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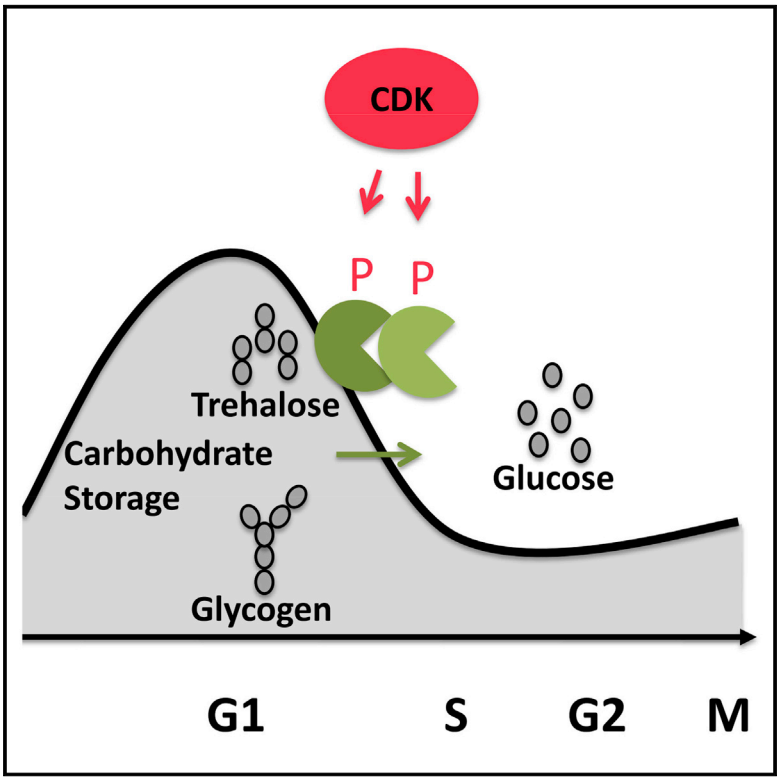
Cyclin-Dependent Kinase Co-Ordinates Carbohydrate Metabolism and Cell Cycle in *S. cerevisiae*

Graphical Abstract



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In Brief

Metabolic and growth processes are co-ordinately regulated by CDK.

Highlights

- Liquidation of stored carbohydrate dependents on cyclin-dependent kinase
- Liquidation of stored carbohydrates depends on CDK sites on catabolic enzymes
- CDK activity controls catabolism co-ordinately with cell-cycle progression
- CDK regulation fine-tunes the final cell division to match nutrient availability

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Cyclin-Dependent Kinase Co-Ordinates Carbohydrate Metabolism and Cell Cycle in *S. cerevisiae*

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SUMMARY

Cyclin-dependent kinases (CDKs) control cell division in eukaryotes by phosphorylating proteins involved in division. But successful proliferation requires co-ordination between division and cellular growth in mass. Previous proteomic studies suggested that metabolic proteins, as well as cell division proteins, could potentially be substrates of cyclin-dependent kinases. Here we focus on two metabolic enzymes of the yeast *S. cerevisiae*, neutral trehalase (Nth1) and glycogen phosphorylase (Gph1), and show that their activities are likely directly controlled by CDK activity, thus allowing co-ordinate regulation of carbohydrate metabolism with cell division processes. In this case, co-ordinate regulation may optimize the decision to undertake a final cell division as nutrients are being exhausted. Co-regulation of cell division processes and metabolic processes by CDK activity may be a general phenomenon important for co-ordinating the cell cycle with growth.

INTRODUCTION

Cell proliferation requires two kinds of processes, growth (macromolecular synthesis, addition of mass) and division. On average, proliferating cells exactly double their mass every round of cell division. Thus, although growth and division processes can be considered separately, long-term cell proliferation nevertheless requires co-ordination between them.

Hartwell demonstrated the separate control of growth and division by studying yeast cell division cycle (*cdc*) mutants, which had defects in cell division (Hartwell et al., 1974; Johnston et al., 1977). A key observation was that *cdc* defects did not stop macromolecular synthesis or growth in mass. Thus, *cdc* mutants, though arrested in the cell cycle, nevertheless continued to add mass, protein, RNA, etc. and grew to extraordinarily large sizes.

Hartwell proposed that yeast cells grow in mass as fast as they can without regard for the cell cycle. Co-ordination is achieved indirectly because cell-cycle entry is triggered only when cells have grown to a sufficiently large size ("size control"). In this view, growth is primary. Dependence of the cell cycle on prior growth gives the observed and necessary co-ordination between division and growth (Hartwell et al., 1974; Johnston et al., 1977). In this view, the cell-cycle machinery need not modulate growth.

This view has been key to the rapid progress in understanding the cell division cycle. Nevertheless, although co-ordination is achieved predominantly because cell division depends on growth, it is also true that it might sometimes be advantageous if cell-cycle regulators could directly control growth as well as division. In mammalian cells, the separation of control between growth and division is less clear than in yeasts; for instance, many growth factors affect both growth and division. Indeed, recent work has highlighted connections between metabolism and cancer (reviewed by Cheong et al., 2012; Vander Heiden et al., 2009; Ward and Thompson, 2012). Therefore, it is worth investigating how, and to what extent, growth and division processes may be co-ordinately regulated by the central cell cycle cyclin-dependent kinase (CDK).

Several proteome-wide screens have been done in yeast to find CDK substrates (Archambault et al., 2004; Holt et al., 2009; Kanshin et al., 2015; Li et al., 2014; Ubersax et al., 2003). Not surprisingly, many putative substrates are involved specifically in cell division, including proteins for cell-cycle transcription, budding, DNA replication, and mitosis (Holt et al., 2009; Ubersax et al., 2003). However, hundreds of other putative substrates are not obviously cell cycle specific (Holt et al., 2009; Ubersax et al., 2003). These substrates could be false positives in these proteome-wide screens. Alternatively, perhaps CDK is genuinely regulating many substrates involved in growth processes, as opposed to division processes.

The role of CDK phosphorylation of these growth proteins has been studied in only a handful of cases. Kurat et al. (2009) showed that activity of the yeast triacyglycerol lipase Tgl4 is controlled by CDK. Harbauer et al. (2014) found that in yeast, mitotic CDK activity directly stimulates mitochondrial protein import. Recently, Aregger et al. (2016) found a link between CDK activity and translation. Other instances have been reviewed (Fajas, 2013).

For yeast, the most important nutrient is sugar. The storage of sugar as glycogen and trehalose is under strong cell-cycle control (Guillou et al., 2004; Küenzi and Fiechter, 1969; Müller et al., 2003; Silljé et al., 1997, 1999). In slowly growing cells (but not in cells growing rapidly on glucose), storage carbohydrate rises to very high levels (up to 20% of dry cell mass) in early G1 phase. Then, in later G1 when cells commit to cell-cycle entry (Start), both glycogen and trehalose are liquidated to glucose (Müller et al., 2003; Silljé et al., 1997). Because the switch from carbohydrate accumulation to liquidation co-incides with the rise in G1 CDK activity, and because some of the enzymes involved are known to be phosphorylated on CDK consensus sites (see below), we speculated that glycogen and trehalose metabolism might be directly controlled by CDK. Thus, to begin validation of the putative metabolic CDK substrates as relevant substrates, we focused on regulation of glycogen and trehalose by CDK activity.

