Title: Branched chain amino acid synthesis is coupled to TOR activation early in the cell cycle in yeast

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ABSTRACT

How cells coordinate their metabolism with cell division determines the overall rate of cell proliferation. Dynamic patterns of metabolite synthesis during the cell cycle are relatively unexplored. Here, we report the first isotope tracing analysis in synchronous, growing budding yeast cells. We found that leucine synthesis, a branched-chain amino acid (BCAA), increased as cells progressed through the G1 phase of the cell cycle, peaking later during DNA replication. Cells lacking Bat1, the mitochondrial aminotransferase, grew slower, were smaller, and were delayed in the G1 phase, phenocopying cells in which the growth-promoting kinase complex TORC1 was moderately inhibited. Loss of Bat1 lowered the levels of BCAAs and reduced TORC1 activity, reported by phosphorylation of Rps6, a TORC1 output. Lastly, for the first time in any system, we show that in wild-type cells, TORC1 activity was dynamic in the cell cycle, starting low in early G1 but increasing as cells progress in the cell cycle. These results suggest a link between BCAA synthesis from glucose to TORC1 activation in the G1 phase of the cell cycle.

INTRODUCTION

How metabolism is coupled to cell division underpins the control of cell proliferation. Except for early embryonic cell divisions, the rate at which cells can divide usually depends not on how fast they can duplicate their genome but on how fast they can synthesize everything else that makes up a newborn cell. Since the discovery of *cdc* mutants in *S. cerevisiae* decades ago, it has been known that cells continue to grow if cell division is blocked (Johnston *et al*, 1977; Hartwell & Unger, 1977). On the other hand, stopping cell growth blocked cell division. In some instances, the cell cycle machinery does trigger specific metabolic pathways, especially in lipid and carbohydrate mobilization (Ewald *et al*, 2016; Kurat *et al*, 2009). By and large, however, metabolism controls division and not the other way around. Similarly, inhibiting the target of rapamycin (TOR) kinase, the 'master regulator' of multiple anabolic pathways, including protein synthesis, arrests cells in early G1 with a small size (Barbet *et al*, 1996). Nonetheless, it is unknown if and how TOR activity is regulated in the cell cycle of continuously dividing cells.

Early reports of cell cycle-dependent metabolic changes in a variety of organisms were later shown to be artifacts of synchronization (Creanor & Mitchison, 1979; Creanor *et al*, 1983). Popular methods to synchronize cells rely on first blocking them at some point in the cell cycle and, after some time, releasing the block so cells can progress synchronously in the subsequent phases of the cell cycle. However, cells continue to grow during arrest, perturbing the physiological coupling between cell growth and division. In recent years, several studies monitored the steady-state levels of metabolites in the cell cycle in yeast (Ewald *et al*, 2016; Blank *et al*, 2020), green alga

(Jüppner *et al*, 2017), fly (Sanchez-Alvarez *et al*, 2015), and human cells (Atilla-Gokcumen *et al*, 2014; Scaglia *et al*, 2014). For example, using synchronous cells obtained by centrifugal elutriation (Gordon & Elliott, 1977; Pretlow & Pretlow, 1979), we had reported an increase in the levels of lipids late in the cell cycle (Blank *et al*, 2020).

However, metabolite abundances do not reveal material flow per unit time through a metabolic pathway (Jang *et al*, 2018). One can infer that information through the use of isotope tracers and the appropriate computational analyses (Weindl *et al*, 2015). Isotope tracing in the cell cycle to monitor the flow through metabolic pathways has remained unexplored. Only one study reported Kreb's cycle flux in dividing human HeLa cells (Ahn *et al*, 2017), synchronized with a double thymidine block, which arrests cells at the G1/S transition (Mitchison, 1971). When the cells were released from their arrest, glucose oxidation peaked late in the subsequent G1 phase, followed by oxidative and reductive glutamine metabolic fluxes in S phase (Ahn *et al*, 2017). It is unknown whether these metabolic changes extend to other systems and how they are connected to molecular pathways that govern cell growth and division.

Here, we queried for the first time with isotope tracing highly synchronous, unarrested, dividing yeast cells. We identified an increase in the synthesis of the branched-chain amino acid (BCAA) leucine in the G1 phase of the cell cycle, and peaking later in the S phase. We found that cells lacking the mitochondrial BCAA aminotransferase Bat1 were smaller and had a much longer G1 phase. Inhibition of TOR activity was epistatic to the slow growth of *bat1Δ* cells. Furthermore, ribosomal protein S6 phosphorylation, which reports on TOR activity, was very low in *bat1Δ* cells. In newborn wild-type daughter cells, TOR activity was also low in early G1 but

increased as cells progressed in the cell cycle. These results provide the first picture of cell cycle-dependent metabolic fluxes in yeast. They also point to dynamic control of TOR activity in unperturbed dividing cells, potentially linking BCAA synthesis to TOR activation in the cell cycle.

RESULTS

Experimental design of isotope tracing in the cell cycle

We used a prototrophic strain, CEN.PK (see Materials and Methods), cultured in synthetic minimal medium (SMM) to avoid biases that could arise from exogenous nutrient supplementation. Inducing synchrony with arrest-and-release approaches will inevitably perturb cellular physiology and potentially introduce artifacts, undermining a study seeking to identify changes in metabolic fluxes during cell division. Instead, we chose a selection method, centrifugal elutriation, to isolate small, early G1 cells. Using uniformly labeled ^{13}C-glucose we generated a sliding pulse-chase window over the entire cell cycle (see Figure 1A and Materials and Methods).

To gauge cell cycle progression and synchrony, we measured cell size with a channelyzer (Figure 1B) and the percentage of budded cells with microscopy (Figure 1C). Note that in *S. cerevisiae* the presence and size of a bud serve as morphological markers of cell cycle progression. The appearance of a bud on the cell surface correlates with the timing of initiation of DNA replication (Hartwell & Unger, 1977; Pringle, 1981). In all experiments, cells progressed synchronously in the cell cycle, increasing in size at a constant rate (Figure 1B), with buds appearing at ~100 min, while by 160-180 min the vast majority of cells were budded and had doubled in size (to >40fL; Figure 1C). By 180 min, new G1 daughter cells were generated, leading to a drop in the percentage of budded cells and cell size (Figure 1B,C). These data suggest that the metabolically labeled samples we prepared were from highly synchronous cells, querying all cell cycle phases.

Synthesis of leucine from glucose changes in the cell cycle

Analyzing the metabolically labeled cell extract samples by gas chromatography-mass spectrometry (GC-MS) revealed few significant changes in the mass isotopomer distributions (MIDs), in most of the species we detected (Figure 2A and File S2). MID changes reflect patterns in the synthesis and turnover of the relevant metabolites. Based on ANOVA statistical analysis, in cells exposed to uniformly labeled ^{13}C-glucose, leucine and pyruvate were the most significantly affected metabolites we detected during the cell cycle of *S. cerevisiae* cells (Figure 2B). The levels of pyruvate (M1) are the highest early in the cell cycle, lower in subsequent phases, but rising again late in the cell cycle (Figure 2B, top panel). We note that pyruvate (M1) likely arises from breakdown pathways. On the other hand, the levels of the M6 form of leucine (containing six ^{13}C carbon atoms) were the lowest at the earliest time point in early G1. Leucine (M6) levels increased significantly (>4-fold) by 100 min, peaking afterward at ~150 min when more than half the cells in that experiment were budded (Figure 1C), during S phase, and declining late in the cell cycle, at ~200 min (Figure 2A,B). Note that for the synthesis of leucine two molecules of ^{13}C-labeled pyruvate and one molecule of ^{13}C-labeled acetyl-CoA provide the carbon input, from the glycolytic breakdown of the ^{13}C-labeled glucose to the leucine (M6) isotopomer. Hence, these results are consistent with an increase in newly synthesized leucine from glucose as cells progress through the G1 phase of the cell cycle.

We also measured the MID values from the media of the same cultures used to prepare the cell extracts. We found that the levels of the glutamate (M4) and glutamine (M2) forms in the media were significantly changed in the cell cycle. Still, these changes

were due to a single time point of the experiment (e.g., glutamate (M4) levels in the media were the highest at the first time point we measured, compared to all subsequent time points), making interpretations difficult. All the MID measurements and data collected on selected ion monitoring (SIM) mode are in File S2.

Cells lacking Bat1 grow slowly, are small and delayed in the G1 phase

To follow up and validate the functional significance of our isotope tracing experiments regarding leucine synthesis, in the remainder of this manuscript we focused on the cell cycle-dependent pattern in BCAA synthesis. The BCAA aminotransferases Bat1 and Bat2 catalyze the synthesis of all BCAAs (Ile, Leu, Val) from the corresponding alpha-keto acids (Figure 3A) (Kispal *et al*, 1996; Eden *et al*, 1996). Bat1 is mitochondrial, while Bat2 is cytoplasmic (Kispal *et al*, 1996; Eden *et al*, 1996). To interrogate the role of BCAA synthesis in the cell cycle, we deleted *BAT1* or *BAT2* in the same prototrophic strain background (CEN.PK; see Materials and Methods) we used in the isotope tracing experiments. There are conflicting reports in the literature about the growth rate of *bat1Δ* cells. One of the studies that identified *BAT1* reported that *bat1Δ* cells grew and divided faster (Schuldiner *et al*, 1996). However, other studies reported that loss of Bat1 reduced growth rate, especially in minimal media (Kispal *et al*, 1996; Takpho *et al*, 2018; Koonthongkaew *et al*, 2020). We found that cells lacking Bat1, but not Bat2, grew poorly on minimal media (Figure 3B). Exogenous supplementation of BCAAs in all combinations suppressed the growth defect of *bat1Δ* cells, especially when valine was present (Figure 3B). Supplementation with the corresponding alpha-keto acids did not suppress the growth defect of *bat1Δ* cells (Figure EV1).

To link the poor growth of *bat1Δ* cells with specific metabolic alterations in these cells, we first measured the intracellular levels of amino acids via a phenylthiohydantoin (PTH)-based detection method (see Materials and Methods). The intracellular levels of isoleucine and, especially, valine were significantly lower in *bat1Δ* compared to *BAT1*$^+$ cells (Figure EV2). Interestingly, the levels of Gly and Ala were also lower in *bat1Δ* cells (Figure EV2). We next compared metabolite levels between *BAT1*$^+$ and *bat1Δ* cells using MS-based approaches to detect biogenic amines and other metabolites (see Materials and Methods). The levels of all three BCAAs (Leu, Ile, and Val) were again significantly lower ($p<0.05$) in cells lacking Bat1 (Figure EV3, FileS1/Sheet8). Overall, however, from the >300 compounds detected and assigned from the mass spectrograms, the levels of only 15 compounds were significantly different between *BAT1*$^+$ and *bat1Δ* cells (1.5-fold change, $p<0.05$; Figure S3, FileS1/Sheet8). Some dipeptides were enriched in either *BAT1*$^+$ or *bat1Δ* cells ($p<0.0001$ with Holm-Bonferroni correction, based on the MetaboAnalyst platform (Chong *et al*, 2019)), while the 'amino acids' metabolite group was enriched in *BAT1*$^+$ vs. *bat1Δ* cells ($p<0.02$ with Holm-Bonferroni correction, based on the MetaboAnalyst platform). Some alterations in TCA cycle metabolites have been reported for double *bat1,2Δ* cells (Kingsbury *et al*, 2015), but these changes were not evident in the single, prototrophic *bat1Δ* cells we evaluated here. In summary, the above results are consistent with the notion that a drop in intracellular BCAAs, and not widespread global metabolite changes, is at least in part responsible for the slow growth of *bat1Δ* cells.

