Intrinsic, periodic and tunable metabolic dynamics: a scaffold for cellular coherence

Papagiannakis, Alexandros

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Autonomous metabolic oscillations robustly gate the early and the late cell cycle

Alexandros Papagiannakis¹, Bastian Niebel¹, Ernst Wit², Matthias Heinemann¹

¹ Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands; ² Probability and Statistics, Johann Bernoulli Institute of Mathematics and Computer Science, University of Groningen, Nijenborgh 9, 9747 AG Groningen, The Netherlands

Summary

Eukaryotic cell division is known to be controlled by the cyclin/CDK machinery. However, eukaryotes have evolved prior to CDKs, and cells can divide in the absence of major cyclin/CDK components. We hypothesized that an autonomous metabolic oscillator provides dynamic triggers for cell cycle initiation and progression. Using microfluidics, cell cycle reporters, and single-cell metabolite measurements, we found that metabolism of budding yeast is a CDK-independent oscillator that oscillates across different growth conditions, both in synchrony with and also in the absence of the cell cycle. Using environmental perturbations and dynamic single-protein depletion experiments, we found that the metabolic oscillator and the cell cycle form a system of coupled oscillators, with the metabolic oscillator separately gating and maintaining synchrony with the early and late cell cycle. Establishing metabolism as a dynamic component within the cell cycle network opens new avenues for cell cycle research and therapeutic interventions for proliferative disorders.
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Highlights

• Metabolic cycles are an intrinsic, growth-condition independent behavior of single cells.

• The metabolic oscillations are not the result of the cell cycle, and thus are autonomous.

• The metabolic oscillator and the cyclin/CDK machinery form a system of coupled oscillators.

• Both the early and late cell cycle operate in coordination with the metabolic oscillator.

Introduction

Initiation and progression of the cell cycle are considered to occur in response to the timely ordered transcriptional, post-transcriptional, and post-translational regulation of the cell cycle (cyclin/CDK) machinery components (Barik et al., 2010; Coudreuse and Nurse, 2010; Tyson and Novak, 2008). However, there is evidence that a cell cycle regulator external to the cyclin/CDK machinery provides triggers for cell cycle initiation or progression. First, cell cycle entry can occur even in the absence of major cell cycle machinery components (e.g., the early cyclins) (Sherr and Roberts, 2004). Second, late cell cycle proteins (e.g. cdc14 and sic1) (Lu and Cross, 2010; Rahi et al., 2016), and possibly also global transcription (Haase and Reed, 1999; Orlando et al., 2008), continue to oscillate in cell cycle–arrested cells. Third, the eukaryotic cell cycle evolved before CDKs and thus the early eukaryotes must have employed non-CDK cell cycle regulators (Krylov et al., 2003).

Because metabolism oscillates in synchrony with (Futcher, 2006; Klevecz et al., 2004; Müller et al., 2003; Silverman et al., 2010; Tu et al., 2005, 2007; Xu and Tsurugi, 2006) and – as suggested – also without the cell cycle (Novak et al., 1988; Slavov et al., 2011), and because metabolic checkpoints exist in the cell cycle program (Jones et al., 2005; Saqcena et al., 2013; Takubo et al., 2013), we conjectured that metabolism operates as an autonomous, cell cycle–independent oscillator, which together with the cell cycle might form a system of coupled oscillators. In response to nutrients, the metabolic oscillator could orbit with different frequencies and provide periodic triggers for cell cycle initiation and
progression. Interactions between metabolites and cell cycle proteins (Buchakjian and Kornbluth, 2010; Lee and Finkel, 2013; Shi and Tu, 2013; Yalcin et al., 2014) could convey those triggers, and in reverse, the cell cycle could entrain metabolism via the regulation of enzyme activity (Ewald et al., 2016; Lee et al., 2014; Tudzarova et al., 2011; Wang et al., 2014; Zhao et al., 2016).

Here, using novel methods for the dynamic quantification of metabolites in single cells, in combination with microfluidics and time-lapse microscopy, we demonstrate that the metabolism of budding yeast is an oscillator, which orbits autonomously of the cell cycle. Perturbation experiments, including dynamic nutrient shifts as well as conditional and targeted depletion of cell cycle proteins, revealed that the metabolic oscillator together with the cell cycle forms a system of coupled oscillators. The metabolic and the cell cycle oscillators accomplish frequency synchrony - required for the activation and progression of the cell division program - only within a certain window of metabolic frequencies - whereas the robust gating of the cell cycle phases by the metabolic dynamics ensures the temporal separation of biomass production (early cell cycle) and segregation (late cell cycle). Our findings demonstrate that the metabolic oscillator is an indispensable component of cell cycle regulation, open new research avenues into cell cycle control, and suggest the metabolic oscillator as a novel and global therapeutic target against proliferative disorders.

Results

Metabolic cycles are an intrinsic, growth-condition independent behavior of single cells

To test our hypothesis, after which metabolism is an oscillator that is coupled to the cell cycle, we used *Saccharomyces cerevisiae* as a model. First, we asked whether a metabolic oscillator exists. Because it has been conjectured that population-level cell-cycle synchronization and cell-to-cell communication artificially induce metabolic oscillations (Aon et al., 2007; Laxman et al., 2010; Sohn et al., 2000), we investigated metabolic and cell cycle dynamics on the single cell level. We used a microfluidic device for the long-term microscopic observation of single budding yeast cells (Huberts et al., 2013), the auto-
fluorescence of the reduced nicotinamide nucleotide NAD(P)H to assess its intracellular levels (Gustavsson et al., 2012; Lloyd et al., 2002), and a protein-based Förster resonance energy transfer (FRET) sensor to measure ATP (Imamura et al., 2009). Optimization of the sensor expression and imaging settings led to adequate signal intensities with marginal cellular photo-damage and -toxicity during the long-term (>12 hours) imaging (Figure S1A-B).

Using these tools, we first investigated whether periodic NAD(P)H and ATP fluctuations occur in single cells, grown on high (10 gL\(^{-1}\)) glucose without cell-to-cell communication or cell-cycle synchronization (Figure S1C). These fluctuations, subsequently identified (Figure 1A-B, Movie S1) and confirmed by autocorrelation analysis (Figure S2A-B), occurred with an average period of approximately 2 hours, which corresponds to the average doubling time under this condition. Through comparative analyses, we validated that the measured single-cell FRET signals reflect intracellular ATP concentrations (Text S1, Figure S1D-H), and that the measured metabolite dynamics are not due to a correlated variability (such as periodic volume chances) (Text S2). Given the absence of cell cycle synchronization (Figure S1C), our findings demonstrate that, contrary to previous reports (Aon et al., 2007; Laxman et al., 2010; Sohn et al., 2000), metabolic cycles with periods in the hour range are an intrinsic behavior of single cells.

To test whether metabolic cycles also occur at other growth conditions, we subjected yeast to different nutrients and metabolic operations (aerobic fermentation, respiration, gluconeogenesis), which varied the doubling time in single cells from 1.4 to 11 h. Despite these different metabolic operations, we consistently identified oscillations in the NAD(P)H and ATP levels (Figure S3A–F), demonstrating that the metabolic cycles occur regardless of growth conditions.

To investigate whether the metabolic cycles occur in synchrony with the cell cycle, we correlated the frequencies of budding with the frequencies of the NAD(P)H or ATP oscillations. Despite the wide range of doubling times across nutrient conditions, the budding frequencies always matched the frequencies of the corresponding NAD(P)H or ATP oscillations (Figure 1C). Thus, the metabolic oscillations and the cell cycle operate in frequency synchrony over the range of growth conditions tested, which suggests a coupling between the two periodic processes.
Figure 1. Metabolism oscillates in single cells, across nutrients and in frequency synchrony with the cell cycle. (A) Oscillating NAD(P)H levels in a single cell. (B) Oscillating ATP levels (three single cells), dynamically measured using the ATeam 1.03 FRET sensor (Imamura et al., 2009). Cells were grown on high glucose (10 gL⁻¹), in the absence of synchronization (Figure S1C). Black lines indicate smoothing splines used for visualization. Vertical orange lines indicate budding. (C) Budding and the frequency of NAD(P)H and ATP oscillations correlate across growth conditions; n = 38 cells with 137 NAD(P)H oscillations (solid circles) and 38 cells with 136 ATP oscillations (solid squares). Time periods between consecutive buddings, or consecutive peaks of NAD(P)H and ATP oscillations, were used to determine budding and oscillation frequency data, respectively. Linear regressions (black diagonals) illustrate the correlations. The greater noise in the ATP oscillation frequency estimation (Figure S2A-B) is reflected in the different R² values for NAD(P)H and ATP.

The metabolic oscillations are not the result of the cell cycle, and thus are autonomous

To determine if the metabolic oscillations are a mere consequence of the cell cycle operation or if they occur in a cell cycle-independent manner and thus are autonomous, we searched in our single cell data for metabolic oscillations that were unaccompanied by cell cycle progression. Such events occurred for all growth conditions (Figure 2A, Figures S4A-B), with an approximate incidence of 1/50 metabolic oscillations on 10 gL⁻¹ glucose. On 0.01 gL⁻¹ glucose, we also found many cells with consecutive metabolic oscillations without cell cycle progression (Figure 2A, Figure S4C-D, Movie S2).

To determine the cell cycle status of the non-dividing cells, we used a strain with fluorescently tagged Whi5, a transcriptional repressor of early cyclins and target of CDK phosphorylation. Whi5 sequesters into the nucleus at late mitosis (hereafter denoted as ‘M
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exit’), and exits upon phosphorylation at late G1 (denoted as ‘START’), reporting an active CDK (Bloom and Cross, 2007; Costanzo et al., 2004; Ferrezuelo et al., 2012) (Figure S5A–C). Using this reporter, we found that cells with metabolic oscillations but without an accompanying cell cycle were either arrested at G1 (i.e. Whi5 in the nucleus, Figure 2B, Movie S3) or occasionally after budding in a non-G1 phase (i.e. Whi5 in the cytoplasm, Movie S4).