Glycogen is a branched polysaccharide synthesized by glycogen synthase (*GSY1* and *GSY2* in yeast) and branching enzyme (*GLC3*) (reviewed by Wilson et al., 2010). Breakdown requires glycogen phosphorylase (*GPH1*) and debranching enzyme (*GDB1*). The vacuolar glucoamylase Sga1 is also involved (Wilson et al., 2010). Trehalose is a disaccharide. Its synthesis requires trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase (*TPS2*). Breakdown to glucose is largely due to neutral trehalase, which is encoded by two genes, *NTH1* and *NTH2*, of which *NTH1* is more active. In addition to transcriptional regulation, many of these activities are subject to post-translational regulation via phosphorylation (Wilson et al., 2010). In many systems, cyclic AMP-dependent protein kinase (protein kinase A [PKA]) leads (directly or indirectly) to phosphorylation of both glycogen synthase and glycogen phosphorylase, inhibiting the former but activating the latter. Thus, high PKA levels lead to low glycogen (because synthesis is inhibited while breakdown is activated), while low PKA levels lead to high glycogen. For example, in yeast, high PKA activity leads to Gsy2 phosphorylation, inactivating the enzyme and glycogen synthesis (Wilson et al., 2010). At the same time, high PKA activity leads to Gph1 phosphorylation on T31, activating the enzyme and glycogen breakdown (Lin et al., 1996). PKA may also activate neutral trehalase (Nth1) by phosphorylating S20, S21, S60, and S83, with S60 and S83 apparently most important (Schepers et al., 2012; Veisova et al., 2012).

However, PKA is not the only protein kinase involved. Glycogen synthase Gsy2 is inhibited by phosphorylation on Ser650 (RPL**S**VPG), Ser654 (VPG**S**PRD), and Thr667 (VYM**T**PGD). While the kinase phosphorylating S650 is not known, S654 and T667 have been identified as substrates of the cyclin-dependent kinase Pho85 (a CDK not primarily involved in cell cycle) in a complex with the cyclins Pcl8 or Pcl10 (Huang et al., 1998; Wilson et al., 2010). S654 is in a sequence context favorable for phosphorylation by the cell cycle CDK Cdc28.

RESULTS

Many Putative CDK Substrates Are Involved in Metabolism

Holt et al. (2009) identified 309 yeast proteins as potential CDK substrates. In Figure S1 and Table S1, we classify these 309 proteins by function into 110 "Cell Cycle," 127 "Metabolism" or 72 "Other or Unknown Function" groups using annotations from the *Saccharomyces* Genome Database. Examples of "Metabolism" proteins include Nth1 (neutral trehalase), Gsy2 (glycogen synthase), Bap2 (leucine permease), Taf4 (TFIID subunit), Tfa2 (TFIIE subunit), and Tif4632 (translation initiation factor eIF4G). Although these functions may be involved in the cell cycle, they are not specific to the cell cycle.

The 127 "Metabolism" substrates included 16 proteins involved in carbohydrate metabolism, seven of them involved in metabolism of trehalose (Nth1, Tps3, Tsl1) or glycogen (Gac1, Gip2, Pig1, Gsy2). In addition, glycogen phosphorylase (Gph1) is a likely CDK target (Ubersax et al., 2003). Therefore, we focused on Nth1 and Gph1, which break down trehalose and glycogen, respectively. In each enzyme, there is one putative CDK phosphorylation site with strong evidence that the site is phosphorylated in vivo. Four different phosphoproteomic studies identified Ser66 of Nth1 (**S**PFK) as being phosphorylated in vivo (Albuquerque et al., 2008; Chi et al., 2007; Holt et al., 2009; Swaney et al., 2013). Holt et al. found Nth1-Ser66 to be the third-most strongly CDK-responsive residue in the proteome. PKA activates Nth1 by phosphorylation on Ser20, 21, 60, and 83 (Schepers et al., 2012; Veisova et al., 2012), so it seemed plausible that phosphorylation of Ser66 in the middle of this cluster of PKA sites might also activate Nth1. Likewise, three phosphoproteomic studies (Albuquerque et al., 2008; Soulard et al., 2010; Swaney et al., 2013) showed that Ser19 of Gph1 (**S**PHQ) is phosphorylated in vivo. It is in the middle of a cluster of six phosphoresidues spread over about 40 amino acids and is close to residue T31, whose phosphorylation, possibly by PKA, activates Gph1 (Lin et al., 1996). Therefore, we focused on Ser66 of Nth1, and on Ser19 of Gph1.

CDK Activity Controls Levels of Glycogen and Trehalose

Drs. D. McCusker and D. Kellogg had previously used the ATP analog 1NMPP1 to inhibit CDK activity in a *CDC28-as* strain ("analog sensitive") (Bishop et al., 2000). They observed accumulation of glycogen and visualized accumulation of presumptive glycogen granules using electron microscopy (McCusker and Kellogg, personal communication). To follow up, we treated asynchronous wild-type (WT) or *CDC28-as* with the 1NMPP1 inhibitor and assayed glycogen and trehalose. Both carbohydrates accumulated to large amounts (Figure 1A). Thus, CDK activity controls accumulation of glycogen and trehalose.

We purified small, slowly growing G1 cells by elutriation and reproduced previous observations (Müller et al., 2003; Silljé et al., 1997) that in such cells, glycogen and trehalose accumulate, then are liquidated at the time of budding (i.e., at Start, the commitment point) (Figure 1B). We again used the *CDC28-as* strain, where CDK activity can be inhibited by 1NMPP1, to show that carbohydrate accumulation, then liquidation at Start, depends on CDK activity. Small unbudded *CDC28-as* cells were isolated by elutriation, and the inhibitory ATP analog was added, or not (Figures 1B and 1C). When Cdc28 was inhibited, glycogen and trehalose accumulated to extremely high levels, and glycogen, at least, continued to accumulate throughout the course of the experiment (Figure 1C). In contrast, when Cdc28 was not inhibited, there was WT accumulation followed by liquidation at Start when CDK activity first