To see how the slow growth of *bat1Δ* cells manifests during cell division, we examined cell cycle-associated phenotypes, both from synchronous and asynchronous

cultures. In budding yeast, changes in the kinetics of cell cycle progression are often accompanied by cell size changes. As nutrient availability and quality are reduced, the birth and mean size of cells are reduced, the rate at which cells increase in size is lowered, and the duration of the G1 phase increases disproportionately compared to the duration of subsequent cell cycle phases (Johnston *et al*, 1977; Soma *et al*, 2014). Loss of Bat1 reduced significantly both the birth and mean cell size in asynchronous cultures in SMM medium (Figure 4A). We then used centrifugal elutriation to obtain highly synchronous, early G1 daughter cell populations. Wild type and *bat2Δ* cells progressed in the cell cycle with similar kinetics (Figure 4B, compare the left and right panels). They had a critical size (defined as the size at which half the cells are budded) of ~35 fL. On the other hand, the slow growth of *bat1Δ* cells made elutriation very challenging, and only about two-thirds of the elutriated cells in the population eventually budded (Figure 4B). The small elutriated *bat1Δ* cells increased in size very slowly, reaching an apparent critical size similar to that of wild type cells (~33fL; Figure 4B, middle panel). Furthermore, based on DNA content analysis, asynchronous *bat1Δ* cultures had at least twice as many cells in the G1 phase, compared to *BAT1*$^{+}$ cells (Figure 4C). Taken together, all the evidence argues that *bat1Δ* cells have a pronounced G1 delay, resembling nutrient-limited cells.

Bat1 is functionally linked to TOR activation in early G1

It has been previously reported that TOR signaling is significantly reduced in loss-of-function *bat* mutants in yeast (Kingsbury *et al*, 2015). Hence, the growth defect of *bat1Δ* cells could at least in part reflect TOR inhibition. Since rapamycin inhibits the TOR

complex 1 (TORC1), which drives cellular anabolic processes (Loewith & Hall, 2011), we queried the growth of *bat1Δ* cells in the presence of sub-lethal levels of rapamycin (Figure 5A). Remarkably, the growth of *BAT1*$^{+}$ and *bat1Δ* cells was equalized by rapamycin added at 30 ng/mL (Figure 5A). These results strongly suggest that the inhibition of TORC1 by rapamycin lies downstream of Bat1, and it is epistatic to the growth defects of *bat1Δ* cells. To further test this notion, we looked at a molecular output of TORC1 signaling. Phosphorylation of ribosomal protein S6 (Rps6) reports on TORC1 activation, and it can be detected in yeast using an antibody against the human phosphorylated S6 protein (Wallace *et al*, 2022; González *et al*, 2015). While phosphorylated Rps6 was readily detected in wild-type cells in minimal medium (Figure 5B), it was barely so in *bat1Δ* cells (Figure 5B, top blot after prolonged exposure). We confirmed that the signal attributed to Rps6 phosphorylation is sensitive to TORC1 inhibition by rapamycin (Figure 5C, left) and phosphatase treatment (Figure 5C, right).

Since we detected an increase in leucine synthesis as cells progress in the cell cycle in wild-type cells (Figure 2), demonstrated a severe G1 delay (Figure 4) and reduced TORC1 activity (Figure 5B) in *bat1Δ* cells, we then examined TORC1 activity in the cell cycle in wild-type cells. We note that despite the well-known G1 arrest of cells upon TORC1 inhibition (Barbet *et al*, 1996), to our knowledge, there is no report of cell cycle-dependent changes in TORC1 activity in dividing, unperturbed, growing cells in any system. We had previously shown that the total abundance of the Rps6 paralogs, Rps6A or Rps6B, or any other ribosomal protein, does not change at all in the cell cycle (Blank *et al*, 2020). Here, we found that Rps6 phosphorylation, which reports on TORC1 activity, rises steadily in synchronous cultures of wild-type prototrophic cells in minimal

medium, increasing ~3-fold by the time cells have exited the G1 phase when they are large (>35 fL) and budded (>60%) (Figure 5D,E). These results provide evidence of cell cycle-dependent changes in TOR signaling for the first time in any system. Together with the other pieces of evidence we presented, they point to a straightforward sequence of events, from glycolytic flux and generation of pyruvate, to BCAA synthesis by the Bat1 aminotransferase, and then TORC1 activation in G1 (Figure 5F).

DISCUSSION

The experiments we described provide the first picture of changing BCAA synthesis in synchronous, proliferating yeast cells. As we discuss below, an important extension of this work is the evidence we presented linking BCAA synthesis to TOR activation in the G1 phase.

It has been known for decades that inhibition of TOR by rapamycin arrests budding yeast (Heitman *et al*, 1991) and mammalian (Albers *et al*, 1993) cells in the G1 phase of the cell cycle. Especially in yeast cells, which get bigger as they progress in the cell cycle, the small size of rapamycin-arrested G1 cells was an essential piece of evidence for TOR to be a ‘master’ regulator of cell growth and anabolism (Barbet *et al*, 1996; Hall, 2017). But just because inhibition of TOR by genetic or chemical means leads to G1 arrest is not enough to conclude that in dividing, unperturbed cells, there is dynamic control of TOR activity in the cell cycle. On the contrary, one could argue that since bulk cell growth and protein synthesis appear to have a constant rate throughout the cell cycle in constant nutrients (Elliott & McLaughlin, 1978), perhaps TOR activity is also constant in these conditions. Yet, our isotope tracing results discussed above argue that despite the apparent constancy of overall cell growth, specific pathways of central metabolism, such as leucine biosynthesis, may show cell cycle-dependent changes (Figure 2).

The BCAA aminotransferases Bat1,2 were reported to be positive regulators of TORC1 in yeast (Kingsbury *et al*, 2015). In that study, after leucine starvation of a leucine auxotrophic strain, the addition of either Leu or α-keto Leu stimulated TORC1 in cells lacking both Bat1 and Bat2. Interestingly, in the same report, only double *bat1,2Δ*

mutants, but not the single mutants (all in the S288c background), were reported to have reduced TORC1 activity (Kingsbury *et al*, 2015). Instead, we found that TORC1 activity was greatly reduced in single *bat1Δ* mutants (Figure 5B). All the strains we used were prototrophs in the CEN.PK background, which has altered sequences in >1400 proteins compared to the S288c background (Nijkamp *et al*, 2012), likely accounting for these differences.

Overall, a consistent picture emerges, regarding the role of BCAA aminotransferases as regulators of TORC1 activity and cell cycle progression in budding yeast, based on the following: First, leucine synthesis from glucose increases as cells progress through the G1 phase (Figure 2). Second, loss of Bat1 reduces cell size and significantly delays cell cycle progression in the G1 phase, phenocopying the cell cycle effects upon TORC1 inhibition (Figure 4). Third, TORC1 inhibition is epistatic to the loss of Bat1 (Figure 5A). Fourth, TORC1 activity does not only change in response to exogenous nutrient changes but is also dynamic in the cell cycle, beginning to rise in G1 (Figure 5). The simplest model to account for these observations, consistent with the existing literature, is the model we outlined in Figure 5F. At least in budding yeast, the model links glycolysis and generation of precursors (e.g., pyruvate) to BCAA synthesis, increased TOR signaling, and cell cycle progression. We note that it is unknown how the activity of Bat1 may be regulated. In the cell cycle, the steady-state levels of the *BAT1* transcript (Spellman *et al*, 1998; Blank *et al*, 2020, 2017), and Bat1 protein (Blank *et al*, 2020), do not change. On the other hand, Bat1 is heavily modified post-translationally at multiple residues by phosphorylation (Lanz *et al*, 2021; Albuquerque *et al*, 2008; Zhou *et al*, 2021), acetylation (Henriksen *et al*, 2012), and

succinylation (Frankovsky *et al*, 2021; Weinert *et al*, 2013), which could affect its enzymatic activity.

Lastly, we note that the mammalian ortholog BCAT1 is activated and necessary for chronic myeloid leukemia (CML) development in humans and mice, exercising its oncogenic function through BCAA production in CML cells (Hattori *et al*, 2017). Interestingly, the therapeutic potential of rapamycin in CML was the objective of a recently completed clinical trial (NCT00780104), but the results are not available yet. Future work, perhaps in CML cell lines, incorporating synchronous cultures and isotope tracing, may test if our yeast results extend to mammals, including humans.

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AUTHOR CONTRIBUTIONS

M.P. and K.H. conceived and designed the study. C.R. and K.S-H. performed and analyzed the isotope tracing experiments. H.B. and M.P. performed all other experiments. All authors evaluated and discussed the data. M.P., H.B., and C.R. wrote the first draft of the manuscript, which was then edited by all authors.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare that could be perceived to influence the presentation or interpretation of the data.

MATERIALS AND METHODS

A Reagent Table is in the Supplementary Files. Where known, the Research Resource Identifiers (RRIDs) are shown in the Reagent Table.

Strains and media

All the strains used in this study are shown in the Reagent Table. Unless indicated otherwise, the cells were cultivated in the standard synthetic minimal media (SMM), containing 0.17% $^{w}/_{v}$ yeast nitrogen base without amino acids and ammonium sulfate, 0.5% $^{w}/_{v}$ ammonium sulfate, 2% $^{w}/_{v}$ glucose at 30°C (Kaiser *et al*, 1994). To generate the *bat1Δ* haploid strain, we used the single-step gene replacement method of Longtine (Longtine *et al*, 1998). Briefly, a PCR product was generated using plasmid pFA6a-kanMX6 as a template (Longtine *et al*, 1998), with primers BAT1-F1 and BAT1-R1, and used to transform strain CEN.PK. Positive colonies resistant to G418 were identified and genotyped by PCR with primers VIII:517387-FWD and VIII:518942-REV, to confirm that *BAT1* was absent and replaced by the kanMX marker. Similarly, using primers BAT2-F1 and BAT2-R1, we generated the *bat2Δ* strain and genotyped it with primers X:705443-FWD and X:706939-REV. During strain construction, the cells were grown in agar plates with the standard rich undefined medium YPD (1% $^{w}/_{v}$ yeast extract, 2% $^{w}/_{v}$ peptone, 2% $^{w}/_{v}$ dextrose) containing G418 (Geneticin) at 100 µg/mL.

