1 **Phosphate budgeting to mitochondria controls glucose-mediated**

2 **mitochondrial repression**

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Abstract

Many cells growing in high glucose repress mitochondrial respiration, as observed in the

Crabtree and Warburg effects. A parsimonious biochemical explanation for this phenomenon

is missing. Using a *Saccharomyces cerevisiae* screen, we identified the conserved

deubiquitinase Ubp3 (Usp10), as necessary for proper mitochondrial repression. Ubp3

mutants have increased mitochondrial activity despite abundant glucose, with

downregulated glycolytic enzymes, rewired glucose metabolism, and increased trehalose.

Utilizing *Δubp3* cells, along with orthogonal approaches, we find that the Crabtree effect is

driven mechanistically by controling mitochondrial access of inorganic phosphate (Pi), as

determined by glycolytic flux. High glycolytic flux consumes free Pi, limiting available Pi for

entry into the mitochondria. Cytosolic Pi-dependent mitochondrial Pi entry is necessary and

sufficient to derepress mitochondria, driving synchronous requirements to sustain

mitochondrial activity. Restricting Pi entry to the mitochondria prevents mitochondrial

derepression. Collectively, we propose a biochemical basis of mitochondrial repression in

high-glucose based on intracellular Pi budgeting.

Introduction

Rapidly proliferating cells have substantial metabolic and energy demands, which are required in order to increase biomass (Buchakjian & Kornbluth, 2010; Cai & Tu, 2011; Zhu & Thompson, 2019). This includes a high ATP demand, obtained from cytosolic glycolysis or mitochondrial oxidative phosphorylation (OXPHOS), to fuel multiple reactions (Nelson et al., 2008). Interestingly, many rapidly proliferating cells have reduced mitochondrial respiration in high glucose. These cells preferentially rely on ATP generated from glycolysis/fermentation over mitochondrial respiration and OXPHOS, even though the net ATP yield per glucose molecule from glycolysis is lower than OXPHOS (López-lázaro, 2008). This occurs even in oxygen replete conditions, and is the well-known Warburg effect (Heiden et al., 2009; Warburg, 1925). A feature of many of these cells is the repression of mitochondrial processes in high glucose, termed glucose-mediated mitochondrial repression or simply the Crabtree effect (Crabtree, 1929; De Deken, 1966). This phenomenon is observed in many cells including some tumors (Heiden et al., 2009), neutrophils (Xia et al., 2021), activated macrophages (Kornberg, 2020), stem cells (Abdel-Haleem et al., 2017; Pacini & Borziani, 2014; Tsogtbaatar et al., 2020), and most famously *S. cerevisiae* (De Deken, 1966). Understanding how such cells growing in high glucose repress their mitochondria is therefore of obvious interest to diverse researchers. Numerous studies have identified signaling programs or regulators involved in glucose mediated mitochondrial repression. However, biochemical programs and regulatory processes in biology typically evolve around key biochemical constraints (Cornish-Bowden, 2016). The minimal biochemical constraints that explain how this phenomenon occurs are unresolved (Diaz-Ruiz et al., 2011; Hammad et al., 2016).

There are two major still unresolved hypotheses on the biochemical origins of the Crabtree effect. The first proposes direct roles for glycolytic intermediates in regulating mitochondrial respiration, by inhibiting specific mitochondrial outputs (Díaz-Ruiz et al., 2008; Lemus et al., 2018). The second proposes that cytosolic and mitochondrial processes compete for a common substrate. For example, a balance between pyruvate utilization in fermentation vs the mitochondrial TCA cycle (mediated by mitochondrial pyruvate transport and the pyruvate dehydrogenase complex) could determine mitochondrial activity under high glucose (Diaz-Ruiz et al., 2011). Relatedly, a competition between glycolytic and mitochondrial processes for mutually required metabolites/co-factors such as ADP or inorganic phosphate (Pi) could also regulate mitochondrial activity (Diaz-Ruiz et al., 2011; Hammad et al., 2016;

Koobs, 1972). This second hypothesis therefore proposes that an imbalance in resource

allocations within the cell might limit access of these metabolites to the mitochondria, causing

mitochondrial repression. These are not all mutually exclusive, and a combination of these

factors might dictate mitochondrial repression in high glucose. However, any hierarchies of

importance are currently unclear (Rodríguez-Enríquez et al., 2001). Collectively, experimental

data for a biochemical basis to explain mitochondrial repression remains incomplete.

One approach to resolve this question has been to identify regulators of metabolic

state under high glucose. Mitochondrial repression occurs rapidly after glucose addition

(Bouchez et al., 2020), and post-translational modifications regulated by signaling systems

might regulate this process (Broach, 2012; Hitosugi & Chen, 2014; Tripodi et al., 2015).

Ubiquitination is a major post-translational modification that regulates global proteostasis

(Hershko & Ciechanover, 1998; Komander & Rape, 2012), but the roles of ubiquitination-

dependent processes in regulating mitochondrial repression are poorly explored.

Ubiquitination itself is determined by the balance between E3-ligase dependent

ubiquitination, and deubiquitinase (DUB) dependent deubiquitination (Hershko et al., 1983;

Pickart & Eddins, 2004). Little is known about the roles of DUBs in regulating metabolic states,

making the DUBs interesting candidate regulators of mitochondrial repression.

In this study, using an *S. cerevisiae* DUB knock-out library based screen, we identified

the evolutionarily conserved Ubp3 protein (Usp10 in mammals) as required for mitochondrial

repression in high glucose. The loss of Ubp3 resulted in mitochondrial activation, and a

reduction in the glycolytic enzymes-phosphofructokinase 1 (Pfk1) and GAPDH isozymes Tdh1

and Tdh2, resulting in decreased glycolytic flux. The consequent rerouting of glucose flux also

increases trehalose biosynthesis. This metabolic rewiring alters the intracellular inorganic

phosphate (Pi) balance, due to both the increased Pi release from trehalose synthesis, and

decreased Pi consumption in glycolysis, to cumulatively increase Pi pools. Using *ubp3Δ* cells

along with independent analysis of wild-type cells, as well as isolated mitochondrial fractions

from these cells, we established that changing intracellular Pi budgeting via this metabolic

rewiring, and consequently increasing Pi allocations to mitochondria is necessary and

sufficient to increase mitochondrial activity. Switching to a respiratory state requires

increased mitochondrial Pi transport, and mitochondrial Pi regulates mitochondrial activity.

Through these data, we propose how intracellular Pi balance, and a cytosolic vs mitochondrial

competition for Pi as controlled by glycolytic flux, is a biochemical basis for the Crabtree
effect.

Results

A deubiquitinase deletion screen identifies Ubp3 as a regulator of glucose-mediated
mitochondrial repression

In Crabtree positive cells such as *Saccharomyces cerevisiae*, high glucose represses
mitochondrial activity as well as OXPHOS-dependent ATP synthesis (De Deken, 1966; Postma
et al., 1989) (illustrated in Figure 1A). Our initial objective was only to identify proteostasis
regulators of glucose-mediated mitochondrial repression. We generated and used a
deubiquitinase (DUB) deletion strain library of *S. cerevisiae* (Figure S1A), to unbiasedly screen
for putative regulators of mitochondrial repression (Figure 1B). For the screen, mitochondrial
activity in wild-type (WT) and mutant cells growing in 2% glucose were assessed based on the
fluorescence intensity of a potentiometric dye Mitotracker CMXRos (which measures the
mitochondrial membrane potential) (illustrated in Figure 1B). Using this screen, we identified
DUB mutants with altered mitochondrial membrane potential (Figure 1C, Figure S1A). Note:
WT cells in a respiratory medium (2% ethanol) were used as an added control to estimate
maximum mitotracker fluorescence intensity (Figure S1B).

A prominent 'hit' was the evolutionarily conserved deubiquitinase Ubp3 (Figure 1C),
which is the homolog of the mammalian deubiquitinase Usp10 (Figure S1C). This protein
shows a degree of conservation across eukaryotes (Figure S1C). Due to this reason, as well as
putative roles in metabolism or mitochondrial function (Isasa et al., 2015; Nostramo et al.,
2016; Ossareh-Nazari et al., 2010), we focused our further attention on this DUB. Cells lacking
Ubp3 showed an ~1.5-fold increase in mitotracker fluorescence (Figure 1C, Figure S1A). To
assess if the increased mitochondrial potential depends on the deubiquitinase activity of
Ubp3, we generated a catalytically dead mutant of Ubp3 - $Ubp3^{C469A}$ and estimated
mitotracker fluorescence. $Ubp3^{C469A}$ cells showed increased mitochondrial potential
comparable to *ubp3Δ* cells (Figure 1D). This data confirmed that the catalytic activity of Ubp3
is required to fully repress mitochondrial activity under high glucose. As a control, we also

assessed steady-state levels of Ubp3 protein (Figure S1D). The mutation in the catalytic site

did not affect steady-state Ubp3 levels.

Next, to assess the requirement of Ubp3 for mitochondrial function, we quantified the

electron transport chain (ETC) complex IV subunit Cox2 (Fontanesi et al., 2006)(Figure 1E).

ubp3Δ cells have significantly higher Cox2 protein than WT cells (Figure 1E). There was no

increase in the protein levels of mitochondrial outer membrane protein Tom70 (Figure S1E),

suggesting that the increased Cox2 is not merely because of an increase in total mitochondrial

content. We next measured the basal oxygen consumption rate (OCR) of *ubp3Δ* cells using

Seahorse XFe24 Analyzer. Notably, basal OCR is significantly higher in *ubp3Δ* cells, indicating

higher respiration (Figure 1F).

Since *ubp3Δ* cells have increased respiration, we asked if ATP synthesis from

mitochondria was higher in these cells. To estimate this, we measured total ATP levels in WT

and *ubp3Δ* cells, in the presence and absence of mitochondrial ETC complex IV inhibitor

sodium azide (Yoshikawa et al., 1998). This would quantify the contribution of mitochondrial

ETC dependent ATP synthesis to the total ATP pools (which come from both glycolysis and

respiration). The total ATP levels in WT and *ubp3Δ* cells were comparable (Figure 1G).

However, upon treatment with sodium azide, ATP levels in WT cells were significantly higher

than *ubp3Δ* cells, contributing to ~60% of the total ATP in these cells (Figure 1H). In contrast,

the ATP levels in *ubp3Δ* cells after sodium azide treatment were ~ 40% of the total ATP levels

(Figure 1H). These data suggest a higher contribution of mitochondrial ATP synthesis towards

the total ATP pool in *ubp3Δ* cells, as compared to WT cells.