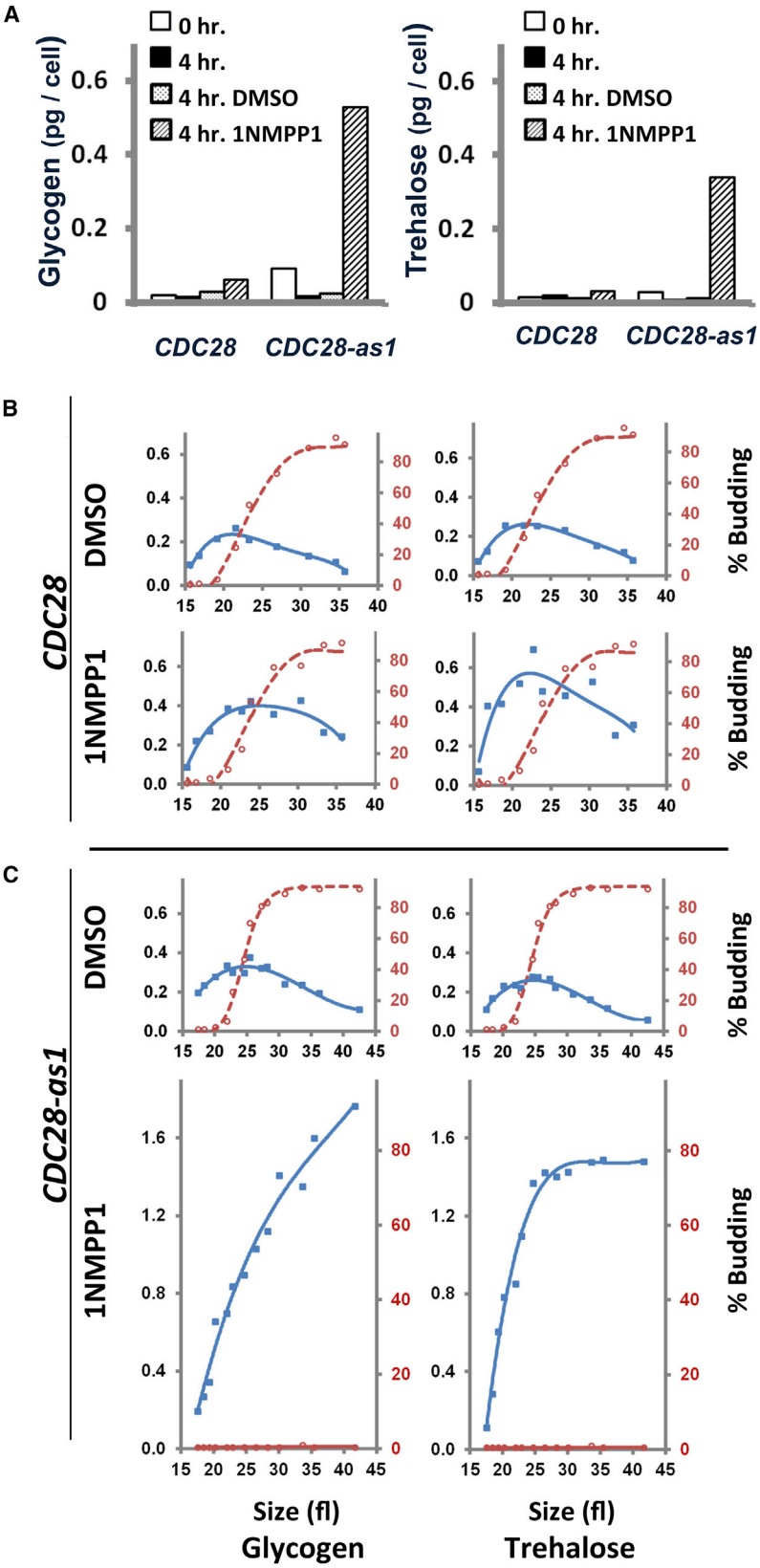

Figure 1. Storage Carbohydrate Breakdown Depends on CDK Activity

(A) Storage carbohydrate accumulates when CDK is inhibited. *CDC28* (GZ317) and *CDC28-as* (GZ328) were grown to 1 × 10^7 cells/ml in YEP+ethanol (EtOH). Cultures were split in three, and these received no addition, DMSO or 1.5 μM 1NMPP1 in DMSO. After 0 and 4 hr, carbohydrates were measured.

(B and C) Carbohydrates accumulate while CDK activity is low, then are liquidated when CDK activity rises after Start. Cells were grown in YEP+EtOH. Small unbudded cells were collected by elutriation, then re-innoculated back into conditioned YEP+EtOH. Cultures were split and received either DMSO alone or 1.5 μM 1NMPP1 in DMSO. Glycogen and trehalose were assayed (Experimental Procedures) and plotted as picograms (pg) glucose equivalent per cell (left axis, blue lines). Budding is the red line.

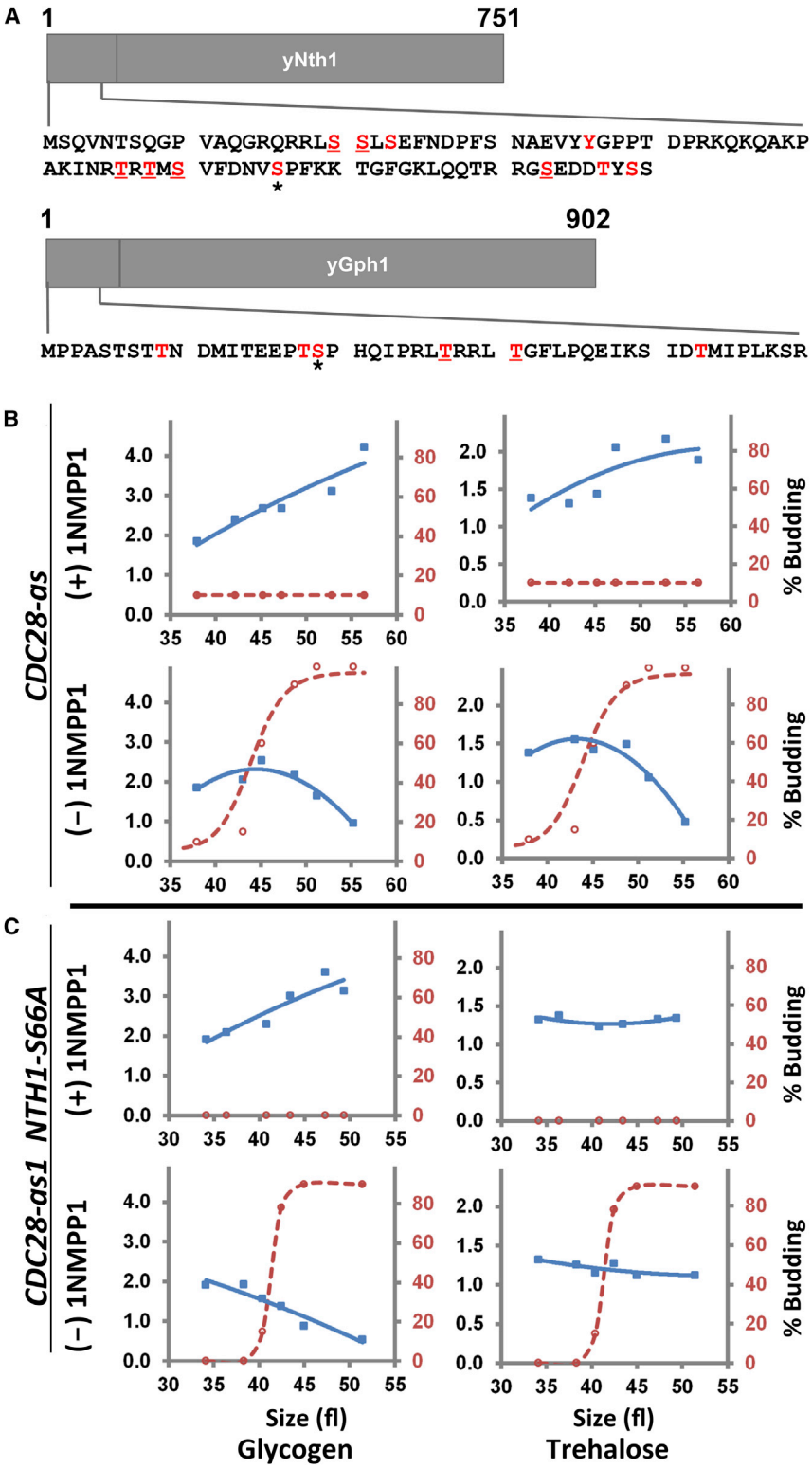

Figure 2. Breakdown of Trehalose Depends on CDK Activity and on Ser66 of Nth1

(A) Structure of yeast Nth1 and Gph1. In vivo phosphosites of Nth1 and Gph1 are colored red. Underlined residues, putative PKA sites; asterisks, putative CDK sites. Other potential metabolic substrates of CDK are shown in Figure S1 and Table S1. (B and C) Liquidation of trehalose depends on residue Nth1-S66. *CDC28-as* (GZ328) and *CDC28-as NTH1-S66A* (GZ329) cells were grown in YEP+EtOH. Small (~17 fL), unbudded cells were collected by elutriation, then re-innoculated back into conditioned YEP+EtOH. All cells were treated with 2.5 μM 1NMPP1 for 3 hr, after which they had grown to ~35 fL, and accumulated high levels of glycogen and trehalose. Cells were washed to remove inhibitor, then aliquoted in two, and inhibitor was added back to one aliquot. Glycogen (left, blue), trehalose (right, blue), size (x axis), and budding (red, y axis on right) were followed with time. Carbohydrate levels are given (y axis, left) in pg/cell. (B) *CDC28-as* cells; (C) *CDC28-as NTH1-S66A* cells.

develops (Figure 1B). (1NMPP1 also had a small effect on carbohydrate accumulation in the WT cells.) Thus, the G1-phase CDK activity promotes the loss of glycogen and trehalose at Start.

