Metabolic-rate dependent cell cycle entry and progression in Saccharomyces cerevisiae
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Chapter 2

The rate of glycolysis dictates cell fate commitment in
Saccharomyces cerevisiae

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Abstract

Despite quiescence being the most abundant cellular state in nature, little is known on the mechanisms that control the transitions in and out of quiescence. Here, using microfluidics and S. cerevisiae strains, in which the rate of glycolysis can be uncoupled from extracellular glucose levels, we show that glycolytic flux controls the transitions in and out of quiescence independently of glucose availability. Using single-cell metabolic reporters, and comparing quiescent with co-existing isogenic dividing cells, we show that energy availability is not the limiting factor for entry to or exit from quiescence. Finally, we show that a burst in NAD[P]H accumulation and a subsequent abrupt increase in the instantaneous growth rate are events that consistently precede the exit from quiescence,

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Chapter 2

whether stochastically or externally induced, and occur also prior to cell cycle initiation in regularly dividing newborn cells. Our results demonstrate that the rate of metabolism dictates cell fate commitment.

Introduction

Despite the fact that life and the process of cell division are inextricably linked, the majority of cells on earth spend most of their lifetime without dividing (Gray et al., 2004; Bergkessel, Basta, and Newman, 2016). In eukaryotes, this state of cell-cycle arrest is called the quiescent or G0 state. While the mechanisms underlying the transition through the various cell cycle phases have been extensively studied, the processes controlling the entry to and exit from quiescence are still poorly understood (De Virgilio, 2012; Cheung and Rando, 2013; Yao, 2014).

In yeast, quiescence is considered to be regulated primarily by nutrient availability: cells enter quiescence when nutrients are scarce, and exit quiescence when nutrients are available (De Virgilio, 2012). However, how nutrients control quiescence is poorly understood. Yeast cells unable to uptake glucose cannot enter growth even when glucose is abundant in the environment (Wieczorke et al., 1999). Furthermore, only when glucose is allowed to be catabolized all the way down to glycolysis, then quiescence-specific cytoskeletal structures (actin bodies), as present in starved cells, are disassembled (Laporte et al., 2011). Finally, very recently it was shown that cell cycle progression might be coupled to an autonomous metabolic oscillator, and that a critical metabolic frequency threshold should be reached for cell cycle initiation and the G1/S transition. (Papagiannakis et al., 2017). Together, these observations suggest that a cell’s decision to enter or exit quiescence (i.e. exit or enter the cell division cycle) might be dominated by its metabolic rates, rather than the extracellular availability of glucose. However, the inherent coupling between glucose levels and the rate of glucose metabolism renders the dissection of the role of these two factors in the regulation of quiescence challenging.

Next to the regulation of quiescence, the phenotype of quiescent cells also remains rather elusive. Cells grown into stationary phase in complex medium have been mostly used so
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far in order to generate and characterize bulks of quiescent yeast cells (Gray et al., 2004, De Virgilio 2012). Under such conditions, quiescent cells have been found to display a set of characteristic features, such as ceased growth and G1 arrest, reduced rates of protein synthesis, and enhanced resilience to various types of stress. However, whether these phenotypic attributes comprise an essential part of the quiescence program, or they are merely an outcome of nutrient shortage, remains an open question (Klosinska et al., 2011). In addition, the coexistence of both quiescent and non-quiescent cells in stationary-phase cultures (Allen et al., 2006; Davidson et al., 2011) further complicates the interpretation of population-level measurements, which reflect the average behavior of the underlying cell sub-populations and can mask quiescence-specific attributes.

Here, exploiting single cell microscopic analyses with microfluidics (Huberts et al., 2013; Lee et al., 2012) and single cell metabolite reporters (NAD[P]H autofluorescence and FRET-based ATP measurements), we show that an engineered Saccharomyces cerevisiae strain with a limited glucose uptake capacity splits into a quiescent population and a population of dividing cells at high glucose concentration. Under these nutrient-rich conditions, specifically the quiescent cells, and not their coexisting dividing counterparts, display features common for starvation-induced quiescent cells, such as ceased growth, reduced protein expression, and G1 arrest, however, without being energy deprived. We show that an increase in the rate of glycolysis, stochastically occurring or externally induced, triggers exit from quiescence, and consistently precedes cells cycle initiation also in regularly dividing daughter cells. Thus, the commitment for cell cycle entry or abstention, is dictated by the rate of glycolysis independently of external nutrients. The dependence of cell fate commitment on metabolic rates highlights glycolysis as an intervention target for cell cycle control.

Results

Glycolytic rates determine quiescence entry and exit

To test whether a limitation in the glucose uptake rate can trigger entry into quiescence even in presence of high [10 gL⁻¹] glucose, we used a yeast strain, in which 21 known
glucose transporter genes were deleted and a single chimeric hexose transporter (HXT) gene was re-introduced (Otterstedt et al., 2004). With this strain, in which the glucose uptake rate (and thus the glycolytic flux) is about 5-fold lower than in the corresponding wild-type (Elbing et al., 2004a), we could uncouple the rate of glycolysis from the extracellular glucose conditions.

**Figure 1. Dividing and non-dividing cells coexist in the presence of glucose.** (A) Schematic representation of the microfluidics-based experimental setup. Cells are trapped underneat PDMS pads and are continuously fed with fresh medium. (B) Boxplot showing the fractions of coexisting dividing and non-dividing cells in the single-HXT (KOYTM6*P) strain (data from 4 biological replicates, 966 cells). Cells were identified as non-dividing after spending at least 40 hours without budding. Error bars indicate the max and min percentages. (C) Time-lapse images of a single dividing and non-dividing cell coexisting at the same nutrient conditions. At the last 20 hours, cells originating by neighboring dividing cells can be seen next to the non-dividing cell.

Cultivating this strain in a microfluidic device (Huberts et al., 2013; Lee et al., 2012), we could monitor hundreds of individual yeast cells and their budding events over at least 40 hours, while cells were constantly fed with glucose-rich medium. Here, we found that while the majority of cells divided, a small fraction of cells [3.06% ± 1.93% (mean ± SD)]
did not divide for at least 40 hours (Figure 1A-C), indicating that despite the abundant presence of glucose, these cells were quiescent. A control experiment with the respective wild-type strain, which achieves high glucose uptake rates at the same conditions (Elbing et al., 2004a), showed that the presence of quiescent cells was not an artifact of the experimental setup, as here all cells divided (Figure 1 – figure supplement 1A). We found, that the quiescent cells in the engineered strain were all arrested in the G1 phase of the cell cycle, as was indicated by their persistent nuclear Whi5-GFP localization (Figure 1 – figure supplement 2A and B), similarly to quiescent cells obtained by nutrient starvation (Klosinska et al., 2011) or in stationary phase cultures (De Virgilio, 2012). Thus, these experiments demonstrated that cells can enter quiescence despite glucose rich-conditions, suggesting that quiescence is controlled by the rate of glucose uptake.

To test whether the glucose uptake rate indeed controls quiescence, we asked whether we could trigger quiescence exit by increasing the influx of glucose. To test this, we introduced a single HXT gene in a strain lacking all known 21 glucose transporters (Otterstedt et al., 2004), under the control of a tetracycline-inducible promoter. In the absence of tetracycline, the HXT was expressed at a very low level (Figure 2 – figure supplement 1A) and allowed only for a slow population-level growth rate (Figure 2 – figure supplement 1B). By growing this strain in the microfluidic device in glucose-rich medium without tetracycline, we again found quiescent cells (≈ 8 % of 220 cells, observed over 16 h), which coexisted with dividing cells. Further, we found that the dividing cells displayed higher levels of HXT-GFP compared to quiescent cells (Figure 2A), suggesting that stochastic differences in the expression of the glucose transporter could account for the different cell cycle fate of the two sub-populations. When we added tetracycline to the medium, we found that almost all (94.9%) quiescent cells started to divide (Figure 2B). The exit from quiescence coincided with the increase in HXT levels upon induction (Figure 2C). Thus, these findings confirmed that an increase in glucose influx triggers quiescence exit, independently of external glucose.

