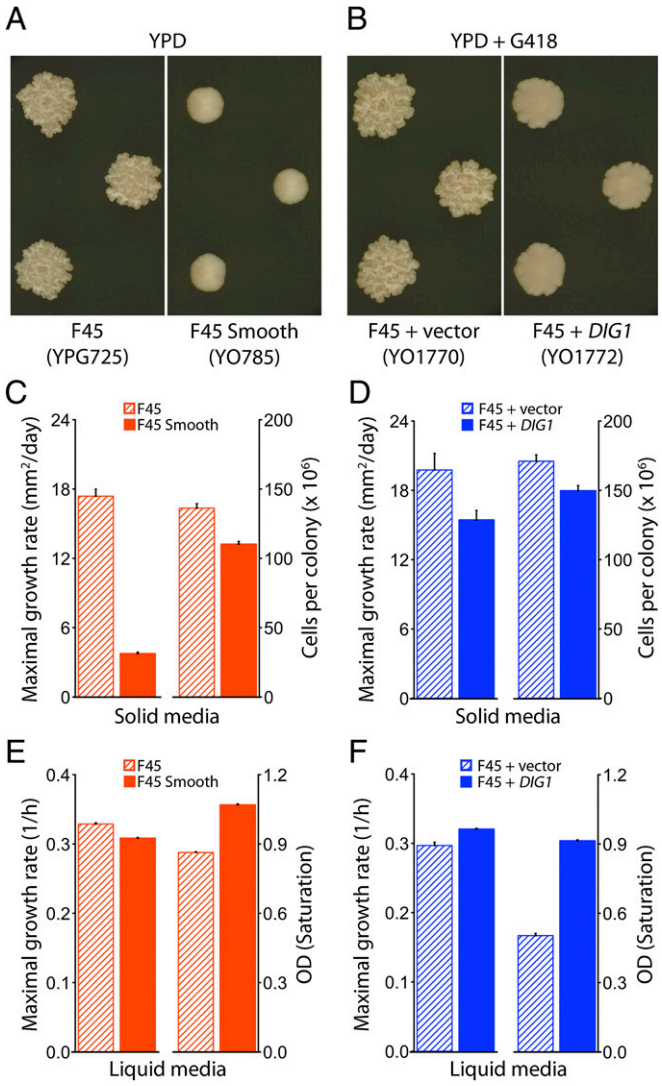

Fig. 6. Growth characteristics of fluffy and smooth strains. (*A* and *B*) Representative colonies of F45 and F45 Smooth growing on YPD agar (*A*), F45 + vector and F45 + *DIG1* growing on YPD + G418 agar as plated in a "checkerboard" pattern (*B*). Images were taken on day 4 of colony growth. (*C*) Maximal growth rate ($P < 10^{-4}$; $n = 12$ or 14 colonies; Wilcoxon rank sum test) and cells per colony ($P < 10^{-7}$; $n = 20$ colonies; Student *t* test) of F45 and F45 Smooth grown on YPD agar. Error bars are SEM. (*D*) Maximal growth rate ($P = 0.025$; $n = 7$ or 10 colonies; Wilcoxon rank sum test) and cells per colony ($P < 10^{-3}$; $n = 20$ colonies; Student *t* test) of F45 + vector and F45 + *DIG1* grown on YPD + G418 agar. Error bars are SEM. (*E*) Maximal growth rates ($P < 10^{-5}$; $n = 28$ or 24 wells; Student *t* test) and saturation OD ($P < 10^{-5}$; $n = 28$ or 24 wells; Student *t* test) of F45 and F45 Smooth grown in liquid YPD. Error bars are SEM and are <1% of the values obtained. (*F*) Maximal growth rates ($P < 10^{-5}$; $n = 14$ or 27 wells; Student *t* test) and saturation OD ($P < 10^{-5}$; $n = 14$ or 27 wells; Student *t* test) of F45 + vector and F45 + *DIG1* grown in liquid YPD + G418. Error bars are SEM and are <5% of the values obtained.

proportional to the relatively large number of pathways required for the fluffy morphology (26–28) or may indicate that the trait is particularly sensitive to changes in gene dosage. In either case, the fact that aneuploidy provides cells multiple routes to the same phenotypic endpoint has fundamental implications for understanding how organisms respond to environmental perturbations. Because aneuploidy is strongly correlated with drug resistance in pathogenic microorganisms (40–43), rampant in cancer (44), and associated with high levels of cancer relapse after drug treatment (45), aneuploidy itself could be an important target for the development of novel antifungal and cancer therapeutics (44). The complexity of the problem is compounded by the fact that the genome sequence (genetic background) of an individual has the potential to affect not only the traits displayed by aneuploid cells, but also how well specific aneuploidies are tolerated. Differences in the ability to recover specific disomes between our strain and one used in a previous study (37) may be one such example.

Phenotypic switching allows organisms to access multiple states with different growth or survival advantages. Here, we examine a system in which *S. cerevisiae* exists in two distinct multicellular states (fluffy and smooth) and is at times able to form heterogeneous populations containing both (Fig. 1*B*). Because the molecular mechanism underlying the fluffy-smooth switch is the mitotic gain and loss of intact chromosomes, it is likely the result of a stochastic process, although we have not directly tested this hypothesis. Furthermore, the fact that these two states exhibit different relative fitness profiles under different environmental conditions (solid and liquid media), suggests that the organism may be leveraging the stochasticity of chromosome missegregation as a bet hedging strategy. Although we identified differences in growth, the two states could easily exhibit different advantages with respect to other traits, such as longevity, resistance to desiccation, and ease of dispersal, which may prove equally or even more beneficial to the organism in the natural environment. The importance of maintaining two states and the ability of the cells to switch between them is further supported by the recent identification a prion-based switch that can modulate colony morphology changes in other strains of *S. cerevisiae* (29).