Figure 2. Metabolic oscillations are independent of the cell cycle. (A) Metabolic oscillations without cell cycle initiation occur spontaneously under all growth conditions. Each line corresponds to a single cell. Diagonal trajectories indicate metabolic cycles (MC) in synchrony with budding and the cell cycle (CC); horizontal trajectories show ”uncoupled” MCs without budding. (B) After two coupled MC/CC event, spontaneous G1 arrest occurred at 245 minutes in a single cell (grown on 10 gL\(^{-1}\) glucose), and the metabolic oscillations continued (also see Movie S3). Arrest persisted for 765 minutes, before resumption of MC/CC synchrony. NAD(P)H oscillations are indicated by grey lines, Whi5-eGFP localization by black lines, and the budding events by orange lines. Red triangles indicate peaks of metabolic oscillations without budding. (C) Mating pheromone alpha factor (added at 240 min) caused cell cycle arrest in G1 and the metabolic oscillations continued (data for a single cell grown on 10 gL\(^{-1}\) glucose).

To substantiate the finding that metabolic oscillations are not the consequence of the cell cycle operation, we added the mating pheromone (alpha factor), which induces G1 arrest
(Bardwell, 2004), to cells growing in the microfluidic device. Also after the pheromone-induced cell cycle arrest, the NAD(P)H levels continued to oscillate (Figure 2C, Figure S6). Together, these findings demonstrate that the metabolic oscillations are not the result of the cell cycle operation and CDK activity, but constitute an autonomous behavior of metabolism, occurring across growth conditions. The autonomy of the metabolic oscillator, and its frequency synchrony with the cell cycle (Figure 1C) in normally dividing cells, suggest metabolism as a separate component in the cell cycle control engine.

The metabolic oscillator and the cell cycle form a system of coupled oscillators

We conjectured that the metabolic and the cell cycle oscillator form a system of coupled oscillators, similar to other instances of synchrony in biology, including the rhythmic flashes of fireflies, or the synchronized discharge of the cardiac pacemaker cells (Strogatz, 2001). Analogously to the fact that an effective contraction of the heart muscle requires a strict synchrony between the cells in the sinoatrial node, also here, cell cycle control could emerge from the coupling and mutual entrainment between the metabolic and the cell cycle oscillator.

To investigate whether the metabolic oscillator and the cell cycle indeed form a coupled oscillator system, we searched for signature features of such systems, by means of steady-state and dynamic perturbations. A common characteristic of coupled oscillators is that their natural frequencies (i.e. the frequency of each individual oscillator when uncoupled) converge to a common compromise frequency (i.e. the common frequency of the oscillators when they are coupled) proportional to the strength of their coupling (Strogatz, 2014). We determined the frequency of the metabolic oscillator in the presence of cell cycle (compromise frequency) in normally dividing cells and in the absence of cell cycle progression (natural frequency of the metabolic oscillator), the latter in cells where the cell cycle was arrested with the alpha factor (Figures S6). In line with the theory of coupled oscillators (Strogatz, 2014), we found a linear correlation between the natural metabolic and compromise frequencies under different conditions (Figure 3A). The compromise frequency was routinely 16% lower than its corresponding natural metabolic frequency.
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(Figure 3A) - a reduction that could be interpreted as a load imposed on the metabolic oscillator by the cell cycle upon coupling.

Another distinctive feature of coupled oscillator systems is that coupling and synchrony are only accomplished when the natural frequencies of the individual oscillators are proximal (Nolte, 2015; Strogatz, 2014). With different nutrient conditions resulting in different natural frequencies for the metabolic oscillator (Figure 3A), we investigated at which natural metabolic frequencies coupling with the cell cycle occurs. Here, we found that at very low metabolic frequencies and at very high ones no coupling occurred. Given the standardized reduction of the compromise frequency (16%) (Figure 3A), we identified the range of natural metabolic frequencies from 0.15 h\(^{-1}\) to 0.96 h\(^{-1}\) to enable coupling (Figure 3B). On 0.01 gL\(^{-1}\) glucose, 75% of the metabolic oscillations without cell cycle (Figure S4C-D) had a natural metabolic frequency below the estimated threshold for coupling and thus cell cycle initiation (Figure 3B), resulting in a substantial fraction of non-dividing cells. A statistical analysis (hazard function analysis – Supplemental Experimental Procedures) of the dynamic NAD(P)H signals and START occurrence further demonstrates the importance of the metabolic frequency for coupling and cell cycle initiation (Figure 3C). These findings reveal a second characteristic of coupled oscillators: coupling between the metabolic oscillator and the cell cycle is only achieved within a window of natural metabolic frequencies; lower or higher metabolic frequencies are not sufficient for coupling and thus for cell cycle initiation.
Figure 3 Coupling slows down the metabolic oscillator, and occurs only within a window of natural metabolic frequencies. (A) Median compromise frequencies measured in dividing cells versus median natural metabolic frequencies measured in alpha factor–arrested cells, under different growth conditions. Both frequencies were estimated from consecutive trough intervals in single cell NAD(P)H oscillations. Median frequencies and their 95% confidence intervals (error bars) were estimated using non-parametric bootstrapping. The slope and intercept of the fitted line with standard errors are indicated. (B) The frequencies of metabolic oscillations with or without cell cycle, measured during growth on high (10 gL\(^{-1}\)) and low (0.01 gL\(^{-1}\)) glucose. Natural metabolic frequencies were measured in cells spontaneously skipping ≥1 cell cycles (e.g. Figure 2B, Figures S4A&C). Crosses indicate distribution means. Natural metabolic frequencies necessary for cell cycle initiation (grey shaded area) were estimated by dividing the fastest (95th percentile – 10gL\(^{-1}\) glucose) and slowest compromise frequencies (5th percentile – 0.01gL\(^{-1}\) glucose) by 0.84 (estimated slope in Figure 3A). (C) The probabilities for cell cycle START based on the dynamics of the NAD(P)H signal are significantly different for dividing (white bars) versus non-dividing cells (grey bars) on low glucose. Probabilities were computed using predictive densities based on a Cox proportional hazards analysis (Cox, 1972) and reflect the hazard of a START event with NAD(P)H dynamics as a time-dependent covariate (hazard function analysis – Supplementary Experimental Procedures). The accuracy of prediction is approximately 70% (Receiver Operating Characteristic - ROC analysis).

Another characteristic of coupled oscillators is phase gating, i.e. the maintenance of a relative phase between oscillators in synchrony (Feillet et al., 2014; Mori et al., 1996). Initially focusing on cells grown on high (10 gL\(^{-1}\)) glucose, we found strict phase gating of the cell cycle events at specific metabolic phases: START and budding (i.e. early S phase; Figure S5D-F) consistently occurred at the ascending part of the oscillating NAD(P)H signal. Mitotic exit always occurred at the signal trough (Figure 4A, Figure S7A–F). ATP oscillations were shifted by approximately 180°, with budding occurring at the descending part of the ATP signals (Figure S8A-B).

Next, we examined whether this pattern of phase gating is maintained under other nutrient conditions. We found that at decreased compromise frequencies START and the early S phase shifted to later NAD(P)H phases (Figure 4B-C). In contrast, M exit always occurred at the troughs of the NAD(P)H oscillations, thus exhibiting a strict, condition-independent phase synchrony with metabolism (Figure 4B-C). The condition-independent gating of the late cell cycle (M exit) indicates its strong coupling to the metabolic oscillator. Adversely, the frequency-dependent phase gating of the early cell cycle elements (START/early S) indicates a weaker coupling.
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Figure 4: Cell cycle events are robustly gated to distinct metabolic phases. (A) Whi5-eGFP translocations (cytosolic (cyt) and nuclear (nuc); black lines), together with the NAD(P)H signals in a single cell growing on high glucose. Orange lines denote budding. (B) The distributions of START, budding and M exit on the NAD(P)H oscillations at different growth conditions; error bars 5-95 percentiles. The same data as in Figure S7A–F were used. (C) Median NAD(P)H phase at START, budding, and M exit with median compromise frequencies under different nutrient conditions. (D) Metabolic response to glucose upshift (from 0.01 gL$^{-1}$ to 10 gL$^{-1}$) at 0 min. NAD(P)H signals from 56 cells normalized to the 100 min signal. (E) Timing of START (triangle), budding (circle), and M exit (square), for 56 cells ordered by the START time closest to the nutrient switch. The average cell cycle durations according to glucose concentration (LG, 0.01 gL$^{-1}$; HG, 10 gL$^{-1}$) and when interrupted by the nutrient switch (Cells 1-16) are indicated. (F) START (green lines) is confined to the ascending part of the NAD(P)H oscillation following the metabolic response (cells 25–40); M exit (red lines) is restricted to the trough (cells 1–16). Single cell (grey symbols) and averaged (black line) NAD(P)H signals are shown. The average NAD(P)H signal of the whole population (blue dashed line) was subtracted from subgroup (cells 25–40 or cells 1–16) averages (black line) to reveal the subgroup-specific NAD(P)H dynamics (labeled as Diff.). (G) The single cell timing of START relative to its cell cycle length (START to M exit), demonstrating the significant prolongation (*** two-tailed t-test p-value < 0.001, Shapiro-Wilk normality test, Welch’s correction for unequal variances) of the cell cycle as a result of the M exit confinement to the trough of the metabolic response. Normal distributions were fitted to data for low (red) and high (blue) glucose, and for cells that were interrupted by the nutrient switch (grey).
Next, to test the robustness of the phase gating, we dynamically perturbed metabolism by switching cells from low (0.01 gL\(^{-1}\)) to high (10 gL\(^{-1}\)) glucose and recorded their cell cycle events. Upon the nutrient upshift, all cells responded with a strong, synchronously occurring peak in the NAD(P)H levels (Figure 4D), independently of their stage in the cell cycle (Figure 4E). Thereafter, the NAD(P)H oscillations continued with periods (START to M exit) matching those on high glucose (Figure 4E). Remarkably, those cells that had completed M exit before the nutrient shift (Figure 4E, cells 25–40), consistent with the phase gating under steady-state conditions (Figure 4B), would START only at the ascending part of the NAD(P)H oscillation following the metabolic response (Figure 4F, left panel), regardless of the timing of their prior M exit (Figure 4E, cells 25–40). Cells interrupted by the nutrient switch after START (Figure 4E, cells 1–16) showed significantly longer (two-tailed t-test, p-value <0.001) cell cycle duration, even compared to those cultured on low glucose (Figure 4G, grey symbols). Consistent with the observed steady-state and frequency-independent phase gating of M exit (Figure 4C), cells would only exit mitosis at the trough of their NAD(P)H oscillation following the metabolic response (Figure 4F, right panel), which prolonged their cell cycle (Figure 4G). Our results show that the gating of the cell cycle phases by the metabolic oscillator is also maintained during dynamic metabolic perturbations and thus is robust. As a result, cell cycle events are delayed even during nutrient upshifts, waiting for the ‘right’ metabolic phase to occur after the metabolic perturbation, in order to maintain synchrony with the metabolic oscillator.