Next, we asked whether it is the rate of glucose uptake that triggers the exit, or rather the rate of glucose breakdown, i.e. the rate of glycolysis. To test this, we cultivated the strain with single chimeric HXT-transporter on the disaccharide maltose as carbon source. On
maltose, this strain attains a high glycolytic rate (Figure 2 – figure supplement 2), since after the uptake by maltose permeases, maltose is cleaved into two glucose molecules, thus feeding glycolysis (Novak, Zechner-Krpan, and Marič, 2004). In contrast to the experiments with glucose, when maltose was used as a carbon source, we could not detect any quiescent cells (Figure 2D). Similarly, when we dynamically switched medium from glucose to maltose after 40 h, the majority of the quiescent cells [65.43% ± 6.02% (mean ± SEM)] exited quiescence in synchrony (Figure 2E; Figure 2 – figure supplement 3A and B), while, interestingly, the remaining quiescent cells died within the same time window (Figure 2 – figure supplement 3B and C).

**Figure 2. An increase in glycolysis triggers quiescent cells to divide.** (A) HXT1-GFP peripheral mean fluorescence in dividing and quiescent cells right after the loading at the microfluidics device. Horizontal yellow lines represent the median value. Two-tailed p-value is shown for Mann-Whitney
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test. All dividing cells budded at least once during the first 5 hours of the experiment. (B) Percentage of quiescent cells from the strain with the inducible HXT (KOYWW100 HXT1) that exit quiescence following the HXT overexpression while the extracellular nutrient conditions remain constant. Cells were supplemented with minimal medium containing 10 gL⁻¹ glucose for 16 hours and then (t = 0), HXT expression was induced by adding 50 ngml⁻¹ tetracycline to the medium. The fate of all cells that remained quiescent for the 16 hours of the pre-tetracycline period was monitored (n = 39 and n = 48 cells for the treated and control experiment respectively). For the control experiment, cells were treated in the same way but no tetracycline was added after the first 16 hours. Dashed lines indicate cells (n = 7 and n = 9 for the treated and control experiment respectively) that were lost from the PDMS pads before showing any response. Because a similar fraction of cells was lost before showing any response in the treated and control experiments, lost cells do not statistically affect the result. (C) Dynamic expression of HXT1-GFP as a result of tetracycline addition determined at the single-cell level (n = 16 to 25) in quiescent cells that exit after the addition of tetracycline. The moment of exit with respect to tetracycline addition is indicated in the histogram. Cells were supplemented for at least 16 hours in minimal medium containing 10 gL⁻¹ glucose and then 50 ngml⁻¹ tetracycline was added to the medium. Inset shows HXT1-GFP levels in the same cells aligned for first bud emergence (dashed blue line). X-axis in the inset spans from -16 to 4 hours from first bud emergence. Data are represented as mean ± SEM. (D) Histogram of time of first division in the microfluidics setup and respective cumulative distribution for single-HXT (KOYTM6*P) (n = 779) cells in 10 gL⁻¹ maltose. Cells were constantly supplemented with fresh minimal medium. (E) Histogram of time of first division in the microfluidics setup for single-HXT (KOYTM6*P) (4 biological replicates, n = 956) cells. Cells were continuously supplemented with fresh minimal medium containing 10 gL⁻¹ glucose for 40 hours, and were subsequently switched to minimal medium containing 10 gL⁻¹ maltose. The dashed yellow line indicates the time threshold after which the entry to division was considered to be stochastic (see Figure 4 – figure supplement 7 and Materials and Methods) and the dashed black line the point where cells were switched to maltose.

Together, these experiments demonstrate that not the extracellular glucose level nor the rate of glucose uptake control quiescence, but rather, it is a limitation or an increase in the rate of glycolysis that trigger the entry into or exit from quiescence respectively.

*Quiescent cells display low metabolic rates, but they are not limited in energy and redox cofactors*

Using the unique possibility of being able to observe quiescent cells in nutrient-rich conditions (instead of starvation-induced (Klosinska et al., 2011) or stationary phase quiescent cells (Allen et al., 2006; Davidson et al., 2011)), we aimed to characterize the state of quiescence, and compare it directly with the simultaneously present dividing cells. To test whether quiescent cells - along with the cell cycle arrest - had ceased cell growth and macromolecular synthesis, we first measured their cell size dynamics. Here, we found that quiescent cells increased in size through time, although with a lower rate than dividing cells (Figure 3A). Further, using a tetracycline inducible system for GFP
expression, we tested their capacity to synthesize protein. Here, we found that the GFP abundance increased in the quiescent cells for at least 15 hours and thereafter plateaued, while dividing cells displayed a continuous, probably aging related, increase in GFP levels (Figure 3B). Thus, despite the arrest of cell division, quiescent cells sustain protein production and grow in size, although with notably decreased rates compared to dividing cells.

Figure 3. Quiescent cells display a distinct phenotype from dividing cells despite their coexistence. (A) Cell size dynamics of dividing (n = 17 to 25) and quiescent (n = 25 to 26) cells. (B) GFP abundance in dividing (n = 20) and quiescent (n = 23 to 24) cells. GFP expression is driven through a tetracycline inducible promoter at an inducer concentration of 300 ng/ml. GFP abundance (straight line) in a cell which dies before the 40 hours (death indicated by the asterisk) is shown for comparison purposes. (C) FRET-based ATP dynamics in dividing (n = 19 to 20) and quiescent (n = 13) cells. (D) NAD[P]H autofluorescence dynamics in dividing (n = 7 to 14) and quiescent (n = 6 to 18) cells. The dynamics at late time points are likely to also reflect aging effects. Dividing cells underwent at least 5 cell divisions. Data are represented as mean ± 95% confidence interval. In all cases cells were constantly supplemented with fresh minimal medium containing 10 gL⁻¹ glucose.
Next, we asked whether the reduced rate of glycolysis and biomass synthesis in the quiescent cells are also reflected in their energy status. To this end, we used an ATP-FRET sensor and the NAD[P]H autofluorescence to measure the level of these metabolites and energy carriers in single quiescent and dividing cells. Unexpectedly, we found that quiescent cells had initially and for approximately 15 hours higher ATP level than dividing cells, which, however, continuously dropped as a function of time, in contrast to dividing cells, which displayed only a minor time-dependent and long-term decrease in ATP, which we again attributed to aging (Figure 3C). Striking differences were also observed in the NAD[P]H levels between quiescent and dividing cells (Figure 3D), with quiescent cells exhibiting significantly higher levels of NAD[P]H compared to dividing cells.

These results show that quiescent cells are metabolically active, as they can sustain protein synthesis and growth, but both processes are attenuated in comparison to dividing cells. Moreover, especially during the first 15 hours, quiescent cells display high ATP and NAD[P]H levels, suggesting that at least during this period, quiescence is not directly forced by energy deprivation.

Metabolism comes first and growth follows

Next, we used these single cell metabolite reporters to shed further light on the dynamic process of quiescence exit. Specifically, to characterize the temporal order of events that occur during quiescence exit, we measured the dynamics of cell size, NAD[P]H, ATP, and Whi5 localization during the transition from quiescence to cell division upon (i) the addition of maltose or (ii) the induction of HXT expression. We used the emergence of the bud cell as a marker of successful quiescence exit.

