A population shift between two heritable cell types of the pathogen *Candida albicans* is based both on switching and selective proliferation

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Differentiated cell types often retain their characteristics through many rounds of cell division. A simple example is found in *Candida albicans*, a member of the human microbiota and also the most prevalent fungal pathogen of humans; here, two distinct cell types (white and opaque) exist, and each one retains its specialized properties across many cell divisions. Switching between the two cell types is rare in standard laboratory medium (2% glucose) but can be increased by signals in the environment, for example, certain sugars. When these signals are removed, switching ceases and cells remain in their present state, which is faithfully passed on through many generations of daughter cells. Here, using an automated flow cytometry assay to monitor white–opaque switching over 96 different sugar concentrations, we observed a wide range of opaque-to-white switching that varied continuously across different sugar compositions of the medium. By also measuring white cell proliferation rates under each condition, we found that both opaque-to-white switching and selective white cell proliferation are required for entire populations to shift from opaque to white. Moreover, the switching frequency correlates with the preference of the resulting cell type for the growth medium; that is, the switching is adjusted to increase in environments that favor white cell proliferation. The widely adjustable, all-or-none nature of the switch, combined with the long-term heritability of each state, is distinct from conventional forms of gene regulation, and we propose that it represents a strategy used by *C. albicans* to efficiently colonize different niches of its human host.

cell-type switching | population shift | *Candida albicans* | microbiology

How different cell types maintain their identity across numerous cell divisions, yet efficiently switch to other cell types when circumstances change, is a central question in biology. White–opaque switching in *Candida albicans*, the major fungal pathogen of humans, provides a simple system to study both the heritability of cell types and the switching between them (1–6). Here, two cell types (white and opaque) retain their specialized characteristics across many cell generations, with switching between the cell types occurring approximately once every 10^4 generations under standard laboratory conditions (4, 5). Although white and opaque cells carry the same genome, they differ in many phenotypes. For example, they have distinct cell morphologies when viewed under a light microscope, they form colonies on Petri dishes that are easily distinguishable by eye, they differ in their ability to mate, and they interact differently with the mammalian immune system (4, 7–14). Approximately 1,000 *C. albicans* genes, of ~6,400 total, show differential regulation (>two fold) between the two cell types (15, 16).

Previous work has shown that white–opaque switching can be pushed in one direction or the other by changes in the environment (13, 17–19). To date, it has been difficult to rigorously distinguish between changes in the switching frequency per se and selective proliferation of one of the two cell types. For example, a new environmental condition that results in a greater fraction of white cells from a starting population of opaque cells could result from 1) the selective proliferation of white cells over opaque cells under the condition, 2) an increase in the opaque-to-white switching frequency, or 3) some combination of the two (Fig. 1).

To distinguish among these possibilities and to accurately monitor switching, we tracked individual opaque cells as they switched to white cells and found that the sugar composition of the growth medium affects both switching frequency and selective proliferation (possibility 3, Fig. 1*C*). Further analysis of the data showed a strong, but unanticipated correlation between opaque-to-white switching and the relative fitness of the resulting white cell in that medium. We propose that adjusting the switching rate to favor a cell type more fit for the new environment may be a general strategy employed by *C. albicans* to adapt rapidly and heritably to new environments.

Significance

The fungal species *Candida albicans* thrives in diverse niches of its mammalian hosts, an ability attributed, in part, to its different morphological forms. We examined one morphological change, known as white–opaque switching, in which cells switch between their typical round-to-oval morphology (white) and an elongated cell type (opaque). Switching is typically rare; however, specific conditions can shift an entire population of opaque cells to white cells. We show that opaque-to-white switching is common in conditions where white cells thrive. We hypothesize that other microbial pathogens may employ similar strategies, matching switching between states to proliferation rates, to thrive in diverse environments.