We next asked if *ubp3Δ* cells required this high mitochondrial activity for growth. For

this, we first assessed growth in high glucose, in the presence and absence of sodium azide.

In high glucose, WT cells expectedly show a minimal growth defect in the presence of sodium

azide, indicating a low reliance on mitochondrial function (Figure 1I). Contrastingly, *ubp3Δ*

cells as well as Ubp3[C469A] cells show a severe growth defect in the presence of sodium azide

(Figure 1I). Next, we reduced mitochondrial respiration by individually deleting several

components of the ETC (Cox2, and the ATP synthase subunits Atp1 and Atp10), and assessed

growth in WT and *ubp3Δ* cells. Loss of these genes results in a severe growth defect in *ubp3Δ*

cells as compared to in WT cells (Figure S1F). Together, these results indicate that the loss of Ubp3 makes cells dependent on mitochondrial ATP synthesis in high glucose.

Collectively these data show that in 2% glucose, *ubp3Δ* cells have high mitochondrial activity and respiration, and rely on this mitochondrial function for ATP production and growth. We therefore decided to use *ubp3Δ* cells in order to delineate necessary requirements for glucose-mediated mitochondrial repression.

Key glycolytic enzymes decrease and glucose flux is rerouted in *ubp3Δ* cells

Glucose-6 phosphate (G6P) is the central node in glucose metabolism where carbon-allocations are made towards distinct metabolic arms, primarily: glycolysis, the pentose phosphate pathway (PPP), and trehalose biosynthesis (Figure 2A). High glycolytic enzymes ensure maximum glycolytic flux when sufficient glucose is present (Grigaitis & Teusink, 2022). We first investigated what happens to glycolytic enzyme amounts in *ubp3Δ* cells. The endogenous protein amounts of key glycolytic enzymes - Phosphofructokinase 1 (Pfk1), GAPDH isozymes (Tdh2, Tdh3), and the enolase isozymes (Eno1, Eno2) were all compared between WT and *ubp3Δ* cells. Pfk1, Tdh2, and Tdh3 proteins were significantly decreased in *ubp3Δ* cells (but not Eno1 and Eno2) (Figure 2B, S2A). This observation was intriguing, because the Pfk and GAPDH steps are critical in determining glycolytic flux. Pfk1 converts fructose 6-phosphate (F6P) to fructose 1,6 bisphosphate (F1,6BP), a committed step of glycolysis (Nelson et al., 2008). GAPDH converts glyceraldehyde 3-phosphate (G3P) to 1,3 bisphosphoglycerate (1,3BPG), which is the major flux regulating step in the lower arm of glycolysis (Nelson et al., 2008; Shestov et al., 2014) (Figure 2A). A reduction in these enzymes could therefore putatively decrease glycolytic flux, and a subsequent reduction in the glycolytic rate could reroute glucose (G6P) allocations via mass action towards other branches of glucose metabolism (Figure 2A). Therefore, we first measured the steady-state levels of key glycolytic and pentose phosphate pathway (PPP) intermediates, and trehalose in WT or *ubp3Δ* cells using targeted LC-MS/MS. Glucose-6/fructose-6 phosphate (G6P/F6P) increased in *ubp3Δ* cells (Figure 2C). Concurrently, trehalose, and the late PPP intermediates ribose 5-phosphate (R5P) and sedoheptulose 7-phosphate (S7P) significantly increased in *ubp3Δ* cells (Figure 2C).

Since steady-state metabolite amounts cannot separate production from utilization,

in order to unambiguously assess if glycolytic flux is reduced in *ubp3Δ* cells, we utilized a pulse

labeling of $^{13}C_6$ glucose, following which the label incorporation into glycolytic and other

intermediates was measured. WT and *ubp3Δ* cells were grown in high glucose, pulsed with

$^{13}C_6$ glucose, and the relative ^{13}C label incorporation into glycolytic intermediates and

trehalose were measured, as shown in the schematic (Figure S2B). Note that because

glycolytic flux is very high in yeast, this experiment requires rapid pulsing and extraction of

metabolites in order to stay in a linear range to avoid label saturation. This experiment is

therefore more extensively described in extended supplementary methods and controls

shown in Figure S2C. We observed increased ^{13}C label incorporation into G6P/F6P as well as

trehalose in *ubp3Δ* cells (Figure 2D). The ^{13}C label incorporation into glycolytic intermediates

F1,6BP, G3P, 3PG and PEP were significantly decreased in *ubp3Δ* cells, suggesting decreased

glycolytic flux (Figure 2D). We next measured ethanol concentrations in WT and *ubp3Δ* cells,

and observed a significant decrease in ethanol concentration in *ubp3Δ* cells (Figure 2E). We

also measured the rate of ethanol formation in WT and *ubp3Δ* cells, by shifting these cells to

fresh, high glucose medium and measuring ethanol concentration over time. We observed a

decrease in rate of ethanol production in *ubp3Δ* cells, suggesting decreased fermentation rate

(Figure 2F).

Collectively, these results suggest that that reduced Pfk1 and GAPDH (in *ubp3Δ* cells)

decrease glucose flux via glycolysis and the resulting overflow diverts glucose flux away from

glycolysis, and towards trehalose biosynthesis and the PPP.

Phosphate accumulates due to altered Pi homeostasis in *ubp3Δ* cells

Since *ubp3Δ* cells had decreased glycolytic flux along with increased flux towards

trehalose synthesis, we next asked if such reallocations of glucose-derived metabolites could

explain the increased mitochondrial activity in *ubp3Δ* cells. Our hypothesis here was based on

the reasoning given below.

An existing idea that proposes a biochemical origin of the Crabtree effect is that there

may be internal competition for shared metabolites/co-factors between (cytosolic) glycolytic

and mitochondrial processes. Here, mitochondria cannot sufficiently access these metabolites and are therefore repressed (Diaz-Ruiz et al., 2011; Koobs, 1972; Rodríguez-Enríquez et al., 2001). Contextually, a plausible role for inorganic phosphate (Pi) in regulating glucose-mediated mitochondrial repression can be conceived. Cytosolic glycolysis requires rapid, high consumption of Pi (Mason et al., 1981; Rodríguez-Enríquez et al., 2001; Van Heerden et al., 2014), and it is therefore possible that this might limit the Pi continuously available for mitochondrial use (Brazy et al., 1982; Koobs, 1972), resulting in mitochondrial repression. In this context, we and others had earlier hypothesized that the balance between reactions that release vs consume Pi might explain global changes in Pi levels (Gupta & Laxman, 2021). On the Pi consumption side, during glycolysis the GAPDH reaction converting glyceraldehyde 3-phosphate (G3P) to 1,3 bisphosphoglycerate (1,3BPG) rapidly converts ADP and Pi to ATP, resulting in a decrease in Pi levels and consequent increase in ATP, in the presence of glucose (Hohmann et al., 1996; Van Heerden et al., 2014). The ATP formed from glycolysis itself is a Pi donor for reactions such as nucleotide biosynthesis, polyphosphate biosynthesis and protein phosphorylation (Gupta & Laxman, 2021; Hunter, 2012). When the glycolytic flux is high, the consumption of Pi will be correspondingly high due to increased ATP, nucleotide and polyphosphate synthesis (Austin & Mayer, 2020; Hohmann et al., 1996; Ljungdahl & Daignan-Fornier, 2012). We therefore wondered if this reaction would limit amounts of cytosolic Pi that remains available for the mitochondria, as hypothesized in an illustration in Figure 3A. Notably, *ubp3Δ* cells have reduced GAPDH levels and decreased glycolytic flux. Separately, reactions resulting in the release of Pi are often underappreciated (Gupta & Laxman, 2021). In this regard, trehalose synthesis is a major Pi releasing reaction, and a significant source of free Pi critical for Pi homeostasis (Gupta et al., 2019; Van Heerden et al., 2014). Notably, flux through this reaction is also substantially higher in *ubp3Δ* cells. Therefore, there appears to be both an increase in Pi release (via trehalose), coupled with decreased Pi consumption, in *ubp3Δ* cells. We therefore asked if this series of events might increase Pi in *ubp3Δ* cells (Figure 3A), and if so could this increased Pi availability lead to increased mitochondrial activity (as hypothesized in Figure 4A)?

To test this, we first assessed Pi levels in *ubp3Δ* and WT cells. *ubp3Δ* cells had significantly higher Pi compared to WT cells, and this increased Pi was comparable to Pi amounts in WT cells grown in excess Pi medium (Figure 3B). Similarly, we compared Pi

amounts in the catalytically inactive Ubp3[C469A] cells, where Pi amounts were increased (Figure

S3A). Therefore, the loss of Ubp3 increases intracellular Pi levels.

Next, we asked if *ubp3Δ* cells exhibit signatures of cells that are in a 'high Pi' state. *S.*

cerevisiae maintain internal Pi balance by concurrently controlling the expression of a set of

genes encoding Pi transporters and enzymes that can increase intracellular Pi, collectively

known as the Pho regulon (Mouillon & Persson, 2006; Secco et al., 2012). The Pho regulon is

induced under Pi limitation, and repressed when Pi is abundant (Gupta et al., 2019; Mouillon

& Persson, 2006). We assessed two major Pho proteins (Pho84: a high-affinity membrane Pi

transporter, and Pho12: an acid phosphatase) in WT and *ubp3Δ* cells in high glucose. WT cells

grown in high Pi were used as a control. *ubp3Δ* cells have lower amounts of Pho84 and Pho12

(Figure 3C). Further, when cells are shifted to low Pi for one hour, Pho84 and Pho12 increased

in *ubp3Δ* cells. This two observations are consistent with the interpretation that reduced

Pho84 and Pho12 amounts in *ubp3Δ* cells are because of increased Pi levels, and not due to

Ubp3 altering Pho regulon function itself (Figure 3C). These data also clarify earlier

observations from *ubp3Δ* cells which noted reduced Pho proteins (Isasa et al., 2015).

Therefore, *ubp3Δ* cells constitutively have higher Pi, and likely a consequent decrease in Pho

proteins.