Here, we found that during both perturbations bud emergence was preceded by a rapid increase in cell size (Figure 4A and B; Figure 4 – figure supplement 1A and B), a burst in NAD[P]H levels (Figure 4C and D; Figure 4 – figure supplement 1C and D), and a rapid increase in ATP content (Figure 4 – figure supplement 2A). As we found similar increases in NAD[P]H levels also when we shifted starved cells to glucose medium, i.e. perturbation leading to an increase of glycolysis (Figure 4 – figure supplement 3A) or directly after
Figure 4. Cell cycle entry is preceded by a metabolically induced burst in cell growth. (A) Cell size dynamics of single-HXT cells that exit quiescence after the switch from 10 gL\(^{-1}\) glucose to 10 gL\(^{-1}\) maltose (KOYTM6*P, \(n = 35\) to 49). Dashed black line (\(t = 0\)) indicates the point of the nutrient switch and solid blue lines the point of first bud emergence for a random subset (\(\approx 60\%\)) of single
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cells. Inset shows cells size dynamics of the same cells aligned for first bud emergence (dashed blue line). X-axis on inset spans from -9.8 to 4.5 hours from first bud emergence. (B) Cell size dynamics of cells aligned for first bud emergence (t = 0, dashed blue line), which exit quiescence as a response to tetracycline addition in 10 gL⁻¹ glucose [KOY VW100 Hxt1:(ph)GFP, n = 22 to 24], or (G) stochastically in 10 gL⁻¹ glucose (KOYTM6*P, n = 44 to 50), (J) and in normally dividing KOYTM6*P daughter cells in 10 gL⁻¹ glucose (KOYTM6*P, n = 20 to 104). (C) NAD[P]H autofluorescence dynamics in cells that exit quiescence after the switch from 10 gL⁻¹ glucose to 10 gL⁻¹ maltose (KOYTM6*P, n = 9 to 20). Dashed black line (t = 0) indicates the point of the nutrient switch and solid blue lines the point of first bud emergence. (D) NAD[P]H autofluorescence dynamics in cells aligned for first bud emergence (t = 0, dashed blue line), which exit quiescence as a response to tetracycline addition in 10 gL⁻¹ glucose [KOY VW100 Hxt1:(ph)GFP, n = 23 to 25], or (H) stochastically in 10 gL⁻¹ glucose (KOYTM6*P, n = 15 to 19), and (K) in normally dividing KOYTM6*P daughter cells in 10 gL⁻¹ glucose (KOYTM6*P, n = 29 to 104). (E) Cross-correlation analysis between the rate of NAD[P]H autofluorescence [N(t)] and cell size [(V(t)] change in cells that exit quiescence as a response to tetracycline addition in 10 gL⁻¹ glucose [KOY VW100 HXT1, n = 24], or (I) stochastically in 10 gL⁻¹ glucose (KOYTM6*P, n = 19). For both (E) and (I) the cross-correlations were computed for the time period of -9.33 hours to exit from quiescence until the moment of exit (bud emergence). In all cases data are represented as mean ± SEM. (F) Schematic representation of the order of events that precede the exit from quiescence after induction of glycolysis with a glucose to maltose switch.

increasing the glucose uptake rate under constant glucose conditions (Figure 4 – figure supplement 3B), we consider the NAD[P]H burst a reflection of an increase in glycolysis. START, the irreversible commitment to cell-cycle entry, which is denoted by the exit of Whi5 from the nucleus (Doncic, Falleur-Fettig and Skotheim, 2011), occurred last, right after the rapid increase in cell growth (Figure 4 – figure supplement 4A and B).

To determine the relative timing of the glycolytic burst, reflected by the change in NADPH dynamics, and the growth burst, we determined the dynamic rates of NAD[P]H autofluorescence change $N(t)$ and the instantaneous cell growth rate $V(t)$ from the data and performed a cross-correlation analysis on these rates. Here, we found positive cross-correlations at approximately -1 to -1.5 hours (Figure 4E), indicating that the burst in NAD[P]H accumulation preceded the increase of the cell growth rate. Because ATP levels increased shortly before NAD[P]H (Figure 4C; Figure 4 – figure supplement 2A) and the Whi5 nuclear exit after the increase in cell size (Figure 4 – figure supplement 4A), these results indicated that the commitment of quiescent cells to cell-cycle entry occurs upon the burst in the rate of glycolysis, and after the subsequent burst in cell growth (Figure 4F).

To confirm that this particular order of events happens during quiescence exit, we allowed wild-type cells to enter quiescence by carbon source depletion in a batch culture
(Figure 4 – figure supplement 5). Then, we loaded these cells in the microfluidics device, provided them with fresh glucose-rich medium, and followed their budding activity, NAD(P)H levels, and cell size dynamics. Here, while we found that after the addition of the fresh medium cells exited quiescence at considerably different times (≈ 1.5-4.5 hours) (Figure 4 – figure supplement 6A), exit from quiescence was always preceded by a burst in NAD(P)H levels (Figure 4 – figure supplement 6B) and a subsequent burst in cell growth (≈ 1 hour after the NAD(P)H burst) (Figure 4 – figure supplement 6C). Importantly, the time of exit was dependent on the timing of the NAD(P)H and the respective growth bursts, rather than the time of the nutrient replenishment (Figure 4 – figure supplement 6B-D).

These findings show that quiescence exit, independently of the means by which the rate of glycolysis is increased, is preceded by a burst in NAD(P)H levels, reflecting an increase in glycolysis, a subsequent burst in cell size growth, and then exit of Whi5 from the nucleus and commitment to the cell cycle.

**Stochastic or programmed metabolic alterations can determine cell fate**

Next, we asked whether the same sequential order of events would also occur in cells that spontaneously exit quiescence without any external induction of glycolysis, which we occasionally noticed in the single-HXT strain (see Materials and Methods) (Figure 2E; Figure 4 – figure supplement 7). Indeed, also here, we found that the exit of Whi5 from the nucleus and budding were preceded by a burst in cell growth (Figure 4 – figure supplement 4C; Figure 4G; Figure 4 – figure supplement 1A). Similar to the induced exits, the burst in cell growth was preceded by a burst in NAD(P)H levels (Figure 4H and I; Figure 4 – figure supplement 1C), and also a cessation of the long-term declining trend of the ATP levels (Figure 3C) just prior to the stochastic exit (Figure 4 – figure supplement 2B), which indicated also an increase in ATP synthesis during this period. Thus, despite the constant extracellular nutrient environment and the lack of external induction, we found here exactly the same order of events to take place prior to quiescence exit.

Next, we asked whether we would find the same order of events to comprise a general requirement for entry into cell division. To test this, we analyzed the cell growth and
NAD[P]H dynamics during the first cell division of cells that were newly born in the microfluidic device. Here, we found that following their birth and prior to their entry to cell division, these newborn cells displayed a burst in NAD[P]H levels and a rapid increase in cell size (Figure 4J and K; Figure 4 – figure supplement 1A and C; Figure 4 – figure supplement 8A-D; Figure 4 – figure supplement 4D), in striking similarity to what was observed for quiescent cells during exit. These findings indicate that an increase in the rate of glycolysis and a subsequent burst in cell growth, not only facilitate the exit from quiescence, but might be also critical for triggering normal cell cycle entry.

Discussion

Using microfluidics, different genetic and environmental perturbations, and single cell analyses, we found that the rate of glycolysis controls the entry of cells to quiescence, and to the cell division cycle, independently of external glucose concentration. We found that quiescent cells in nutrient-rich conditions are not limited in energy (ATP) and redox (NAD[P]H) cofactors and that they are metabolically active, as they can grow in size and sustain protein synthesis, but with rates that are attenuated in comparison to dividing cells. We found that after the induction of glycolysis, cells undergo a defined series of events prior to exit: they transiently increase their ATP and NAD[P]H levels and they exhibit a burst in their previously attenuated cell growth rate. Similar events occur at steady-state nutrient-rich conditions when cells exit quiescence stochastically or when regularly dividing daughters initiate their cell cycle, suggesting that also in these cases fate commitment is dominated by the rate of metabolism (Figure 5).