The random gain and loss of complete chromosomes has many favorable properties as a mechanism for phenotypic switching, both in the context of a preemptive bet-hedging strategy as well as a rapid means for coping with unfamiliar environmental stresses (46). Although gene expression patterns can modulate phenotypic states (11), aneuploidy provides a more direct mode of inheritance. Additionally, because regulatory networks involve complex sets of molecular interactions that must evolve over time, aneuploidy might be a useful bet-hedging strategy for responding to environmental perturbations that are not frequently encountered. As aneuploidy perturbs hundreds of genes simultaneously, it has the potential to affect larger numbers of genes and processes per event relative to a spontaneous mutation. Importantly, unlike a spontaneous mutation, aneuploidy and any associated growth defects can also revert at a high frequency. This reversibility makes aneuploidy a particularly useful mechanism in conditions that are encountered infrequently and are likely to change. The inherent properties of aneuploidy thus first allow organisms to rapidly and extensively survey the fitness landscape imposed by new environmental conditions. Aneuploidy then allows organisms the flexibility of reverting to their previous state if conditions return, or stability as they fine-tune their strategy to over-express only the subset of genes responsible for the growth advantage if conditions remain stable (46). Thus, aneuploidy is surprisingly well suited to provide a relatively quick, heritable, and reversible mechanism through which cells and organisms can toggle between phenotypes while searching for optimal fitness solutions.

Materials and Methods

Yeast Strains, Media, and Manipulation. Unless noted, standard media and methods were used for growth and genetic manipulation of yeast (47). Strains used in this study are listed in *SI Appendix*, Table S1. Colony morphology was assayed on YPD (2% glucose, wt/vol) plates. Respiratory competency was assayed by growth on YPG (3% glycerol, vol/vol) plates. The conditional centromere experiments used minimal medium (SC-Ura-His, Sunrise Science Products) to enrich for disomes and 5-FOA resistance (SC, 1 g/L 5-FOA, Zymo Research) to select euploid revertants. To ensure consistency in colony growth, all images of yeast colonies presented are from colonies arrayed into an ordered grid either by micromanipulation with 10-mm colony separation

GENETICS

(Figs. 1–5) or FACS sorting (*SI Appendix, SI Materials and Methods*) at 12.7-mm spacing (Fig. 6).

Isolation of Smooth Colony Strains. To isolate strains with a smooth colony phenotype, cells from a fluffy colony were grown overnight at 30 °C in liquid YPD (2% glucose, wt/vol). Cells were then plated onto YPD solid agar at a density of 200 cells per plate. Colonies were grown for 3 d at 30 °C and then screened for the smooth colony morphology.

Imaging. All images were taken with a Canon PowerShot SX10IS, F8.0, 1/8-s shutter speed, ISO80 under consistent lighting conditions, imaging distances and zoom. Images were cropped and scaled with Adobe Photoshop CS5.1 with no further modifications to the images.

Molecular Karyotyping by RAD-seq Coverage. The molecular karyotype of each strain was determined by multiplexed RAD-seq (31) of yeast genomic DNA as described (48). For each strain, the coverage ratio of each marker is compared with the haploid reference and plotted against a DNA marker index ordered by genomic position (chromosomes I to XVI). Marker-to-marker variation was normalized using a panel of euploid strains. Detailed descriptions of calculations can be found in *SI Appendix, SI Materials and Methods*. Raw RAD-seq reads of strains for which ploidy was analyzed have been deposited in the National Center for Biotechnology Information Sequence Read Archive (www.ncbi.nlm.nih.gov/sra/) with accession no. ERP002462.

Conditional Centromeres. Replacement of the native centromeres of chromosomes with a conditional centromere was adapted from a protocol using a well-characterized construct (33) that has been modified further (32). Briefly, galactose induction was performed by first growing the strains in YP-Raffinose for 48 h at 30 °C to saturation and diluting 1:2,000 for growth overnight at 30 °C. Cultures were then diluted to 5×10^6 cells per mL, split into two aliquots, and galactose (1.5% wt/vol final concentration) was added to one. Cultures were monitored by cell count until cell density had increased 2.5-fold (~3–4 h). 1×10^7 cells were then plated onto SC-His-Ura plates to select for yeast disomic for the chromosome that contains the conditional centromere. In our hands, ~$1–3 \times 10^3$ colonies were recovered on each selection plate. Additionally, we see a high frequency of respiratory deficient colonies on our selection plates. Because respiratory competency is generally required for the fluffy phenotype, we checked all colonies obtained for growth on glycerol plates and only analyzed respiratory competent strains. For 5-FOA selection, 10^7 cells from colonies growing up on SC-His-Ura plates were resuspended in PBS and plated onto 5-FOA plates using glass beads. Plates were allowed to grow for 3 d before colonies were selected for further analysis. An average of ~$1–2 \times 10^2$ colonies were recovered on each selection plate.

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