We next asked if the increased Pi in *ubp3Δ* cells is because of altered G6P allocations

towards different end-points. In particular, the Pi release from trehalose synthesis

(conversion of trehalose 6-phosphate to trehalose) can be a major source of cytosolic Pi

(Gupta et al., 2019; Gupta & Laxman, 2021). We therefore assessed the contribution of

increased trehalose synthesis towards the high Pi levels observed in *ubp3Δ* cells, by estimating

Pi levels in WT and *ubp3Δ* cells in the absence of trehalose 6-phosphate phosphatase (Tps2),

which catalyzes the Pi releasing step in trehalose synthesis. Notably, loss of Tps2 in *ubp3Δ*

cells resulted in a significant decrease in Pi (Figure 3D). There was no further difference in Pi

between *tps2Δ* and *ubp3Δtps2Δ* cells (Figure 3D). As an added control, we assessed trehalose

in WT and *ubp3Δ* cells in the absence of Tps2, and found no difference (Figure S3B). These

data show that increasing G6P flux towards trehalose biosynthesis is a major source of the

increased Pi in *ubp3Δ* cells.

Since the major GAPDH isozymes, Tdh2 and Tdh3, are reduced in *ubp3Δ* cells, we

asked if a reduction in GAPDH can also decrease Pi consumption and increase Pi. To assess

this, we generated a strain where Tdh2 and Tdh3 are deleted. *tdh2Δtdh3Δ* cells exhibit a

growth defect, but are viable, permitting further analysis. *tdh2Δtdh3Δ* cells had higher Pi

levels in high glucose (Figure 3E). Expectedly, we also observed a decrease in free ethanol in

tdh2Δtdh3Δ cells (Figure S3C), along with an accumulation of glycolytic intermediates F1,6 BP

and G3P, and decreased 3PG and PEP (Figure S3D). However, G6P and trehalose levels

between WT and *tdh2Δtdh3Δ* cells were comparable (Figure S3D, S3E). These data sugest that

unlike *ubp3Δ* cells, the increased Pi in *tdh2Δtdh3Δ* cells primarily comes only from decreased

Pi consumption (GAPDH step), and not from a combination of increased Pi synthesis (from

increased trehalose synthesis) as well as decreased Pi consumption (due to decreased

GAPDH). To further confirm that the increase in Pi in *ubp3Δ* and *tdh2Δtdh3Δ* cells in high

glucose is because of altered glucose flux, we measured the Pi in these cells growing in a

gluconeogenic medium – 2%, ethanol. In this scenario, the GAPDH catalyzed reaction is

reversed, converting 1,3BPG to G3P, and this would release and not consume Pi. Compared

to WT cells, the Pi levels decreased in *ubp3Δ* and *tdh2Δtdh3Δ* cells (Figure S3F), suggesting

that the changes in Pi in these mutants is driven by change in the balance of Pi release or

consumption.

These results indicate that the combined effect of increased Pi release coming from

trehalose synthesis, and decreased Pi consumption from reduced GAPDH collectively increase

the Pi levels in *ubp3Δ* cells. We therefore wondered, could the availability of Pi in the

cytoplasm, based on these arms of glucose metabolism determine mitochondrial repression?

Phosphate availability dictates mitochondrial activity

In other words, could there be Pi budgeting between cytosolic glycolysis and

mitochondria, to regulate Pi availability within the mitochondria, and therefore mitochondrial

activity (Figure 4A)? To test this, we first asked if the mitochondria in *ubp3Δ* cells have

increased Pi. Mitochondria were isolated by immunoprecipation from WT and *ubp3Δ* cells,

the efficiency of mitochondria isolation was analyzed (Figure S4A) and relative Pi levels

compared. Pi levels were normalised to the protein levels of the mitochondrial matrix protein

Idh1 (isocitrate dehydrogenase) in isolated mitochondria, since Idh1 protein levels did not

decrease in *ubp3Δ* cells (Figure S4B), and also consistent with (Isasa et al., 2015). We observed

significantly higher mitochondrial Pi in *ubp3Δ* cells (Figure 4B). We next asked if the increased

mitochondrial activity in *ubp3Δ* cells is a consequence of high Pi. If this were the case, the

tdh2Δtdh3Δ cells should partially phenocopy *ubp3Δ* cells with respect to mitochondrial

activity. We first assessed mitochondrial membrane potential in *tdh2Δtdh3Δ* cells.

tdh2Δtdh3Δ cells have higher mitotracker intensity compared to WT cells (Figure S4C). Next,

we measured Cox2 protein, and observed that Cox2 levels are significantly higher in

tdh2Δtdh3Δ cells (Figure 4C). Finally, we measured basal OCR in *tdh2Δtdh3Δ* cells. Consistent

with the mitotracker and Cox2 results, a high basal OCR in *tdh2Δtdh3Δ* cells was also observed

(Figure 4D). Therefore, *tdh2Δtdh3Δ* cells have high mitochondrial activity, comparable to

ubp3Δ cells.

We next asked if increased Pi is necessary to increase mitochondrial activity in both

ubp3Δ and *tdh2Δtdh3Δ* cells. While glycolysis is defective in *ubp3Δ* and *tdh2Δtdh3Δ* cells,

there are key differences in the nodes of regulation in these respective cells. Therefore, it is

necessary to distinguish the effect of high Pi vs. only the effect of low glycolysis in activating

mitochondria in these mutants. Since *ubp3Δ* cells have an ~30% increase in Pi levels, we asked

what would happen to mitochondrial activity if we brought down the levels of Pi in *ubp3Δ*

cells to that of WT cells. Logically, if only decreased glycolysis (independent of Pi) is sufficient

to activate mitochondria, merely bringing down the Pi levels in *ubp3Δ* cells (while maintaining

the low glycolysis in *ubp3Δ* cells) should not affect mitochondrial activity. Notably, *ubp3Δ* cells

grown in low (1 mM) Pi have Pi levels similar to WT cells in standard (normal Pi) medium

(Figure S4D). *ubp3Δ* cells grown in low Pi also had decreased ethanol, suggesting reduced

glycolysis (Figure S4E). Therefore, we used this condition to further understand the role of Pi

in inducing mitochondrial function. We first measured the mitochondrial membrane potential

in *ubp3Δ* cells and *tdh2Δtdh3Δ* cells. Mitotracker fluorescence decreased in both *ubp3Δ* cells

and *tdh2Δtdh3Δ* cells in low Pi (Figure S4F). This suggests that increased Pi in *ubp3Δ* and

tdh2Δtdh3Δ cells is required for increasing mitochondrial membrane potential. Note: basal

mitotracker fluorescence in WT cells also decreases in low Pi, consistent with a necessary role

of Pi levels in regulating mitochondrial activity (Figure S4F). Next, we measured Cox2 protein

in *ubp3Δ* cells and *tdh2Δtdh3Δ* cells in low Pi. Cox2 levels were significantly reduced in both

ubp3Δ and *tdh2Δtdh3Δ* cells in this condition (Figure 4E). Finally, we measured OCRs in these

cells under the same conditions. Consistent with both reduced mitotracker intensity and Cox2

levels, basal OCR also decreased in both *ubp3Δ* and *tdh2Δtdh3Δ* cells in low Pi (Figure 4F).

These data collectively indicate that high intracellular Pi is necessary to increase

mitochondrial activity in *ubp3Δ* and *tdh2Δtdh3Δ* cells.

Next, we asked how mitochondrial Pi transport regulates mitochondrial activity. Mir1

and Pic2 are yeast mitochondrial Pi transporters, with Mir1 being the major Pi transporter

(Murakami et al., 1990; Zara et al., 1996). We first limited mitochondrial Pi availability in

ubp3Δ cells by knocking-out only *MIR1*. In *mir1Δ* cells, the increase in Cox2 observed in *ubp3Δ*

cells was no longer observed (Figure 4G). Consistent with this, we observed no increase in the

basal OCR in *mir1Δubp3Δ* cells compared to *mir1Δ* cells (Figure 4H). This indicates that

mitochondrial Pi transport is necessary for increasing mitochondrial activity in *ubp3Δ* cells.

Furthermore, the loss of Mir1 resulted in a significant decrease in Cox2 protein as well as basal

OCR even in WT cells (Figure 4G, Figure 4H). These data suggest that mitochondrial Pi

transport (via Mir1) is critical for maintaining basal mitochondrial activity even in high glucose.

We also assessed what happens to mitochondrial Pi transporters Mir1 and Pic2 in

ubp3Δ cells, compared to WT cells. No significant increase in Mir1 and Pic2 could be observed

(Figure S4G). This data is consistent with the scenario where *ubp3Δ* cells increase

mitochondrial Pi primarily by increasing available Pi pools, and not by merely increasing the

Pi transporters.

Collectively, these data suggest the possibility that increased cytosolic Pi can increase

the mitochondrial Pi pool, which in turn increases mitochondrial activity. Decreasing

mitochondrial Pi by either reducing total Pi, or by selectively blocking mitochondrial Pi

transport decreases mitochondrial activity.

Mitochondrial Pi availability controls glucose-mediated mitochondrial repression

So far, our data suggest a direct role for the pool of Pi available for the mitochondria,

in regulating mitochondrial activity. Could this, therefore, be a general mechanism of

controlling glucose-mediated mitochondrial repression?

To test this, we first asked how important mitochondrial Pi transport was for the

switch to increased respiration. Highly fermentative yeast cells increase respiration in low

glucose (Broach, 2012; Gasmi et al., 2014). We measured the basal OCR in WT and *mir1Δ* cells

after switching from high (2%) to low (0.1%) glucose. WT cells showed a significant increase

in the basal OCR (Figure 5A) while *mir1Δ* cells did not show an increase in basal OCR (Figure

5A). An alternate scenario wherein cells increase mitochondrial activity is after glycolytic

inhibition. We asked what happens upon 2DG treatment, in the context of mitochondrial Pi

transport and mitochondrial activation. WT cells increased their basal OCR upon a 1-hour

treatment with 2DG, indicating increased respiration (Figure 5B). However, this increase was

not observed in *mir1Δ* cells upon 2DG treatment (Figure 5B). We next measured the

mitochondrial membrane potential with increasing concentrations of 2DG. Mitotracker

fluorescence increased with an increase in 2DG in WT cells. However, this was not observed

in *mir1Δ* cells (Figure S5A). These data indicate that mitochondrial Pi transport is necessary

for increasing mitochondrial activity under glucose derepression.

Next, we asked if the switch to higher respiration involves upregulating the

mitochondrial Pi transporter. We measured Mir1 protein in WT cells in a high (2%) glucose

and after shifting cells to low (0.1%) glucose. Mir1 increases significantly upon a shift to low

glucose, suggesting that Mir1 is glucose repressed (Figure 5C). High Mir1 was also observed

in cells grown in 2% ethanol (where glucose is absent), compared to that of glucose-grown

cells (Figure S5B), further reiterating that Mir1 is glucose-repressed. These data collectively

suggest that decreased Pi (due to high Pi consumption in glycolysis), coupled with decreased

Mir1 protein levels, results in reduced mitochondrial Pi under high glucose.