Together, the proportionality between the natural metabolic and compromise frequencies, the critical bandwidth of natural metabolic frequencies required for coupling and cell cycle initiation, and the phase gating of the cell cycle phases on the metabolic oscillator indicate that the metabolic and the cell cycle oscillator form a system of coupled oscillators. Further, the autonomous nature of the metabolic oscillator, the robust phase gating of the cell cycle events even during dynamic metabolic perturbations, and the dependency of cell cycle initiation on the metabolic frequency suggest that the oscillating metabolism is an indispensable component in the cell cycle regulation machinery, determining the timing of the cell cycle phases and setting the pace of cell division.
The early and the late cell cycle are separately coupled and in coordination with the metabolic oscillator

On the basis of our data, we derived an interaction topology for the system of coupled oscillators, where the metabolic oscillator is coupled to, and gates the phase the early cell cycle (biomass duplication) and the late cell cycle (biomass segregation) (Figure 5A), operating in addition to the classic CDK-centric connections (Figure 5B). Because we observed the phase gating of M exit to be condition-independent (Figure 4C) and to be maintained even during dynamic perturbations (Figure 4F – right panel), we postulate that a strong connection exists between the autonomous metabolic oscillator and the late cell cycle. Consequently, if indeed strongly connected to the metabolic oscillator, the late cell cycle should also oscillate when the early cell cycle is halted. This notion is supported by the observations of Lu and Cross, who found periodic nucleolar/cytoplasmic localization of the Cdc14 phosphatase (an essential activator of the Anaphase Promoting Complex and mitotic exit) in metaphase-arrested cells (i.e. in cells with fixed cyclin/CDK activity accomplished through stable non-degradable Clb2kd) (Lu and Cross, 2010). These findings demonstrate that the late cell cycle can oscillate even in the absence of the early cell cycle oscillations, supporting its strong coupling to the autonomous metabolic oscillator.

Figure 5 Interaction topology between the metabolic oscillator and the cell cycle elements. (A) The reconstructed topology of cell cycle regulation. The black lines indicate couplings between the metabolic oscillator (‘MET’) and the cell cycle elements (START, early S and M exit), scaled to their phase-difference (Figure 4C). The grey lines indicate the classical connections between the cell cycle
phases. \( \omega_m \) denotes the frequency of the metabolic oscillator. This model extends the classic, CDK-centric view (B) of cell cycle regulation.

Further, according to the inferred interaction topology between the metabolic oscillator and the cell cycle elements (Figure 5A), the metabolic oscillator should also be connected to the early cell cycle. If this is indeed true, the early cell cycle should continue to oscillate when the late cell cycle is halted. To test this, we halted the late cell cycle by dynamic depletion of Cdc14 (Movie S5) using the yeast-adapted auxin-based degron system (Nishimura et al., 2009), an orthogonal system for the conditional and targeted protein degradation. First, following the dynamics of NAD(P)H, we found that the metabolic oscillations persisted after the arrest of the late cell cycle (Figures 6A-B, Movie S6, arrest of the late cell cycle confirmed by the absence of cytokinesis: cf. Figure 6C, Movie S5), also here witnessing the cell-cycle autonomous nature of the metabolic oscillator. Second, analyzing the dynamics of the cell size, we found that during the late cell cycle arrest biomass synthesis continued to occur in waves. Each wave was accompanied by one metabolic oscillation (cf. Figure 6B-C, Movie S6). The clockwise phase correlation between the oscillating NAD(P)H rates and the oscillating cell volume increase rates in Cdc14-depleted cells (Figures 6D & S9) similarly to dividing cells (Figure 6E & S9) confirm the coordination between the metabolic oscillator and early S also in the Cdc14-depleted cells. Remarkably, biomass synthesis waves ceased once the metabolic cycling stopped, or its amplitude was low (Figure 6B-C).

To substantiate the connection between the metabolic oscillator and the early cell cycle, we tested whether DNA replication, an S phase reporter, also persists in the Cdc14-depleted cells. As a dynamic single-cell measure of DNA replication we used the histone H2A, tagged with mRFP1, previously (Rattray and Müller, 2012) and here (Figure 6F-G) shown to correlate with the DNA content. After the auxin-induced Cdc14-depletion and late cell cycle arrest we found that the DNA amount continued to increase (Figure 6F-H). The identified chromosomal bridges (Figure 6I), indicating spindle defects, absence of chromosomal abscission and nuclear division (Amaral et al., 2016), additionally confirm late cell cycle arrest. Each metabolic oscillation, also during late cell cycle arrest, resulted in one additional genome (Figure 6G-H). Together, the sustained metabolic oscillations in
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in the absence of late cell cycle activity, and the maintenance of their synchrony with the cell size (biomass synthesis) and DNA content dynamics evidence the direct coupling of the metabolic oscillator to the early cell cycle.

**Figure 6. The metabolic oscillator is directly coupled to the early cell cycle.** In this experiment, we arrested the late cell cycle (M exit) by dynamic depletion of Cdc14-AID at 310 min. (A) mGFP-AID expressing cells, present in the same microfluidic chip, were used to determine the timing of Cdc14-AID depletion upon the addition of auxin (as in Movie S5). (B) Single cell NAD(P)H levels continued to oscillate after the depletion of Cdc14 (grey line, smoothing spline for visualization). (C) The rate of perimeter increase (right y-axis), estimated from the smoothed cell perimeter (left y-axis), used as a proxy of the biomass synthesis rate. Yellow areas indicate periods with rapid biomass synthesis where budding (vertical orange lines for first two cycles) occurs or would occur (in the cycles after Cdc14 depletion) (see also Movie S6). Three consecutive biomass synthesis waves occur after the last cytokinesis (last bud release before Cdc14 depletion) at 235 min, each in synchrony with the oscillating metabolism (cf. Figure 6B). The oscillating NAD(P)H and perimeter increase rates are coordinated in (D) late cell cycle arrested cells similarly to (E) dividing cells (see also Figure S9). The respective single cell signals are plotted against each other for each time-point (grey markers). Exemplary trajectories from four single cells are presented for each condition (colored lines and markers). (F) The histone Hta2-mRFP1 abundance, a reporter for the DNA content, was measured in...
the same cell (as in Figure 6B-C) prior and after the late cell cycle arrest. After every metabolic oscillation (from trough to trough of the NAD(P)H signal) the DNA content (Hta2-mRFP1 abundance – left y-axis) increased by the same amount, before and also after Cdc14 depletion. The number of genomes were determined by dividing the Hta2 abundance at any given time point by the value at 240 min (just after cytokinesis), when cells have exactly one copy of their genome. Vertical black arrows extending from Figure 6B mark the troughs of each metabolic oscillation. (G) Per metabolic oscillation, the Hta2-mRFP1 abundance increases by an amount corresponding to approximately one genome in dividing cells (before Cdc14-depletion – 26 oscillations from 15 single cells) and in Cdc14 depleted cells (17 oscillations from 11 single cells). Data from the metabolic oscillations, which were interrupted by the Cdc14-AID depletion (at 310 min) were not included in the analysis. Means and the standard deviation are presented. Both distributions passed the Shapiro-Wilk normality test. (H) Microscopy images for certain time points (as indicated by grey lines) in the Hta2-mRFP1 and DIC channels show the increase in the DNA content and cell volume in the same single cell as in Figure 6B-C&F (scale, 10 μm). After Cdc14 depletion, in the absence of cytokinesis, each of the three consecutive metabolic oscillations occurs in synchrony with one biomass production cycles (yellow numbers) and one DNA replication cycle. (I) Chromosomal bridges, found in Cdc14-depleted cells using 100x magnification, confirm the late cell cycle arrest (scale, 10 μm).

Discussion

Through dynamic monitoring of NAD(P)H and ATP levels as well as cell cycle events in single cells, we found that metabolism is a cell cycle–independent, nutrient-responsive oscillator, which together with the cell cycle forms a system of coupled oscillators. By means of steady-state and dynamic perturbations, we revealed that the metabolic oscillator is separately coupled to the early and the late cell cycle, globally orchestrating biomass formation and segregation. Our findings demonstrate the biological significance of the metabolic oscillator. The cells are unable to progress through the cell cycle in the absence of an oscillating metabolism, and the oscillating metabolism dynamically gates the occurrence of the cell cycle events (e.g. START and M exit) in response to nutrient changes.

The idea of endogenous oscillations in respiration or global protein synthesis setting the pace of the cell cycle was already suggested in the 1980s (Klevecz, 1969; Klevecz and Ruddle, 1968; Lloyd et al., 1982; Novak and Mitchison, 1987; Novak et al., 1988), but was abandoned soon after the discovery of the cyclin/CDK machinery (Watts, 2001), likely because the respective analyses of synchronized cultures did not generate broadly convincing data. Here, through exploiting latest advances in single cell technology (Huberts
et al., 2013) and single cell metabolite measurements to circumvent cell cycle synchronization, in combination with targeted protein depletion (Morawska and Ulrich, 2013; Nishimura et al., 2009) to replace the classic temperature-sensitive mutants (Hartwell et al., 1974), we could for the first time demonstrate that metabolism is an autonomous oscillator, which together with the cell cycle forms a system of coupled oscillators. With these tools, not only we avoided potential synchronization (Aon et al., 2007; Laxman et al., 2010; Sohn et al., 2000) or temperature-related (Murray et al., 2001) artifacts, but also used the inherent cell-to-cell variability to unravel the interactions between metabolism and the cell cycle.