Centrifugal elutriation, cell size and DNA content measurements

General methods have been described previously (Hoose *et al*, 2012; Soma *et al*, 2014). For the isotope tracing experiments, the elutriated early G1 cells were split into

eight samples and allowed to proceed in the cell cycle in a medium with ^{12}C-glucose for varying times, as indicated in Figure 1A. Then, to label newly synthesized metabolites, we switched the medium to one with uniformly labeled ^{13}C-glucose, and the cells were cultured for a 20 min 'pulse' period. The culture was then switched back to a ^{12}C-glucose medium for a 20 min 'chase' period, and the cells were harvested for metabolite extraction (He *et al*, 2014). The experiment was repeated four more times from independent elutriation experiments, generating separate pulse-chase time courses. Cell size and budding measurements from these experiments are in File S1/Sheet1.

Metabolite extraction for isotope tracing

The lyophilized cell extracts were first quenched by adding to the sample microcentrifuge tubes 0.4 ml methanol (kept at -20 °C) and 400 µl double-distilled water (ddH_2O) with ^{13}C-glutaric acid (1µg/ml, 4 °C) used as internal standard. Then, 400 µl chloroform (at -20 °C) was added, and the samples were mixed on a rotating shaker (at 1400 rpm) for 20 min at 4 °C. The samples were centrifuged for 5 min at 16,000 *g* at 4 °C, and 300 µl of the supernatant were transferred to GC glass vials (Klaus Trott Chromatographiezubehör, Germany) and dried under vacuum at 4 °C overnight (Labconco, United States). The vials were then capped with magnetic caps (Klaus Ziemer GmbH, Germany) and stored at -80 °C until measurements.

Before derivatizing the supernatant samples, 50 µl of a ^{13}C glutaric acid solution (10 µg/ml) in ddH_2O was added as an internal standard to each sample and dried under vacuum as described above.

Derivatization and GC-MS measurements

The sample derivatization was performed by adding 30 µl 2% methoxyamine hydrochloride (MeOX) in pyridine (Roth, Germany) for 90 min at 55 °C, followed by the addition of 30 µl N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) and incubated for 60 min at 55 °C. The tert-butyldimethylsilyl (TBDMS) derivatized samples were analyzed with a gas chromatograph connected to a mass spectrometer (GC-MS) (Agilent 7890A and 5975C inert XL Mass Selective Detector). For each measurement, 1 µl of the sample was injected in splitless mode into an SSL injector at 270 °C. The GC was equipped with a 30 m DB-35MS + 5 m Duraguard capillary column (0.25 mm inner diameter, 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min, and the GC-oven was run at the following temperatures and times per sample: 6 min 80 °C; 6 min 80 to 300 °C; 10 min 300 °C; 2.5 min 300 to 325 °C; 4 min 325 °C. Each GC-MS run lasted 60 minutes. The transmission temperature from GC to MS was 280 °C, and the MS and the quadrupole temperatures were 230 °C and 150 °C, respectively. The ionization in the mass detector was performed at 70 eV. The detector was operated in SCAN-mode for full scan spectra from 70 to 800 m/z with 2 scans/s. We used a C10-C40 alkane standard mixture (Sigma-Aldrich) to calibrate retention indices.

Isotope tracing data analysis

Data analysis was performed with the Metabolite Detector (Hiller *et al*, 2009), RStudio, and LibreOffice software packages. After calibration with the alkane mixture, a

normalization by the internal standard ^{13}C-Ribitol was performed to eliminate variations in sample volumes. Batch quantifications were performed with Metabolite Detector. Non-targeted analysis was performed with an in-house library and the following settings: Peak threshold 5; minimum peak height 5; 10 bins per scan; deconvolution with 5; no baseline adjustment; compound reproducibility 1.00; maximum peak discrimination index 100.00; min # ions 20.

All the data from these experiments are in File S2. The MID values from the cell extracts, and the associated p-values from ANOVA analysis to detect significant differences, are in File S2/Sheet1, and File S2/Sheet2, respectively. Similarly presented are the data for the MID values from culture supernatants (File S2/Sheet3 and File S2/Sheet4), and the SIM data from cell extracts (File S2/Sheet5 and File S2/Sheet6) and culture supernatants (File S2/Sheet7 and File S2/Sheet8). To normalize and display the data in the cell cycle as shown in Figure 2, we processed the values as described previously (Spellman *et al*, 1998), yielding “expressed ratios”. Briefly, the value of a species at any one time point in the cell cycle was divided by the average of all values of that species at all time points in the series. These expressed ratios were then Log2-transformed and used to generate the graphs in Figure 2.

Statistical analysis, sample-size and replicates

For sample-size estimation, no explicit power analysis was used. All the replicates in every experiment shown were biological ones, from independent cultures. A minimum of three biological replicates was analyzed in each case, as indicated in each corresponding figure's legend. The robust bootstrap ANOVA was used to compare

different populations via the t1waybt function, and the posthoc tests via the mcppb20 function, of the WRS2 R language package (Wilcox, 2011; Mair & Wilcox, 2020). The non-parametric Kruskal-Wallis and posthoc Nemenyi tests were done with the posthoc.kruskal.nemenyi.test function of the PMCMR R language package. No data or outliers were excluded from any analysis.

Immunoblot analysis

For protein surveillance, protein extracts were made as described (Wallace *et al*, 2022), and resolved on 12% Tris-Glycine SDS-PAGE gels, unless indicated otherwise. Loading was measured with an anti-Pgk1p primary antibody (at 1:5,000; ThermoFisher; Cat#: 459250), followed by a secondary antibody (at 1:5,000; ThermoFisher; Cat#: 31431). Ribosomal protein S6 phosphorylation was detected by a specific rabbit monoclonal antibody against Ser235/236 of the human protein (at 1:2,500; Cell Signaling, Cat#: 4858), followed by a secondary antibody (at 1:5,000; ThermoFisher; Cat#: 31466). Imaging and quantification was done as described previously (Blank *et al*, 2020, 2017; Maitra *et al*, 2020, 2022). Signal intensities in the cell cycle (see Figure 5E) are shown as Log2-transformed expressed ratios, as described above (Spellman *et al*, 1998).

Amino acid analysis

The HPLC-based measurements of amino acid levels was done at the Protein Chemistry Laboratory of the Department of Biochemistry and Biophysics, as described previously (Maitra *et al*, 2020; He *et al*, 2014).

Steady-state metabolite profiling

The untargeted, primary metabolite and biogenic amine analyses were done at the NIH-funded West Coast Metabolomics Center at the University of California at Davis, according to their established mass spectrometry protocols, from 1-2E+07 cells, as described previously (Blank *et al*, 2020; Maitra *et al*, 2020, 2022). The raw data for the primary metabolite measurements are in File S3. The raw data for the primary amine measurements are in File S4. To identify significant differences in the comparisons among the different strains, we used the robust bootstrap ANOVA, as described above. The input values we used were first scaled-normalized for input intensities per sample. Detected species that could not be assigned to any compound were excluded from the analysis.

Data availability

Strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. The Expanded View, Supplementary Material and Source data includes the following:

Figure EV1: Alpha-keto acid supplementation does not suppress the growth defect of *bat1Δ* mutants.

Figure EV2: Amino acid composition of *BAT1*$^{+}$ and *bat1Δ* strains from PTH-based analysis.

Figure EV3: Volcano plot of steady state abundances of primary metabolites and biogenic amines.

Reagent Table

File S1: Source data for the graphs in Figures 1,2,4,5,S2.

File S2: Metabolite measurements from the GC-MS measurements during the cell cycle, from the isotope tracing experiments.

File S3: Steady-state primary metabolite measurements in *BAT1*$^{+}$ vs. *bat1Δ* cells.

File S4: Steady-state biogenic amine measurements in *BAT1*$^{+}$ vs. *bat1Δ* cells.

File S5: Source immunoblots for Figures 5B,C.

File S6: Source immunoblots for Figures 5D,E.

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FIGURES

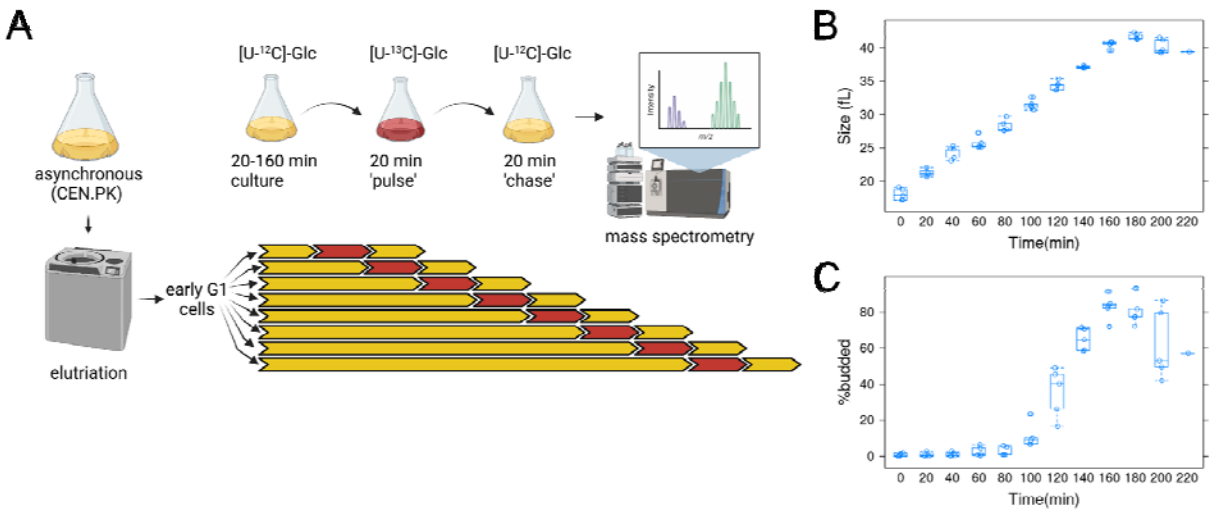


FIGURE 1. Overview of the approach to obtain samples for metabolic flux analysis in highly synchronous, dividing, budding yeast cells. A, For each experiment, early G1 daughter cells of a prototrophic strain (CEN.PK; see Materials and Methods) were obtained by centrifugal elutriation. The elutriated culture was split into eight aliquots and cultured for a varying amount of time, from 20 to 160 min, in minimal, [U-^{12}C]-glucose medium. They were then transferred to a medium with [U-^{13}C]-glucose for 20 min (pulse) and then incubated for another 20 min in [U-^{12}C]-glucose medium (chase). Metabolite extracts from these cells were analyzed by mass spectrometry. Five such independent experiments were performed. The figure was generated with Biorender.com. B, Boxplots showing the cell size (y-axis) over time (x-axis) of all the samples as they progressed in the cell cycle. C, Boxplots showing the percentage of budded cells (y-axis) from the same samples shown in B. The boxplot graphs were generated with R language functions. The values used to draw the plots are in File S1/Sheet1.