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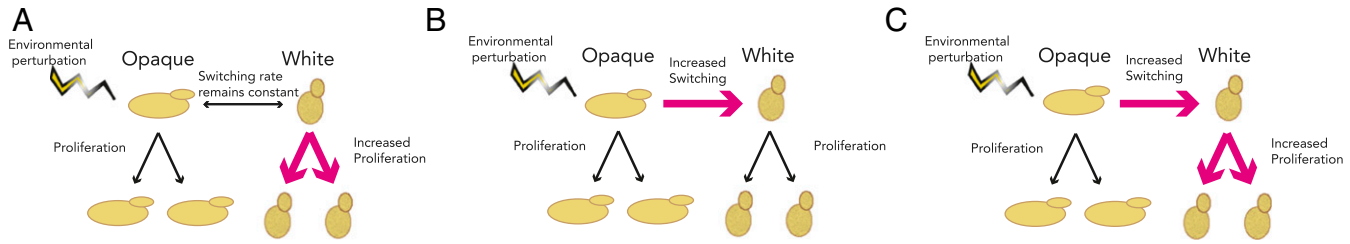

Fig. 1. In response to a new environment, a population of opaque cells can, in principle, become a population of white cells in one of three ways (dark magenta arrows). (*A*) The new environment could selectively favor white cell proliferation so they outcompete their opaque counterparts. In the extreme case, the shift of environments could simply kill opaque cells. (*B*) The environmental perturbation could increase the opaque-to-white switching rate such that opaque cells are rapidly transformed into white cells. (*C*) The switch to the new environment could both increase the opaque-to-white switching rate and selectively favor the proliferation of white cells.

Results

Development of a Flow Cytometry Approach to Monitor the Dynamics of Opaque-to-White Switching. To observe both opaque-to-white switching and proliferation of the two cell types, we considered two factors. First, we needed a way to track both types of events in the same culture under a wide variety of conditions. Second, because switching appears stochastic (2, 4, 5), it was important to use an assay with single-cell resolution.

We constructed a *WH11* fluorescent reporter (20, 21) and confirmed it as a suitable proxy for the single-cell analysis of opaque-to-white switching (ref. 22 and *SI Appendix*). We first determined the level of *WH11* reporter fluorescence that marked a cell as having switched from opaque to white. It has been known for many years that a temperature increase from 25 °C to 37 °C causes opaque cells to switch to white cells en masse (13). Using a pure population of opaque cells in which the *WH11* promoter was fused to a fluorescent reporter [YFP (23)], we tracked fluorescence over time as individual opaque cells switched to white cells in response to such a temperature shift (Fig. 2*A* and *SI Appendix*, Fig. S2). During the experiment, we also plated cells and scored for white and opaque colonies; this traditional bulk switching assay measures the median commitment point for opaque-to-white switching across the population (see, for example, refs. 4 and 20).

As shown in Fig. 2, the two assays tracked well with each other; populations that remained opaque (as measured by the plating assay) showed no expression of the *WH11* YFP reporter, and populations where a fraction of cells had committed to switching (as determined by the plating assay) showed a similar fraction of YFP-expressing cells (Fig. 2*B* and *SI Appendix*, Fig. S2). We empirically determined a YFP expression threshold (YFP/side scatter > 30 A.U.) that correlated well with the commitment and employed it in our subsequent analysis to distinguish white from opaque cells (*SI Appendix*, Fig. S2). These observations show it is possible to accurately track the switching of individual cells using flow cytometry.

De Novo White Cell Appearance Rates Positively Correlate with White Cell Proliferation Rates. We next analyzed the effects of a wide range of sugar concentrations on the opaque-to-white switching rate as well as on the proliferation rates of white cells. To visualize efficient switching in a large population of individual cells, we carried out opaque-to-white switching experiments where the temperature of the culture was raised from 25 °C to 37 °C. This temperature shift provided a large dynamic range to monitor the effect of different media conditions on switching rates. Here opaque cells were grown at 25 °C in rich media containing glucose, *N*-acetylglucosamine (a monosaccharide derivative of glucose, abbreviated GlcNAc), or a wide range of different combinations of these two sugars. These cultures were analyzed with a flow cytometer every 20 min for 4 h, at which point the temperature was raised to 37 °C to induce opaque-to-white switching. Following the temperature shift, cells were tracked every 20 min for an additional 17 h (Fig. 3*A*). As can be seen in Figs. 3*B* and 4, there is a