So far, these results indicate that mitochondrial Pi transport is required for

mitochondrial activity, and that decreasing mitochondrial Pi availability represses

mitochondria. Therefore, we next asked whether increasing mitochondrial Pi is sufficient to

increase mitochondrial activity in high glucose. Since WT cells in high Pi have higher Pi levels

(Figure 3B), it could be prematurely assumed that directly adding Pi to cells grown in high

glucose might increase the Pi accessible to mitochondria, and thereby mitochondrial

respiration. Alternately, since glycolytic flux is maximal in high glucose, merely supplementing

Pi to the cells might be insufficient to increase Pi access to the mitochondria, but might only

further fuel glycolysis (as was originally obseved by Harden and Young in 1908. Interestingly,

supplementing more Pi to the medium did not result in an increase in mitochondrial Pi.

Furthermore, WT cells grown in a high Pi medium have further decreased levels of

mitochondrial Pi (Figure S5C). Consistent with this, directly adding Pi to cells grown in high

glucose have decreased basal OCR (Figure S5D). This suggests that in high glucose, further

supplementing Pi might only serve to sustain glycolysis, but not allow increased Pi access to

the mitochondria.

To further dissect this possibility, we treated cells with glycolytic inhibitor 2DG to

inhibit glycolysis, and then subsequently added Pi and measured the OCR (Figure S5E). In this

case, we observed a significant increase in basal OCR upon supplementing Pi (Figure S5E).

Collectively this suggests that decreasing glycolysis, and increasing Pi levels can now increase

respiration. Next, to unambiguously test the extent of mitochondrial activation based on Pi

availability, we isolated mitochondria, and estimated activity *in vitro* with increasing Pi.

Isolated mitochondria were incubated with 1 mM pyruvate, 1 mM malate, 0.5 mM ADP and

increasing Pi (KH_2PO_4) concentrations, and mitochondrial activity was estimated.

Mitochondrial activity increased with increased Pi, with maximum activity observed with 25

mM Pi (Figure 5D). This reconstitution reveals the extent of mitochondrial activation based

purely on the availability of accessible external Pi. Next, in a complementary experiment, we

overexpressed Mir1 in wild-type cells (under a G6PD promoter), in order to forcibly increase

Pi within mitochondria. For this experiment, we selected clones with an ~2-fold increase in

Mir1 protein (Figure S5F). We first measured Cox2 protein in the Mir1 overexpressing (Mir1-

OE) cells and observed an increase in Cox2 (Figure 5E). Next, we measured the basal OCR in

Mir1-OE cells and observed a significant increase in basal OCR (Figure 5F). Therefore,

increasing mitochondrial Pi transport alone is sufficient to increase mitochondrial activity in

high glucose.

The transport of pyruvate into the mitochondria is required for mitochondrial

respiration (McCommis & Finck, 2015). We asked where Pi availability stands in a hierarchy

of necessary constraints with respect to mitochondrial pyruvate transport, as a requirement

for mitochondrial derepression. We measured the amounts of the Mpc3 subunit (critical for

mitochondrial respiration) of the mitochondrial pyruvate carrier (MPC) complex (Bender et

al., 2015; Timoń-Gómez et al., 2013). Mpc3 protein increases in *ubp3Δ* cells (Figure S5G),

correlating with the increased mitochondrial activity. Interestingly, when *ubp3Δ* cells are

grown in low Pi, Mpc3 protein further increased (Figure S5G), but as shown earlier this
condition cannot increase OCR or mitochondrial activity (Figure 4F, S4F). Next, we measured
the levels of Mpc3 in WT and *mir1Δ* cells in 2% glucose and upon shifting to low (0.1%)
glucose. The basal levels of Mpc3 decrease in *mir1Δ* cells, but upon shifting to low glucose,
Mpc3 levels increase in both WT and *mir1Δ* cells, with comparatively higher levels in *mir1Δ*
cells (Figure S5H). These data now allow a precise interpretation. Even under conditions
where Mpc3 levels are high (*ubp3Δ* cells in low Pi medium, and *mir1Δ* cells in low glucose),
mitochondrial activity remains low if Pi is restricted (Figure 4F, 4H). This indicates that even
though changes in Mpc3 levels accompanies the change in mitochondrial activity,
mitochondrial Pi availability is the primary driver of mitochondrial activity and respiration,
superseding Mpc3 protein. To test if Mpc3 is required for the increase in basal OCR upon
shifting from high to low glucose, we measured the basal OCR in WT and *mpc3Δ* cells. There
was no decrease in basal OCR in *mpc3Δ* cells in high glucose, and the basal OCR increased to
the same level as that of WT cells after shifting to 0.1% glucose (Figure S5I). These data,
suggest that Mpc3 is not required for the increase in basal OCR upon shifting to low glucose.
Finally, since Mpc3 changes with mitochondrial Pi availability (Figure S5G, S5H), we measured
Mpc3 in the Mir1OE strain. No further changes in Mpc3 were observed in Mir1OE cells (Figure
S5J), indicaging that increasing mitochondrial Pi alone need not increase Mpc3 levels. The
increase in Mpc3 levels instead best correlates with decreased glycolysis (*ubp3Δ* cells-Figure
S5G, *ubp3Δ* cells in low Pi-Figure S5G, low glucose medium-Figure S5H), suggesting that Mpc3
protein is repressed during glycolysis. Overall, even though Mpc3 levels respond to changes
in glycolytic rate, increased Mpc3 is insufficient to increase mitochondrial activity and
respiration in the absence of adequate mitochondrial Pi. Contrastingly, increasing
mitochondrial Pi is necessary and sufficient to drive increased mitochondrial activity and
respiration.

Finally, we asked how dependent growth under 2DG treatment is towards
mitochondrial Pi transport. In the presence of 2DG, WT cells exhibit a small decrease in
growth. In contrast, *mir1Δ* cells exhibit a severe growth defect upon 2DG treatment, unlike
WT cells (Figure 5G), revealing a synergetic effect of combining 2DG with inhibiting
mitochondrial Pi transport. This, along with data shown in Figure 5B, show that mitochondrial
Pi transport is required for cells to switch to more respiration upon glycolytic inhibition, and

limiting mitochondrial Pi makes cells hypersensitive to 2DG. Therefore, the combined

inhibition of glycolysis and mitochondrial Pi transport can effectively restrict the growth of

highly glycolytic cells.

Collectively, we find that mitochondrial Pi availability controls glucose-mediated

mitochondrial repression. Increasing available pools of Pi to enter the mitochondria is

sufficient to induce mitochondrial activity regardless of glucose availability.

Discussion

In this study, we highlight a central role for Pi allocations between cytosolic glycolysis

and mitochondrial processes - both of which compete for Pi - as a driver of glucose-mediated

mitochondrial repression. This concept is schematically illustrated in Figure 5H. Ubp3 can

control this process (Figure 1), by allowing high glycolytic flux by maintaining the amounts of

the glycolytic enzymes phosphofructokinase (Pfk1) and GAPDH (Tdh2 and Tdh3). At high rate

of fermentation and growth in *S. cerevisiae*, glycolytic enzymes levels are optimal for

maintaining the high glycolytic rate, and therefore, change in the enzyme levels will have a

direct, proportionate effect on flux (Grigaitis & Teusink, 2022). The loss of Ubp3 results in

decreased glycolytic flux, due to a decrease in these key enzymes, collectively resulting in a

systems-level, mass-action based rewiring of glucose metabolism where more G6P is routed

towards trehalose synthesis and the PPP (Figure 2, Figure 5H). Indeed, previous studies also

find that inhibiting phosphofructokinase reroutes glucose flux from glycolysis to PPP

(Hollinshead et al., 2016; Miyazawa et al., 2017; Yi et al., 2012), and our study may permit

additional, contextualized interpretations of these results. Such reallocations of glucose flux

collectively result in an overall Pi increase, coming from the combined effect of increased Pi

release (from trehalose synthesis), and decreased Pi consumption (via reduced GAPDH). This

changes the intracellular Pi economy to increase the Pi pool available to mitochondria, and

thereby increases mitochondrial activity (Figure 4, Figure 5H). Notably, increased

mitochondrial Pi is necessary and sufficient to increase respiration in high glucose (Figure 4,

Figure 5). Mitochondrial Pi transport is necessary to maintain mitochondrial activity in high

glucose, and also to increase mitochondrial activity in low glucose (Figure 4, Figure 5). Finally,

the mitochondrial Pi transporter Mir1 itself decreases in high glucose (Figure 5). Therefore,
along with high Pi consumption, this glucose-dependent repression of Mir1 restricts
mitochondrial Pi availability (and thereby activity) in high glucose. These data reveal a central
role for Pi budgeting in regulating mitochondrial activity, and mediating mitochondrial
repression.

Traditionally, loss-of-function mutants of metabolic enzymes are used to understand
metabolic state regulation. This approach makes nuanced investigations impossible, since
metabolic enzymes are often essential for viability. Furthermore, metabolic pathways have
multiple, contextually regulated nodes, through which cells maintain their metabolic state.
Therefore, alternate approaches to identify global regulators of metabolic states (as opposed
to single enzymes), might uncover ways via which multiple nodes are simultaneously tuned,
and reveal unanticipated systems-level principles of metabolic state rewiring. Such regulators
can include kinases/phosphatases, ubiquitin ligases/ deubiquitinases,
acetyltransferases/deacetylases, etc., all of which can dynamically regulate multiple nodes. In
this context, the proteostatic regulation of metabolism by reversible ubiquitination (and the
roles of DUBs therein) remain underexplored, perhaps because of the challenges of
identifying specific substrates. In this study of glucose-mediated mitochondrial repression,
the loss of the DUB Ubp3 decreases glycolytic flux by reducing the enzymes at two critical
nodes in the pathway - Pfk1 and GAPDH. A consequence of both these steps is that while they
are coupled to Pi consumption, unlike complete loss of function mutants, a reduction in
amounts will not kill cells but rewire metabolic flux. By 'hitting' multiple steps in glycolysis
simultaneously, *ubp3Δ* cells have decreased Pi consumption, as well as increased Pi release.
Such a cumulative phenomenon reveals more than, for example, inhibiting only GAPDH alone,
where the increase in Pi is only due to reduced Pi consumption, and not from increased
trehalose biosynthesis. Similarly, 2DG shuts down a majority of glycolysis (instead of
dampening the PFK and GAPDH steps), and this results in an acute metabolic rewiring. Our
serendipitous identification of a general regulator, which regulates multiple steps to change
the metabolic environment, suggests a general basis of mitochondrial regulation that would
have otherwise remained hidden. Separately, finding the substrates of Ubp3 and how Ubp3
regulates glycolytic enzymes are exciting future research questions requiring concurrent
innovations in accessible chemical-biological approaches.