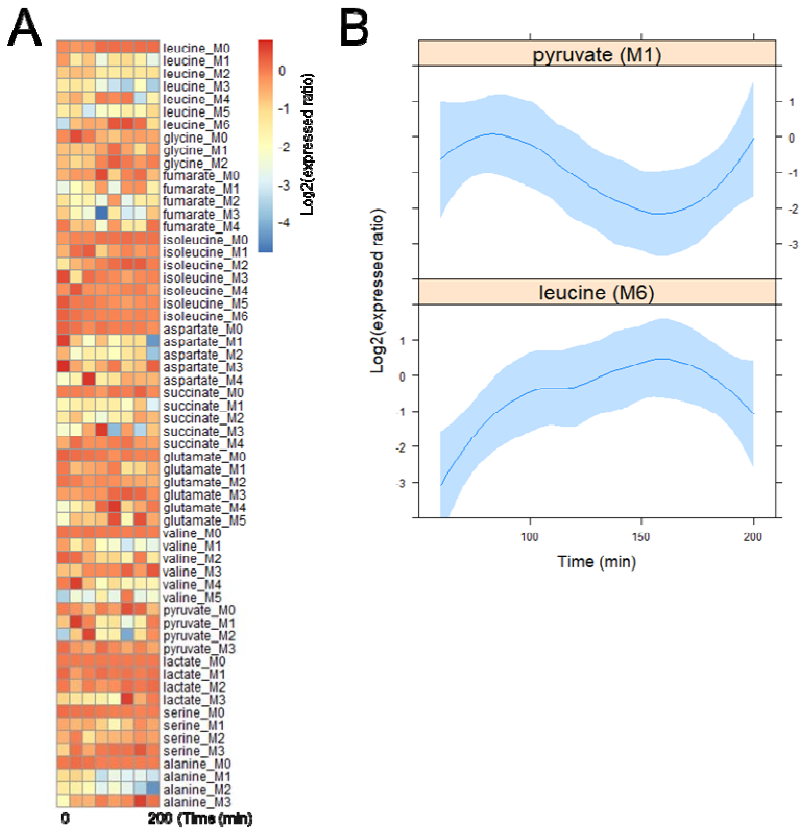


FIGURE 2. Synthesis of leucine from glucose changes in the cell cycle. A, Heatmap of all mass isotopomer distributions (MID) for metabolite species detected from the cell extracts of highly synchronous *S. cerevisiae* cells progressing in the cell cycle. The levels of each species were the average of five independent measurements for the time indicated at the bottom of the heatmap, which was then divided by the average value of the entire time series for that species. These 'expressed ratios' were then Log2-transformed. The Log2(expressed ratios) values were displayed with the *pheatmap* R language package. The different columns of the heatmap correspond to the different times in the cell cycle, from Figure 1. The values used to generate the heatmap are in File S1/Sheet2. B, The Log2(expressed ratios) of the two species

(pyruvate-M1, and leucine-M6) which showed the most significant changes ($p<0.1$, see File S2/Sheet2) in cells across the cell cycle is shown on the y-axis (see Materials and Methods), as a function of time (x-axis). Loess curves and the std errors at a 0.95 level are shown. The values used to draw the plots are in File S1/Sheet3.

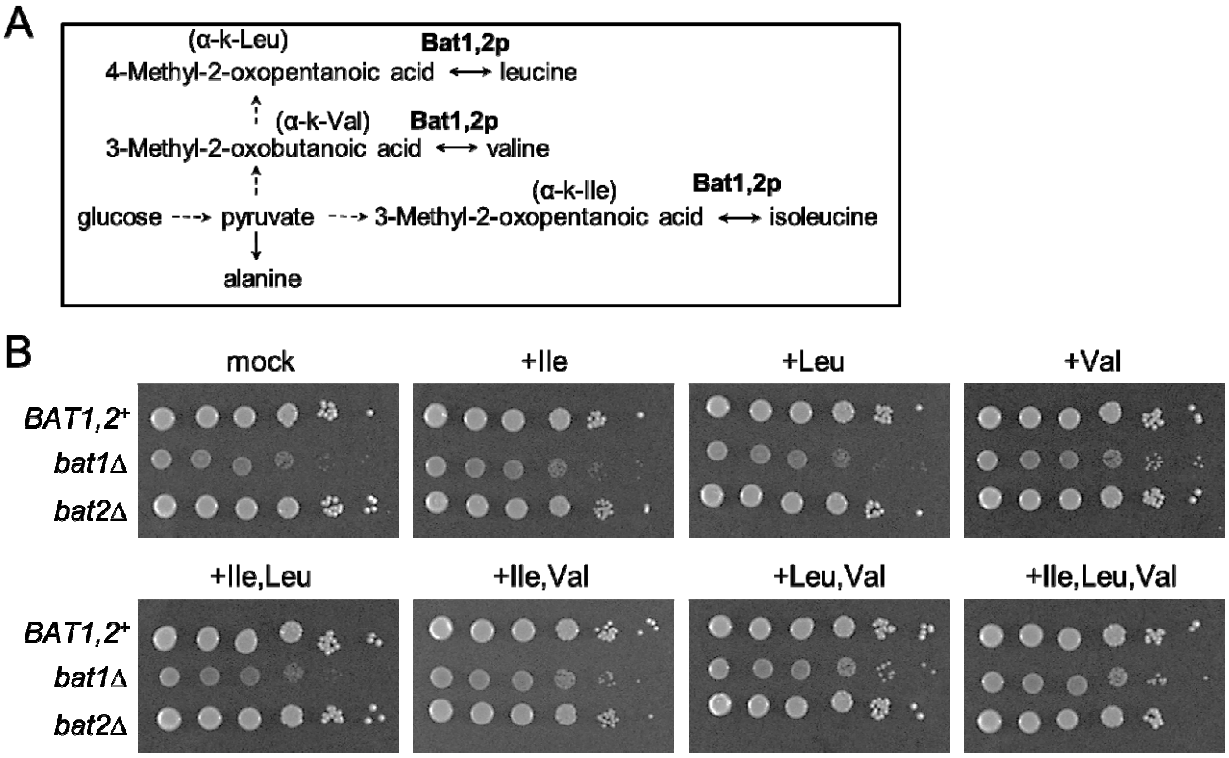


FIGURE 3. BCAA supplementation suppresses the growth defect of *bat1Δ* mutants. A, Diagram of the reactions leading to BCAAs from the corresponding alpha-keto (α-k) acids, catalyzed by Bat1,2p. B, The indicated strains (all in the prototrophic CEN.PK background; see Materials and Methods) were spotted at 10-fold serial dilutions on solid Synthetic Minimal Medium (SMM) agar plates. Exogenous amino acids were added at 1mM final concentration, as indicated in each case. The plates were incubated at 30 °C and photographed after 3-days.

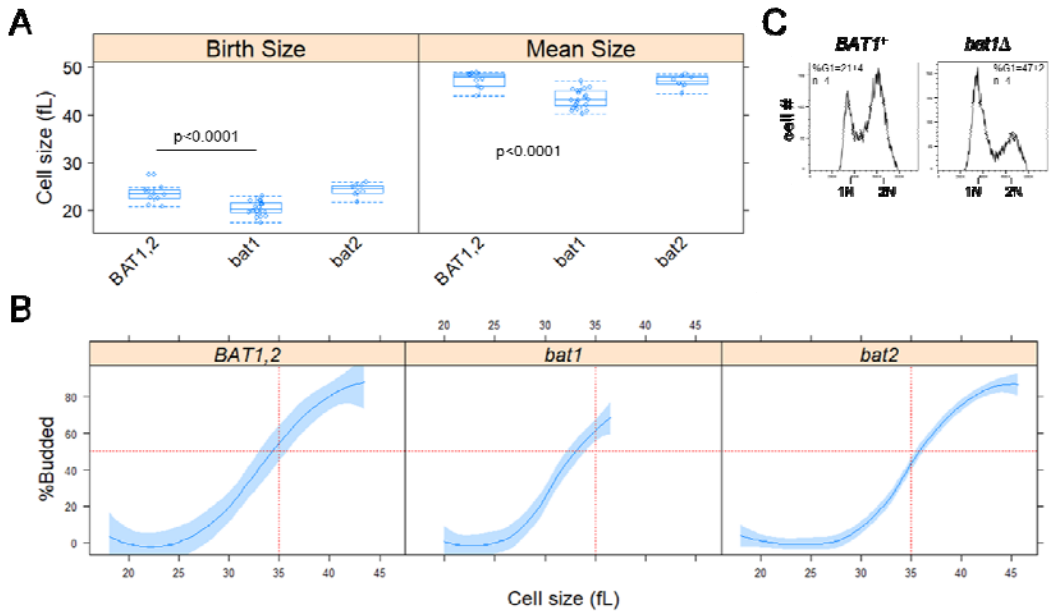


FIGURE 4. Cells lacking Bat1 are delayed in the G1 phase of the cell cycle. A. Boxplots showing the size of the cells (in fL). The measurements were taken from asynchronous cultures in synthetic minimal media (SMM) without amino acid supplementation. The values used to draw the plots are in File S1/Sheet4. B, Plots of the percentage of budded cells (y-axis) as a function of size (x-axis). The measurements were from daughter cells of the indicated strains, obtained by centrifugal elutriation, progressing in the cell cycle in SMM medium. Loess curves and the std errors at a 0.95 level are shown. The values used to draw the plots are in File S1/Sheet5. C, DNA content profiles of *BAT1* and *bat1Δ* cells from asynchronous cultures, in SMM medium. On the x-axis of the histograms is fluorescence per cell, while on the y-axis is the cell number. Peaks corresponding to cells in G1 with unreplicated (1N) and cells in G2 and M phases with fully replicated (2N) DNA are indicated. The percentage of cells with G1 DNA content (%G1) from 4 independent measurements is shown in each case (mean and sd).