Accordingly, cell cycle control is not just the result of the cyclin/CDK machinery, but emerges from the collective synchrony between coupled and mutually entrained oscillators. While it has been suggested that the CDK oscillator locks the phase of ‘peripheral oscillators’, namely the Cdc14-release, the global transcription and centrosome duplication oscillators (Cross et al., 1989; Oikonomou and Cross, 2010), our work establishes the metabolic oscillator as an additional, key component within the cell cycle control network. The metabolic oscillator and the cyclin/CDK machinery independently are not sufficient for cells to divide. Only the ensemble of the metabolic oscillator and cell cycle oscillators (and eventually also the cell cycle transcription program (Banyai et al., 2016; Hillenbrand et al., 2016; Rahi et al., 2016)) exerts the higher order function of cell cycle control. Thus, our discovery does not undermine the importance of the cyclin/CDK machinery, but expands the current view on cell cycle regulation.

The role of the metabolic oscillator in cell cycle control is supported by evolutionary findings. An amino acid sequence-based reconstruction of the maximum-likelihood phylogeny of cell cycle regulating kinases has shown that the CDKs emerged late in the evolution of eukaryotes (Krylov et al., 2003). The metabolic oscillator could constitute the previously conjectured ancestral non-CDK controller driving DNA replication and segregation (Murray, 2004). We envisage that during evolution, the CDK oscillator was grafted onto the metabolic oscillator to finely-tune the coordination between biomass formation and segregation (Pines, 2011; Tyson and Novak, 2008), to reduce noise in the duration of the cell cycle phases (Talia et al., 2007) and to robustly order the cell cycle phases.
Through our work we open a new avenue for the investigation of cell cycle control. Future research will need to unravel the nature of the metabolic oscillator, which we found to oscillate on all nutrient conditions. Such research efforts require the development of novel methods to dynamically assess the activity of metabolic pathways in single cells. Only when the nature of the metabolic oscillator is discovered, it will be possible to unravel the precise molecular functioning of the proposed system of coupled oscillators, incorporating the recently identified connections from the cyclin/CDK machinery towards metabolic enzymes (Ewald et al., 2016; Zhao et al., 2016), or in the opposite direction (Shi and Tu, 2013) and the cyclic expression of metabolic enzymes (Silverman et al., 2010; Wyart et al., 2010).

Once fully unraveled, the metabolic oscillator and its connections to the CDK-machinery could serve as targets for the manipulation of cell fate (dormancy or proliferation) (Vander Heiden et al., 2009; Pearce et al., 2009; Wang and Green, 2012) or therapeutic targets against proliferative disorders (Galluzzi et al., 2013).

**Experimental Procedures**

**Strains**

All strains (Table S1) were constructed on the background of the prototrophic YSBN6 (Kümmler et al., 2010) and its HIS auxotrophic version (YSBN16). The construction of all stains is described in the Supplemental Experimental Procedures. Primer sequences are included (Table S2).

**Cultivation**

Cells were grown in minimal medium (Verduyn et al., 1992) supplemented with the appropriate carbon source (glucose, galactose or pyruvate). Exponentially growing cells were loaded in the microfluidic dissection platform as described (Huberts et al., 2013; Lee
et al., 2012). A detailed description of the pre-culturing and culturing schemes is provided in the Supplemental Experimental Procedures.

**Microscopy**

We used Nikon-Ti inverted microscopes with either an Andor 897 Ultra EX2 EM-CCD camera, or 2x Andor LucaR EM-CCD cameras (dual camera system for FRET measurements), together with the CooLED pE2 excitation system. For NAD(P)H measurements, cells were excited at 365 nm (15 % LED intensity / 200 msec exposure), using a 350/50 nm bandpass filter, a 409 nm beam-splitter and a 435/40 nm emission filter. For GFP measurements, cells were excited at 470 nm (15 % / 200 msec), using a 470/40 nm bandpass filter, a 495 nm beam-splitter and a 525/50 nm emission filter. For RFP measurements, cells were excited at 565 nm (15 % / 200 msec for mRFP1, 50 % / 600 msec for mCherry), using a 560/40 nm bandpass filter, a 585 nm beam-splitter and a 630/75 nm emission filter. For FRET measurements, the donor (CFP) was excited at 440 nm (5 % / 50 msec) using a 438/24 nm bandpass filter. Donor emission was recorded using a 458 nm beam-splitter and a 483/32 nm emission filter. Acceptor (YFP) emission was recorded using a 535/30 emission filter. Only for FRET imaging, 2x2 pixel binning and 3x EM-gain (within the linear amplification range) were applied during image acquisition. In the DIC channel, the light of a halogen lamp was passed through a 420 nm beam-splitter, to exclude UV light and minimize cell damage during long-term acquisition. For the FRET measurements 60x Nikon Plan Apochromat objective was used. For the rest of the measurements a 40x Nikon Super Fluor Apochromat was used. Images were taken every 5 min for fast growth conditions, 10 min during slow growth or 20 min during growth on 0.01 gL⁻¹ glucose. The NIS elements software was used to control the microscope.

**Image Analysis**

The BudJ plug-in (Ferrezuelo et al., 2012) for ImageJ (Schneider et al., 2012) was used to segment, track single cells, and measure the NAD(P)H, Cln2-eGFP, mGFP-AID, Cdc14-mCherry-AID fluorescence. The Whi5-eGFP localization was manually determined for
each single cell. The FRET donor and acceptor signals were manually determined, separately in each fluorescent channel. For the determination of the cellular perimeter and Hta2-mRFP1 abundance before and after Cdc14-AID depletion, cells were manually segmented using the DIC channel (as in Figure 6H) and imageJ (Schneider et al., 2012). A detailed description of image analysis, including background estimation and clustered fluorescence quantification is provided in the Supplemental Experimental Procedures.

**Signal analysis**

All single cell fluorescent signals (NAD(P)H, ATP-FRET, Cln2-eGFP) presented in Figures 2B-C, 4A, S4C, S5D and S6, were de-trended by dividing with a fitted smoothing spline in order to remove low frequency variations. In Figures 1A-B, 2B-C, 4A, S4A-B&E and S6, a spline functions (fitted to the raw, or de-trended NAD(P)H or ATP signals) were added only for the visualization of the metabolic dynamics and were not used in further analysis. In the Cdc14-depletion experiments, spline functions (parameter value 5e-4) were used to de-noise the perimeter and the NAD(P)H oscillations, and estimate the rate of perimeter increase and NAD(P)H in single cells (Figure S9). Smoothing splines were fitted using the MATLAB Curve Fitting Toolbox™.

**Author contributions**

AP designed the study, developed all reporters, performed all experiments, and analyzed all data. BN contributed to the development of the study and performed the autocorrelation function analysis. EW performed the hazard function and the autocorrelation function analysis. MH conceived, designed and supervised the study. AP and MH wrote the manuscript.
Acknowledgements

Financial support is acknowledged from the EU ITN project ISOLATE. The authors thank Jakub Leszek Radzikowski and the Molecular Systems Biology group for helpful discussion, Andrew Millar, Marcel van Vugt, Peter van Haastert, Maarten Linskens, and Bayu Jayawardhana for discussion and critical comments on the manuscript, and Marti Aldea for useful advice and support on image analysis (BudJ toolbox).

References


Autonomous metabolic oscillations gate the cell cycle


as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell 12, 49–61.


Supplemental Information for

Autonomous metabolic oscillations robustly gate the early and the late cell cycle

Alexandros Papagiannakis¹, Bastian Niebel¹, Ernst Wit², Matthias Heinemann¹¶

¹ Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
² Probability and Statistics, Johann Bernoulli Institute of Mathematics and Computer Science, University of Groningen, Nijenborgh 9, 9747 AG Groningen, The Netherlands
Supplemental Experimental Procedures

Chemicals

All regular chemicals were purchased from Sigma-Aldrich, Acros-Organics, Formedium or Merck-Millipore. Anhydrotetracycline hydrochloride, α1-mating factor acetate salt, potassium phthalate monobasic and 3-indoleacetic acid sodium salt (auxin) were purchased from Sigma-Aldrich.

Strains and strain construction

The prototrophic, S288c-derived strain of *Saccharomyces cerevisiae* YSBN6 (Kümmel et al., 2010) and the corresponding HIS⁺ strain (YSBN16) were used. For an overview on strains, refer to Table S1. ATP levels in single cells were measured using the Ateam 1.03 ATP FRET sensor (Imamura et al., 2009), codon-optimized version for yeast (Geneart). Two expression cassettes were constructed and tested for the expression of the ATeam 1.03 FRET sensor: (i) the PFY1p based inverter device (Blount et al., 2012) and (ii) a constitutive expression cassette via the strong TEF1p promoter. For the development of the first construct, the whole tetracycline inducible system (i.e. the tetracycline repressor expression cassette, the yEGFP coding sequence, the iPFY1p-tetO promoter) was amplified from the pINV plasmid (Blount et al., 2012) and incorporated into the HO-poly-KanMX-HO plasmid (Voth et al., 2001) using circular polymerase extension cloning (CPEC) (Quan and Tian, 2009, 2011) DNA assembly method and the primer pairs HO and HO-TetEX (Table S2), resulting in the vector pC6B. Next, we used Gibson DNA assembly (Gibson et al., 2009, 2010) and the primer pairs TetEX and ATP (Table S2) to incorporate the ATP FRET sensor coding sequence into the pC6B vector, replacing the yEGFP coding sequence to yield the plasmid pG5A. The pC6B and pG5A plasmids were amplified in *Escherichia coli*, and checked using restriction digestion and sequencing (primers Seq1-3 – Table S2). After linearization of the vector pG5A using the primer pair Seq4, we transformed YSBN6 cells, using an established protocol (Gietz and Schiestl, 2007), with the resistance to the G418 antibiotic as a selection marker. Correct homologous recombination in the HO region
located in chromosome IV was tested with PCR (primers Seq6Fwd-Seq6Rev and seq5Fwd-seq5Rev, see Table S2).