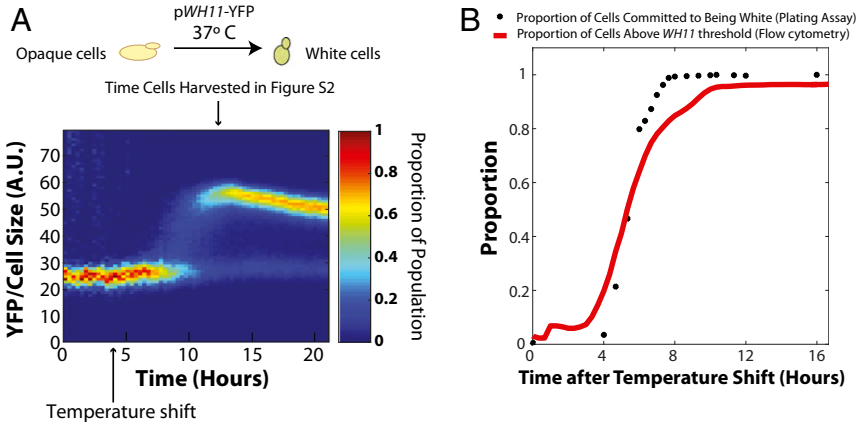

Fig. 2. Expression of p*WH11*-YFP serves as an accurate proxy for the point at which opaque cells become committed to white cells. (*A*) In response to a 37 °C temperature shift, opaque cells switch to white cells en masse. The units of *WH11* expression on the *y* axis are arbitrary and represent fluorescent expression divided by side scatter, which corrects for cell size. The *x* axis represents time; the temperature shift to 37 °C happened after 4 h. The data are plotted as a heatmap with the color representing the proportion of cells that express *WH11* at a specific value (the *y* axis) and time (the *x* axis). (*B*) To correlate *WH11* expression (red line) with commitment to the white cell state (black circles), the cells from the experiment in *A* were plated at 25 °C (the low temperature) and the resulting opaque and white colonies were counted. *SI Appendix*, Fig. S2 shows that this correlation holds across a broad range of sugar concentrations.

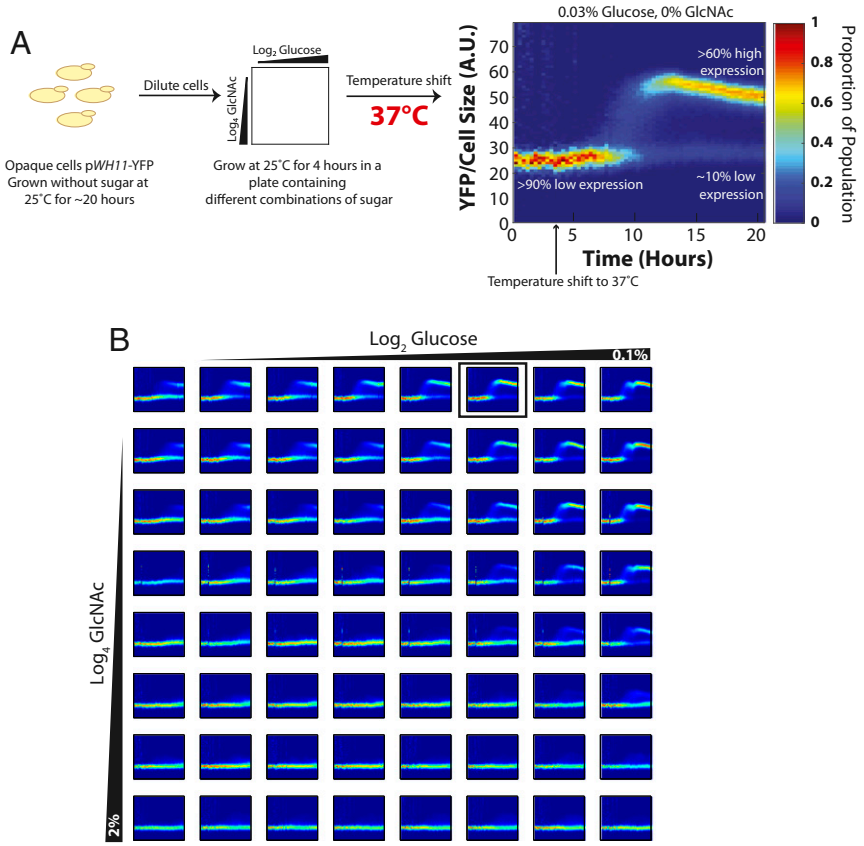

Fig. 3. The p*WH11*-YFP reporter permits single-cell analysis of opaque-to-white switching. (*A*) Opaque cells were cultured at 25 °C in rich media lacking sugar, diluted, and grown in rich media containing the specified combination of glucose and GlcNAc for 4 h and then subjected to a temperature shift to 37 °C. Throughout this experiment, *WH11* expression was monitored in individual cells. The proportion of cells expressing *WH11* at the value indicated in the *y* axis is plotted as a heatmap across time (the *x* axis). This plot shows that *WH11* expression is induced in nearly all cells in response to a temperature shift. The very small fraction of cells that do not become fluorescent are nonetheless white cells as revealed by the plating assay. Although we do not know why these cells do not fluoresce, their numbers are sufficiently low that they do not complicate any of the interpretations. This plot represents a time course of *WH11* expression in a single combination of glucose and GlcNAc. (*B*) Similar time courses were collected for multiple wells, where each well had a different concentration of glucose and GlcNAc. The well displayed in *A* is boxed.