This study highlights the importance of intracellular Pi balance in maintaining metabolic states. Because phosphates are required everywhere, it is challenging to identify hierarchies of Pi-dependent processes in metabolic state regulation (Gupta & Laxman, 2021). Phosphate transfer reactions are the foundation of metabolism, driving multiple, thermodynamically unfavorable reactions (Kamerlin et al., 2013; Westheimer, 1987). Contextually, the laws of mass action predicts that the relative rates of these reactions will regulate overall Pi balance, and contrarily the allocation of Pi to these reactions will determine the rate of these Pi-dependent reactions (Gupta & Laxman, 2021; Van Heerden et al., 2014). One way by which cells might control Pi allocations for different reactions comes from compartmentalizing Pi in organelles, to spatially restrict Pi availability (Booth & Guidotti, 1997; Solesio et al., 2021; Vila et al., 2022). Alongside this, the rates of reactions that release and utilize Pi would also determine relative Pi allocations (Gupta & Laxman, 2021; Van Heerden et al., 2014). Our data collectively suggest a paradigm where the combination of these factors regulates mitochondrial activity. In high glucose, in cells with high glycolytic flux (like yeast), mitochondrial Pi availability becomes restricted due to the greater utilization of Pi in glycolysis compared to mitochondria. A consequent limitation in mitochondrial Pi could thereby cause mitochondrial repression. Consistent with this idea, a rapid decrease in intracellular Pi upon glucose addition has been observed (Koobs, 1972; Rodríguez-Enríquez et al., 2001; Sussman et al., 1980). Further, supplementing Pi correlates with decreased mitochondrial repression in tumors (Brin & McKee, 1956; Koobs, 1972; Wu & Racker, 1959). Concurrently, the levels of the mitochondrial Pi transporter Mir1 is also glucose-repressed, further limiting mitochondrial Pi uptake. By increasing Pi through a systems-level rewiring of glucose metabolism (such as in *ubp3Δ* cells), cells collectively increase mitochondrial access to Pi. Since Pi is critical for multiple mitochondrial processes, this relative Pi budgeting can determine mitochondrial activity. Notably, even though directly supplementing Pi decreases mitochondrial repression in tumors (Koobs, 1972), our data suggests that this alone might not be sufficient in highly glycolytic cells such as yeast. In these conditions, adding Pi further increases glycolysis, as was first observed during the beginnings of modern biochemistry (Harden & Young, 1908). Indeed, directly supplementing Pi to glycolytic cells decreases mitochondrial Pi and respiration (Figure S5). Interestingly, supplementing Pi under conditions of low glycolysis (where mitochondrial Pi transport is already enhanced), as well as directly supplementing Pi to isolated mitochondria, increases respiration (Figure 5, S5). Therefore, in

order to deprepress mitochondria, a combination of increasing Pi along with decreasing glycolysis is required. An additional explanation for this phenomenon is the change in intracellular pH that accompanies the changes in glycolytic state. The cytosolic pH in highly glycolytic cells is ~7 and decreasing glycolysis results in cytosolic acidification (Dechant et al., 2010; Orij et al., 2011). Since mitochondrial Pi transport is itself dependent on the proton gradient, a low cytosolic pH would favour mitochondrial Pi transport (Hamel et al., 2004). Therefore, under conditions of decreased glycolysis (2DG treatment, deletion of Ubp3, and decreased GAPDH activity), where cytosolic pH is acidic, increasing cytosolic Pi might indirectly increase mitochondria Pi transport, thereby leading to increased respiration. Alternately, increasing mitochondrial Pi transporter amounts to enhance mitochondrial Pi uptake can also achieve the same result, as seen by overexpressing Mir1 (Figure 5). A similar observation has been reported in *Arabidopsis*, reiterating an evolutionarily conserved role for mitochondrial Pi in controlling respiration (Jia et al., 2015). Relatedly, glycolytic inhibition can suppress cell proliferation in Warburg-positive tumors (Pelicano et al., 2006), or inflammatory responses (Soto-Heredero et al., 2020). The glucose analog 2-deoxyglucose (2DG) inhibits the hexokinase and phosphoglucose isomerase catalyzed steps (Laussel & Léon, 2020), and has been used to inhibit 'Warburg' positive cancer cells (Aft et al., 2002; O'Neill et al., 2019). However, these cells survive by switching to mitochondrial respiration (Lu et al., 2015; Oliveira et al., 2021; Shiratori et al., 2019), requiring alternate approaches to prevent their proliferation (Cheng et al., 2012). Inhibiting mitochondrial Pi transport in combination with glycolytic inhibition could effectively restrict the proliferation of Warburg/Crabtree positive cells. As seen in *ubp3Δ* cells, this mode of regulating Pi balance will therefore have systems-level changes in the metabolic milieu, enhancing certain types of metabolic reactions to thereby control metabolic states.

Since its discovery in the 1920s, the phenomenon of accelerated glycolysis with concurrent repression of mitochondrial activity has been intensely researched. Yet, a parsimonious explanation of the biochemical basis of glucose-mediated mitochondrial repression remains missing. One hypothesis suggests that the availability of glycolytic intermediates might determine a biochemical basis of the Crabtree effect. F1,6BP inhibits complex III and IV of the electron transport chain (ETC) in Crabtree positive yeast (Diaz-Ruiz et al., 2011; Díaz-Ruiz et al., 2008; Hammad et al., 2016; Lemus et al., 2018). Similarly, the

ratio between G6P and F1,6BP regulates the extent of mitochondrial repression (Díaz-Ruiz et al., 2008; Lemus et al., 2018). Therefore, glycolytic intermediates might trigger the Crabtree effect (Hammad et al., 2016). Our study now clarifies these hypotheses, placing Pi centre stage. Although G6P/F6P accumulates in *ubp3Δ* cells (Figure 2C), this is not the case in *tdh2Δtdh3Δ* (GAPDH mutant) cells (Figure S3D), suggesting that G6P/F6P accumulation in itself is not the prime criterion to increase mitochondrial activity. Separately, the competition for common metabolites/co-factors between glycolysis and respiration (such as ADP, Pi or pyruvate) could drive this phenomenon (Diaz-Ruiz et al., 2011; Koobs, 1972). In *ubp3Δ* cells growing in low Pi, there is decreased glycolysis (Figure S4E) concurrent with increased mitochondrial pyruvate transporter (Mpc3) levels (Figure S5G). According to the above hypothesis, this should increase mitochondrial activity. However, these cells have decreased mitochondrial activity. Similarly, switching to low glucose increases Mpc3 levels in *mir1Δ* cells, but cannot increase mitochondrial respiration (Figure S5H, 5A), and basal OCR in *mpc3Δ* cells increases in low glucose similar to WT cells (Figure S5I). Therefore, in alternate scenarios, such as contexts where amino acids can fuel TCA cycle, Mpc3 is not critical to switch to high respiration. These collectively reiterate a dominant role of mitochondrial Pi transport (and Pi access) in regulating mitochondrial respiration. Our data also clarifies or contextualizes observations from other model systems where mitochondrial Pi transport seems to regulate mitochondrial respiration (Poncet et al., 2006; Scheibye-Knudsen & Quistorff, 2009; Seifert et al., 2015).

Relying on parsimony, we propose that Pi access to the mitochondria provides a simple explanation for the biochemical basis of the Crabtree effect. A single-step event in evolution, of either reducing mitochondrial Pi transporter amounts, and/or increasing glycolytic flux (to deplete cytosolic Pi), will result in whole-scale metabolic rewiring to repress mitochondria. Such an event need not invoke the evolution of elaborate regulatory mechanisms where glycolytic intermediates allosterically inhibit mitochondrial processes, and such regulatory events can easily be imagined as subsequent adaptations to enforce mitochondrial repression. Given the central role played by Pi in cells, it is not surprising that something as fundamental as access to Pi could determine mitochondrial activity. The basis of the Crabtree effect can be explained by this simple competition for Pi between glycolysis and mitochondrial processes. The rapid incorporation of Pi into faster glycolysis gives cells a

606 proliferation advantage, while also sequestering Pi in the form of usable ATP. Over the course

607 of evolution, this can drive other regulatory mechanisms to enforce mitochondrial repression,

608 plausibly leading to the currently observed complex regulatory networks and signaling

609 programs and the Crabtree effect we know today.

Materials and methods

Yeast strains, media, and growth conditions

A prototrophic CEN.PK strain of *Saccharomyces cerevisiae* (WT) (J.P. van Dijken et al., 2000)

was used. Strains used in this study are listed in Table S1. Gene deletions and chromosomal C

terminal tagged strains were generated by standard PCR mediated gene deletion or tagging,

using drug resistance markers (Longtine et al., 1998). Cells were grown overnight in a standard

high glucose, at 30°C, and subcultured in the required medium. For experiments involving a

shift to a no-Pi or low glucose medium, cells were subcultured in high glucose till OD_{600}~0.6,

pelleted by centrifugation (1000 x g, 2 min at RT), washed, and shifted to no-Pi or low glucose

medium for 1 hour. The detailed media compositions and growth conditions are described in

extended methods (supplement file 1).

Mitotracker fluorescence assay

Cells were grown to OD_{600}~0.6, 1 OD_{600} cells were collected by centrifugation (1000 x g, 1 min

at RT), and resuspended in 1 ml fresh media. Mitotracker CMXRos (M7512 ThermoFisher) was

added to a final concentration of 200 nM and incubated at 30°C in a shaking incubator. Cells

were washed and resuspended in fresh media and fixed with 2% formaldehyde for 20

minutes. Fixed cells were washed in 1xPBS, pH 7.4, and resuspended in 1 ml 1xPBS. 300 µl of

this was aliquoted onto 96 well plates in replicates. Mitotracker fluorescence was measured

using Thermo Varioscan™ LUX multimode plate reader at 579/599 excitation/emission

wavelengths. Mitotracker fluorescence readings were normalized using OD_{600} of each sample

and the relative fluorescence intensity was calculated. Statistical significance was calculated

using unpaired Student's t-test (GraphPad prism 9.0.1).