For the development of the second construct, the pG5A plasmid was linearized using the primer pair TEFATP (Table S2). The linearized plasmid was then self-ligated in a single step phosphorylation-ligation reaction to generate the pTEF:ATP plasmid with the TEF1p constitutive yeast promoter upstream the ATP FRET sensor coding sequence. The pTEF:ATP recombinant plasmids was amplified in *Escherichia coli*, and checked using restriction digestion and sequencing (primers Seq1Fwd, Seq2Rev & Seq4Rev – Table S2). The pTEF:ATP plasmid was then linearized using the Seq4 (Table S2) primer pair and was used to transform YSBN6 cells, using an established protocol (Gietz and Schiestl, 2007), with the resistance to the G418 antibiotic as a selection marker. Correct homologous recombination in the HO region was tested with PCR (primers Seq6Fwd-Seq6Rev and seq5Fwd-seq5Rev, see Table S2). We checked for functional Förster energy transfer between the donor (CFP) and acceptor (YFP) modules in the ATeam 1.03 FRET sensor by measuring the emission spectrum from 465 to 599 nm in cells expressing the ATP sensor and excited at 420 nm.

To generate cell cycle reporter strains, we PCR-amplified the eGFP-tagged Whi5 and Cln2 gene from the yeast GFP collection (Invitrogen) (Huh et al., 2003) including the HIS3MX6 auxotrophic selection marker and sequences upstream and downstream the STOP codon of the respective genes, necessary for homologous recombination (primers Whi5 and Cln2, see Table S2). The PCR products were then transformed into the YSBN16 HIS strain, using an established protocol (Gietz and Schiestl, 2007), for the generation of the YSBN16 Whi5-eGFP and YSBN6 Cln2-eGFP prototrophic yeast strains. Correct integration was checked with PCR (primers Whi5Ver, CLn2Ver, see Table S2).

To dynamically deplete the Cdc14 phosphatase, an essential activator of the Anaphase Promoting Complex (APC) and late mitosis, we used the auxin-based degron system adapted for yeast (Morawska and Ulrich, 2013). First, we implemented the auxin-based degron system in our yeast strains. Therefore, we generated the pG1J plasmid, which contained a gene for mGFP (monomeric GFP) tagged at the C terminus with the AID\textsuperscript{71-114} auxin-induced sequence (Morawska and Ulrich, 2013). Specifically, the backbone of the pC6B plasmid, including the pTEF1 promoter and CYC1 transcriptional terminator, was
amplified (primer pair C6B – Table S2). The degron tag (S3-5GA-AID71-114) was amplified from the #2189 plasmid (Morawska and Ulrich, 2013) (primer pair AID Fwd/5GA Rev – Table S2), and the mGFP coding sequence from the pHIPZ-mGFP-fusionator plasmid (Saraya et al., 2010) (primer pair mGFP – Table S2). The three amplified pieces were assembled using Gibson DNA assembly for the construction of the pG1J plasmid (Table S1), which was validated by restriction digestion and sequencing (primers Seq1Fwd, Seq9Rev, Seq7Rev & Seq8Rev). Next, the *Oryza sativa* TIR1 (Os-TIR1) coding sequence, together with its regulatory elements (ADH1p) were amplified from the BY25598 yeast strain (Table S1) provided by the NBRP, Japan (Nishimura et al., 2009) (primers pADH1Fwd/AtTIR1Rev – Table S2), and inserted into the amplified backbone of the pG1J plasmid (primer pair G1JBkbn), together with the transcriptional terminator ADH1tt, which was amplified from the plInverter plasmid (Blount et al., 2012) (primers ADH1TTFwd / AtTIRADH1TT Rev – Table S2). The three amplicons were assembled using Gibson DNA assembly, yielding the pG2J plasmid. The pG2J plasmid was amplified in *Escherichia coli* and validated by restriction digestion and sequencing (primers Seq1Fwd, Seq1Rev, Seq9Rev, Seq7Rev & Seq8Rev – Table S2). The recombinant pG2J plasmid was linearized (primer pair Seq4 – Table S2) and transformed into YSBN6 wild type yeast cells for integration into the HO locus (Chromosome IV), with the resistance to the G418 antibiotic as a selection marker, thus resulting in the YSBN6.G2J strain, which contained the S3-5GA-AID71-114 tagged version of mGFP. Correct homologous recombination into the HO locus was tested with PCR (primers Seq5Fwd / Seq6Rev – Table S2). In our single cell time-lapse microscopy measurements, we used this an internal control to monitor the protein depletion dynamics as a result of auxin (IAA) addition. Finally, to generate a plasmid encoding only for the OsTIR1 expression cassette for future use with any fluorescently labeled protein, we removed the mGFP expression cassette from the G2J plasmid using a phosphorylation ligation protocol. Specifically, the pG2J plasmid was linearized (primer Prim1Fwd/Prim2Rev – Table S2). Then, the linearized plasmid was self-ligated in a single step phosphorylation-ligation reaction to generate the pOsTIR1w/oGFP plasmid encoding for the OsTIR1 module but no fluorescence reporters. The pOsTIR1w/oGFP recombinant plasmid was amplified in *Escherichia coli*, and checked using restriction digestion and sequencing (primers Seq1Fwd, Seq1Rev, Seq9Rev, Seq7Rev & Seq8Rev – Table S2). The pOsTIR1w/oGFP plasmid was then linearized using the Seq4 primer pair (Table S2) and was used to transform YSBN6 cells, using an established
protocol (Gietz and Schiestl, 2007), with the resistance to the G418 antibiotic as a selection marker, for the construction of the YSNB6.OsTIR1w/oGFP strain. Correct homologous recombination in the HO region was tested with PCR (primers Seq5Fwd, Seq6Rev – Table S2). The YSNB6.OsTIR1w/oGFP strain was then used as a scaffold for the dynamic depletion of fluorescently labeled proteins tagged with the degron sequence (S3-5GA-AID$^{71-114}$). To tag Cdc14 (phosphatase and essential activator of M exit) with the AID* degron sequence into the YSNB6.OsTIR1w/oGFP strain, we constructed the pG23A plasmid. We first, amplified the S3-5GA-AID$^{71-114}$ degron sequence from the #2189 plasmid (Morawska and Ulrich, 2013) (primer pair IAA – Table S2), the mCherry coding sequence from the pBS35 plasmid ((Hailey et al., 2002) – Yeast Resource Center, University of Washington) (primer pair mCherry – Table S2), the natMX expression cassette offering resistance to clonNAT from the pAG36 vector ((Goldstein and McCusker, 1999) – AddGene) (primer pair Nat – Table S2), the flanking sequences upstream and downstream the STOP codon of the Cdc14 ORF from the wild type YSNB6 genome (primer pairs Cdc14CDS & Cdc14DOWN respectively – Table S2) and the backbone of the pG2J plasmid (primer pair Amp – Table S2). The seven amplicons with complementary overhangs were used in a Gibson DNA assembly reaction for the construction of the pG23A plasmid. The pG23A plasmid was amplified in *Escherichia coli*, and checked using restriction digestion. The pG23A plasmid was then used to transform the YSNB6.OsTIR1w/oGFP strain using clonNAT and G418 antibiotics as selection markers, for the construction of YSNB6.OsTIRw/oGFP.G23A. Correct homologous recombination in the Cdc14 region was tested with PCR (primers Cdc14CDSFwd / Cdc14DOWNRev – Table S2). The YSNB6.OsTIRw/oGFP.G23A strain expresses Cdc14 fused with mCherry and tagged with the AID$^{71-114}$ sequence. Using this strain, we could dynamically deplete Cdc14 by the addition of indole-3-acetic acid (IAA) into the culture medium.

In order to combine conditional Cdc14 depletion and late cell cycle arrest experiments with RFP labeled cell cycle reporters, we constructed a strain which expressed the degron-tagged Cdc14 (Cdc14-AID$^{71-114}$) without any fluorescent label. First, we linearized the pG23A plasmid (primer pair Cdc14LinRFPex – see Table S2) excluding the coding sequence of mCherry. The linearized plasmid was then self-ligated in a single step phosphorylation-
ligation reaction to generate the pG23ARFPex plasmid. The pG23ARFPex recombinant plasmid was amplified in *Escherichia coli*, and checked using restriction digestion and sequencing (primers Seq1Rev & Seq101 – Table S2). The pG23ARFPex plasmid was then linearized using the G23Lin (Table S2) primer pair and used to transform YSBN16.Whi5-eGFP (Table S1) cells, applying an established protocol (Gietz and Schiestl, 2007), with the resistance to the clonNAT antibiotic as a selection marker, to generate the YSBN16.Whi5-eGFP.G23ARFPex strain (Table S1). Correct homologous recombination in the Cdc14 region was tested with PCR (primers Cdc14CDSFwd / Cdc14DOWNRev - see Table S2).

Next, the Os-TIR1 module was incorporated to assemble the auxin inducible protein degradation machinery. Specifically, the linearized pOsTIR1w/oGFP plasmid (primer pair Seq4 - Table S2) was transformed in the YSBN16.Whi5-eGFP.G23ARFPex strain (Table S1) (Gietz and Schiestl, 2007) with the resistance to the G418 and ClonNAT antibiotics as selection markers, for the construction of the YSBN16.Whi5-eGFP.OsTIR1w/oGFP.G23ARFPex strain (Table S1). Correct homologous recombination in the HO region was tested with PCR (primers Seq5Fwd, Seq6Rev – Table S2). Complete integration of the constructs was also confirmed by the absence of cytokinesis upon the addition of auxin, as the physiological effect of Cdc14 depletion.