marked dependence of white cell number increase on sugar composition, ranging from virtually no increase in GlcNAc alone (Figs. 3 *B*, *Lower Left* and 4 and *SI Appendix*, Figs. S3 and S4) to nearly 100% of the population composed of white cells in glucose alone (Figs. 3 *B*, *Upper Right* and 4 and *SI Appendix*, Figs. S3 and S4). Different combinations of the two sugars produced widely different numbers of white cells in the population (Figs. 3 and 4 and *SI Appendix*, Figs. S3 and S4).

We next measured white-to-opaque switching in our experimental setup. Since we do not know exactly how switching occurs, some caution is needed in setting up equations that assume a particular mechanism of switching. For example, we observed that, even when switching occurs at high frequency, the number of opaque cells does not decline (*SI Appendix*, Fig. S5). This argues against a model where switching occurs through an opaque cell "turning into" a white cell. It seems more likely that switching occurs when an opaque cell buds off a new white cell while the mother remains an opaque cell; however, it is possible that the switching process is more complex than this simple model. Because of these uncertainties, our analysis focuses on the rate of appearance of white cells that cannot be accounted for by the proliferation of existing white cells. We refer to this parameter as the rate of de novo white cell appearance. An advantage of this parameter is that it is largely independent of any particular model of switching, but it allows a qualitative assessment of the contribution of switching to the population structure.

We can calculate the expression for the rate of de novo white cell appearance with the equation

$$\frac{dW}{dt} = qW + \frac{dW(denovo)}{dt}O, \qquad [1]$$

where W is the number of white cells, O is the number of opaque cells, and q is the proliferation rate of white cells. Since de novo white cells derive from opaque cells, they are multiplied together in the expression. Solving for $\frac{dW(de\ novo)}{dt}$ gives the equation

$$\frac{dW(de\ novo)}{dt} = \frac{\frac{dW}{dt} - qW}{O}. \qquad [2]$$

Eq. **2** shows that the de novo rate of white cell appearance is meaningful only if opaque cells are present in the population. (If the entire population is white, then the white cell increase derives solely from proliferation and $\frac{dW(de\ novo)}{dt}$ is undefined.)

We empirically determined that the most accurate time to measure de novo white cell appearance is during a 3-h window, 4 to 7 h after the temperature shift (*SI Appendix*), the time window where opaque-to-white switching is observable across all of the sugar conditions we tested (*SI Appendix*, Fig. S5). Assuming that the de novo white cell appearance rate is relatively constant