The notion that cells can undertake decisions on the basis of their intracellular metabolic fluxes has recently gained recognition (Kochanowski et al., 2013; Kotte et al., 2014; Kotte, Zaugg and Heinemann, 2010; Schmidt-Glenewinkel and Barkai, 2014; Elbing et al., 2004b; Huberts, Niebel and Heinemann, 2012; Folmes et al., 2012; Agathocleous and Harris, 2013). For example, the levels of persister cells in *Escherichia coli* cultures were found to be modulated in a metabolic flux-dependent manner independently of external nutrient conditions (Kotte et al., 2014). In addition, glycolytic gene expression was found
to be set by the rate of glycolysis independently of the extracellular concentration of glucose (Elbing et al., 2004b), and observations also suggest that yeast cells determine their mode of metabolism on the basis of the level of their glycolytic rate (Huberts, Niebel and Heinemann, 2012). Further, it was shown that yeast undergoes cell-cycle-autonomous periodic changes in metabolic activity at constant nutrient environments, and that these metabolic oscillations are coupled to the cell cycle when sufficient metabolic rates are attained (Papagiannakis et al., 2017). In line with what we demonstrate here, these findings suggest that a periodic increase in metabolic activity might be essential and even sufficient for triggering cell cycle entry independently of changes in the extracellular nutrient environment.

Figure 5. Schematic model for the regulation of quiescence. The level of glycolytic flux determines whether cells reside in quiescence or enter cell division. High flux levels could dictate exit by promoting the growth-dependent dilution of cell-cycle inhibitors or by directly inducing the expression of cell-cycle activators. The role of extracellular glucose availability in the regulation of quiescence is mainly restricted on allowing glycolysis to operate within a certain range of rates. At conditions of low enzyme or transporter abundance, stochastic variations in gene expression can determine fate commitment by influencing the rate of glycolysis. Further, periodic bursts in metabolic activity occurring under constant nutrient conditions can also influence cell fate.

How cells translate metabolic rates to fate decisions? We found that the increase in glycolytic flux is followed by an increase in the cellular growth rate, which is highly predictive for commitment. While an increased rate of size growth could promote fate commitment by leading to the attenuation of a critical cell size required for entry to the cell cycle, we found no correlation between absolute cell size and cell cycle entry (Figure 5 – figure supplement 1) suggesting that the rate by which the size changes, and not the
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absolute cell size, is relevant for commitment. From this perspective, our results support inhibitor-dilution models for cell cycle entry, where for example the rate of cell growth would exceed the rate of synthesis of a cell cycle inhibitor, leading thus to a decrease in its concentration. Recent findings provided evidence that the commitment of yeast cells to the cell-cycle is controlled by inhibitor-dilution models (Schmoller et al., 2015; Soifer, Robert and Amir, 2016).

Next, it will be necessary to unravel how metabolic rates are mechanistically translated into cell cycle control. The high degree of conservation of G1 control across eukaryotes (Bertoli, Skotheim, and Bruin, 2013), together with the fact that proliferative disorders are often coupled to altered metabolic activity (Levine and Puzio-Kuter, 2010; DeBerardinis et al., 2008; Calvo-Vidal and Cerchietti, 2013; Altman and Dang, 2012), could render our results of a metabolic flux triggered cell fate decision relevant also for eukaryotes other than yeast.

Materials and Methods

Strains and media

Yeast strains used in this study are all prototrophic and of the CEN.PK2-1C strain-background, with the exception of YSBN6 (S288C strain-background). All the strains are summarized in Table A. All experiments were performed using minimal medium with composition (modification from Verduyn et al., 1992) per liter of 5 g of (NH₄)₂SO₄, 3 g of KH₂PO₄, 0.5 g of MgSO₄ · 7H₂O, 4.5 mg of ZnSO₄ · 7H₂O, 0.3 mg of CoCl₂ · 6H₂O, 1.0 mg of MnCl₂ · 4H₂O, 0.3 mg of CuSO₄ · 5H₂O, 4.5 mg of CaCl₂ · 2H₂O, 3.0 mg of FeSO₄ · 7H₂O, 0.4 mg of NaMoO₄ · 2H₂O, 1.0 mg of H₃BO₃, 0.1 mg of KI, 1.5 or 15 mg of EDTA, 0.05 mg of biotin, 1.0 mg of calcium pantothenate, 1.0 mg of nicotinic acid, 25 mg of inositol, 1.0 mg of pyridoxine, 0.2 mg of para-aminobenzoic acid, and 1.0 mg of thiamine. The minimal medium was further supplemented with either 10 g L⁻¹ or 0.01 g L⁻¹ glucose, or 10 g L⁻¹ maltose as a carbon source. Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), Acros-Organics (Geel, Belgium), Formedium (Hunstanton, United Kingdom), or Merck-Millipore (Billerica, Massachusetts).

Strain construction

To monitor global protein dynamics in single cells, we introduced a yeGFP (yeast codon optimized version of eGFP) under the regulation of a tetracycline inducible system (Blount et al., 2012) to the HO genomic locus of the KOY TM6*P strain (strain KOY TM6
C6B C). The integration cassette with the tetracycline inducible system [pINV plasmid, (Blount et al., 2012)], the KanMX4 gene, and overhangs with homology to the HO region were amplified from the pC6B plasmid (Papagiannakis et al., 2017) using primers Seq4 (all the primers used in this study are summarized in Table B). Transformants were selected in the presence of G418 (Formedium) in YPD (Formedium) (20 gL⁻¹ glucose (Sigma-Aldrich)) plates and were tested for correct cassette integration using the Seq5 primers.

**Table A.** List of yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>KOY PK2-1C83 (wild-type)</td>
<td>MATa MAL2-8′ SUC2</td>
<td>Otterstedt et al., 2004</td>
</tr>
<tr>
<td>YSBN6 (wild-type)</td>
<td>MATa FY3 ho::HphMX4</td>
<td>Canelas et al., 2010</td>
</tr>
<tr>
<td>KOY VW100 (hxt null strain)</td>
<td>MATa MAL2-8′ SUC2 hxt1Δ ura3-52 gal2Δ::loxP stl1 Δ::loxP agt1Δ::loxP ydl247w Δ::loxP yjr160c Δ::loxP hxt13 Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12 Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10 Δ::loxP hxt8 Δ::loxP hxt514 Δ::loxP hxt2 Δ::loxP hxt367 Δ::loxP Integration cassette at former HXT367 site containing the truncated, constitutive promoter of HXT7 (Hauf et al., 2000), the KIURA3 open reading frame for counter selection, and the HXT7 terminator</td>
<td>Otterstedt et al., 2004</td>
</tr>
<tr>
<td>KOY VW100 Hxt1</td>
<td>KOY VW100P ho::KanMX4-pINV-HXT1</td>
<td>This study</td>
</tr>
<tr>
<td>KOY VW100 Hxt1:(ph)GFP</td>
<td>KOY VW100 ho::KanMX4-pINV-HXT1-(ph)GFP</td>
<td>This study</td>
</tr>
<tr>
<td>KOY VW100 Hxt1 Whi5-mGFP</td>
<td>KOY VW100P ho::KanMX4-pINV-HXT1 whi5Δ::WHI5-mGFP-zeoR</td>
<td>This study</td>
</tr>
<tr>
<td>KOY TM6*P</td>
<td>KOY.VW100P Integration into the cassette: HXT7prom-TM6*::HXT7term, ura3-52::URA3</td>
<td>Otterstedt et al., 2004</td>
</tr>
<tr>
<td>KOY TM6 C6B C</td>
<td>KOY TM6*P ho::yEGFP-TetR-KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>KOY TM6* Whi5-mGFPa</td>
<td>KOY TM6*P whi5Δ::WHI5-mGFP-zeoR</td>
<td>This study</td>
</tr>
<tr>
<td>KOY.TM6 TEF:ATP1</td>
<td>KOY TM6*P ho::TEF1p-ATeam1.03-KanMX4</td>
<td>This study</td>
</tr>
</tbody>
</table>