Since Ser66 of Nth1 is a putative Cdc28 phosphorylation site (Figure 2A), we investigated trehalose breakdown in an Nth1-S66A mutant. As before, small *CDC28-as* cells were isolated by elutriation, but in this case with or without the *NTH1-S66A* mutation. These cells were then treated with CDK inhibitor for 3 hr to allow accumulation of glycogen and trehalose. When the CDK inhibitor was washed away, CDK activity was restored (as shown by budding), and in the *NTH1* WT strain, both storage carbohydrates were liquidated (Figure 2B). But in the *NTH1-S66A* strain, though CDK activity was again restored (see budding index), only glycogen, but not trehalose, was liquidated (Figure 2C).

As a second approach, we specifically regulated the G1-phase CDK activity. We used a strain lacking WT G1 cyclins, but containing the G1 cyclin *CLN1* under control of a *GAL* promoter (i.e., *cln1 cln2 cln3 GAL-CLN1*) (Schneider et al., 2004). Importantly, this strain is a *gal1* (galactokinase) *gal10* (epimerase) mutant, rendering galactose a non-metabolizable inducer. The strain was grown on ethanol, with galactose to induce *CLN1* for cell-cycle progression. After shutting off *GAL-CLN1* by removing galactose, we obtained small G1 cells by elutriation. These were grown in YEP+ethanol without galactose; because of the lack of G1 cyclins, these cells could not activate CDK. After the cells had grown to a moderately large size, they were split into two aliquots, and galactose was added to one

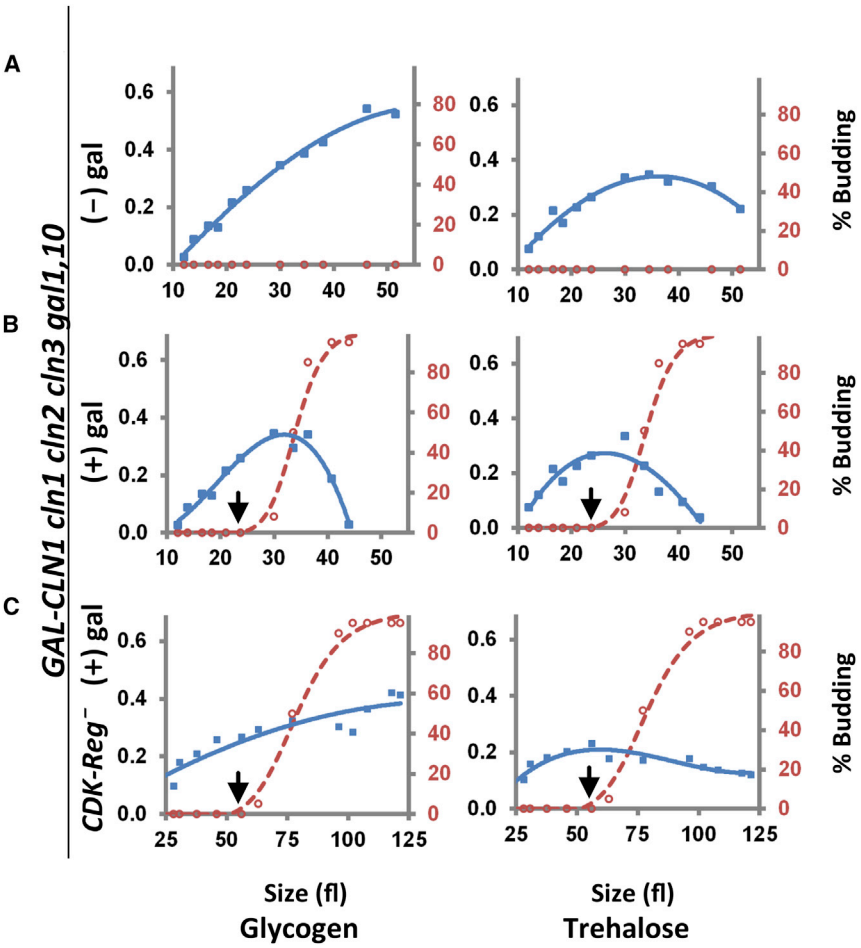

Figure 3. Breakdown of Glycogen and Trehalose Depends on Cln-CDK Kinase and on Gph1-Ser19 or Nth1-Ser66

(A–C) Cells of strain BS111 (*GAL-CLN1 cln1 cln2 cln3 gal1 gal10*) ([A], [B]) or the same strain carrying *NTH1-S66A* and *GPH1-S19A* (C) were grown in YEP+EtOH+galactose, washed, shifted to YEP+EtOH for 1 hr, and then small unbudded cells were collected by elutriation. These were re-inoculated into the conditioned YEP+EtOH medium. Glycogen (left) and trehalose (right) were assayed and indicated in pg/cell (blue lines, y axis, left).

(A) No galactose added after elutriation.

(B) Half of the same sample as in (A), but with galactose added at 22 fL to induce *GAL-CLN1* (arrow) (3 hr after elutriation). Note that the first five data points in (A) and (B) are the same data re-plotted.

(C) BS111 *NTH1-S66A GPH1-S19A* (GZ320) elutriated as above was grown in YEP+EtOH for 3 hr, then 0.5% galactose was added 3 hr after elutriation (arrow) to induce *GAL-CLN1*. Addition of galactose does not activate PKA-dependent events in this *gal1 gal10* background (Figure S2).

to induce *CLN1*. The cells without galactose (i.e., without CDK activity) did not bud and accumulated glycogen and trehalose to very high levels (Figure 3A). However, when *CLN1* was induced, cells budded, and both carbohydrates were quickly liquidated (Figure 3B). Thus, the liquidation of glycogen and trehalose requires G1 cyclin. Finally, we examined a mutant of this strain that lacked both Ser66 of Nth1 and Ser19 of Gph1 and found that liquidation of glycogen was prevented, and liquidation of trehalose was largely but not entirely prevented. As a control, we showed that addition of galactose to a similar *gal1 gal10* strain that did not have the *GAL-CLN1* construct did not activate PKA or cause liquidation of trehalose (Figure S2).

Gph1 and Nth1 Can Be Phosphorylated by CDK In Vitro

We then asked whether CDK could phosphorylate Gph1 or Nth1 in vitro. The two enzymes were purified from *E. coli* (Experimental Procedures), then treated in vitro with recombinant human cyclin B/CDK1. Both Gph1 and Nth1 were phosphorylated as shown by the appearance of a low mobility band on a Phos-tag gel (Figure 4A). At least in the case of Nth1, where phosphorylation was most robust, mass spectrometry showed that phosphorylation was occurring on the consensus CDK site Ser66 (**S**PFKK) (Figure 4B), the same site shown to be phosphorylated and CDK dependent in vivo (Holt et al., 2009), and whose mutation prevented liquidation of trehalose (Figures 2 and 3). No other phosphopeptides were detected. This in vitro phosphorylation caused a roughly 7-fold increase in the in vitro activity of Nth1 (Figure 4C) (though absolute levels of activity were very low, likely because of the absence of PKA and 14-3-3 proteins) (Veisova et al., 2012). We did not see a significant increase in the in vitro activity of Gph1 after CDK treatment; however, activation of Gph1 may also require phosphorylation by PKA (Lin et al., 1996).