In order to quantify the DNA content during late cell cycle arrest (after Cdc14-AID\textsuperscript{71-114} depletion), we labeled histone H2A with mRFP1 (Hta2-mRFP1). Specifically, the Hta2-mRFP1 cassette and the phleomycin resistance cassette (Ble) were amplified in one piece from the KOY.TM6*P hxx2-GFP hta2-mRFP1 strain (Schmidt, 2014) (Table S1) using the primer pair Hta2Lin. The linearized cassette was transformed in the YSBN16.Whi5-eGFP.OsTIR1w/oGFP.G23ARFPex strain (Table S1), using an established protocol (Gietz and Schiestl, 2007), with the resistance to the G418, ClonNAT and Phleomycin antibiotics as selection markers, for the construction of the YSBN16.Whi5-eGFP.Hta2-mRFP1.OsTIR1w/oGFP.G23ARFPex strain (Table S1). Correct homologous recombination in the Hta2 region was verified with PCR (primer pair Hta2Lin – Table S2) and the fluorescence in the RFP channel clearly corresponding to chromatin structures.
Cultivation

Single yeast colonies growing on YPD 20 gL\(^{-1}\) glucose agar plates were used to inoculate 10 mL minimal 10 gL\(^{-1}\) glucose medium (pH was adjusted with 10 mM K-Phthalate-KOH to pH 5) (Verduyn et al., 1992) in 100 mL shake flasks, and grown (at 30°C, 300 rpm) overnight past the diauxic shift (to OD between 5 and 10). The overnight culture was used to inoculate fresh 10 gL\(^{-1}\) glucose medium to an OD of 0.1, which was grown to an OD between 1 and 1.5 (still exponential growth on 10 gL\(^{-1}\) glucose). For the expression of the ATeam 1.03 sensor via the tetracycline inducible the iPFY1p promoter, 300 ng mL\(^{-1}\) of anhydrous tetracycline were applied (also in the pre-culture).

For microscopy experiments on 10 gL\(^{-1}\) glucose, the exponentially growing cells were again diluted to an OD of 0.1 in fresh 10 gL\(^{-1}\) glucose medium, and then used to load the microfluidic chip as described (Huberts et al., 2013; Lee et al., 2012). For microscopy experiments on pyruvate or galactose, the exponentially growing cells on high glucose (10 gL\(^{-1}\)) were diluted in 20 gL\(^{-1}\) pyruvate or galactose medium to an OD of 0.1. The cells were grown to an OD of 1 to 1.5 (still exponential growth on pyruvate or galactose), diluted to an OD of 0.1 in pyruvate or galactose medium, and loaded onto the microfluidic chip. For microscopy experiments on 0.05, 0.025 or 0.01 gL\(^{-1}\) glucose, the exponentially growing cells on 10 gL\(^{-1}\) glucose were used to inoculate a 0.05, 0.025 or 0.01 gL\(^{-1}\) glucose medium to an OD of 0.05, and grown to an OD of 0.2 to 0.8. This exponentially growing culture was used to inoculate a fresh 0.05, 0.025 or 0.01 gL\(^{-1}\) glucose medium to an OD of 0.05, where the cells were grown for 2 hours and then used to load the microfluidic chip. In the Cdc14 depletion experiments, the Cdc14-AID or Cdc14-AID-mCherry expressing cells were always mixed with cells expressing mGFP-AID (YSBN6.G2J - Table S1) to determine the timing of Cdc14 depletion (Movie S5).

For cell culturing in the microfluidic chip, cells were fed at 4.8 μL min\(^{-1}\) with the same media as in the culture just before loading. The provided media were pre-warmed to 30°C and saturated with atmospheric air by shaking at 300 rpm for at least two hours prior to be used. For the metabolic perturbation experiment (switch from 0.01 gL\(^{-1}\) to 10 gL\(^{-1}\) glucose), the carbon source was switched 12-15 h hours after cell loading on the chip. In alpha factor perturbation experiments, the medium was switched to a medium supplemented with 5 μg mL\(^{-1}\) alpha factor (Futcher, 1999) after 4 h of growth. After a 12 h arrest, we switched back
to the medium without pheromone. In the cell cycle perturbation experiments, we depleted the Cdc14 phosphatase by adding 0.1 mM of indole-3-acetic acid (IAA) to the medium after 4-7 h of normal growth on minimal 10 g L⁻¹ glucose medium.

**Image Analysis**

For segmentation and tracking of single yeast cells, the BudJ plug-in (Ferrezuelo et al., 2012) for ImageJ (Schneider et al., 2012) was used. The plug-in was used to determine the background-corrected average cytoplasmic pixel fluorescence intensity from single cells, for the GFP and NAD(P)H channels. Specifically, cells to be tracked over time were selected by clicking at the center of the desired cell and its boundaries were estimated by BudJ based on the pixel intensity change at the perimeter of the cell, as visualized in the DIC image. The average fluorescence intensity of the pixels contained within the specified cell boundaries was determined for each cell by BudJ for the NAD(P)H channel, and the GFP channel (Cln2-eGFP, mGFP-AID<sub>71-114</sub>). The modal grey value of the whole image area in each fluorescent channel determined for each time-point was subtracted from the fluorescence of each monitored cell. A comparison of the automated background estimation (i.e. the modal grey value) with a manual background acquisition (average intensity of the pixels around the cell covering the pad area) showed no significant differences between the two methods. Cells with large vacuoles (occasionally occurring) were manually excluded from the data set. The Whi5 nuclear or cytoplasmic localization was manually determined for each single cell. The time point of budding was also manually determined as the moment of first bud appearance on the cell surface, which we monitored in the DIC channel. The perimeter of cells (in µm) was manually measured in each single cell and each individual frame, using the white cellular borderline in the DIC channel (as in Figure 6H).

The ATP FRET images were manually analyzed using ImageJ (Schneider et al., 2012). Specifically, the average pixels intensity within the area of a cell was determined, separately for the YFP and CFP channels. The average pixel intensity of the area around the monitored single cell was measured at each time point and subtracted from the cellular fluorescence for each of the fluorescent channels. The FRET signals were determined by dividing the background-corrected YFP by the background-corrected CFP fluorescence.
For the quantification of the Cdc14-mCherry-AID nuclear localization and intracellular abundance, we used the BudJ plug-in (Ferrezuelo et al., 2012) for ImageJ (Schneider et al., 2012) and specifically the Cluster Index function, setting the cluster threshold at 1 standard deviation (brightest pixels by 1 standard deviation at the foci), the minimum fluorescence change at foci at 5%, the minimum foci size at 2 pixels, and the maximum foci size at 10 pixels. The background corrected average fluorescence intensity of Cdc14 at the foci was used as a marker of Cdc14 localization in and out of the nucleus, as well as its dynamic depletion as a result of the indole-3-acetic acid (IAA) addition.

For the quantification of the intracellular Hta2-mRFP1 abundance, cells in the DIC images were manually segmented using ImageJ (Schneider et al., 2012) (as in Figure 6H). The whole cell median fluorescence was used as the cellular background fluorescence, and subtracted from the fluorescence images. The DNA specific fluorescence abundance was then quantified by multiplying the segmented whole cell area in μm2 (number of scaled pixels: 0.4 μm per pixel – 40x objective), by the mean fluorescence intensity.

**Hazard function analysis**

To investigate the importance of the metabolic oscillation frequency for cell cycle initiation, we investigated whether we can predict START in cells solely on the basis of their NAD(P)H dynamics. First, we separated the recorded NAD(P)H oscillations into those with and without cell cycle progression, based on a computational algorithm that segments each NAD(P)H oscillation in six discrete segments. Segment 1 started at the NAD(P)H trough and extended over the first 25% of the ascending part, segment 2 covered 50% of the ascending part, whereas segment 3 covered the final 25% of the ascending part to the peak of the de-trended NAD(P)H signal. The same pattern was used to segment the descending part from the peak to the next trough of the NAD(P)H oscillation. To identify these six segments, we applied the computational algorithm in R on the de-trended NAD(P)H signals. The background-corrected NAD(P)H signals were de-trended by subtracting an adaptive smoothing function, fitted using a window smoother (runmed) in the computing environment R. We used the segmented NAD(P)H signals to automatically identify those metabolic oscillations, where no cell cycle progression takes place. Specifically, in case six
segments past the last cell cycle event (START, budding or M exit) occurred without any progression to the next cell cycle event, then this metabolic cycle was flagged as a metabolic oscillation without cell cycle. The NAD(P)H signals from the beginning of each experiment until the first mitotic exit were omitted.

Using a Cox proportional hazard model, we identified on the basis of the NAD(P)H signals and the associated cell cycle progressions, which frequency-related and time-dependent NAD(P)H characteristics would be most predictive for START. We tested the (i) NAD(P)H levels: amplitude of the de-trended signal, (ii) NAD(P)H slope: segment-specific derivative of the de-trended signal, (iii) NAD(P)H rate: derivative of the de-trended and smoothed NAD(P)H signal, and (iv) the NAD(P)H integral: area under the dynamic de-trended NAD(P)H curve. Specifically, we defined a Cox proportional hazard model (Cox, 1972) for START by means of its hazard function, \( \lambda(t | Z) = \lambda_0(t) e^{\beta Z(t)} \), where \( Z(t) \) is the relevant characteristic of the time-dependent signal and \( \lambda_0 \) is the baseline hazard function. This model described the instantaneous cell cycle transition probability as a combination of an underlying baseline event probability across time and a covariate dependent hazard multiplier. The analysis was performed in R, using the coxph function in the survival package. The correlation and thus predictive power of the estimated metabolic NAD(P)H characteristics on cell cycle progression was statistically evaluated, using the Akaike Information Criterion (AIC), R2 and the likelihood ratio test. Here, we found the NAD(P)H integral to have the highest predictive power for START. Next, we estimated the probability for START as a function of the time-dependent metabolic integral of the NAD(P)H signal from the last M exit, separately in the dividing and the non-dividing cells. The sensitivity of our model predictions was validated using a ROC (receiver operating characteristic) analysis (Pepe, 2007).
## Supplemental Tables

### Table S1: Yeast strains developed and/or used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>(Kümmel et al., 2010)</td>
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Autonomous metabolic oscillations gate the cell cycle

<table>
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<tr>
<th>Prime Sequence Used</th>
<th>YSBN16.YSBN16</th>
<th>YSBN16 ho::ADH1p-OsTIR1-KanMX4 Whi5::eGFP-HIS3MX6 Hta2::mRFP1-Blé Cdc14::mCherry-AID71-114-natMX</th>
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</table>

Table S2: Primer sequences used for the development of all recombinant strains.

The underlined sequences correspond to the overhangs designed for CPEC and/or Gibson assembly. The term “gDNA” stands for purified genomic DNA.