To determine the cell cycle phase of the non-dividing cells, we generated a genomic fusion of the Whi5 gene with mGFP in the KOY TM6*P and KOY VW100 Hxt1 strains (strains KOY TM6* Whi5-mGFPa and KOY VW100 Hxt1 Whi5-mGFP). An integration cassette with long (≈ 500 bp) overhang sequences homologous to the regions upstream
and downstream the stop codon of Whi5 was generated using an adaptation of the method described here (Wach, 1996). First, the mGFP sequence together with the zeoR (zeocin resistance) gene were amplified from the pSNA10 plasmid (Saraya et al., 2010) using primers with short overhangs homologous to the region upstream and downstream the stop codon of Whi5 (Whi5-mGFP For, Whi5-ZEO Rev). Then, the sequences spanning ≈ 500bp upstream and ≈ 500 bp downstream the stop codon of Whi5 were amplified from genomic KOY TM6*P DNA using the Whi5-CDS For & Rev and Whi5-DN For & Rev primers respectively. All 3 amplification products were subsequently fused to a single linear fragment in an additional PCR step and the primers Whi5-CDS For and Whi5-DN Rev were used to amplify the final product of the mGFP-zeoR cassette carrying the long integration overhangs. Transformants were selected in the presence of zeocin (Thermo Fisher Scientific, Waltham, Massachusetts) in YPD (20 gL⁻¹ glucose or maltose) plates and correct cassette integration was confirmed by microscopically observing the expected cell-cycle dependent Whi5 localization cycles (Doncic, Falleur-Fettig and Skotheim, 2011) using the Whi5-mGFP screen For & Rev and Whi5-ZEO screen For & Rev primers.

To monitor ATP dynamics in single cells, we introduced a yeast codon optimized version of the Ateam 1.03 ATP FRET sensor (Imamura et al., 2009) to the HO genomic locus of the KOY TM6*P strain (strain KOY.TM6 TEF:ATP1). The integration cassette with codon optimized version of the Ateam 1.03 ATP FRET sensor and overhangs with homology to the HO region were amplified from the pTEF:ATP plasmid (Papagiannakis et al., 2017) using primers Seq4. Transformants were selected in the presence of G418 in YPD (20 gL⁻¹ glucose) plates.

To dynamically modulate glucose influx while retaining the extracellular glucose concentration constant, we introduced the HXT1 gene under the regulation of a tetracycline inducible system to the HO genomic locus of the hxt null strain KOY VW100P (strain KOY VW100 Hxt1). The integration cassette was generated using Gibson DNA assembly (Gibson et al., 2009). First, the tetracycline inducible system [(pINV plasmid (Blount et al., 2012)] without the yeGFP coding sequence, together the KanMX4 gene and the HO integration overhangs, were amplified from the pC6B plasmid (Papagiannakis
et al., 2017) using primers with short overhangs homologous to the prime ends of the HXT1 gene (hxt1-TETEX For & Rev). Then, the HXT1 gene was amplified from KOY PK2-1C83 genomic DNA using primers with short overhangs homologous to the prime ends of the PCR product generated at the previous step (tetex-HXT1 For & Rev). The two PCR products were reconstituted to a single plasmid (pHxt1) using Gibson DNA assembly (New England Biolabs, Ipswich, Massachusetts) and the plasmid was amplified in *Escherichia coli*. The integration cassette was amplified from pHxt1 using the Seq4 primers. Yeast transformants were selected in the presence of G418 in YPD (20 gL⁻¹ maltose) plates and were tested for correct cassette integration using the Seq5 primers.

For the characterization of KOY VW100 Hxt1, a version of the strain with a GFP tag fused to the HXT1 transporter was generated (strain KOY VW100 Hxt1:(ph)GFP). The integration cassette was generated using a 3-piece Gibson DNA assembly. In specific, the same part of the pC6B plasmid (Papagiannakis et al., 2017) as above was amplified using the hxt1-TETEX For and phgfp-TETEX Rev primer combination, the HXT1 gene was amplified using phgfp-HXT1 For and tetex-HXT1 Rev primer combination, and a GFP variant with reduced pH sensitivity was amplified from genomic DNA of the KOY WT Hxk2-pH-GFP strain (Schmidt, 2014) using primers tetex-PHGFP For & hxt1-PHGFP Rev. The three PCR products were reconstituted to a single plasmid (pHxt1-(pH)GFP) using Gibson DNA assembly and the plasmid was amplified in *Escherichia coli*. The integration cassette was amplified from pHxt1-(pH)GFP using the Seq4 primers. Yeast transformants were selected in the presence of G418 in YPD (20 gL⁻¹ maltose) plates and were tested for correct cassette integration using the Seq5 primers.

**Physiological characterization on glucose and maltose**

For physiology measurements, 250-ml flasks containing 25 ml of 10 gL⁻¹ glucose or maltose minimal medium were used. Media were inoculated with a single colony of KOY KOY PK2-1C83 or KOY TM6*P and cultures were incubated overnight at 30°C, 300 rpm. Cultures were then diluted in 25ml fresh medium of the same initial composition and cells were allowed to attenuate exponential growth. While at exponential phase, cultures were diluted in fresh medium at a final OD₆₀₀nm of ≈ 0.1 and after completing at least 3
The rate of glycolysis dictates cell fate commitment in *Saccharomyces cerevisiae* divisions, cells were further diluted in fresh medium at a final OD$_{600\text{nm}}$ of $\approx 0.1$ before measurements were initiated.

**Table B.** List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Used for strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq4 Forward</td>
<td>AATTATCCTGGGCACGAG</td>
<td>KOY TM6 C6B C; KOY.TM6 TEF;ATP1; KOY VW100 Hxt1</td>
</tr>
<tr>
<td>Seq4 Reverse</td>
<td>ACTGTAAGATTCGCCAC</td>
<td>KOY TM6 C6B C; KOY.TM6 TEF;ATP1; KOY VW100 Hxt1</td>
</tr>
<tr>
<td>Seq5 Forward</td>
<td>CTGGGACAATCGTTATCC</td>
<td>KOY TM6 C6B C; KOY VW100 Hxt1</td>
</tr>
<tr>
<td>Seq5 Reverse</td>
<td>CCACAACTCTTATGAGGC</td>
<td>KOY TM6 C6B C; KOY VW100 Hxt1</td>
</tr>
<tr>
<td>Whi5-mGFP Forward</td>
<td>CCGAAGTGGAGACGTCTGTGAGC AAGGCGAG</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>Whi5-ZEO Reverse</td>
<td>GGAGAAAAAACCTCGTACTACCACA CCGTACCCTCGTCTGAC</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>Whi5-CDS Forward</td>
<td>AGACGACTCTTACCTCGG</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>Whi5-CDS Reverse</td>
<td>TGTGTTAGTACGAGTTTTTCTCC</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>Whi5-DN Forward</td>
<td>TGGTGCCGAGTCTGC</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>Whi5-DN Reverse</td>
<td>TGGTGCCGAGTCTGC</td>
<td>KOY TM6* Whi5-mGFPa; KOY VW100 Hxt1 Whi5-mGFP</td>
</tr>
<tr>
<td>hxt1-TETEX Forward</td>
<td>CGGGAGTTGAATTCCATTTTTAATAA CTAGTAGTTGGGTCTCTATC</td>
<td>KOY VW100 Hxt1; KOY VW100 Hxt1:(ph)GFP</td>
</tr>
<tr>
<td>hxt1-TETEX Reverse</td>
<td>GTTTAGCAGAATAAACCTCGAGTA AGCTTGGTACCG</td>
<td>KOY VW100 Hxt1</td>
</tr>
<tr>
<td>tetex-HXT1 Forward</td>
<td>AAGCTTACTCAGGTTATTTTCTCTGC TAAACAACCTCTTG</td>
<td>KOY VW100 Hxt1</td>
</tr>
<tr>
<td>tetex-HXT1 Reverse</td>
<td>CTACTAGTTATAAAATGAATTCAA CTCGCCGATC</td>
<td>KOY VW100 Hxt1; KOY VW100 Hxt1:(ph)GFP</td>
</tr>
<tr>
<td>phgfp-TETEX Reverse</td>
<td>GATGAGCTCTACAAATAACCTCGAG TAAGCTTGGTACCG</td>
<td>KOY VW100 Hxt1:(ph)GFP</td>
</tr>
<tr>
<td>phgfp-HXT1 Forward</td>
<td>TTCTCGTTTACTCATTTTTCTCTG CTAAACAACCTCTTG</td>
<td>KOY VW100 Hxt1:(ph)GFP</td>
</tr>
<tr>
<td>tetex-PHGFP Forward</td>
<td>AGCTTACTCAGGTTATTTTCTCTG AGCTCATCCATGC</td>
<td>KOY VW100 Hxt1:(ph)GFP</td>
</tr>
<tr>
<td>hxt1-PHGFP Reverse</td>
<td>TTGTTTACAGGAAAATAGGT AAACGAGAAGAACCCTTAC</td>
<td>KOY VW100 Hxt1:(ph)GFP</td>
</tr>
</tbody>
</table>