Mitotracker based screen to identify DUBs that regulate mitochondrial repression

The deubiquitinase deletions were made by PCR mediated gene deletion, DUB deletion mutants generated were grown in high glucose and the mitochondrial membrane potential measured by the mitotracker assay described earlier. For performing the screen, the 19 DUB knockouts were divided into two groups- each containing 10 and 9 DUB KOs respectively and the screen was performed separately for each group in replicates. WT cells grown in high glucose indicated the basal mitochondrial potential and WT cells grown in ethanol was used as a positive control for each group. The mitotracker fluorescence intensity were normalised to OD_{600} of each mutant and the fluorescence intensity relative to the WT cells were calculated. The relative fluorescence intensity for the replicate samples are shown in supplementary figure S1A, mean fluorescence intensities of the replicates (relative to WT) were calculated and plotted as heat maps (Figure 1C).

CRISPR-Cas9 based mutagenesis

To generate the Ubp3^{C469A} strain, cells were transformed a constitutive Cas9 expressing plasmid (Addgene plasmid 43802, (Dicarlo et al., 2013)). Guide RNA (gRNA) sequences (forward and reverse) were annealed and cloned into a pMEL13 plasmid. Strains were then transformed with gRNA plasmid and homology repair (HR) fragments with the base pair mutation. The HR fragment was generated by site-directed mutagenesis PCR. Positive clones were selected using drug, and sequenced to confirm the mutation. Oligonucleotides used are listed in Table S2.

Serial dilution growth assay

For serial dilution-based growth assays, cells were grown in standard high glucose to OD_{600}~0.8, collected by centrifugation (1000 x g, 1 min at RT), washed with water, serial dilutions made (OD_{600}=1, 0.1, 0.01, 0.001, 0.0001), 5 µl of each dilution were spotted onto agar plates, incubated at 30°C and growth monitored.

Protein extraction and Western blotting

The cells were grown in the indicated medium to OD_{600}~0.8 and pelleted by centrifugation (1000 x g, 2 min at RT). Total protein was precipitated and extracted using trichloroacetic acid (TCA), and resuspended in SDS/glycerol buffer as described earlier (Vengayil et al., 2019). For

loading controls, the same gel fragments were stained using Coomassie R-250. All western

blot quantifications were done using ImageJ software. Graphs were plotted using GraphPad

prism 9.0.1. Detailed protocol is described in extended methods (supplement file 1).

Basal OCR measurement using Seahorse assay

The basal OCR was measured using Agilent Seahorse XFe24 analyzer. 1 ml of the XF Calibrant

solution was aliquoted into each well of the utility plate and the sensor cartridge plate was

hydrated overnight as per the manufacturer's instructions. The sensor cartridge was loaded

with complex IV inhibitor sodium azide and equilibrated in the Seahorse XFe24 analyzer one

hour prior to the start of the assay. The culture plate was coated with 50 µl poly-L-lysine and

incubated for 1 hour at RT. Excess poly-L-lysine was removed and the plate dried at 30°C for

30 minutes. The cells were grown in standard high glucose to an OD_{600}~0.6. The samples were

aliquoted to poly-L-lysine coated wells at a final cell number in each well of ~3x10^5. The

plate was centrifuged for 2 min at 100xg (acceleration 2, brake 2) and incubated at 30°C for

30 minutes. The plate was loaded to the Seahorse XFe24 analyzer and basal OCR measured

over time. A minimum of three measurements were taken with intermittent 2 min mixing and

waiting steps. This was followed by sodium azide injection to the sample wells, and three

measurements were taken with intermittent 2 min mixing and waiting steps. The basal OCR

readings were normalized to the OD_{600} of samples in each well (for cell numbers), and graphs

were plotted using GraphPad prism 9.0.1.

ATP assay

The ATP levels were measured by ATP estimation kit (Thermo Fisher A22066). Detailed sample

collection protocol is described in extended methods (supplement file 1). The reaction

mixture was prepared as per manufacturer's instructions and 10 µl of sample was mixed with

90 µl of reaction mix and luminescence was measured using Sirius luminometer (Tiertek

Berthold). The ATP standards prepared in 20 mM Tris-HCl, pH 7.0 was used to plot the

standard curve and to estimate the ATP concentration in the samples. The relative ATP

concentrations were calculated, graphs were plotted and using GraphPad prism 9.0.1.

Metabolite extraction and analysis by LC-MS/MS

The steady state levels as well as relative ^{13}C label incorporation into metabolites were

estimated by quantitative LC-MS/MS methods as described in (Walvekar et al., 2018). The

detailed methodology is described in extended methods (Supplement file 1). All the peak

area measurements for the steady state and ^{13}C glucose flux experiments are listed in

supplement file 2.

Ethanol estimation assay

696   The ethanol concentration in the medium was estimated using potassium dichromate based

697   assay as described in (Sriariyanun et al., 2019) with modifications. The detailed protocol is

698   described in extended methods (Supplement file 1). The statistical significance was calculated

699   using unpaired Student's t-test (GraphPad prism 9.0.1).

Malachite green based Pi estimation

The cells were grown to OD_{600}~0.8, pelleted by centrifugation (1000 x g, 2 min at RT), pelleted

cells were washed and resuspended in 1 ml ice-cold HPLC grade water (to avoid Pi

contamination) and lysed by bead beating (3 x 20s, 1 min intermittent cooling). Lysates were

centrifuged (14000 rpm, 1 min at 4°C) and 800 µl of the clear supernatant collected. Protein

concentrations of the supernatant were estimated using BCA assay (BCA assay kit, G-

Biosciences). Lysates corresponding to 1µg total protein was used for the Pi estimation assay.

Pi estimation was done using a malachite green phosphate assay kit (Cayman chemicals,

10009325) as per the manufacturer's instructions. Statistical significance was calculated using

unpaired Student's t-test (GraphPad prism 9.0.1).

Mitochondrial isolation by immunoprecipitation

Mitochondria was isolated by immunoprecipitation as described in (Chen et al., 2017; Liao et

al., 2018) with modifications. The detailed sample preparation protocol is described in

extended methods (supplement file 1). The eluted mitochondria were used in malachite

green assay for Pi estimation or boiled with SDS-glycerol buffer for western blots.

For mitotracker assays with isolated mitochondria (Figure 5D), the mitochondria enriched

fraction resuspended in SEH buffer was centrifuged twice- 700 x g for 5mins and 1500 x g for

5 mins at 4°C. The supernatant was collected and centrifuged at 12000 x g for 10 mins at 4°C

and the pellet was resuspended in ice-cold SEH buffer. The protein concentration of this crude mitochondria was estimated by BCA protein estimation assay and mitochondria corresponding to 5 µg protein was incubated in 200 µl SEH buffer containing 1 mM pyruvate, 1 mM malate, 0.5 mM ADP and $0 - 50$ mM KH_2PO_4 for 30 minutes at 30°C. Mitotracker CMXROS was added at a final concentration of 200 nM, followed by incubation at 30°C for 30 minutes. The fluorescence intensities were measured using Thermo Varioscan™ LUX multimode plate reader at 579/599 excitation/emission, and fluorescence intensities relative to samples containing 0 mM KH2PO4 was calculated. Statistical significance was calculated using unpaired Student's t-test (GraphPad prism 9.0.1).

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Author contributions

VV and SL conceptualized the study and designed experiments. VV, SN, SA, SV performed experiments. All data were analyzed by VV and SL, and specific experiments were analyzed by SN, SA and SV. VV and SL wrote the manuscript, including inputs from SN, SA and SV. SL secured funding for this study.

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 Figure legends

 Figure 1: A deubiquitinase deletion screen identifies Ubp3 as a regulator of glucose-
 mediated mitochondrial repression

A) Schematic depicting glucose-mediated mitochondrial repression (Crabtree effect).
Crabtree positive cells have high fermentation and repressed mitochondrial activity in high
glucose.

B) Schematic describing the screen with a yeast library with individual knock-outs of
deubiquitinase enzymes (DUBs), to identify regulators of glucose-mediated mitochondrial
repression.

C) Identifying DUB deficient cells with altered mitochondrial potential. The heat map shows
the relative mitochondrial membrane potential of 19 deubiquitinase deletion mutants in high
glucose. The cells were grown in high (2%) glucose, stained with mitotracker CMXRos and
fluorescence intensity measured. Mean mitotracker intensity obtained from 2 biological
replicates is shown in the heatmap. Also see supplementary figure S1A, S1B.

D) The deubiquitinase activity of Ubp3 and glucose-mediated repression of mitochondrial
membrane potential. A catalytically inactive mutant of Ubp3, Ubp3^{C469A} was generated. WT,
ubp3Δ, and Ubp3^{C469A} cells were grown in high glucose and the relative mitochondrial
membrane potential was measured. Data represent mean ± SD (n=3). Also see supplementary
figure S1D.

E) Effect of loss of Ubp3 on the electron transport chain complex IV subunit protein Cox2. WT
and *ubp3Δ* cells were grown in high glucose and Cox2 protein was measured by western blot
using an anti-Cox2 antibody. A representative blot (out of 3 biological replicates, n=3) is
shown in the panel.

F) Basal oxygen consumption rate (OCR) in high glucose in *ubp3Δ* cells. WT and *ubp3Δ* cells
were grown in high glucose medium and the basal OCR was measured using a seahorse XFe24
analyzer. Sodium azide (complex IV inhibitor) injection was done after three basal OCR
measurements to estimate the non-mitochondrial respiration. Basal OCR corresponding to ~3

x 10^5 cells, from two independent experiments (n=2), normalized to the OD_{600} is shown. Bar

graph representations of the data are shown in the inset.

G) Total ATP levels in *ubp3Δ* and WT cells. WT and *ubp3Δ* cells were grown in high glucose

medium, and the total ATP were measured. Data represent mean ± SD (n=3).

H) Dependence of *ubp3Δ* cells on mitochondrial ATP. WT and *ubp3Δ* cells were grown in high

glucose medium, and treated with 1 mM sodium azide for 45 minutes. The total ATP levels in

sodium azide treated and untreated cells were measured. Data represent mean ± SD (n=3).

I) Requirement for mitochondrial respiration in high glucose in *ubp3Δ* cells. Serial dilution

growth assay in high glucose in the presence and absence of ETC complex IV inhibitor sodium

azide (1 mM) are shown. Also see supplementary figure S1F.

The Coomassie-stained gel for total protein was used as a loading control for all western blots.

All western blot quantifications were done using ImageJ software. The graphs were plotted

using GraphPad prism 9.0.1. The statistical significance was calculated using an unpaired-

student t-test. *p<0.05, **p<0.01, ***p<0.001.