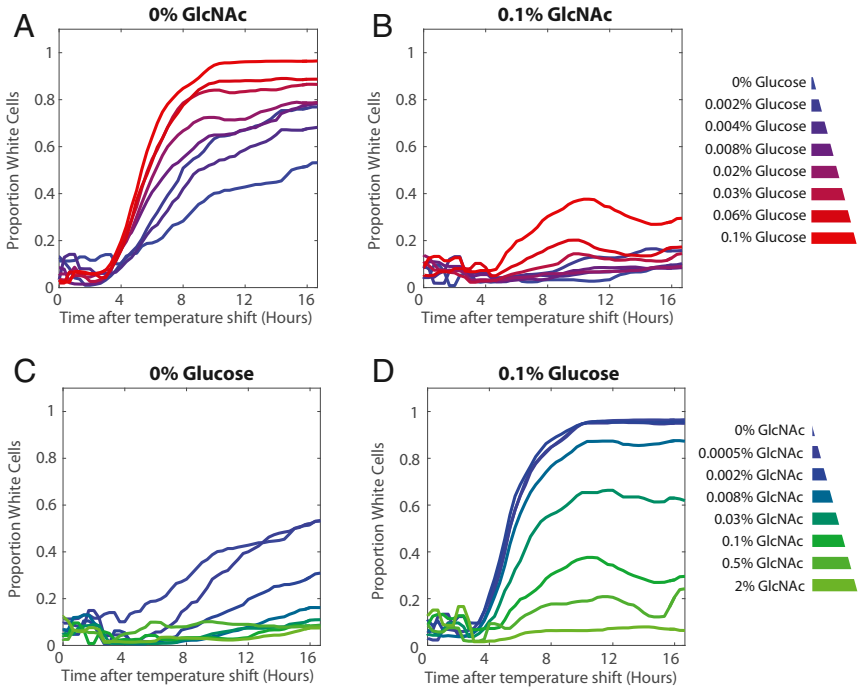

Fig. 4. Glucose promotes opaque-to-white switching while GlcNAc prevents it. (*A* and *B*) Switching in response to a 37 °C temperature shift by opaque cells grown in different concentrations of glucose with no GlcNAc (*A*) or with 0.1% GlcNAc (*B*). The proportion of cells that have switched (the proportion of cells expressing *WH11*; Fig. 2) is plotted on the *y* axis while time after the 37 °C temperature shift is plotted on the *x* axis. Each colored line in the plot represents a different concentration of glucose, as indicated. (*C* and *D*) Switching in response to a 37 °C temperature shift by opaque cells grown in GlcNAc in the absence of glucose (*C*) or with 0.1% glucose (*D*). As in *A* and *B*, the proportion of cells that have switched is plotted as a function of time; the different colored lines represent different concentrations of GlcNAc, as indicated. See *SI Appendix*, Figs. S3 and S4 for opaque-to-white switching line plots for all glucose and GlcNAc combinations tested.

during this timeframe (*SI Appendix*, Fig. S5), we calculated the de novo white cell appearance rates across all ninety-six 3-h windows, each consisting of 10 time points, in our assay.

Before solving for the rate of de novo white cell appearance, we must calculate q, the proliferation rate of white cells. To determine q, we carried out an independent experiment where a pure population of white cells was subjected to the same grid of 96 glucose and GlcNAc conditions in a flow cytometer (4 h at 25 °C followed by a shift to 37 °C for 17 h). In other words, the experimental conditions mimic the switching experiment as closely as possible. Under these conditions, white-to-opaque switching is expected to be negligible; consistent with this expectation, we did not detect any cells whose fluorescence changed in the course of the experiment. The increase of white cells is then

$$\frac{dW}{dt} = qW - eW, \qquad [3]$$

where W is the number of white cells, q is the proliferation rate of white cells, and e is the death rate of white cells.

For each of the 96 conditions, W is measured at each time point and $\frac{dW}{dt}$ can be determined by the change in cell number as a function of time. If we assume death rates are negligible (see below), q can be calculated from the data for each of the 96 conditions; this calculation was made for the 3-h window 4 to 7 h after the temperature shift and is plotted in Fig. 5*A*.

Solving the equations, we conclude that the rate of de novo white cell appearance varied dramatically (over 100-fold), ranging from none observable (<0.1% of the population switched from opaque to white within the 3-h time window in high GlcNAc and low glucose) to 100% of the population having switched when glucose is abundant and GlcNAc levels are low (Fig. 5*B*). Intermediate combinations of the sugars elicit intermediate rates over a broad range (Fig. 5*B*).

An unanticipated correlation was found between the de novo appearance of white cells and the proliferation rates of white cells in that medium ($r^2 = 0.3$, Pearson correlation, Fig. 5*C*). For example, in high concentrations of glucose, de novo white cells appear at a high rate (Fig. 5*B*), and the resulting white cells proliferate rapidly (Fig. 5 *A* and *C*). The implication of this correlation is considered in *Discussion*.

The assumption that death rates are negligible for this analysis is consistent with previous literature measuring the growth of white and opaque cells (for example, ref. 20). However, we cannot rule out the possibility that low levels of cell death occur under the present experimental setup. Therefore, we computationally tested whether different levels of cell death could confound our conclusions. To do so, we modeled hypothetical cell death rates of 10%, 30%, and 50% of proliferation rates (*SI Appendix*, Fig. S6) and found that, although the Pearson correlation decreased somewhat as hypothetical death rates were increased, our conclusions (Fig. 5*C*) did not qualitatively change.