Phenotypes of Storage Carbohydrate Mutants

Storage carbohydrates accumulate through G1 and then are degraded at S-phase in slowly growing cells (Guillou et al., 2004; Müller et al., 2003; Silljé et al., 1997, 1999), but the role of this oscillation is unknown. Liquidation of stored carbohydrate could provide a burst of glucose for synthesis of the cell wall, RNA, DNA, etc., and also a burst of energy via glycolysis. Accordingly, one theory is that mobilization of storage carbohydrate speeds progression into the next cell cycle (Futcher, 2006; Guillou et al., 2004; Müller et al., 2003; Silljé et al., 1997, 1999). Another theory is that liquidation of carbohydrate allows cells to switch from respiration to glycolysis while DNA is synthesized, allowing escape from mutagenic effects of reactive oxygen species (Chen et al., 2007; Klevecz et al., 2004; Tu et al., 2005).

To evaluate the quantitative plausibility of these hypotheses, we grew cells in ethanol medium (Figure 5A) to see how much carbohydrate was stored at different phases of growth. This was motivated by batch studies in cells grown in glucose, which showed very low levels of storage carbohydrate at low cell titers, but high levels when cells approached stationary phase (Lillie and Pringle, 1980). Similarly, we found cells grown in ethanol

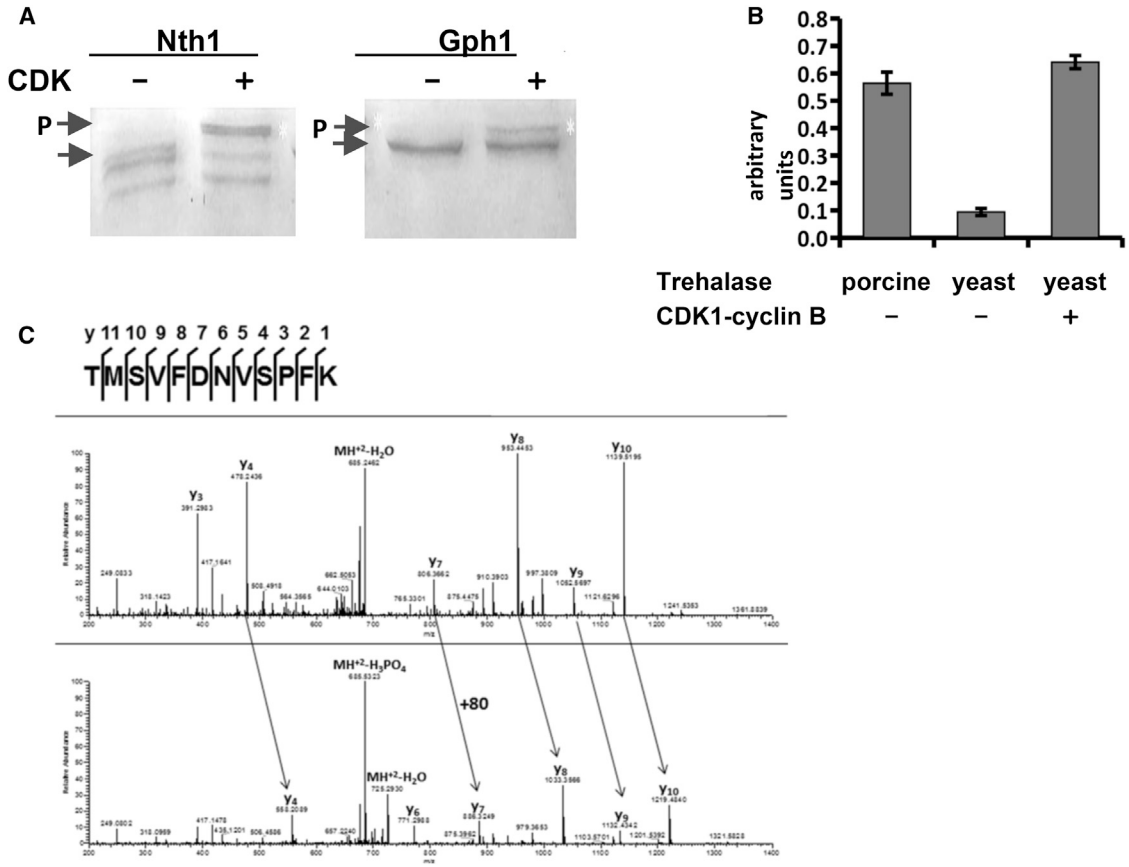

Figure 4. Yeast Nth1 and Gph1 Proteins Are Phosphorylated by CDK In Vitro

(A) CDK phosphorylates Nth1 and Gph1 in vitro. Nth1 and Gph1 were purified from *E. coli* and treated with hCDK1-cyclin B in vitro, then analyzed on Phos-tag SDS-PAGE gels. Full-length Nth1 and Gph1 are marked with arrows; phosphorylated forms are marked with a "P" and an arrow. The faster migrating band under Nth1 is an N-terminally truncated fragment lacking the phosphorylation site.

(B) CDK activates Nth1 trehalase in vitro. Porcine trehalase (positive control), and yeast Nth1 with and without CDK treatment, were assayed for trehalase activity.

(C) CDK phosphorylates Nth1 in vitro on S66. Nth1 protein bands with and without mobility shifts in panel A were extracted from a gel and subjected to mass spectrometry to assay phosphorylation. Ser66 was the only phosphosite identified in Nth1.

had low levels of glycogen and trehalose at low cell titers (totaling about 50 fg per cell), but the amount of stored carbohydrate increased steadily, then dramatically, with cell titer, reaching extremely high levels as cells approached stationary phase (Figure 5A; Table S2). Many previous experiments on carbohydrate storage in respiring cells used glucose-limited chemostats (Guillou et al., 2004; Müller et al., 2003; Silljé et al., 1999); at low growth rates, amounts of stored carbohydrate were high, while at higher growth rates, stored carbohydrates decreased (Silljé et al., 1999).

To put the quantities in Figure 5A into perspective, at low cell titers (1 to 2.5 × 10^7 cells/ml) cells store about 50 fg carbohydrate per cell (Table S2). Although this is 2- to 4-fold more than a rapidly growing cell in YEP glucose (our measurements, data not shown; Lillie and Pringle, 1980), it is still not very much. We measured the dry cell mass of WT strain GZ240 (in stationary phase) to be 17.2 pg/cell, similar to literature values of 16.5 pg for exponential cells (Klis et al., 2014) (see also http://bionumbers.hms.harvard.edu/). A 17 pg cell contains about 3,000 fg of carbohydrate in its cell wall, about 400 fg in the ribose sugar of RNA, about 7 fg in the deoxyribose sugar of DNA, and additional carbohydrate in other molecules (Klis et al., 2014) (http://bionumbers.hms.harvard.edu/). Our elutriations show that the 50 fg (average) per cell is mainly in the large, unbudded G1 cells, where it may reach a peak of perhaps 300 fg per cell (Figure 1). Although this is more than enough for DNA synthesis; it is not quite enough for RNA synthesis and is negligible from the point of view of constructing a new cell wall. In terms of energy, 300 fg of glucose, if used solely by glycolysis, could supply the cell's ATP needs for about 20 s (assuming an ATP flux of 0.6 pmole/cell hr) (Slavov et al., 2014). Thus, from the point of view of either structural components or energy, the amount of carbohydrate stored by low titer respiring cells is small, perhaps inconsequential.