OD$_{600\text{nm}}$ was measured by spectrophotometry and absolute cell counts were determined by flow cytometry (BD Accuri C6; BD Biosciences, Franklin Lakes, New Jersey). Levels of
glucose and extracellular metabolites were determined by sampling the supernatant of the cultivation medium every 60 or 120 min for the whole duration of the exponential growth phase. In specific, 0.2 ml of culture were collected in 1.5-ml eppendorf tubes (Eppendorf, Hamburg, Germany) and samples were centrifuged for 5 min at 13200 rpm. The cell-free supernatant was transferred to filter columns of 0.22 pore size (SpinX; Corning Inc., Corning, New York), was spinned for 15 seconds, and the flow-through was transferred to an HPLC vial. HPLC (Agilent 1290 LC System; Agilent Technologies, Santa Clara, California) analysis for glucose, maltose, pyruvate, glycerol, acetate, and ethanol concentrations was performed using the Hi-Plex H column with 5 mM H₂SO₄ as eluent at a constant flow rate of 0.6 ml/min and a constant column temperature of 60°C. 10 ul of each sample and appropriate standards were injected for analysis. Substrate concentrations were detected with refractive index and UV (210 nm) detection and chromatogram integration was done with Agilent Open Lab CDS software. Data were obtained from at least 3 biological replicates for each stain at each tested condition.

Dry cell weight was determined by filtering at least 20 ml of culture at an OD₆₀₀nm of 7.5 or higher through pre-weighed nitrocellulose filters of 0.2 um pore size (Whatman; GE Healthcare Life Sciences, Chicago, Illinois). After filtration, filters were washed with distilled water and were subjected to dry-incubation at 80°C. Following the incubation, the filters were weighed again to obtain the dry weight of the filtered population. The absolute cell count of the culture right before filtering was used to calculate the dry weight per cell of the examined strain at the respective nutrient condition.

Oxygen and carbon dioxide transfer rates were determined at a shaking bioreactor using an online gas exhaust monitoring system termed RAMOS (Kühner AG, Basel, Switzerland) (Anderlei and Büchs, 2001; Anderlei et al., 2004). Cultivation in the RAMOS device was performed in 250-ml flasks containing 25 ml of minimal medium with 10 gL⁻¹ of the appropriate carbon source. Media were inoculated at a final OD₆₀₀nm of ≈ 0.1 from the same cultures used to inoculate the cultures for the determination of glucose and extracellular metabolites concentrations, and cultivations were run in parallel. Measurements with RAMOS were performed every 30 min. Every measurement cycle was composed of a 10-min measurement phase during which the partial oxygen and
carbon dioxide pressures were monitored, and a 20-min rinsing phase during which measuring flasks were rinsed with air in order to achieve headspace gas concentrations that are similar to those of regular shake flasks.

Regression analysis was performed in gPROMS using an exponential growth model consisting of the following equations:

\[ \frac{d}{dt} X = \mu * X \]

\[ \frac{dc_s}{dt} = -X * q_s \]

\[ q_s = \frac{\mu}{Y_{xs}} \]

For each extracellular metabolite the following equations were used:

\[ \frac{dc_p}{dt} = X * q_p \]

\[ q_p = \frac{Y_{ps}}{Y_{xs}} \]

Where:

- \( X \): \([g_{dcw}/l]\) biomass concentration
- \( c_s \): \([g/l]\) substrate concentration
- \( c_p \): \([g/l]\) product concentration
- \( \mu \): \([h^{-1}]\) biomass growth rate
- \( q_s \): \([g/(g_{dcw} * h)]\) specific glucose uptake rate
- \( q_p \): \([g/(g_{dcw} * h)]\) specific production rate
- \( Y_{xs} \): \([g_{dcw}/g]\) biomass yield
- \( Y_{ps} \): \([g/g]\) product yield
Chapter 2

Characterization of the tetracycline inducible system

To determine the effect of tetracycline (Sigma-Aldrich) on HXT1 expression through the tetracycline-inducible system and the respective effects of HXT1 expression on growth, the KOY VW100 Hxt1:(ph)GFP strain was grown in 50 ml 10 gL\(^{-1}\) maltose minimal medium until an OD\(_{600nm}\) of ≈ 1. The culture was centrifuged for 4 min at 300 rpm at room temperature, growth medium was removed, and cells were resuspended at 1 ml 10 gL\(^{-1}\) glucose minimal medium. 15-ml tubes containing 5ml of 10 gL\(^{-1}\) glucose minimal medium and a range of different anhydrous tetracycline concentrations were inoculated with the resuspended cells and cultures were incubated at 30°C, 300 rpm. HXT1-GFP fluorescence and growth rate were followed by flow cytometry with measurements starting 90 min following the inoculation and taking place each 60 min for the rest 12 hours. Steady state HXT1-GFP expression was achieved 2 hours after the initiation of measurements (3.5 hours after inoculation in the media containing the inducer), and the 3-8 hours period of the measurements was used to estimate the mean HXT1-GFP fluorescence and growth rate per condition. Data from 3 technical replicates were generated at each tetracycline concentration. As a control for leaky HXT1-GFP expression, cultures without KOY VW100 Hxt1:(ph)GFP tetracycline were followed. As a control for cellular autofluorescence, a culture of KOY VW100 Hxt1 without tetracycline was followed. To correct for tetracycline-related fluorescence at the GFP spectrum, KOY VW100 Hxt1 cells were incubated for ≈1 hour in 10 gL\(^{-1}\) minimal medium containing all the range of the tested tetracycline concentrations. An effect of tetracycline on cellular autofluorescence was observed at tetracycline concentrations of 200 ngml\(^{-1}\) and higher, and the level of tetracycline-induced autofluorescence was subtracted from the KOY VW100 Hxt1:(ph)GFP measurements at the respective concentrations.

Microfluidics

For microfluidics experiments, single colonies were used to inoculate 100-ml shake flasks containing 10ml of the cultivation medium and cells were incubated overnight at 30°C at a shaking speed of 300 rpm. Unless indicated otherwise, the cultures were then subjected to at least one round of dilution in fresh medium of the same initial composition and further incubated at 30°C, 300 rpm, until attenuation of exponential growth. Cells from the
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exponentially growing cultures at an OD$_{600nm}$ of 0.1-0.5 were used to load the microfluidics device. During the microfluidics cultivation, cells were continuously supplemented with fresh medium of the same initial composition as during the batch cultivation. When a nutrient-shift was performed, the switch medium was pre-incubated at 30°C.

Specifically for the null-HXT strains (KOYVW100 Hxt1 and KOYVW1-Hxt1:(ph)GFP), single colonies were used to inoculate 10 gL$^{-1}$ maltose minimal medium. After overnight incubation, the cultures were diluted at a final OD$_{600nm}$ of roughly 0.025 in 10 gL$^{-1}$ glucose minimal medium, and were further incubated for 12 hours. Cells were then used to load the microfluidics device and were thereafter continuously supplemented with fresh 10 gL$^{-1}$ glucose minimal medium. After 16-18.5 hours of cultivation in the microfluidics device, 50 ngml$^{-1}$ anhydrous tetracycline was added to the medium to induce the expression of the HXT1 transporter.