Opaque Cell Proliferation and Opaque-to-White Switching Are Both Necessary for the Observed Population Shift. Upon finding that opaque-to-white switching and white cell proliferation show an unanticipated correlation, we wanted to investigate how switching and proliferation contribute to the rapid population shift of opaque cells to white cells. First, we asked whether opaque cells needed to proliferate at all for opaque-to-white switching to occur. To address this question, we cultured opaque cells as before, but upon shifting the temperature to 37 °C, cells were grown in water or minimal media lacking amino acids (where they could not proliferate) or rich media with amino acids (where they actively proliferated) and plated at various time

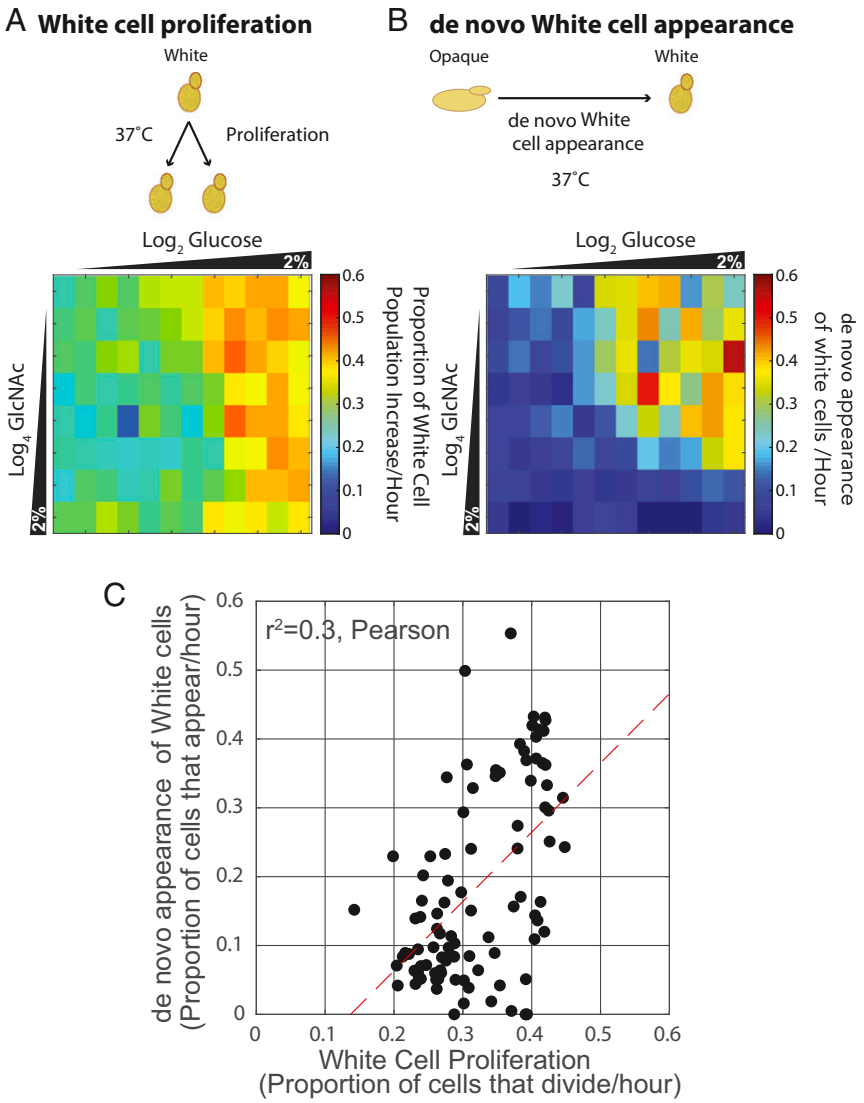

Fig. 5. White cell proliferation and de novo white cell appearance. (*A*) White cell proliferation. White cells were inoculated into a grid of glucose and GlcNAc concentrations, and proliferation rates were determined by counting individual cells. The results are shown as a heatmap where each box represents the proliferation of white cells under a specific glucose and GlcNAc combination during a 3-h window 4 to 7 h after the 37 °C temperature shift. The units of proliferation are expressed as the proportion of the white cell population that increased per hour and can vary from zero to one; in other words, if the value is 0.5, that means that the population of white cells increased 50% in 1 h. (*B*) De novo white cell appearance is plotted as a heatmap with the color representing the value of de novo white cell appearance during the time period 4 to 7 h after the 37 °C temperature shift. This value represents the proportion of cells that increase per hour and can vary from zero to one. (*C*) To determine the correlation between de novo white cell appearance and proliferation rates, we plotted the proliferation of white cells (as determined from *A*) against the de novo appearance of white cells (as determined in *B*), where each black circle represents a specific concentration of glucose and GlcNAc. A first-degree polynomial was fitted (least squares) to the data and plotted as a dashed red line. The linear correlation between the two variables was measured with a Pearson coefficient.