We assayed three kinds of storage carbohydrate mutants for phenotypes: the *tps1 gsy1 gsy2* mutant (the "CHO-" mutant), which cannot make trehalose or carbohydrate; the *nth1 gph1* mutant (or sometimes the *nth1 nth2 gph1* mutant), which can store carbohydrates but not liquidate them, and, most specifically, the *NTH1-S66A GPH1-S19A* double point mutant (the

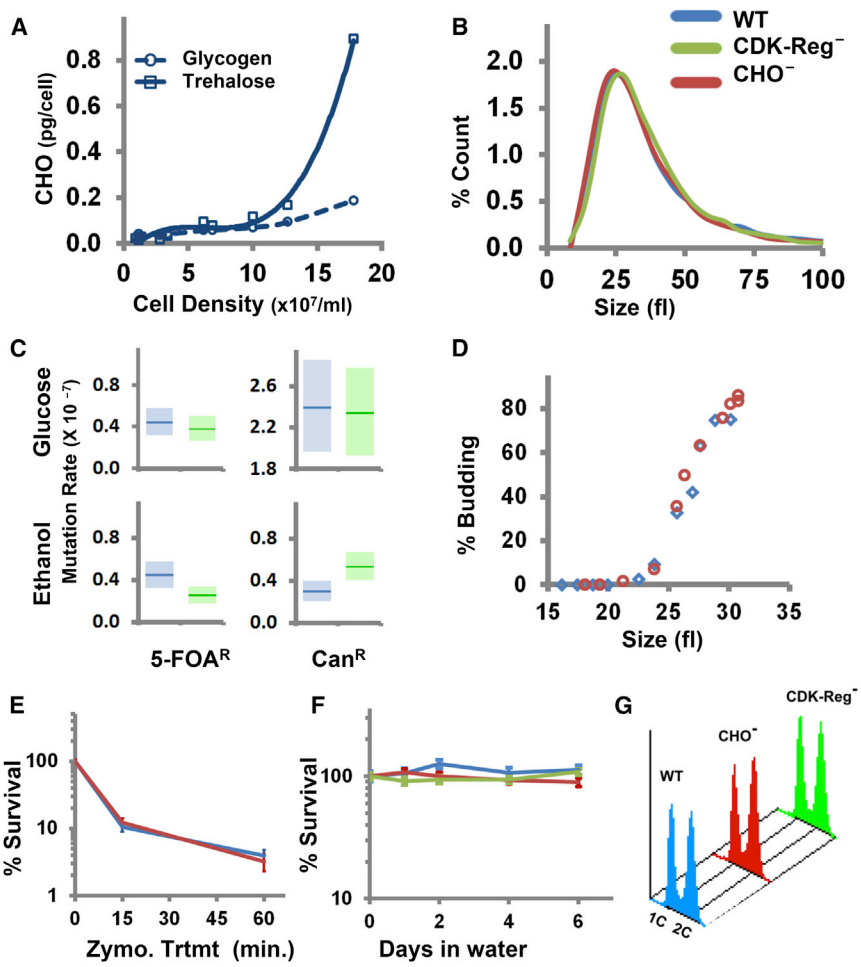

Figure 5. Storage Carbohydrate Mutants Have Little Phenotype When Carbon Source Is Abundant

(A) Carbohydrate levels. WT cells (GZ238) were grown in YEP+ 1.5% EtOH and sampled at various cell titers. Glycogen and trehalose were assayed. Raw data are given in Table S2.

(B) Cell size distributions. WT (GZ240), CHO- (GZ364), and CDK-Reg- (8BDA-U+) cells were grown to 1 × 10^7 cells/ml in YEP+0.5% glycerol and cell volume distributions were measured.

(C) Mutation rate. 95% confidence intervals are shown (shaded). Fluctuation tests were used to analyze mutation rates to 5-FOA and canavanine resistance in WT (GZ240, blue) and CDK-Reg- (8BDA-U+, green) cells after growth in either YEP+4% glucose or YEP+1.5% EtOH.

(D) Size at Start. Small unbudded WT cells (GZ238, blue) and *gsy1 gsy2 tps1* cells (GZ364, red) ("CHO-") were obtained by elutriation after growth in YEP+EtOH. Cell size and cell-cycle progression (budding) were followed after re-innoculation. Both strains had a critical cell size for Start of about 27 fl. Transcription at Start is also not affected by carbohydrate storage (Figure S3).

(E) Zymolyase resistance. WT (GZ240, blue) and CDK-Reg- (8BDA-U+) cells (red) were grown to 1 × 10^7 cells/ml in YEP+1% EtOH and exposed to 100 μg/ml Zymolyase for indicated times, then briefly to 0.02% SDS, then plated for viability (Experimental Procedures).

(F) Survival in water. WT (GZ240, blue), CHO- (GZ364, red), and CDK-Reg- (8BDA-U+, green) cells were grown in YEP+1% EtOH to 3 × 10^7 cells/ml. Cells were washed, then resuspended in water and incubated with agitation at 30°C. Samples were plated for viability daily onto YEP galactose plates.

(G) DNA content. WT (GZ238), CHO- (GZ364), and CDK-Reg- (8BDA-U+) cells were grown to 1 × 10^7 cells/ml in SM+EtOH. DNA content was analyzed by flow cytometry.

"CDK-Reg- " mutant), which specifically lacks CDK control of carbohydrate liquidation (but still has CDK control of carbohydrate synthesis). We assayed for phenotypes in cells grown at low titres (1 to 3 × 10^7 cells/ml) in YEP ethanol or YEP glycerol, and to some extent also in synthetic media with ethanol as the carbon source. Perhaps unsurprisingly, in view of the small amount of carbohydrate stored, we failed to see any significant phenotype. Mutants were indistinguishable from WT in terms of doubling time (not shown), cell size distribution during asynchronous growth (Figure 5B), mutation rate (Figure 5C), cell size at Start (Figures 5D and S4), Zymolyase resistance (Figure 5E), survival in water (Figure 5F), and DNA content (Figure 5G).

Two of these negative results deserve special mention. We previously proposed that stored carbohydrates promoted an early Start in respiring cells (Futcher, 2006). According to this hypothesis, cells lacking carbohydrate (the CHO- mutant) or unable to liquidate carbohydrate in response to CDK activity (the CDK-Reg- mutant) should have a delayed Start, and hence abnormally large cells. We used elutriation to test this hypothesis in three strain backgrounds (CEN-PK, S288C [Figure 5D] and W303) and using the CHO- mutant (5D), the CDK-Reg- mutant (not shown), and the *nth1 gph*1 mutant (not shown). In most experiments there was little or no effect of the mutations. In the CEN-PK background, the CHO- mutant had slightly larger cells than the WT, but on the other hand, in the W303 background, there was a small effect in the opposite direction, and in S288C there was no effect (Figure 5D). In addition to elutriation, we assayed size at Start in single cells (Figure S4) and assayed Start gene expression as a function of size (Figure S4), again seeing no change in the mutants. Overall, we feel there is little evidence for delayed Start in these mutants at low cell titers, contrary to our earlier hypothesis (Futcher, 2006). Nevertheless, in cells growing more slowly in carbon-limited conditions, and accumulating larger amounts of carbohydrate, there is good evidence that stored carbohydrates do potentiate the next cell cycle (Guillou et al., 2004; Silljé et al., 1999).