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Figure 2: Key glycolytic enzymes decrease and glucose flux is rerouted in *ubp3Δ* cells

A) A schematic illustrating directions of glucose-6-phosphate (G6)) flux in cells. Glucose is

converted to G6P, which is a precursor for trehalose, the pentose phosphate pathway (PPP),

and glycolysis. In high glucose, *S. cerevisiae* cells have high glycolysis, and pyruvate is

converted to ethanol (fermentation). Phosphofructokinase 1 (Pfk1) catalyzes the conversion

of fructose-6 phosphate (F6P) to fructose-1,6 bisphosphate (F1,6 BP) which is the committed

step of glycolysis. F1,6 BP is converted to glyceraldehyde-3 phosphate (G3P) which is then

converted to 1,3 bisphosphoglycerate (1,3 BPG) by the enzyme glyceraldehyde phosphate

dehydrogenase (GAPDH). Two major isozymes of GAPDH in yeast- Tdh2 and Tdh3 are shown

in the schematic.

B) Effect of loss of Ubp3 on key glycolytic enzymes. WT and *ubp3Δ* cells containing epitope

tagged Pfk1, Tdh2, and Tdh3 were grown in high glucose and the protein levels were

measured by western blot using an anti-FLAG antibody. A representative blot (out of n=3) is

shown. Also see supplementary figure S2A.

C) Steady-state metabolite amounts in WT and *ubp3Δ* cells in high glucose. The relative

steady-state levels of trehalose, major glycolytic, and PPP intermediates were estimated using

targeted LC-MS/MS in WT and *ubp3Δ* cells grown in high glucose. Data represent mean ± SD

(n=3). Also see table S3.

D) Relative glycolytic and trehalose synthesis flux in WT and *ubp3Δ* cells. The relative ^{13}C label

incorporation into trehalose and glycolytic intermediates, after a pulse of 1% $^{13}C_6$ glucose is

shown. Data represent mean ± SD (n=3). Also see table S3, figures S2B, S2C.

E) Ethanol production in *ubp3Δ* cells. WT and *ubp3Δ* cells were grown in high glucose and the

ethanol concentration in the media was measured. Data represent mean ± SD (n=3).

F) Relative rate of ethanol production in WT vs *ubp3Δ* cells. WT and *ubp3Δ* cells were grown

in high glucose and at OD_{600} ~ 0.6, equal number of cells were shifted to fresh medium (high

glucose) and the ethanol concentration in the medium was measured temporally. Data

represent mean ± SD (n=3)

Statistical significance was calculated using unpaired-student t-test. *p<0.05, **p<0.01,

***p<0.001.

1127

Figure 3: Phosphate (Pi) accumulates due to altered Pi homeostasis in *ubp3Δ* cells

A) Schematic showing the maintenance of Pi balance during glycolysis. Trehalose synthesis

from G6P releases free Pi, and the conversion of G3P to 1,3BPG by GAPDH is a major Pi

consumer. This balance between Pi release and consumption maintains cytosolic Pi levels. In

ubp3Δ cells, there is an increase in trehalose biosynthesis (which releases Pi). *ubp3Δ* cells have

decreased GAPDH, which will decrease Pi consumption. This increase in Pi release along with

a decrease in Pi consumption could increase cytosolic Pi.

B) Intracellular Pi levels in WT and *ubp3Δ* cells. WT and *ubp3Δ* cells were grown in high glucose

and the total free phosphate (Pi) levels were estimated. WT cells grown in high Pi (2% glucose,

10mM Pi) was used as the positive control. Data represent mean ± SD (n=3). Also see

supplementary figure S3A.

C) Pho regulon responses in WT and *ubp3Δ* cells. Protein levels of Pho84-FLAG and Pho12-

FLAG were compared between WT cells grown in high glucose and in high Pi, *ubp3Δ* cells in

high glucose with or without a shift to a no-Pi medium for one hour, by western blot using an

anti-FLAG antibody. A representative blot (out of n=3) is shown.

D) Contribution of trehalose synthesis as a Pi source in *ubp3Δ* cells. WT, *tps2Δ*, *ubp3Δ,* and

ubp3Δtps2Δ cells were grown in high glucose and the total Pi levels were estimated by

malachite green assay. Data represent mean ± SD (n=3). Also see supplementary figure S3B.

E) Loss of GAPDH isozymes Tdh2 and Tdh3 and effect on Pi levels. WT, *ubp3Δ,* and *tdh2Δtdh3Δ*

cells were grown in high glucose and the total Pi levels were estimated. Data represent mean

± SD (n=3). Also see supplementary figure S3F.

The statistical significance was calculated using unpaired-student t-test. *p<0.05, **p<0.01,

***p<0.001.

1151

Figure 4: Phosphate availability dictates mitochondrial activity

A) Schematic depicting a hypothetical mechanism of cytosolic free Pi controling mitochondrial

activity by regulating mitochondrial Pi availability. In *ubp3Δ* cells, the increased Pi release

coupled with the decreased Pi consumption results in increasing Pi, which can increase the Pi

available for transport to mitochondria and enhance mitochondrial activity.

B) Mitochondrial Pi amounts in WT vs *ubp3Δ* cells. Mitochondria were isolated by

immunoprecipitation from WT and *ubp3Δ* cells and mitochondrial Pi was estimated. The Pi

levels in mitochondria, normalised to Idh1 protein levels are shown. Data represent mean ±

SD (n=3). Also see supplementary figure S4A, B.

C) Cox2 protein in *tdh2Δtdh3Δ* cells. WT, *ubp3Δ,* and *tdh2Δtdh3Δ* cells were grown in high

glucose and Cox2 protein was estimated by western blot. A representative blot (out of n=3)

is shown in the panel.

D) Basal OCR levels in high glucose in *tdh2Δtdh3Δ* cells. WT, *ubp3Δ,* and *tdh2Δtdh3Δ* cells were grown in high glucose and the basal OCR was measured from two independent experiments (n=2). Also see supplementary figure S4C.

E) Pi amounts and Cox2 protein levels in *ubp3Δ* and *tdh2Δtdh3Δ* vs WT cells. WT cells were grown in high glucose, *ubp3Δ* and *tdh2Δtdh3Δ* cells were grown in high glucose and low Pi medium, and Cox2 protein was estimated by western blot. A representative blot (n=3) is shown. Also see supplementary figure S4D, S4F.

F) Pi amounts and basal OCR in *ubp3Δ* and *tdh2Δtdh3Δ* vs WT cells. WT cells were grown in high glucose, *ubp3Δ* and *tdh2Δtdh3Δ* cells were grown in high glucose and low Pi medium, and the basal OCR was measured from two independent experiments (n=2).

G) Effect of loss of mitochondrial Pi transporter Mir1 on Cox2 protein. WT, *ubp3Δ*, *mir1Δ,* and *mir1Δubp3Δ* cells were grown in high glucose and Cox2 protein was estimated by western blot. A representative blot (n=3) is shown.

H) Relationship of mitochondrial Pi transport and basal OCR in WT vs *ubp3Δ* cells. WT, *ubp3Δ*, *mir1Δ,* and *mir1Δubp3Δ* cells were grown in high glucose and the basal OCR was measured from two independent experiments (n=2).

The statistical significance was calculated using unpaired-student t-test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure 5: Mitochondrial Pi availability controls glucose-mediated mitochondrial repression.

A) Relationship of mitochondrial Pi transport and respiration after glucose removal. WT and *mir1Δ* cells were cultured in high glucose (2% glucose) and shifted to low (0.1%) glucose for 1 hour. The basal OCR with and without a shift to low glucose was measured. The basal OCR corresponding to ~3 x 10^5 cells, normalized to the OD_{600}, from two independent experiments (n=2) are shown.

B) Requirement of mitochondrial Pi transport for cells to switch to respiration upon glycolytic inhibition by 2DG. WT and *mir1Δ* cells were cultured in high glucose and treated with or

without 0.25% 2DG for 1 hour. The basal OCR was measured from two independent

experiments (n=2). Also see supplementary figure S5A.

C) Glucose-dependent regulation of the mitochondrial Pi transporter Mir1. Cells containing

endogenous Mir1 chromosomally tagged at the C terminus with a 6x HA epitope tag were

grown in a high glucose and shifted to low glucose (0.1% glucose) for 1 hour. The protein

levels of Mir1 were estimated and compared. A representative blot (out of n=3) is shown in

the panel. Also see supplementary figure S5B.

D) Increasing Pi concentrations and mitochondrial activity in isolated mitochondria.

Mitochondria were isolated from WT cells grown in high glucose, incubated with 1 mM

pyruvate, 1 mM malate, 0.5 mM ADP and 0-50 mM KH_2PO_4. The mitochondrial activity was

estimated by measuring mitotracker fluorescence intensity, and relative intensities

normalised to the sample with 0 mM KH_2PO_4 is shown. Data represent mean ± SD (n=3).

E) Effect of overexpressing Mir1 on Cox2 protein. Mir1-HA was overexpressed under a G6PD

promoter. WT (containing empty vector) and Mir1 overexpressing (Mir1 OE) cells were grown

in high glucose and Cox2 protein was estimated by western blot. A representative blot (out

of n=3) is shown in the panel. Also see supplementary figure S5F.

F) Effect of overexpressing Mir1 on basal OCR. WT (containing empty vector) and Mir1 OE

cells were grown in high glucose medium, and the basal OCR was measured from two

independent experiments (n=3).

G) Mitochondrial Pi transport requirement for growth after 2DG treatment. Shown are serial

dilution growth assays in high glucose in the presence and absence of 0.1% 2DG, using WT

and *mir1Δ* cells. The results after 40hrs incubation at 30°C are shown.

I) A model illustrating how Pi availability to the mitochondria controls mitochondrial activity.

In high glucose, the decreased Pi due to high Pi consumption in glycolysis, along with the

glucose-mediated repression of mitochondrial Pi transporters, results in decreased

mitochondrial Pi availability. This results in reduced mitochondrial activity. In low glucose,

increased mitochondrial Pi transporters (and lower glycolytic flux) increases the

mitochondrial Pi, leading to enhanced mitochondrial activity. In *ubp3Δ* cells in high glucose,

high trehalose synthesis and lower glycolytic flux results in an increase in Pi levels. This increases the mitochondrial Pi availability and thereby increases the mitochondrial activity.

The statistical significance was calculated using unpaired-student t-test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Supplement figures:

Figure S1:

A) A list of the known/identified *S. cerevisiae* deubiquitinase enzymes is shown. The DUBs which were used in this study, and the mitotracker intensity of two biological replicates (n=2) relative to WT are shown.