points. We found that opaque-to-white switching did not occur without opaque cell proliferation (*SI Appendix*, Fig. S7). In rich media, however, an entire population of opaque cells could shift to white cells 3 to 5 h after the temperature shift, consistent with what we observed in our flow cytometry setup (*SI Appendix*, Fig. S7). Hence, opaque cells need to be actively proliferating to switch to white cells.

From our data in Fig. 5*A*, we know the rate of proliferation of white cells under these conditions. White cells divide at most two times within 3 h at 37 °C. In a hypothetical case in which opaque cells do not proliferate and opaque-to-white switching does not occur, a population that started out 50% white and 50% opaque would be only 80% white and 20% opaque after 3 h at 37 °C. In our cytometry experiments, we observed populations of cells that were 100% opaque 4 h after a temperature shift. Three hours later (7 h after a temperature shift), the entire population switched to be nearly 100% white. Hence, even if white cells have a proliferative advantage over opaque cells, that selective advantage is not sufficient to account for the observed population shift in the timeframe we observed. Instead, both white cell proliferation and opaque-to-white switching are needed for an entire population of opaque cells to rapidly switch to white cells.

Opaque-to-White Switching in Response to Other Stimuli Is Also Dependent on Glucose and GlcNAc Concentrations. To test the generality of the dependence of opaque-to-white switching on sugar concentration, we measured bulk switching in response to conditions other than a temperature shift. Using the traditional plating assay (which measures the population-based commitment

point of opaque-to-white switching), we found that various environmental factors (including geldanamycin, rapamycin, and sodium chloride; *SI Appendix*, Fig. S8) increased de novo white cell appearance in the absence of a temperature shift. In every condition we tested, opaque cells switched to white cells more frequently when grown in glucose compared to GlcNAc (*SI Appendix*, Fig. S8). Although we could not accurately monitor the de novo appearance rates of white cells in these experiments (as we could for the flow cytometry experiments), we are of the impression that the major effects in these experiments are due to changes in switching per se and not to differences in cell proliferation. In support of this idea, the total number of colonies observed in these experiments was similar across all conditions tested, ruling out the possibility that proliferation differences (or cell death differences) could be solely responsible for the effects of sugar composition. The results of these experiments indicate that the dependency of opaque-to-white switching on sugar composition is a general property of the switching system and is not particular to the temperature shift.

Discussion

We used single-cell measurements to investigate white–opaque switching in *C. albicans*, a component of the human microbiome and also the most prevalent fungal pathogen of humans (24). White–opaque switching produces two distinctive types of cells (white and opaque) from the same genome; each cell type is stable through many rounds of cell division and switching between them appears stochastic (2, 6). White cells and opaque cells differ in many characteristics and are thought to be specialized for different niches in the human host (reviewed in refs. 2, 3, and 25–28).

Our principal findings are as follows: 1) Opaque-to-white switching frequencies (as monitored by de novo white cell appearance) can vary continuously over at least a factor of 1,000, depending on the growth media. Specifically, alterations in the concentrations of two sugars, glucose and GlcNAc, produce a broad range of reproducible switching frequencies. Despite the large dynamic range of switching frequencies, the switching itself remains all or none. 2) Across a broad range of glucose and GlcNAc concentrations, opaque-to-white switching correlates well with the relative fitness of white cells in that medium. 3) The population shift from opaque to white requires actively proliferating opaque cells, opaque-to-white switching, and white cell proliferation. Taken together, these findings support a model where nutritional conditions more favorable to white cells stimulate a rapid population shift of opaque cells to white cells. An increase in opaque-to-white switching, in combination with the relative fitness advantage of white cells under these same conditions, ensures that when conditions change, a purely opaque cell population can rapidly and completely convert to a white population.