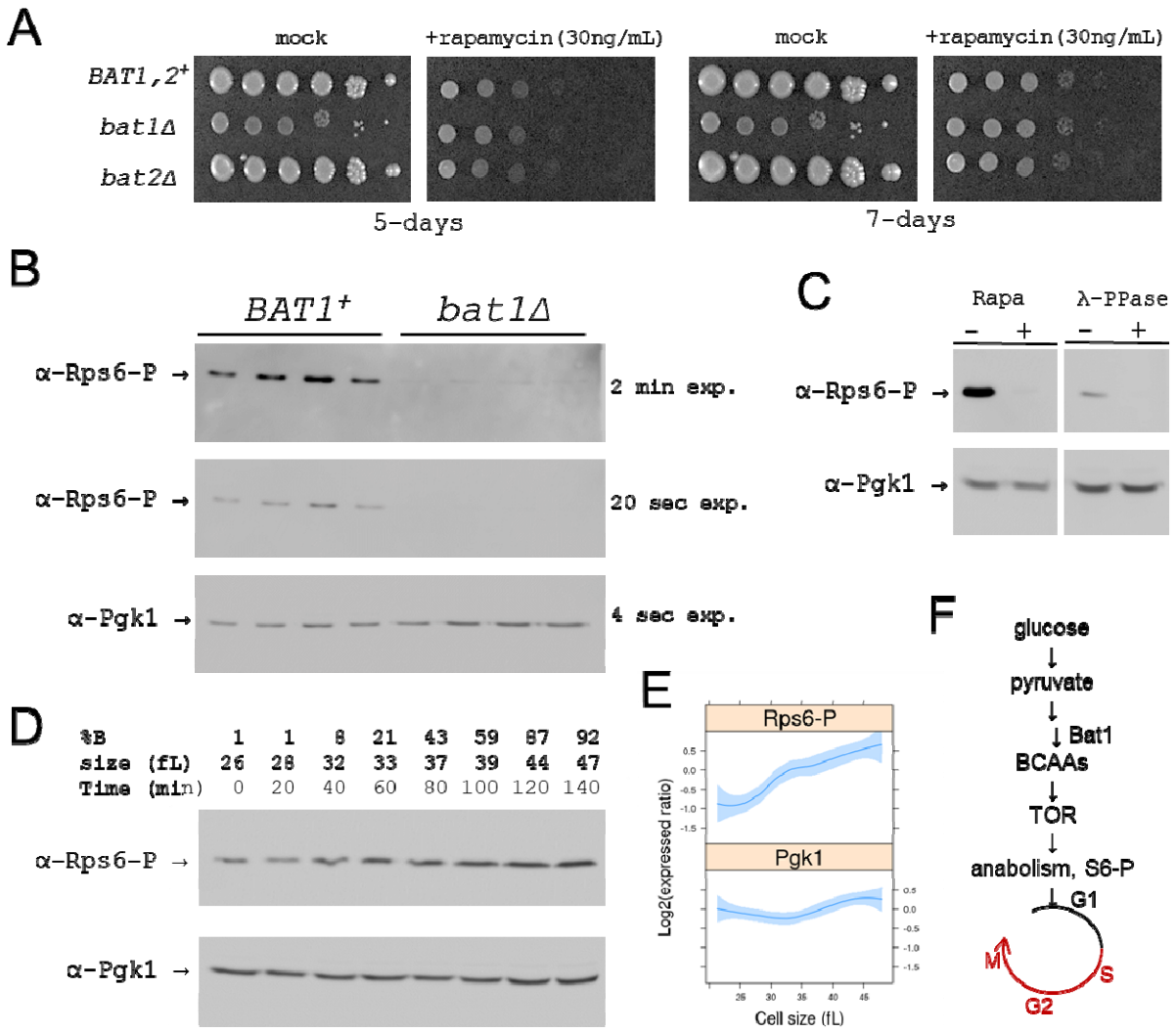


FIGURE 5. Bat1 is functionally linked to TOR activation in early G1. A, The indicated strains were spotted at 10-fold serial dilutions on solid Synthetic Minimal Medium (SMM) agar plates. Rapamycin was added at 30 ng/mL, as shown in each case. The plates were incubated at 30 °C and photographed after 5 and 7 days, as indicated. B, Immunoblots of total cell extracts from asynchronous *BAT1* and *bat1Δ* cells, from four independent experiments. The signal from Pgk1 (α-Pgk1) is shown on the blot at the bottom, and from phosphorylated Rps6 (α-Rps6-P) is on the blot above,

indicated for two exposures (2 min, top; 20 sec, middle). C, On the left are immunoblots from wild-type (CEN.PK) cells treated (+), or not (-), with rapamycin (Rapa) at 200 ng/mL for 1 h before cell extract preparation. On the right are immunoblots of wild-type (CEN.PK) cell extracts. The extracts were treated (+), or not (-), with λ-phosphatase for 1 h (see Materials and Methods). The levels of phosphorylated Rps6 (α-Rps6-P) and Pgk1 (α-Pgk1) are shown in each case. D, Immunoblots of total cell extracts from synchronous, elutriated wild-type (CEN.PK) cells. At the top, the percent of budded cells (%B), cell size (in fL), and time (in min) are indicated. The levels of phosphorylated Rps6 (α-Rps6-P) and Pgk1 (α-Pgk1) are shown in each case. E, Quantification of the levels of phosphorylated Rps6 and Pgk1 from four independent experiments done as in D. The relative levels of each protein across the cell cycle is shown on the y-axis (see Materials and Methods), as a function of cell size (x-axis). Loess curves and the std errors at a 0.95 level are shown. The values used to draw the plots are in File S1/Sheet4. The source immunoblots are in Files S5 and S6. F, schematic of our findings, linking BCAA synthesis to TOR activation early in the cell cycle.

EXPANDED VIEW FIGURES

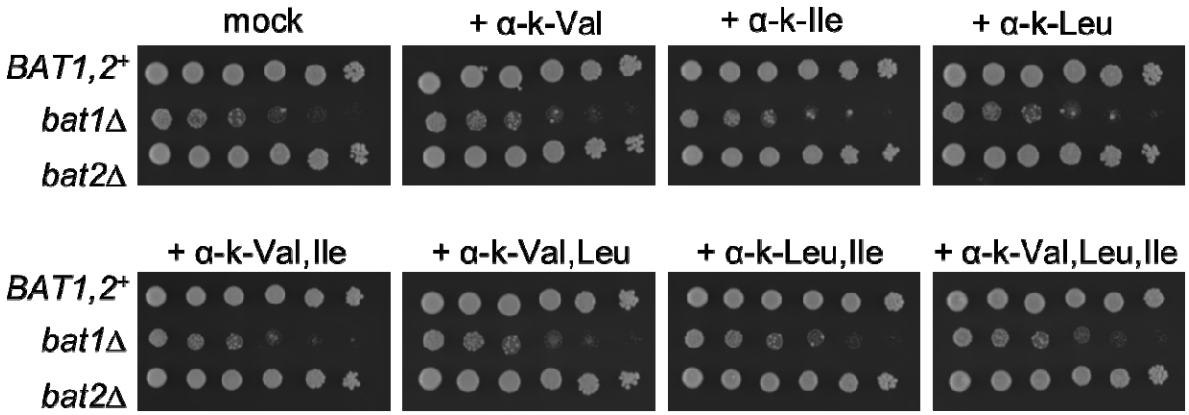


FIGURE EV1. Alpha-keto acid supplementation does not suppress the growth defect of *bat1Δ* mutants. The experiment was done as in Figure 3. Exogenous alpha-keto acids were added at 1mM final concentration, as indicated in each case.

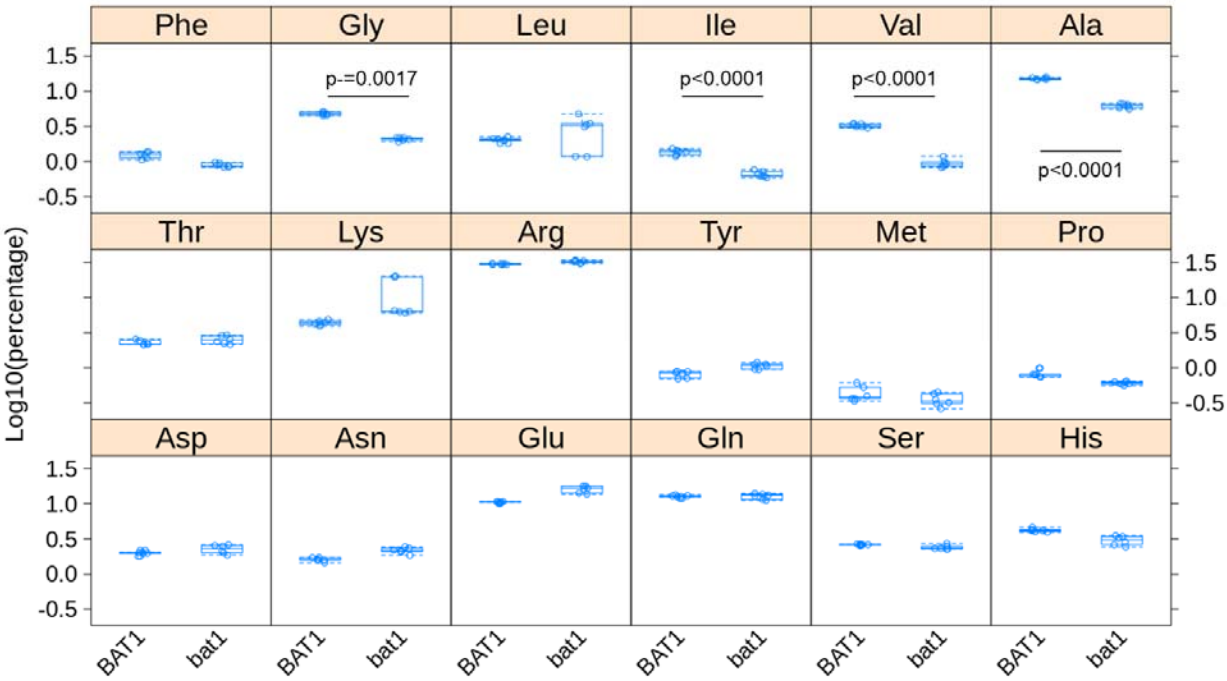


FIGURE EV2. Amino acid levels in *BAT1*$^{+}$ and *bat1Δ* cells. Intracellular amino acid levels were measured as described in Materials and Methods from exponentially growing cultures in SMM media. The fraction of each amino acid is on the y-axis. The four amino acids (Gly, Ile, Val, Ala) with significantly altered fractional abundance (>2-fold, $p<0.05$; from six independent samples in each case) are indicated. The values used to draw the plots are in File S1/Sheet7.