<table>
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### Table 1: Oligo Primers

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### Notes

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Supplemental Text

Text S1: Validation of FRET-based dynamic ATP measurements in single, unsynchronized yeast cells.

So far, the ATeam 1.03 FRET sensor has only been applied for the short-term (approx. 17 min) measurement of the intracellular ATP levels in single yeast cells (Bermejo et al., 2010). Here, we used a yeast codon-optimized version of the ATeam 1.03 FRET sensor to investigate if ATP levels oscillate. Because there could be numerous issues with FRET sensors (for instance sensitivity to redox or pH), we checked whether the FRET signals that we measured indeed reported intracellular ATP levels.

First, we estimated the intracellular ATP concentration range in our cells using the measured FRET ratios and a calibration curve from the literature (Imamura et al., 2009) (cf. Figure S1D). Here, we found that the thereby estimated intracellular concentrations of ATP (between 3.5 mM and 6 mM; Figure S1E) matched those determined in metabolomics experiments (Canelas et al., 2009; Walther et al., 2010). Second, we compared our measured ATP dynamics with the enzymatically determined ATP oscillations observed in synchronized chemostat cultures (Müller, 2006) (Figure S1F). For the comparison, we used the budding indexes as reported in these chemostat experiments (Figure S1G), which we converted into budding rates (Figure S1H). In the chemostat culture the budding rate was highest when the ATP levels dropped (Figure S1F-H). This behavior is consistent with our single-cell ATP measurements where budding occurred at the descending part of the ATP oscillators, after the peak (Figures S1E and S8B). These comparative analyses demonstrate that our single-cell ATP FRET measurements indeed reflect intracellular ATP levels.
Text S2: The metabolic oscillations are not caused by the process of budding.

One could speculate that the observed NAD(P)H and ATP oscillations that we found in frequency synchrony with budding might just be a consequence of volume variations during the cell cycle (e.g. budding and cytokinesis). Here, we demonstrate that the metabolite oscillations are not caused by volume oscillations, but constitute an intrinsic cyclic metabolic behavior: Firstly, the NAD(P)H and ATP oscillations occur also in the absence of budding and cell division, when no periodic changes in cell morphology or volume occur (Figure S4A-C, Movie S2). Secondly, the NAD(P)H and ATP oscillations occur oppositely in phase (Figure S8A-B). If the metabolic oscillations were the result of volume variations and budding, rather than an intrinsic cyclic metabolic behavior, all measured metabolites would have shown oscillations that are in phase.
Supplemental Figures

Figure S1: Establishing and validating the FRET-based, dynamic ATP measurements in single, unsynchronized yeast cells. This supplementary figure demonstrates that with optimized expression and light exposure ATP FRET signals can be obtained from single cells with limited negative effects on cell growth (A), shows the fluorescence spectra of the cells expressing the FRET sensor compared to the cellular background fluorescence (B), demonstrates that in the microfluidic chip cells are not synchronized (C), shows the linear range used for the ATP FRET signal calibration (D), and finally shows the estimated ATP concentration range and the related budding occurrence in our single cell measurements (E) in comparison to previously reported ATP (F) and budding (G, H) time series measured in synchronized yeast populations. Specifically: (A) Optimization of imaging settings to measure NAD(P)H and ATP levels in single cells. The NAD(P)H excitation does not have any
significant effect on the viability of single yeast cells as shown by a comparison of the average number of buddings during the first 1000 min of growth in the microfluidics device, estimated from 28 YSN6 wild type cells subjected to NAD(P)H and brightfield imaging every 5 min (purple box), and 17 YSN6 wild type cells imaged only in the brightfield channel every 5 min (white bar). The error bars represent the 5% to 95% percentiles and the numbers in each box correspond the number of cells used for the respective analysis. Towards optimizing imaging of ATP levels, we developed two expression cassettes for the ATeam 1.03 ATP FRET sensor (Imamura et al., 2009) (G5A.2B and TEF ATP.1B — Table S1, see panel) and integrated them into the yeast genome. The construct with the tetracycline inducible moderate promoter iPFY1 (Blount et al., 2012) (grey box in panel) allowed for tuning the expression levels of the sensor by changing the tetracycline concentration in the medium. In the other genetic construct (red boxes), the sensor’s expression was driven by the strong constitutive TEF1 promoter, thus allowing for a strong expression of the sensor. As a quantitative measure of the phototoxic effect of CFP excitation, we defined a ‘stress factor’, estimated as the product of the percent light intensity of the excitation light source and the exposure time in milliseconds for a fixed image acquisition frequency of 1 image per 5 minutes. We found the ‘stress factor’ to have a significant effect on viability which was defined as the average number of buds produced during 1000 min of image acquisition. However, by expressing the ATP sensor via the strong constitutive promoter (TEF1p), we achieved sufficient signal intensities with marginal effects on growth (number of buddings per 1000 minutes). Thus, through a combination of excitation light intensities and exposure times, and optimized expression of the ATeam 1.03 ATP sensor, we managed to identify imaging conditions (5%, 50msec, pTEF1-ATeam1.03) for the long-term ATP measurements in single yeast cells. **(B)** The emission spectrum of YSN6.ATPTEF.1B cells (Table S1) (red) was measured using a plate reader (TECAN Spark 10M) to confirm the excitation of the acceptor (YFP) by the donor (CFP) module in the ATeam1.03 FRET sensor, and thus its functionality in vivo. **(C)** The random distribution of the budding events between cells, over the time course of 1000 min, demonstrates the absence of cell cycle synchronization in our microfluidic device. Budding occurrence was measured in 20 cells, all grown in the same microfluidic device on high glucose (10 gL⁻¹). Each marker (black line) corresponds to a budding event and each row to a monitored single cell. **(D)** The previously published (Imamura et al., 2009) calibration curve of the ATeam 1.03 ATP sensor, and fitted a linear regression in the YFP/CFP ratio range from 1.2 to 2.2, with which we estimated the ATP levels in our cells. **(E)** The calibration curve was used to estimate the oscillating ATP levels, as shown here in six single cells growing on 10 gL⁻¹ glucose in the microfluidics device. **(F)** Published ATP data from synchronized yeast populations in glucose-limited chemostat cultures determined enzymatically (Müller, 2006). Here, the ATP levels oscillate in synchrony with the **(G)** budding index, namely the percentage of the budding cells. We converted the time in phase, by defining 360 phase degrees between two consecutive peaks of the reported oscillating ATP signal and budding index. **(H)** Using the first derivative of the budding index as a proxy of the budding frequency in the synchronized population, we found the highest budding frequencies (yellow shaded region) to coincide with decreasing part of the ATP oscillations, similar to what we found with the ATP FRET sensor in single cells, cf. (E).
Figure S2: An autocorrelation function analysis confirmed that NAD(P)H and ATP signals oscillate in single cells. We performed the autocorrelation function analysis on (A) NAD(P)H (137 metabolic oscillations from 29 single cells) and (B) ATP (66 metabolic oscillations from 12 cells) signals from cells growing in high (10gL⁻¹) glucose, using the R software and the acf function. Here, we found a period of approximately 125 min for NAD(P)H and 165 min for ATP (time lag required for the correlogram to complete a full oscillation). The longer estimated periodicity for the ATP compared to the NAD(P)H signals is the result of the stress caused by the CFP (ATP-FRET donor) excitation (Figure S1A – stress factor 250). The black vertical lines correspond the sampled autocorrelations for each time lag. The blue dashed lines indicate the 95% significance bounds, outside of which the null hypothesis that there is no auto-correlation is rejected. Consequently, only results above or below the blue dashed lines indicate a significant periodicity, and all results within the blue dashed lines are due to noise. The autocorrelation coefficient (AR1) corresponds to the correlation of the first order auto-regression of our time series, and was estimated by regressing the observation at time t-1 onto the observation at time t. The AR1 coefficient shows the amount of self-similarity of one signal to the next, from which the amount of noise in the signal can be deduced. From the AR1 coefficients, it can be seen that the noise levels of the NAD(P)H signals (AR1=0.914) are lower compared to the ATP signals (AR1 = 0.814). As a result, the estimation of the periodicity in the ATP compared to the NAD(P)H signals is more noisy (blue dashed lines in Figure S2A-B). This can be explained by the fact that the NAD(P)H levels are directly estimated on the basis of the metabolite’s auto-fluorescence, whereas the ATP levels are determined indirectly using a radiometric optical sensor and involves the ratio of two noisy measurements (acceptor/donor fluorescence). The higher noise in the estimation of ATP compared to NAD(P)H oscillation periods is responsible for the lower R² value in the ATP-budding correlation compared to the NAD(P)H budding correlation as shown in Figure 1C.
Figure S3: Metabolic oscillations occur also under respiratory growth on glucose and gluconeogenic growth on pyruvate. Single-cell background corrected NAD(P)H oscillations during (A) respiratory growth on low glucose and (B) gluconeogenic growth on pyruvate. Autocorrelation function analyses demonstrated the existence of metabolic oscillations with an average period of (C) 700 min on low glucose (94 cells) and (D) 270 min on pyruvate (12 cells) (time lag required for the correlogram to complete a full oscillation). Single-cell ATP oscillations measured using the ATP FRET sensor (E) during respiratory growth on low glucose, or (F) gluconeogenic growth on pyruvate.
Figure S4: Metabolic oscillations in the absence of budding occur in all tested growth conditions, but most frequently on 0.01 g L\(^{-1}\) glucose. (A) Oscillatory NAD(P)H signals from three single cells growing on high glucose (10 g L\(^{-1}\) glucose - top), pyruvate (20 g L\(^{-1}\) pyruvate – middle) or low glucose (0.01 g L\(^{-1}\) glucose – bottom), and the related budding events (vertical orange lines), where at certain metabolic oscillations no budding occurred. (B) Oscillatory ATP signals from single cells growing on high glucose (10 g L\(^{-1}\) glucose - top), pyruvate (20 g L\(^{-1}\) pyruvate – middle), or low glucose (0.01 g L\(^{-1}\) glucose – bottom), and the related budding events (vertical orange lines), where at certain metabolic oscillations no budding occurred. (C) A collection of four cells with sustained metabolic oscillations without budding during growth on low glucose (0.01 g L\(^{-1}\)) (see also Movie S2). The NAD(P)H signals were de-trended by dividing by a fitted smoothing spline. (D) Histogram of the frequency distribution of the periods of 63 metabolic oscillations without budding from 14 cells growing on low glucose and exhibiting multiple subsequent oscillations without budding. The period of each oscillation was estimated from the time between two consecutive troughs. This data shows that the autonomous metabolic oscillator can cycle at broadly different periods ranging from 3 to 25 hours with periods of 12 hours occurring most frequently in cells without cell cycle on low glucose.
Figure S5: Illustration and validation of single cell reporters used to resolve the three cell cycle events START, early S and Mitotic exit. (A) Whi5-eGFP transfer to the cytoplasm and transfer to the nucleus is used to indicate START of the cell cycle in the late G1 phase and mitotic exit (Ball et al., 2011; Costanzo et al., 2004; Ferrezuelo et al., 2012), respectively. Budding has been shown to correlate with the early S phase (Ball et al., 2011; Fatima and Kim, 1993). (B) Illustration of how DIC images were used to determine the time-points were budding occurred (white arrows) and thus the early S phase. In the top left corner, a magnified image of a budding cell is shown. (C) Exemplary image of the GFP channel used to capture the Whi5-eGFP localization in the nucleus (red arrows) or the cytoplasm (white arrows). (D) NAD(P)H and Cln2-eGFP oscillations in a single yeast cell. The orange vertical lines indicate budding. (E) Comparison of the distribution of the Cln2-eGFP peaks with the NAD(P)H phase. 50 NAD(P)H oscillations from 4 single cells were de-trended by dividing over a fitted smoothing spline to remove the low frequency noise. The de-trended signals were aligned by converting time into phase and superimposed. The green vertical lines indicate the peaks of the Cln2-eGFP oscillations on the respective NAD(P)H phases. A lognormal distribution (black solid line) describes the density distribution of the Cln2-eGFP peaks. (F) A Tukey box plot indicating
the NAD(P)H phase, at which budding and the Cln2-eGFP peak occurred on high glucose. 31 buddings are included from 5 single cells, and 50 Cln2-eGFP oscillation peaks from 4 single cells. With the levels of the Cln2 reaching a maximum in the early S phase (Ball et al., 2011; Fatima and Kim, 1993), and budding occurring at the same moment as Cln2 peaks, these observations suggest that budding is a good indicator of the early S phase. Because the Cln2-eGFP signals are noisy and peak identification is thus difficult, and recording of Cln2-eGFP signals also requires additional light exposure of the cells, we used the easy-to-identify bud appearance as reporter for the early S phase.
Figure S6: Metabolism continues to oscillate in alpha factor arrested cells, across nutrient conditions. Single cell NAD(P)H signals in dividing (left column) and cell cycle arrested cells (alpha factor, middle column), on 10 gL\(^{-1}\) glucose, 20 gL\(^{-1}\) galactose, 0.05 gL\(^{-1}\) glucose, 0.025 gL\(^{-1}\) glucose and 20 gL\(^{-1}\) pyruvate (top to bottom). Autocorrelation function analyses was performed to confirm periodicity in the cell cycle-arrested cells at each condition (right column). The autocorrelation function analysis was first applied for each single cell, and then the average correlogram was plotted (error bars: standard error of the mean).
Autonomous metabolic oscillations gate the cell cycle
Figure S7: The measured cell cycle events are in synchrony with the NAD(P)H oscillations, and occupy specific NAD(P)H phase regions, partly varying with nutrient condition. (A) Occurrence of the cell cycle phases (START, Early S, M exit) determined through Whi5-eGFP transfer and budding with respect to the NAD(P)H oscillations on high glucose conditions (10 gL⁻¹). 31 de-trended NAD(P)H oscillations from 5 different cells expressing Whi-eGFP were superimposed by converting the time into phase. Lognormal distributions describe the occurrence of Whi5 location change (red, into the nucleus; green, out of the nucleus) and budding events (orange). (B) The same as for cells grown on galactose (20 gL⁻¹) with 31 NAD(P)H oscillations from 8 cells, (C) on 0.05 gL⁻¹ glucose with 46 NAD(P)H oscillations from 16 cells, (D) on 0.025 gL⁻¹ glucose with 44 NAD(P)H oscillations from 4 cells, (E) on pyruvate (20 gL⁻¹) with 27 NAD(P)H oscillations originating from 26 single cells and (F) on low glucose conditions (0.01 gL⁻¹) with 20 NAD(P)H oscillations originating from 13 single cells.
Autonomous metabolic oscillations gate the cell cycle