Second, it has been proposed that respiring cells liquidate stored carbohydrates and switch to glycolysis at Start to avoid mutagenic effects of reactive oxygen species during S-phase (Chen et al., 2007; Klevecz et al., 2004; Tu et al., 2005). Mutants that may replicate outside of the putative protected window of

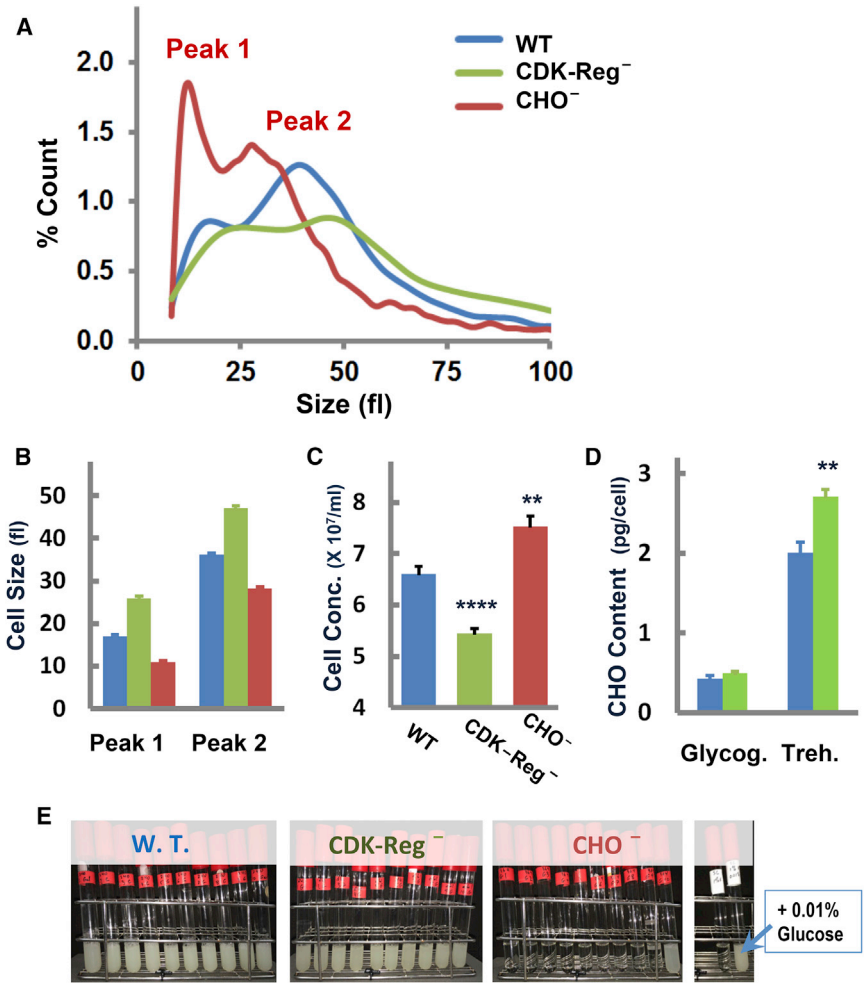

Figure 6. Storage Carbohydrate Mutants Have Strong Phenotypes when Exhausting Carbon Source

(A–C) Cell size and cell number in stationary phase. Ten independent cultures each of WT (GZ238, blue), CHO- (GZ364, red), and CDK-Reg- (8BDA-U+, green) cells were grown to saturation in YEP+0.5% glycerol.

(A) Cell volume distributions. One representative of each strain is shown.

(B) The mode cell volume of cells in the "peak 1" (small cells) or "peak 2" (larger cells) was assayed for 10 independent cultures of the WT (blue), CDK-Reg- (green), and CHO- (red) strains. In a few cultures (~3 per strain), the two peaks were not resolved, and no value was recorded. The mean of the ~7 modes is presented. All means are significantly different from all other means ($p < 0.01$). Error bars are SEM (C). The mean number of cells/ml for each strain. WT (blue), CDK-Reg- (green), and CHO- (red). Asterisks show significance of difference from WT (***$p < 10^{-5}$; **$p < 0.01$). Error bars are SEM.

(D) Glycogen and trehalose were measured for three WT (blue) and three CDK-Reg- (green) cultures grown to saturation in YEP 0.5% glycerol. Trehalose levels are significantly different ($p < 0.01$). Error bars are SEM.

(E) CHO- mutants have difficulty re-entering the cell cycle. Ten cultures each of WT, CHO-, or CDK-Reg- mutants were grown to stationary in YEP+1% EtOH and washed, and 10^5 cells were re-innoculated into 10 tubes each of 5 ml SC medium with 1% galactose. Cultures were incubated with rolling at 30°C for three days. Right, supplementation with trace (0.01%) glucose allows proliferation.

low respiration have been reported to have mutation rates elevated more than 10-fold (Chen et al., 2007). However, we find that the CDK-Reg- strain, which has little ability to liquidate stored carbohydrates at Start, has mutation rates similar to WT (Figure 5C). The mutation rate at *CAN1* is about 2-fold elevated (comparing mutant to WT in ethanol), but the mutation rate at *URA3* is about 2-fold reduced, and overall there is little evidence for increased mutation in the CDK-Reg- strain during growth on ethanol. Moreover, calculations suggest there is not nearly enough stored carbohydrate to allow these cells to cease respiring for a significant length of time (see above). The near-normal mutation rates further suggest there is no significant DNA replication stress in these strains, as also suggested by the DNA content (Figure 5G).

On the other hand, when we looked at cells approaching stationary phase, when much larger amounts of carbohydrate were stored, multiple phenotypes appeared. These were most dramatic when carbon source was limiting, such as YEP with 0.5% glycerol (and were barely seen, if at all, when carbon source was not limiting, as in YEP with 2% ethanol). Perhaps most interestingly from a cell-cycle perspective, the cell size distribution, and the number of cells per ml at stationary phase, changed markedly in response to the various mutations. The CHO- mutant grows to a larger number of cells per ml than the WT (Figure 6C), presumably because it uses all the carbohydrate in the medium without storing any, and this extra division potential is reflected in the fact that this strain has small (Wee-) cells in stationary phase (Figures 6A and 6B). Conversely, the CDK-Reg- strain grows to a smaller number of cells than WT (a selective disadvantage) (Figure 6C), and this is reflected in the fact that the CDK-Reg- strain has extra-large cells in stationary phase (Figures 6A and 6B). The altered cell size distributions are specific to stationary phase (compare to Figure 5B). Our interpretation of these effects is that CDK activity plays a key role in managing consumption versus storage of carbohydrate during the last cell divisions. Without CDK regulation, excessive carbohydrate is stored, not enough is consumed, a final cell division (the "final lap") is not undertaken, and the final cell titer is sub-optimal. Indeed, the CDK-Reg- strain stores more trehalose and glycogen at stationary phase than does the WT (Figure 6D); in this mutant, in stationary phase in YEP 0.5% glycerol, stored carbohydrate reaches a remarkable 3,200 fg/cell (the highest storage we have measured), about 20% of dry cell mass. This is an excellent example of how CDK activity can modulate metabolism to optimize cell division.