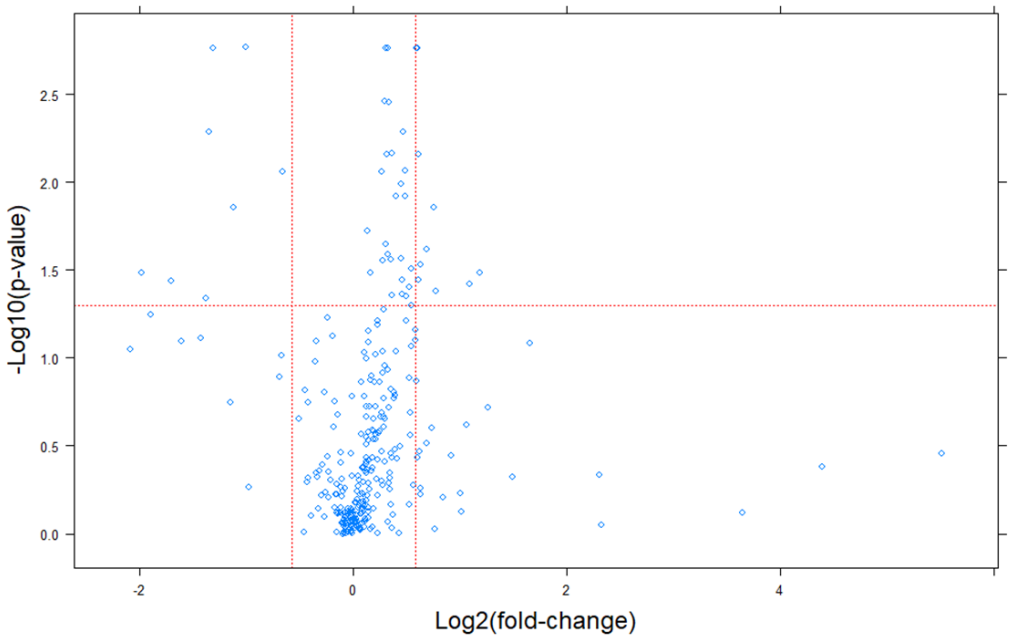
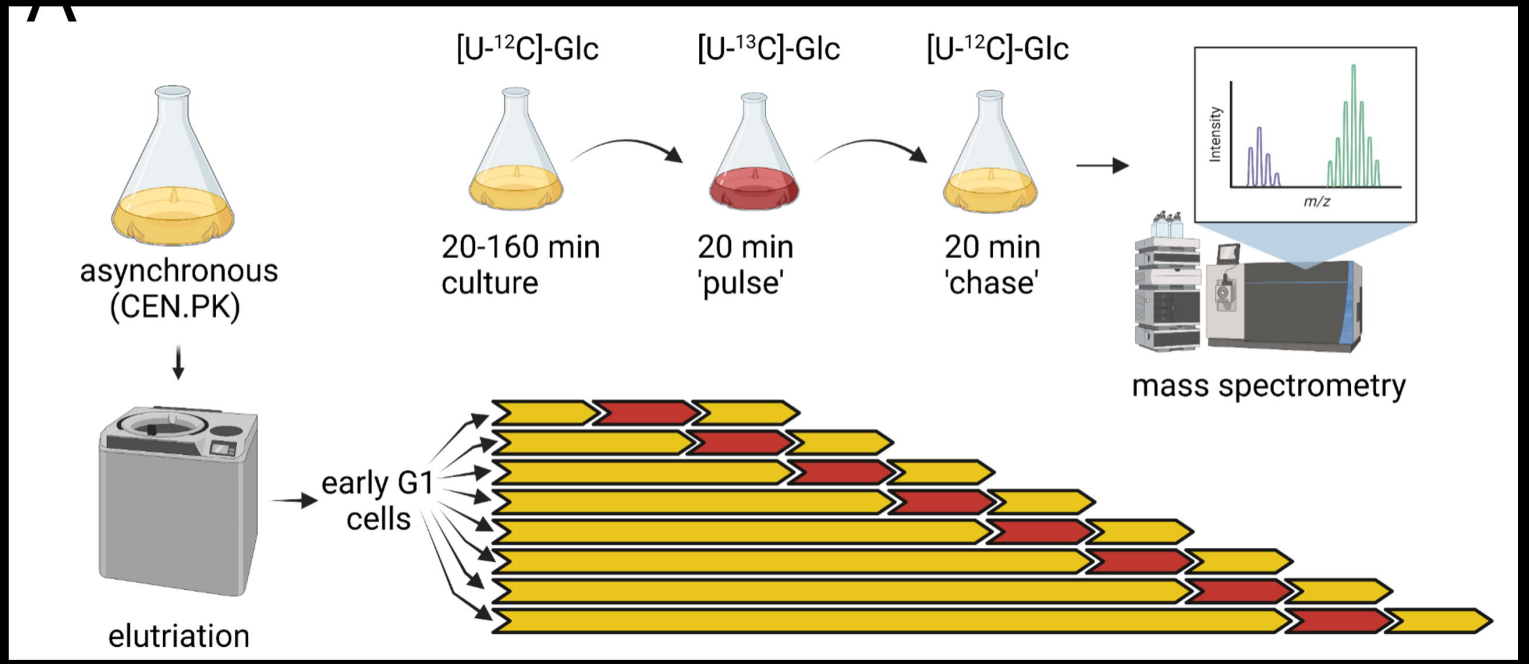
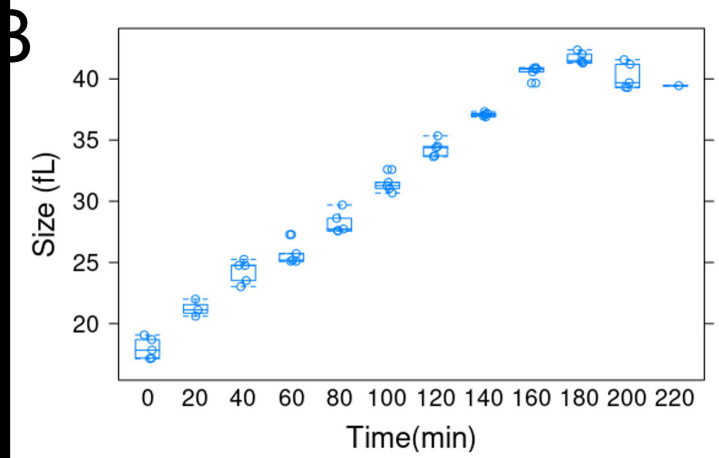
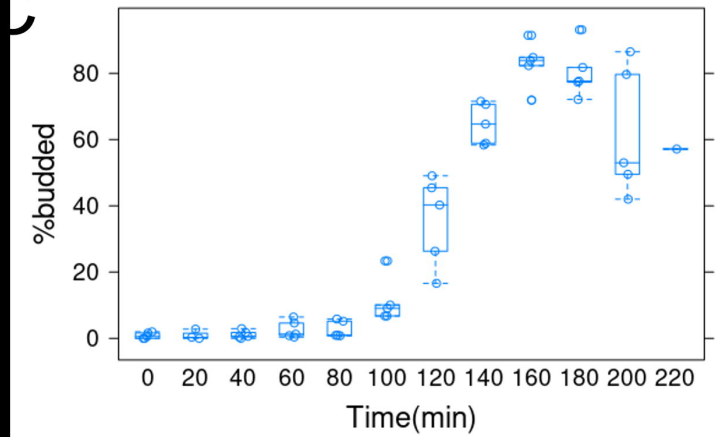


FIGURE EV3. Changes in primary and biogenic amine metabolites in cells lacking Bat1. Metabolites whose levels changed were identified from the magnitude of the difference (x-axis; Log2-fold change in *BAT1*$^{+}$: *bat1Δ* cells) and statistical significance (y-axis), indicated by the red lines. The analytical and statistical approaches are described in Materials and Methods. The values used to generate the graphs are in File S1/Sheet8.


A
[U-12C]-Glc
[U-13C]-Glc
[U-12C]-Glc
asynchronous
(CEN.PK)
20-160 min
culture
20 min
'pulse'
20 min
'chase'
Intensity
m/z
mass spectrometry
elutriation
early G1
cells


B
Size (fL)
40
35
30
25
20
0 20 40 60 80 100 120 140 160 180 200 220
Time(min)


C
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80
60
40
20
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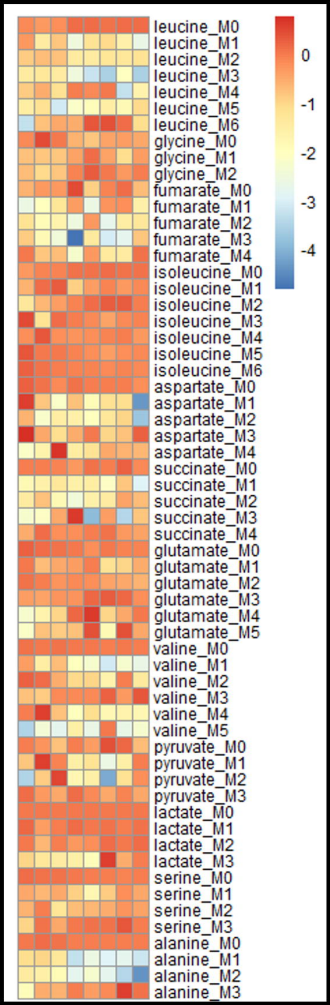
0 20 40 60 80 100 120 140 160 180 200 220
Time(min)