For the nutrient upshift experiments using the wild-type strain YSBN6, cells from exponentially growing cultures on 10 gL$^{-1}$ glucose minimal medium were used to load the microfluidics device, and were thereafter supplemented with 0.0010 gL$^{-1}$ glucose minimal medium. After the first 3 hours on 0.01 gL$^{-1}$ glucose in the microfluidics device, cells were supplemented with minimal medium containing 10 gL$^{-1}$ glucose.

For the nutrient upshift experiments using the wild-type strain KOY PK2-1C83, single colonies were used to inoculate 10 gL$^{-1}$ glucose minimal medium and cells were incubated for 64 hours at 30°C, 300 rpm. The cultures were then diluted at an OD$_{600nm}$ of 0.1 in minimal medium without carbon source and cells were immediately loaded at the microfluidics device. During cultivation in the microfluidics device, cells were continuously supplemented with 10 gL$^{-1}$ glucose minimal medium.

For the experiments with the KOY TM6 C6B C strain, the medium for both the batch and the microfluidics cultivation was supplemented with 300 ngml$^{-1}$ anhydrous tetracycline.

*Microscopy*
Chapter 2

Microscopy experiments were performed using a microfluidics dissection platform (Huberts et al., 2013) mounted to an inverted fluorescence microscope (Eclipse Ti-E; Nikon instruments, Amsterdam, The Netherlands). A custom-made microscope incubator (Life Imaging Services GmbH, Basel, Switzerland) was used to retain the temperature constant at 30°C. For the determination of budding activity and cell size measurements, brightfield images were captured every 10 minutes using either a 60x (CFI Plan Apo; NA = 1.4; Nikon, Tokyo, Japan) or a 40x (CSI S Fluor Oil; NA = 1.3; Nikon) objective and a UV blocking filter. For fluorescence imaging, an LED-based system (pE2; CoolLED Limited, Andover, United Kingdom) was used. GFP (excitation at 470 nm using a 470/40 nm bandpass filter and a 495 nm or 491 nm beam-splitter, 100 ms exposure time, 10 % light intensity, 525/50 nm emission filter) was measured every 60 minutes for protein abundance measurements (EM gain 3), every 40 minutes for detection of Whi5-GFP localization and determination of the cell cycle phase (EM gain 300), and every 10 minutes (200 ms exposure time, 10 % light intensity, EM gain 3) for detection of Whi5-GFP localization and determination of START during exit. NAD[P]H autofluorescence (excitation at 365 nm using a 350/50 nm bandpass filter, 200 ms exposure time, 15 % light intensity, 435/40 nm emission, EM gain 0) was measured every 40 minutes in the mutant strains and every 10 minutes in the wild-type strains. ATP-FRET measurements (CFP: 440 nm with 438/24 bandpass filter, 100 ms exposure time, 10 % light intensity, 483/32 nm emission filter, EM gain 3, YFP: 535/30 nm emission filter, EM gain 3) were performed every 40 minutes. All fluorescence measurements were performed using the 60x objective with the exception of NAD[P]H auto-fluorescence for which the 40x objective was used. Brightfield and GFP images were captured using a Luca EM R DL-604 (Andor Technology Ltd, Belfast, United Kingdom) or an iXon Ultra 897 DU-897-U-CD0-#EX (Andor Technology Ltd) camera while for NAD[P]H the latter was exclusively used. FRET measurements were recorded using a dual-camera setup (iXon Ultra 897 DU-897-U-CD0-#EX; Andor Technology Ltd). Readout speed for fluorescence imaging was set to 1 MHz. Axial focus fluctuations during time-lapse imaging were corrected using automated hardware (PFS; Nikon).

Image and data analysis
Cell size and fluorescence intensity measurements were performed in a semi-automated manner using the ImageJ plugin BudJ (Ferrezuelo et al., 2012). Unless explicitly indicated, time-series data from each cell were normalized to the mean value of the signal during the respective time period, to avoid artefacts arising from extreme behaviours of individual cells. Measurements of fluorescence intensity were corrected for background autofluorescence. Whi5-GFP localization was evaluated on the basis of the presence (nuclear) or absence (cytoplasmic) of a single bright cluster within the cell. For comparison of HXT-GFP levels between quiescent and dividing cells in the absence of tetracycline right after the loading of the microfluidics device, the mean fluorescence at the periphery of each cell was measured manually using ImageJ. For time-series HXT-GFP measurements, the mean fluorescence of the cell was measured using BudJ. The FRET-based ATP levels were obtained by calculating the YPF to the CFP ratio of each cell for a specific time point after correction of both signals for background autofluorescence. The de-trending of the ATP signal was performed by fitting a linear regression to the ATP signal of each individual cell and subtracting the regression values from the raw signal at each time point. For the calculation of the cross-correlations between the NAD[P]H accumulation rate $N(t)$ and cell instantaneous growth rate $V(t)$, a LOWESS curve was fitted to the raw NAD[P]H and respective cell size data and the 1st derivative of the smoothed signals was calculated. Then, the cross-correlation between the derivatized signals was computed. To determine the timing of the NAD[P]H and growth bursts in single cells following the addition of fresh nutrients in the wild-type strain, a LOWESS curve was fitted in the NAD[P]H and respective cell size data and the 1st derivative was calculated. The time point where cells attained the highest rate of NAD[P]H increase or cell size growth prior to exit from quiescence was denoted as the point of the NAD[P]H or growth burst respectively. Curve fitting, derivatization, and statistical analyses were performed in GraphPad Prism. The cross-correlations were computed using the ccf function in the R package.

**Determination of stochastic exits**

In the 40-hour microscopic experiments with the single-HXT strain, we occasionally observed cells that did not divide for even a day or more, but would spontaneously start
to divide while the nutrient conditions were retained constant (Figure 2E). To identify whether these cells display a regularly long lag between cell birth and division, or whether these cells are quiescent and exit quiescence stochastically, we determined the time between birth and first cell division for hundreds of daughter cells of the single-HXT strain (Figure 4 – figure supplement 7). While it would typically take 1.17 ± 3.33 (median ± IQR) hours between cell birth and the first cell division, Tukey’s outlier detection (Tukey, 1977) suggested that cells which spent at least 9.17 hours without dividing are in a quiescent state, rather than in a long lag between birth and division.

Acknowledgements

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Competing interests

Authors declare no competing interests.

References


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Supplementary material

Figure 1 – figure supplement 1. No quiescent cells are observed in the wild-strain at the same nutrient conditions. Histogram of time of first division in the microfluidics setup and respective cumulative distribution for wild-type (KOY PK2-1C83, n = 789) cells in 10 gL\(^{-1}\) glucose. Cells were constantly supplemented with fresh minimal medium.

Figure 1 – figure supplement 2. Quiescent cells are arrested in G1. (A) Schematic representation of the cell cycle phase to Whi5 localization relationship (Doncic, Falleur-Fettig and Skotheim, 2011). (B) Whi5-GFP localization in dividing and quiescent cells. Merged phase contrast and fluorescent images. Whi5-GFP localization dynamics were followed for at least 40 hours for 23 quiescent cells.
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Figure 2 – figure supplement 1. Response of KOYVW100 HXT1 cells in the addition of tetracycline. (A) Steady-state expression of HXT1-GFP as a function of tetracycline concentration in minimal medium containing 10 gL⁻¹ glucose. HXT1-GFP levels were determined with flow cytometry. (B) Population growth rate as a function of HXT1-GFP fluorescence in minimal medium containing 10 gL⁻¹ glucose, determined with flow cytometry. In both cases, data are represented as mean ± SD for data were derived from 3 technical replicates.
Figure 2 – figure supplement 2. Physiology of KOYTM6×P on glucose and maltose. (A) Rates of carbon uptake, ethanol production, CO₂ production and O₂ depletion, respective respiratory quotient, and rates of acetate, glycerol and pyruvate production in the single-HXT (KOYTM6×P) strain on glucose and maltose. All parameters were determined in cells growing exponentially during butch cultivation on minimal medium containing 10 gL⁻¹ of the respective carbon source. For comparison purposes, the same parameters for the wild-type (KOY PK2-1C83) strain on 10 gL⁻¹ glucose were determined. Error bars indicate the propagated standard error of the parameter estimation. (B) Respective carbon balance.
The rate of glycolysis dictates cell fate commitment in *Saccharomyces cerevisiae*.