B) WT cells grown in a respiratory, ethanol medium and mitotracker fluorescence intensity compared to glucose medium. WT cells were grown in high glucose medium (2% glucose) and ethanol medium (2% ethanol), and the mitochondrial membrane potential was measured. Data from two independent experiments (n=2) is shown.

C) Schematic representation of the conserved domains of Ubp3 and its mammalian ortholog Usp10. The catalytic cysteine of Usp10 and Ubp3 is highlighted.

D) The catalytically inactive mutant of Ubp3, Ubp3^{C469A} does not have altered Ubp3 protein levels. WT and Ubp3^{C469A} cells containing endogenous Ubp3 tagged with 3xFLAG (C terminus) were grown in high glucose and the protein levels of Ubp3 were measured. A representative blot (n=3) is shown in the panel.

E) Tom70 protein amounts do not change in *ubp3Δ* cells. WT and *ubp3Δ* cells containing endogenous Tom70 tagged at the C terminus with a 3xFLAG epitope were grown in high glucose and the protein levels of Tom70 were measured by western blot. A representative blot (n=3) is shown.

1243   F) Loss of proteins regulating mitochondrial respiration and growth defects in *ubp3Δ* cells in

1244   high glucose. Serial dilution growth assay was done in high glucose. The results after 24 hours

1245   of incubation at 30°C are shown.

1246   The statistical significance was calculated using unpaired-student t-test. *p<0.05, **p<0.01,

1247   ***p<0.001.

1248

Figure S2:

A) Loss of Ubp3 does not change the protein levels of enolase isozymes Eno1 and Eno2. WT

and *ubp3Δ* cells containing Eno1 and Eno2 C terminus endogenously tagged with a 3x FLAG

epitope, were grown in high glucose and protein amounts were estimated. A representative

blot (out of n=3) is shown in the panel.

B) Schematic showing the experimental design for measuring ^{13}C label incorporation into

glycolytic intermediates and trehalose using a $^{13}C_6$ glucose-pulse.

C) Changes in ^{13}C label incorporation into glycolytic intermediates and trehalose with time,

and linearity of label incorporation after a pulse of 1% $^{13}C_6$ glucose is shown.

The statistical significance was calculated using unpaired-student t-test. *p<0.05, **p<0.01,

***p<0.001.

1260

Figure S3:

A) Loss of Ubp3 deubiquitinase activity and Pi levels. WT, *ubp3Δ,* and Ubp3^{C469A} cells were

grown in high glucose and the total free phosphate (Pi) levels were estimated. Data from two

independent experiments (n=2) is shown.

B) Trehalose levels in WT and *ubp3Δ* cells in the absence of Tps2. *tps2Δ* and *ubp3Δtps2Δ* cells

were grown in high glucose, and the trehalose levels were estimated using targeted LC-

MS/MS. Data represent mean ± SD (n=3).

C) Loss of GAPDH isozymes Tdh2 and Tdh3 and ethanol production. WT and *tdh2Δtdh3Δ* cells were grown in high glucose and the ethanol concentration in the medium was measured. Data are represented as mean ± SD (n=3). Note: The WT shown here is same as the WT in Figure 2E, since the ethanol assays in Figure 2E and Figure S3C were done together and the common WT control was used.

D) Steady state levels of glycolytic intermediates: *tdh2Δtdh3Δ* cells have unaltered steady state levels of G6P/F6P, increased levels of F1,6 BP and G3P and decreased levels of 3PG and PEP. WT and *tdh2Δtdh3Δ* cells were grown in high glucose and the steady state amounts of glycolytic intermediates were estimated using targeted LC-MS/MS. Data represent mean ± SD (n=3). Also see table S3.

E) Trehalose levels in *tdh2Δtdh3Δ* cells. WT and *tdh2Δtdh3Δ* cells were grown in high glucose and trehalose amounts were estimated using targeted LC-MS/MS. Data represent mean ± SD (n=3).

F) Pi levels in *ubp3Δ* and *tdh2Δtdh3Δ* cells in ethanol medium. WT, *ubp3Δ,* and *tdh2Δtdh3Δ* cells were grown in ethanol medium and the total Pi levels were estimated. Data represent mean ± SD (n=3).

The statistical significance was calculated using unpaired-student t-test. *p<0.05, **p<0.01, ***p<0.001.

Figure S4:

A) Lack of vacuolar contamination in mitochondria isolated by immunoprecipitation. Mitochondria were isolated from WT cells grown in high glucose and the protein levels of Tom20, Cox2, Idh1 and Vph1 and were measured in cell lysate and immunoprecipitated mitochondria by western blot.

B) Idh1 levels in *ubp3Δ* cells and WT cells grown in high Pi. WT cells were grown in high glucose, or high glucose and high Pi medium (10mM Pi) and *ubp3Δ* cells were grown in high

glucose, and the Idh1 protein levels were estimated by western blot. A representative blot (out of n=3) is shown.

C) Mitochondrial membrane potential in *tdh2Δtdh3Δ* cells. WT, *ubp3Δ,* and *tdh2Δtdh3Δ* cells were grown in high glucose and the mitochondrial membrane potential was measured. Data represent mean ± SD (n=3)

D) Pi levels in *ubp3Δ* cells grown in low Pi (1mM Pi) vs WT cells. WT cells were grown in high glucose and *ubp3Δ* cells were grown in high glucose or high glucose-low Pi, and the total Pi levels were estimated. Data represent mean ± SD (n=3).

E) Ethanol production in *ubp3Δ* cells grown in a low Pi medium. WT cells were grown in high glucose and *ubp3Δ* cells were grown in high glucose or high glucose-low Pi, and the ethanol concentration in the medium was measured. Data represent mean ± SD (n=3).

F) Pi amounts and mitochondrial membrane potential in WT, *ubp3Δ,* and *tdh2Δtdh3Δ* cells. The cells were grown in high glucose or high glucose-low Pi, and the mitochondrial membrane potential was measured. Data from two independent experiments (n=2) is shown.

G) The protein levels of mitochondrial Pi transporters Mir1 and Pic2 in *ubp3Δ* cells. WT and *ubp3Δ* cells containing endogenously tagged Mir1 and Pic2 at their C terminus with a 6x HA epitope tag were grown in high glucose and Mir1 and Pic2 proteins were measured by western blot. A representative blot (out of n=3) is shown.

The statistical significance was calculated using unpaired-student t-test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure S5:

A) Requirement of Mir1 for switching to increased mitochondrial activity upon 2-deoxyglucose (2-DG) treatment. WT and *mir1Δ* cells were grown in high glucose and treated with 0.1% and 0.25% 2-DG for one hour. The mitochondrial membrane potential was measured from two independent experiments (n=2).

B) Mir1 protein levels and glucose repression. Cells containing Mir1 endogenously tagged at the C terminus with a 6x HA epitope tag were grown in high glucose or a respiratory medium (2% ethanol). Mir1 protein was estimated by western blot. A representative blot (n=3) is shown in the panel.

C) Effect of supplementing Pi in the medium on mitochondrial Pi. WT cells were grown in high glucose, and high glucose, high Pi medium (10 mM Pi), mitochondria was isolated and the mitochondrial Pi levels were estimated. The mitochondrial Pi levels normalised to Idh1 protein levels is shown. Data represent mean ± SD (n=3). Also see supplementary figure S4B.

D) Increasing the Pi concentration in a high glucose medium results in a decrease in basal OCR. WT cells were grown in high glucose medium (YPD-Pi medium), supplemented with 5 mM Pi and at OD_{600}~0.6, the medium was supplemented with Pi (10 mM and 25 mM final concentrations). The basal OCR was measured one hour after Pi supplementation. Data represent mean ± SD (n=3).

E) Effect of increasing the Pi concentration in a high glucose medium, in the presence of a glycolytic inhibitor, on basal OCR. WT cells were grown in high glucose medium (YPD-Pi medium), supplemented with 5 mM Pi and at OD_{600}~0.6, the medium was supplemented with Pi (25 mM final concentration) and 2DG (0.25%) for one hour and the basal OCR was measured. Data represent mean ± SD (n=3).

F) Overexpression of Mir1 under the G6PD promoter. C terminal 6x HA epitope-tagged Mir1 was expressed under G6PD promoter in cells with Mir1 endogenously tagged at the C terminus with a 6x HA epitope tag. WT cells (carrying an empty vector, and expressing Mir1-HA under the endogenous Mir1 promoter) and Mir1-OE cells were grown in high glucose. The protein levels of Mir1 were estimated by western blot, and clones with an ~2 fold increase in Mir1 were selected. A representative blot (n=3) is shown.

G) Mpc3 protein levels in *ubp3Δ* cells. WT and *ubp3Δ* cells containing endogenously tagged Mpc3 at the C terminus with a 3x FLAG epitope tag, were cultured in high glucose (2% glucose) and high glucose-low Pi (2% glucose, 1 mM Pi). The Mpc3 protein levels were measured by western blot. A representative blot (n=3) is shown in the panel.

H) Mpc3 protein amounts upon shifting WT and *mir1Δ* cells to a low-glucose medium. WT and *mir1Δ* cells containing Mpc3-FLAG, were cultured in high glucose (2% glucose) and shifted to low (0.1%) glucose for 1 hour. Mpc3 protein was measured by western blot. A representative blot (n=3) is shown in the panel.

I) Requirement of Mpc3 for the increase in basal OCR upon shifting to low glucose. WT and *mpc3Δ* cells were cultured in high glucose (2% glucose) and shifted to low (0.1%) glucose for 1 hour. The basal OCR from two independent experiments (n=2), normalized to the OD_{600} is shown.

J) Mpc3 protein levels and Mir1 overexpression. WT cells and Mir1-OE cells containing endogenously tagged Mpc3 ate the C terminus with a 3x FLAG tag were grown in high glucose. The protein levels of Mpc3 were estimated by western blot. A representative blot (n=3) is shown in the panel.

The statistical significance was calculated using unpaired-student t-test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Figure 1: A deubiquitinase deletion screen identifies Ubp3 as a regulator of glucose-mediated mitochondrial repression

Figure 2: Key glycolytic enzymes decrease and glucose flux is rerouted in *ubp3Δ* cells

Figure 3: Phosphate accumulates due to altered Pi homeostasis in *ubp3Δ* cells

Figure 4: Phosphate availability dictates mitochondrial activity

Figure 5: Mitochondrial Pi availability controls glucose-mediated mitochondrial repression