Figure S8: The NAD(P)H and ATP oscillations are opposite in phase. (A) Averaged and de-trended NAD(P)H signals from 8 cells (each with 4 oscillations) growing on 10 gL$^{-1}$ glucose, aligned with budding (at 0 min). Error bars represent the 95% confidence intervals of the mean. (B) Averaged and de-trended ATP signals from 17 cells (each with 4 oscillations) growing on 10 gL$^{-1}$ glucose, aligned with budding (at 0 min). Error bars represent the 95% confidence intervals of the mean. The grey dashed lines allow for a comparison between the phases of the NAD(P)H and ATP signals. These data showed that the ATP oscillations are opposite in phase to the NAD(P)H oscillations and as such demonstrated that the observed oscillations in the metabolite levels are not the mere result of volume variation and cell division.

Figure S9: The rate of NAD(P)H oscillates in synchrony with the perimeter increase rate, in normally dividing and late cell cycle arrested cells. Dynamic time derivate of NAD(P)H and perimeter from 6 single cells: 3 normally dividing cells (before Cdc14-AID depletion - left) and 3 late cell cycle arrested cells (after Cdc14-AID depletion – right). The NAD(P)H rates correspond to the first derivative of the detrended and smoothed NAD(P)H signals. The perimeter derivatives were estimated on the basis of the smoothed single cell perimeter data. The NAD(P)H rates oscillate in synchrony with the waves of perimeter increase. The corresponding phase plots (clockwise trajectories) demonstrate the condition-independent (before and after Cdc14-AID depletion) phase correlation between the two signals.
Supplemental Movies

Movie S1: NAD(P)H oscillations in synchrony with cell division in a single yeast cell. The NAD(P)H auto-fluorescence (left – NAD(P)H channel) of a single yeast growing on 20g L⁻¹ pyruvate is presented in synchrony with budding (right – DIC channel). The background corrected NAD(P)H signal of the same cell is also plotted at the bottom. Orange vertical lines indicate budding occurrence.

Movie S2: NAD(P)H oscillations measured in non-dividing, quiescent cells on 0.01 gL⁻¹ glucose – the metabolic oscillations are not the result of budding or cell volume fluctuations. Four cells supplemented with 0.01 g L⁻¹ glucose minimal medium. The cells exhibit metabolic oscillations in the absence of periodic volume fluctuations (e.g. budding – vertical orange lines). The same metabolic signals are presented in Figure S4C.

Movie S3: NAD(P)H oscillations in the absence of budding during the G1 phase. Microscopy time-lapse movies of a single cell, expressing the eGFP tagged transcriptional repressor Whi5, in the DIC (left movie) and GFP (right movie) channels. In the GFP channel, the cycles of Whi5 localization in and out of the nucleus indicative of the G1 and non-G1 phases of the cell cycle, respectively, can be seen; below the NAD(P)H signal of the same single cell. Between 500 and 1250 min, and while Whi5 is localized in the nucleus indicating the G1 phase, NAD(P)H oscillations persist in the absence of budding and thus cell cycle (see also Figure S2B).

Movie S4: NAD(P)H oscillations in the absence of budding during the non-G1 phase. Microscopy time-lapse movies of a single cell, expressing the eGFP tagged transcriptional repressor Whi5, in the DIC (left movie) and GFP (right movie) channels. In the GFP channel, the cycles of Whi5 localization in and out of the nucleus indicative of the G1 and non-G1 phases of the cell cycle, respectively, are shown; below the NAD(P)H signal of the same single cell. After 1200 minutes, the cell continues with NAD(P)H oscillations in the absence of budding in the non-G1 (Whi5-eGFP in the cytoplasm) as well as G1 (Whi5-eGFP in the nucleus) cell cycle phases.

Movie S5: Demonstration of dynamic Cdc14 depletion. 2 populations of single yeast cells are grown and monitored in the microfluidic device. The green cells (YSBN6.G2J) constitutively expressed mGFP tagged with the AID<sup>71-114</sup> degron sequence. They were used as a marker of protein depletion due to the addition of 0.1 mM of auxin at 04:00 (hh:mm). The rest of the cells (YSBN6.OsTIR1w/oGFP.G23A – Table S1) expressed Cdc14 fused with mCherry and tagged with the AID<sup>71-114</sup> degron sequence. The localization of Cdc14 in the nucleolus (red signal) is evident. Cdc14-mCherry-AID and mGFP-AID are depleted at the same time (at 05:30 hh:mm) as a result of auxin addition. After the depletion of Cdc14-mCherry-AID, M exit is not activated in the respective strain, meaning that they cannot complete mitosis and cytokinesis, thus exhibiting filamentous growth. The cells that had mGFP tagged with the degron sequence, continued to grow normally.
Movie S6: When M exit is halted (Cdc14 depletion) cycles of biomass synthesis continue in synchrony with the metabolic oscillations. A single cell expressing Cdc14 tagged with mCherry and the degron sequence AID^{71-114}. The auxin (0.1 mM IAA) induced Cdc14 depletion, as monitored in the indicated (white arrow) single cell, is plotted (top left). Despite the Cdc14 depletion and halting of the late cell cycle (M exit), metabolic oscillations persisted (middle left). At the same time the cellular perimeter increased in waves (bottom left), indicating cycles of biomass synthesis even in the absence of M exit. Each wave of biomass synthesis was accompanied by one metabolic oscillation. The yellow shaded regions across all three plots mark the increasing rate of cellular perimeter, the same region where budding occurs in normally dividing cells (Figure 6C). The time stamp (top right corner) corresponds to the time axis of the depicted plots in minutes.

Supplemental References