leucine_M0
leucine_M1
leucine_M2
leucine_M3
leucine_M4
leucine_M5
leucine_M6
glycine_M0
glycine_M1
glycine_M2
fumarate_M0
fumarate_M1
fumarate_M2
fumarate_M3
fumarate_M4
isoleucine_M0
isoleucine_M1
isoleucine_M2
isoleucine_M3
isoleucine_M4
isoleucine_M5
isoleucine_M6
aspartate_M0
aspartate_M1
aspartate_M2
aspartate_M3
aspartate_M4
succinate_M0
succinate_M1
succinate_M2
succinate_M3
succinate_M4
glutamate_M0
glutamate_M1
glutamate_M2
glutamate_M3
glutamate_M4
glutamate_M5
valine_M0
valine_M1
valine_M2
valine_M3
valine_M4
valine_M5
pyruvate_M0
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lactate_M3
serine_M0
serine_M1
serine_M2
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alanine_M2
alanine_M3
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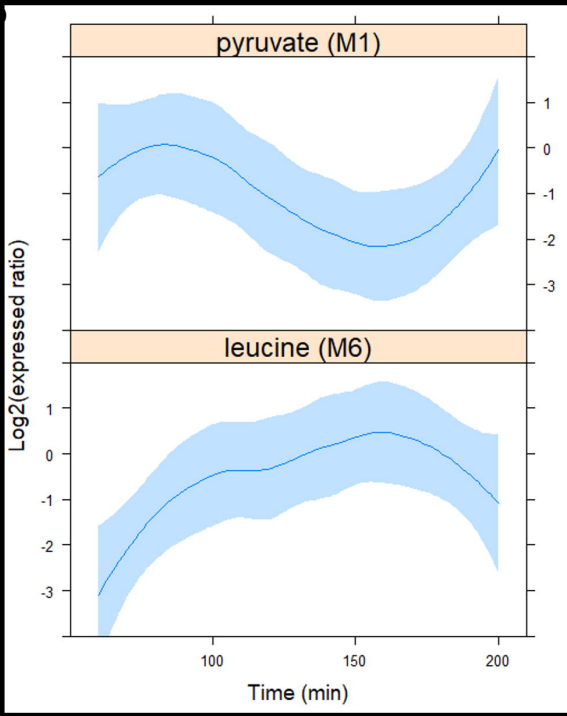
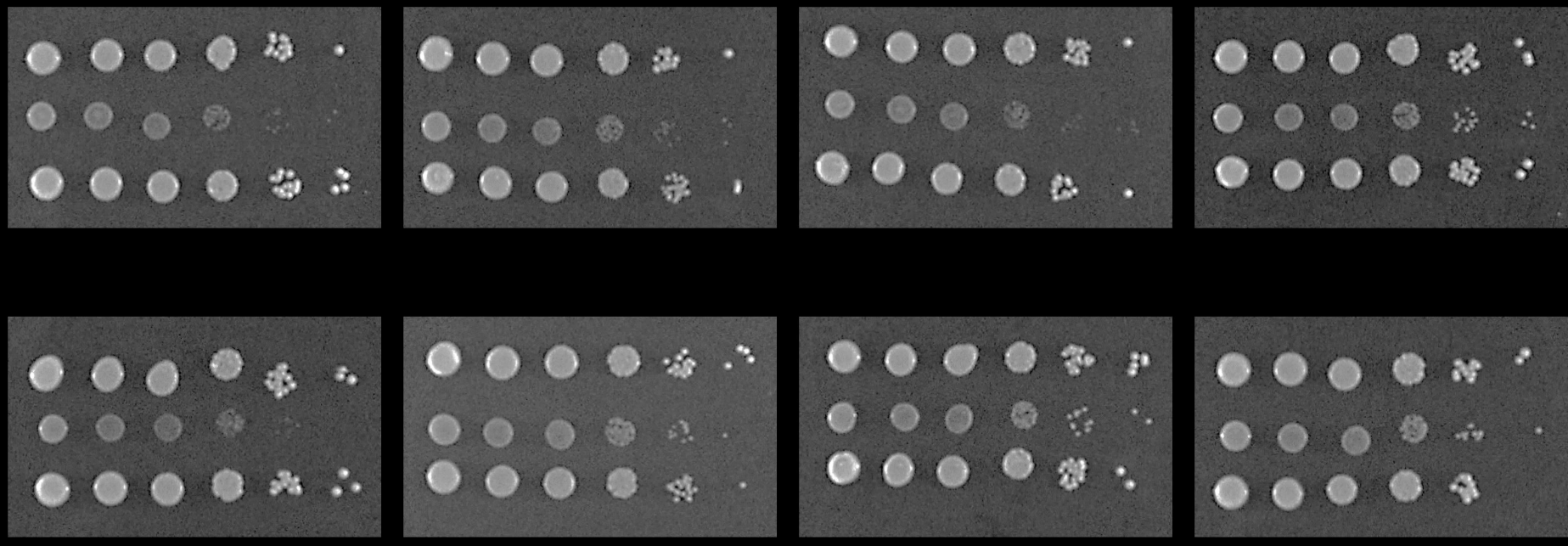
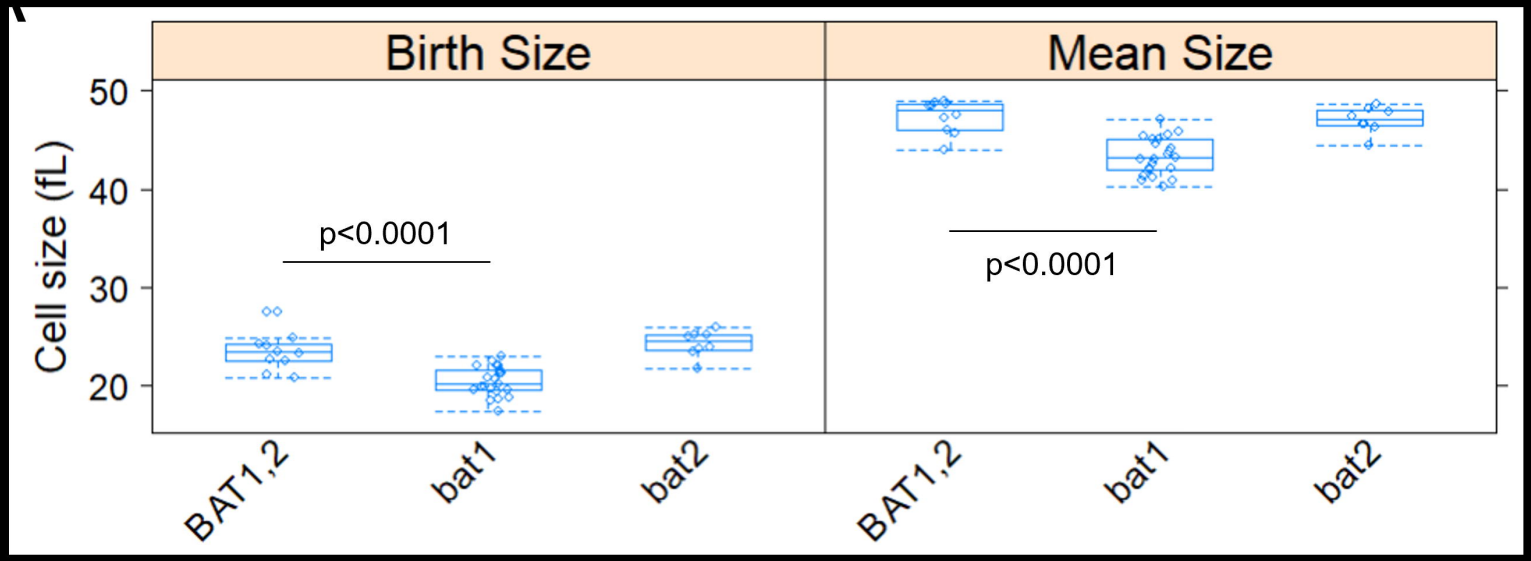
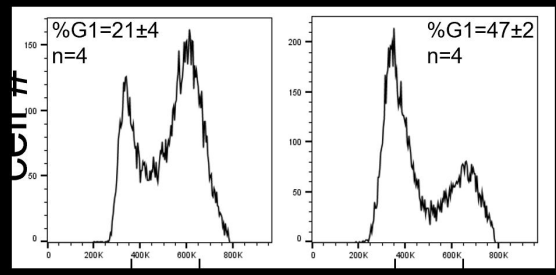
-2
-3
-4


pyruvate (M1)
leucine (M6)
Log2(expressed ratio)
Time (min)
100
150
200
1
0
-1
-2
-3




Birth Size
Mean Size
Cell size (fL)
50
40
30
20
p<0.0001
p<0.0001
BAT1,2
bat1
bat2
BAT1,2
bat1
bat2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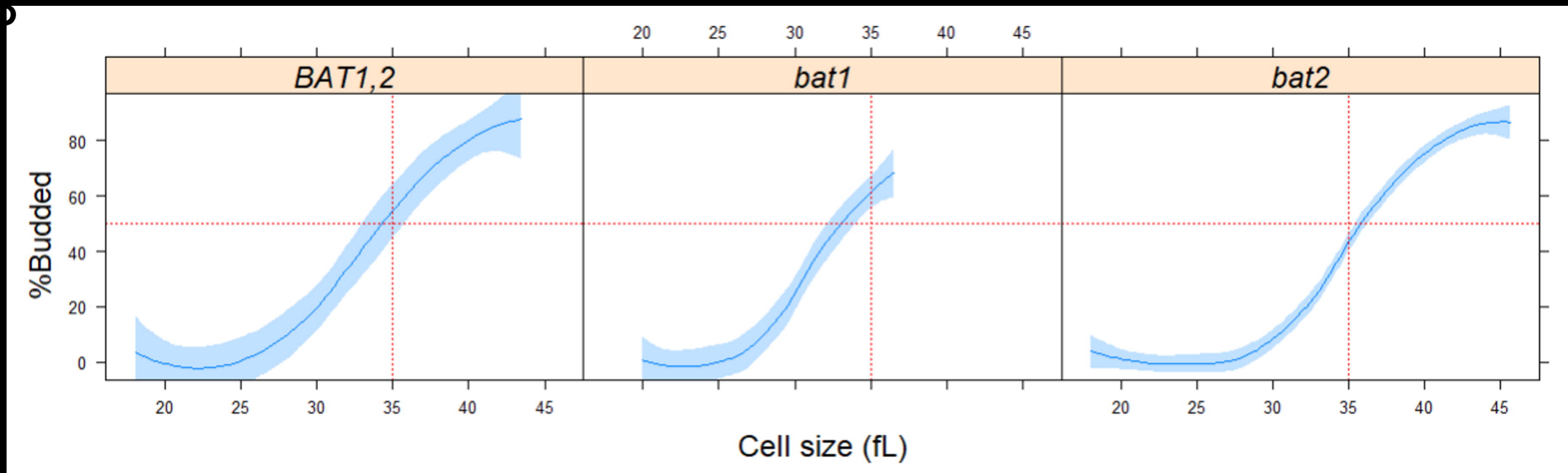
%G1=47±2
n=4


BAT1,2
bat1
bat2
%Budded
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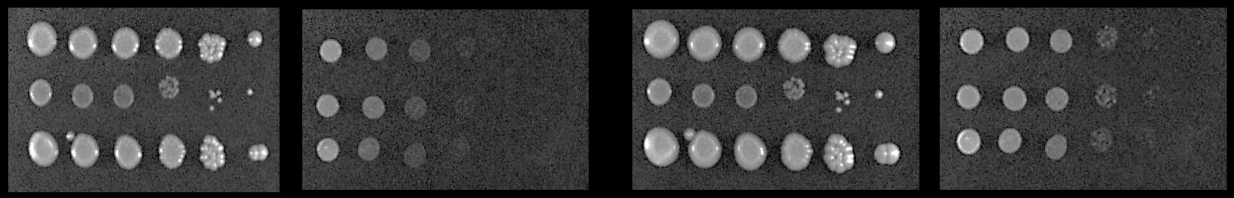
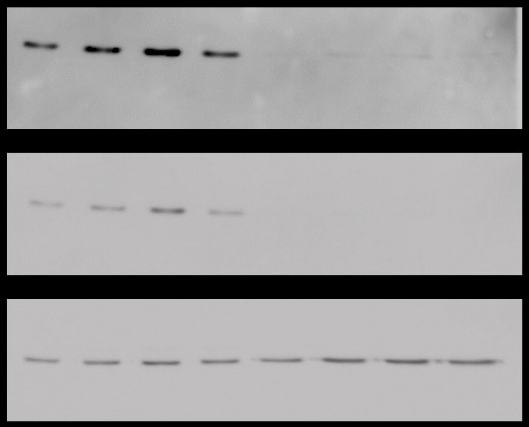
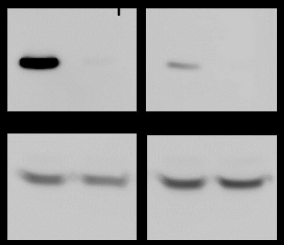
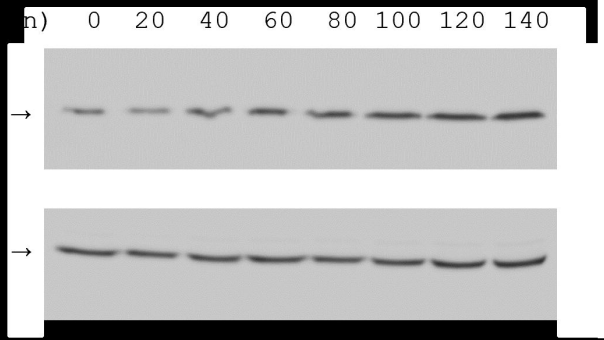
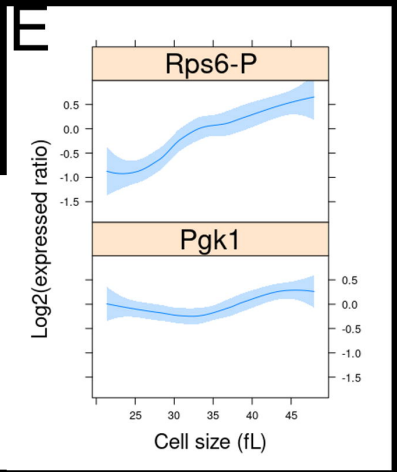
60
40
20
0
20
25
30
35
40
45
Cell size (fL)