**Figure 2 – figure supplement 3.** Response of single-HXT cells in the switch from glucose to maltose. (A) Time of first division in the microfluidics setup for 956 (KOYTM6*P*) single cells that were used in Figure 2A. Each color corresponds to a biological replicate. Cells were continuously supplemented with fresh minimal medium containing 10 gL\(^{-1}\) glucose for 40 hours, and were subsequently switched to minimal medium containing 10 gL\(^{-1}\) maltose. The dashed black line indicates the point where cells were switched to maltose. (B) Time of first division (n = 18) or death (n = 8) of previously non-dividing cells after the switch from 10 gL\(^{-1}\) glucose to 10 gL\(^{-1}\) maltose (Mann–Whitney U test, P-value: 0.678). (C) Phase contrast images showing morphological phenotypes associated to cell death following the switch from 10 gL\(^{-1}\) glucose to 10 gL\(^{-1}\) maltose. Cell death was identified as the point of cell lysis (Cell 1) or as the point where cells acquired a sudden osmotic-stress-like phenotype (Cell 2) which was typically also followed by cell lysis.
Figure 4 – figure supplement 1. Single cell dynamics during exit. Single cell traces for (A, B) cell size (C, D) and NAD[P]H autofluorescence dynamics. The arrows indicate the time point of first bud emergence for each cell.
The rate of glycolysis dictates cell fate commitment in Saccharomyces cerevisiae.

Figure 4 – figure supplement 2. FRET-based ATP dynamics during exit from quiescence. (A) FRET-based ATP dynamics in single-HXT (KOYTM6*P) quiescent cells (n = 10 to 13) that exit quiescence as a response to the switch from 10 gL⁻¹ glucose to 10 gL⁻¹ maltose. Before the switch, cells were on glucose for at least 40 hours. Dashed black line (t = 0) indicates the point of the nutrient switch and solid blue lines the point of first bud emergence of single cells. (B) Detrended FRET-based ATP dynamics in single-HXT (KOYTM6*P) quiescent cells (n = 9) that exit quiescence stochastically in 10 gL⁻¹ glucose. Cells are aligned for first bud emergence (t = 0, dashed blue line). In both cases data are represented as mean ± SEM.

Figure 4 – figure supplement 3. NAD[P]H autofluorescence dynamics as a response to increased glycolytic flux. (A) Absolute raw NAD[P]H autofluorescence dynamics in wild-type (YSBN6) cells (n = 93 to 95) following a nutrient upshift from 0.01 gL⁻¹ to 10 gL⁻¹ glucose concentration in the minimal medium. For the control experiment (blue line), glucose concentration was retained constantly to 0.01 gL⁻¹ (n = 56). (B) Absolute NAD[P]H autofluorescence dynamics in KOY VW100 Hxt1:(ph)GFP cells (n = 29 to 57) following the addition of 50 ngml⁻¹ tetracycline to the minimal medium while retaining the glucose concentration constant at 10 gL⁻¹. In all cases data are represented as mean ± SEM.
Figure 4 – figure supplement 4. START occurs after the burst in cell growth. (A) Cell size dynamics of single-HXT cells aligned for START (t = 0, dashed blue line), which exit quiescence as a response to maltose addition (KOYTM6*P Whi5-mGFPa, n = 4 to 5), or (B) as a response to tetracycline addition in 10 gL⁻¹ glucose (KOY VW100 Hxt1 Whi5-mGFP, n = 32 to 37), or (C) stochastically in 10 gL⁻¹ glucose (KOYTM6*P Whi5-mGFPa, n = 5), (D) and in normally dividing daughter cells in 10 gL⁻¹ glucose (KOYTM6*P Whi5-mGFPa, n = 13 to 22). Data are represented as mean ± SEM.
Figure 4 – figure supplement 5. Carbon source depletion in wild-type cells during batch cultivation. Extracellular glucose and ethanol concentration during batch cultivation of wild-type (KOY PK2-1C83) cells. 10 ml minimal medium with 10 gL⁻¹ glucose were inoculated at an OD₆₀₀nm of ≈ 0.05. At these conditions, glucose and ethanol are fully depleted after ≈ 28 hours. Cells were subjected to batch cultivation for at 64 hours without replenishment of nutrients before their usage in further experiments.
Figure 4 – figure supplement 6. The glycolytic and growth burst requirements for exit explain the variability during exit of starvation-induced wild-type quiescent cells. (A) Cumulative distribution of quiescent wild-type (KOY PK2-1C83) cells (n = 36) that exit quiescence as a response to addition of fresh medium. Cells were batch cultivated in minimal medium containing 10 gL⁻¹ glucose and were allowed to enter quiescence by naturally depleting their nutrients. 64 hours after the inoculation of the batch culture, cells were transferred to the microfluidics device and were supplemented with fresh medium containing 10 gL⁻¹ glucose (t = 0). The group of Fast cells contains the 50% of cells that exited quiescence first after the addition of the fresh medium. The group of Slow cells contains the remaining fraction. (B) Dynamics of NAD[P]H autofluorescence and (C) cell size of cells in (A). (D) Correlation of time between the NAD[P]H and growth bursts and time of exit from quiescence (bud emergence) in single cells. Apart from the population response where only mean values are shown, data in (B) and (C) are represented as mean ± SEM.
Figure 4 – figure supplement 7. Determination of stochastic exits. Time from birth (detachment from mother cell) till first division (bud emergence) for KOYTM6*P daughter cells (n = 242). Cells that spent at least a period equal to the 3rd quartile plus 1.5 times the interquartile range of the data set (t = 9.17 hours) without dividing, were identified as quiescent. After this time threshold, cells start to divide at random time points while the extracellular conditions remain constant, and therefore, these exits from quiescence were categorized as stochastic. Birth was identified by the characteristic darkening of the bud neck followed by the displacement of the daughter away from the mother cell. Onset of division was identified by the appearance of the bud protrusion. During the determination of the time between birth and first division cells were continuously supplemented with minimal medium containing 10 gL⁻¹ glucose.
Figure 4 – figure supplement 8. Cell size and NAD[P]H autofluorescence dynamics in normally dividing daughter cells. (A) Cell size and respective (B) NAD[P]H autofluorescence dynamics in single-HXT (KOYTM6*P) daughter cells (n = 82 to 104) aligned for birth (t = 0, dashed line). Histogram in (A) and (B) shows the time of first bud emergence for the respective cells. (C) Cell size and (D) NAD[P]H autofluorescence dynamics in single-HXT (KOYTM6*P) daughter cells aligned for first bud emergence (t = 0, dashed line). Cells were categorized in groups according to the time they spent between birth and first bud emergence [n = 23 to 35 (light grey), 5 to 11 (grey), and 8 to 15 (dark grey)]. Data are represented as mean ± SEM.
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Figure 5 – figure supplement 1. Absolute cell size at bud emergence does not correlate with entry to cell division. Absolute cell size at first bud emergence versus time of first bud emergence for normally dividing daughter cells (open circles) and cells which exit quiescence stochastically (solid circles) during constant supplementation of 10 gL⁻¹ glucose minimal medium, and cells which exit quiescence after induction with 10 gL⁻¹ maltose minimal medium (triangles) in the single-HXT (KOYTM6*P) strain. Each color corresponds to a different biological replicate.