Understanding the role of opaque cells is increasingly important in light of recent studies showing that, contrary to previous expectation, between 35% and 69% of MTL heterozygous (a/α) strains (which compose more than 90% of *C. albicans* clinical isolates) are capable of forming opaque cells (29–32). Unfortunately, the role of opaque cells in *C. albicans* biology has been difficult to understand because opaque cells do not proliferate well under 1) conditions used to isolate *C. albicans* samples from patients, 2) many common laboratory conditions in vitro, or 3) standardized animal model protocols in vivo (8, 17, 33). Here, we show that opaque-to-white switching, in combination with the resulting white cell proliferation, can shift an entire population of cells in just a few hours. Comparing opaque cells and white cells by relying only on their relative proliferation rates under standard conditions may therefore yield an incomplete picture of the importance of opaque cells: Their genomes can continue to propagate, as white cells. To this point, a recent study found that opaque cells thrive just as well as white cells in mouse models of *C. albicans* infections when the standard animal models were slightly modified (33).

Our results indicate that both opaque-to-white switching and white cell proliferation combine to shift an entire population from opaque cells to white cells. There are two broad models that can account for this behavior: 1) Switching and proliferation rates are independently responsive to the environment; in other words, they act in parallel. 2) The two responses act in series with one directly influencing the other. The structure of the transcriptional circuit controlling white–opaque switching supports the second model. The circuit is composed of several nested feedback loops (*SI Appendix*, Fig. S9), and the same transcriptional regulators involved in switching are also responsible for maintaining the white and opaque states (11, 34, 35). Because proliferation rates in different media are determined by whether a cell is white or opaque, they must be controlled (directly or indirectly) by these same regulators and therefore be dependent on any switching event. A simple way to change the switching frequency in response to an environmental cue would be through the up- or down-regulation of one or more of these transcriptional regulators. For example, the rate of opaque-to-white switching could be increased through the down-regulation of the master regulator Wor1, which is highly expressed in opaque cells and required for their maintenance. A model where Wor1 levels decline in response to the proliferative rates of the opaque cell type in a given environment could account for switching rates favoring white cell formation in that environment.

We propose that the complexity of the transcriptional circuit controlling white–opaque switching permits switching to be all or none with respect to single cells, yet allows the switching frequency to vary widely in accordance with the environment. The success of *C. albicans*, as both a pathogen and a member of the human microbiota, requires it to occupy many niches (for example, the gut, mucosal surfaces, and the bloodstream), and it has been appreciated for many years that white and opaque cells differentially express many proteins, including metabolic enzymes, cell-surface receptors, and transcription regulators. It seems likely that the ability to rapidly alter population structure in response to the environment contributes to *C. albicans*' ability to colonize and proliferate in many different niches of its human hosts.

Experimental Procedures

Automated Flow Cytometry. A 25 °C overnight YEP (10 g/L yeast extract and 20 g/L Bacto-peptone) culture was diluted to an optical density (OD_{600}) of ∼0.05 to 0.2 and grown for an additional ∼2 to 3 h in YEP media. A 30-μL sample was removed from the culture for measurement on the cytometer at each time point and 30 μL of fresh YEP media containing the appropriate 1× concentration of glucose and/or GlcNAc was used to maintain a constant culture volume for the duration of the experiment (12 to 18 or more hours). Every 20 min, the flow cytometer (BD Biosciences LSR II Analyzer) measured the fluorescence of cells in each well of a 96-well plate (each well was measured for 8 s). For additional information on the hardware, software, and data processing of the automated flow cytometry system, see *SI Appendix, Extended Experimental Procedures* and Zuleta et al. (22).

Plate-Based White–Opaque Switching Assays. During automated flow cytometry measurements, cells were collected right after analysis by the flow cytometer, diluted, and spotted onto SD+AA+Uri plates (see *SI Appendix, Extended Experimental Procedures* for media composition). Opaque-to-white switching assays were performed as previously described (11, 12, 35).

Liquid-Based White–Opaque Switching Assays. To assess whether we could observe switching dynamics in our automated flow cytometry system in the absence of a temperature shift, we developed a liquid-based switching assay during this study. Here, strains were grown overnight at 25 °C in YEP and diluted in the morning. They were grown for 4 to 6 more hours until they reached an OD_{600} of 0.5 to 2. Cells were then treated with one of several environmental or chemical perturbations and grown for the desired amount of time (usually 16 h) at 25 °C in YEP. They were then diluted and spotted onto SD+AA+Uri plates and analyzed as described above for the plate-based assay.

Data Availability. Raw flow cytometry data are plotted in *SI Appendix*, Fig. S5. The raw data files are available upon request.

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