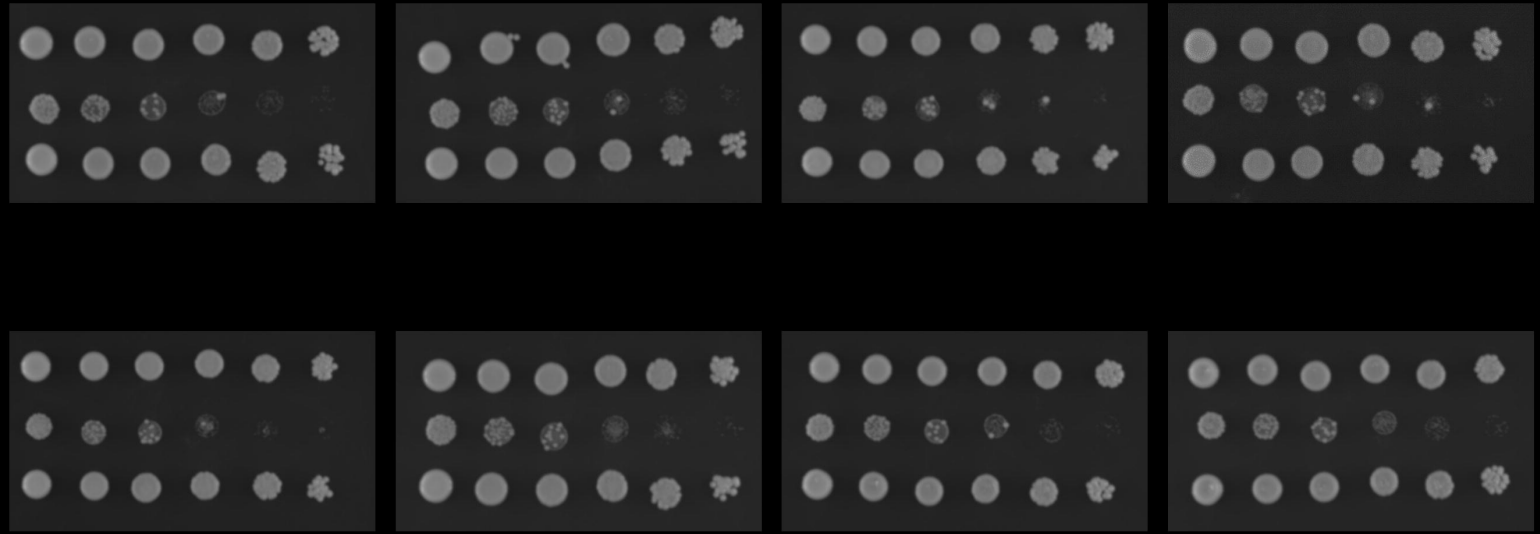


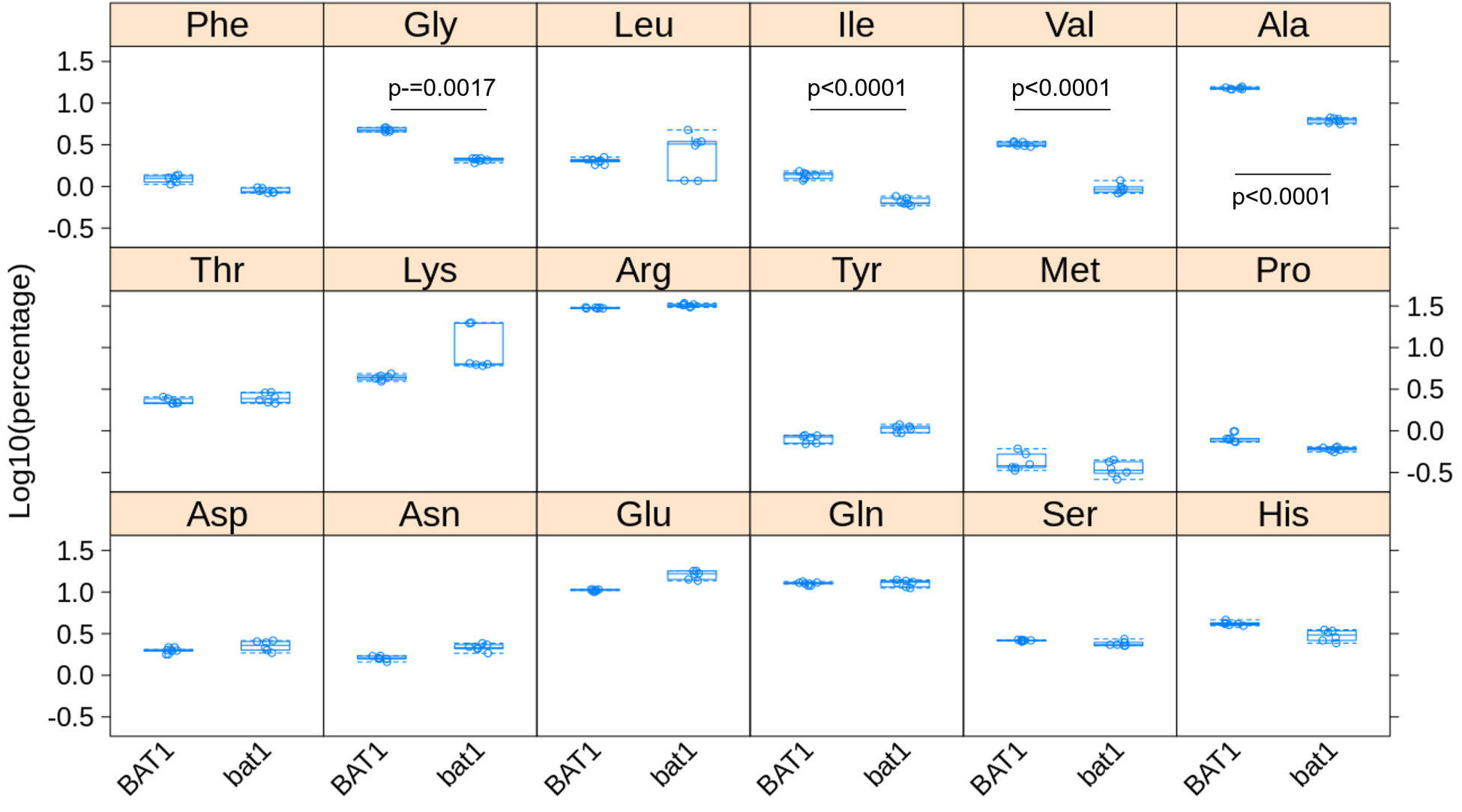


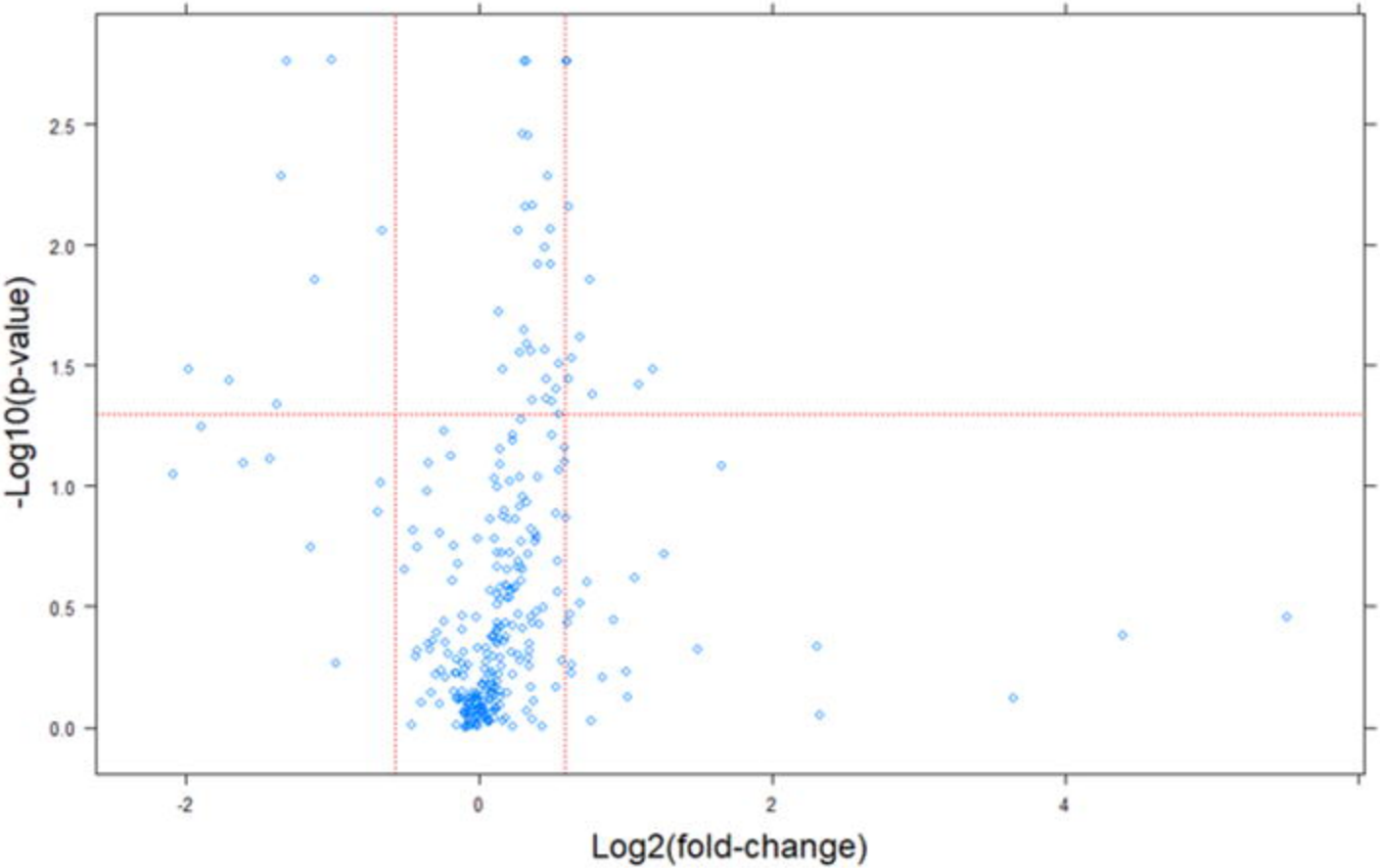

n) 0 20 40 60 80 100 120 140
→
→


E
Rps6-P
Pgk1
Log2(expressed ratio)
0.5
0.0
-0.5
-1.0
-1.5
25
30
35
40
45
Cell size (fL)

M
S
G2




Phe
Gly
Leu
Ile
Val
Ala
Thr
Lys
Arg
Tyr
Met
Pro
Asp
Asn
Glu
Gln
Ser
His
p-=0.0017
p<0.0001
p<0.0001
p<0.0001
Log10(percentage)
1.5
1.0
0.5
0.0
-0.5
BAT1
bat1


-Log10(p-value)
2.5
2.0
1.5
1.0
0.5
0.0
-2
0
2
4
Log2(fold-change)