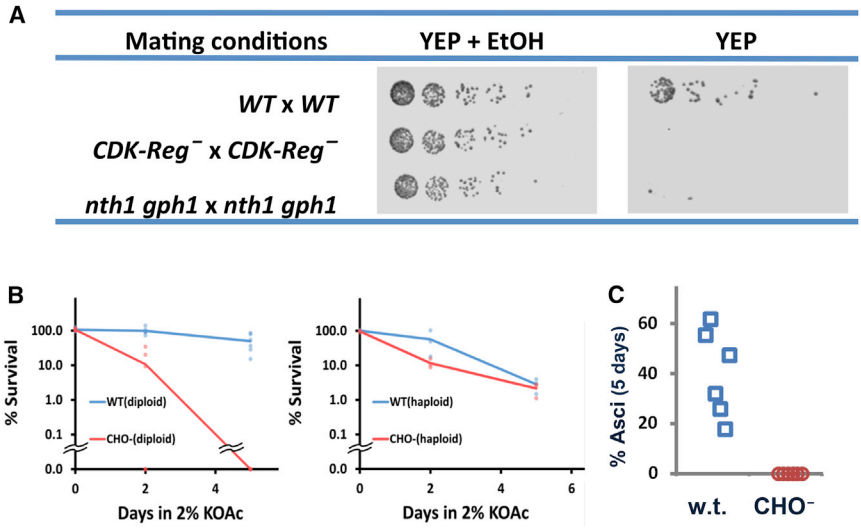

Figure 7. Storage Carbohydrate Mutants Have Mating Defects and Are Completely Unable to Sporulate

(A) CDK-Reg- and *nth1 gph1* cells mate poorly in the absence of carbon source. WT (GZ238-U+, GZ239-L+), CDK-Reg- (8BDA-U+, 9BDA-L+), and *nth1 gph1* (GZ358-U+, GZ359-L+) haploids of each mating type were grown to 5 × 10^7 cells/ml in YEP+1% EtOH, then harvested and washed. The two mating types were mixed either on YEP+1% EtOH plates or YEP (no carbon source) plates at high density and left to mate for 12 hr. They were resuspended and spotted on SC –ura –leu plates to select for complementation (i.e., mating). Under these conditions, even the WT mates poorly (around 10^{-4}).

(B) Diploid CHO- cells die rapidly in sporulation media. Haploid or diploid CHO- cells (diploid isolates of 8BDA x 9BDA) were subjected to a pre-sporulation growth protocol, then shifted into sporulation medium (2% potassium acetate), and viability was monitored daily. On day 5, 10^5 diploid CHO- cells were plated, yielding zero colonies.

(C) Diploid CHO- cells do not sporulate. Six diploid CHO- strains were isolated from a cross of 8BDA x 9BDA and were monitored for ascus formation in parallel with six diploids from a WT cross, GZ238 x GZ239. No asci were seen in any of the CHO- cultures.

If storing carbohydrate leads to a low final cell titer, would the cell's best strategy be to store no carbohydrate, like the CHO- mutant, and have the maximum cell titer? Unfortunately, CHO- cells are sensitive to several kinds of stress (De Virgilio et al., 1994; Elliott et al., 1996; Hottiger et al., 1994; Singer and Lindquist, 1998; Tapia and Koshland, 2014; Wiemken, 1990) (but see Petitjean et al., 2015 for a different view).

We found several additional phenotypes. First, after growth to stationary phase, CHO- cells have difficulty re-entering the cell cycle upon a switch to poor nutrients. When CHO- cells were grown to stationary phase in YEP ethanol, then re-innoculated into synthetic medium with galactose, they often had long lag phases before resuming proliferation. In some experiments, they failed to proliferate at all (Figure 6E). Cells remained viable, as they formed colonies after plating onto rich medium. The lag phase/inability to proliferate was suppressed by trace amounts of glucose (Figure 6E). Similar lag phases could sometimes also be seen for WT cells, but the defect was more common and pronounced for the CHO- mutant. Related phenotypes have been noted (Guillou et al., 2004; Shi et al., 2010; Silljé et al., 1999).

Second, the CDK-Reg- cells and the *nth1 gph1* cells (Figure 7A) have a mating defect in conditions lacking a carbon source (CHO- cells were not tested). Both mutants mate like WT on media containing carbon source, but when the two mating types are grown separately, then mixed on carbon-free media, the WT mates much better (albeit at a much lower efficiency than on nutrient-rich media) than either of the mutants. The phenotype is bilateral—both partners must be mutant for a strong phenotype.

If stored carbohydrates assist with mating, one has to ask how the carbohydrate would be released, since CDK is repressed by mating factor. However, mating factor activates the MAP kinases Kss1 and Fus3. Strikingly, the regulatory CDK site in Gph1 has the sequence PT**S**PHQ. This serine is not only a CDK site but also a consensus MAP kinase site. We have very preliminary evidence (not shown) that glycogen stored during G1 can be partially released by α factor treatment.

In addition, the CHO- mutant has a complete sporulation defect. Thousands of cells in six independent CHO- cultures (from independently constructed diploids) were examined microscopically in Figure 7C, and not a single ascus was seen. This is a much more severe phenotype than when either trehalose or glycogen alone are lacking (De Silva-Udawatta and Cannon, 2001; Walther et al., 2014). Although CDK is not active in early meiosis, there are other possible routes for the liquidation of carbohydrate, perhaps involving the meiotic protein kinase Ime2 or the sporulation-specific glucoamylase Sga1.

The defect of diploid CHO- cells is not simply their failure to sporulate. In addition, they rapidly and virtually completely die in sporulation medium (2% potassium acetate) in what may be an active process (Figure 7B). Conceivably, cells in sporulation conditions with too little carbohydrate undergo apoptosis to release nutrients for their luckier kin. This rapid and complete death does not occur in the haploid CHO- mutant (Figure 7B); it is specific for the a/α diploid, suggesting it is related to the meiotic defect.

DISCUSSION

For the last four decades, it has been thought that regulation of the cell cycle, and regulation of growth and metabolism, are largely independent processes, ultimately linked by a cell size control on division. However, we show here that the central cell cycle regulatory CDK directly modulates metabolism of storage carbohydrates. Glycogen and trehalose accumulate to very high levels in slowly growing G1-phase yeast when either G1 cyclin expression or Cdc28 kinase activity is blocked but are liquidated to glucose when Cln-Cdc28 activity is provided. Neutral trehalase, Nth1, is phosphorylated on a CDK consensus site in vivo (Albuquerque et al., 2008; Chi et al., 2007; Holt et al., 2009; Swaney et al., 2013). We find this same site can

be directly phosphorylated in vitro by CDK and that such phosphorylation increases enzymatic activity in vitro. Lack of the site, or lack of the kinase, decreases trehalose mobilization in vivo. Finally, we note that this site (SPFKK) is not only an excellent site for CDK phosphorylation, it is also an essentially perfect consensus site (S-P-x-K/R-K/R-K/R) for dephosphorylation by the phosphatase Cdc14 (Bremmer et al., 2012). The observed pattern of trehalose abundance (high in early G1, low at other times) is consistent with a simple model in which trehalase is activated by any form of CDK, followed by inactivation by Cdc14 in late M/early G1. Thus, direct control by CDK can explain the mechanism by which stored carbohydrate oscillates with the cell cycle (Küenzi and Fiechter, 1969; Silljé et al., 1997; Müller et al., 2003).

In cells at low titers respiring in plentiful non-fermentable carbon source such as ethanol, we found that a relatively small amount of carbohydrate was stored, perhaps 300 fg in a G1-phase cell. This is only enough to fully support ATP generation via glycolysis for about 20 s. Accordingly, mutants that could not store carbohydrate had no observable phenotypes while growing under such conditions. This finding poses some challenges to the idea of a yeast metabolic cycle (Chen et al., 2007; Klevecz et al., 2004; Tu et al., 2005), which is proposed to allow respiring yeast to switch to glycolysis for the duration of S-phase, to avoid the mutagenic effects of reactive oxygen species. Yet, the amount of stored carbohydrate (at low cell titer) seemed too small to support such glycolysis, and we saw no convincing increase in mutation rate in strains that could not liquidate carbohydrate during S-phase.

On the other hand, in cells approaching stationary phase, glycogen and trehalose accumulated to as much as 3000 fg/cell, or about 20% of dry cell mass (Figure 6) (Guillou et al., 2004; Müller et al., 2003). At the beginning of a new cell cycle, this carbohydrate can be liquidated in a CDK-dependent way (PKA may also be required). Under these circumstances, CDK activity is presumably having a very major effect on the overall metabolism of the cell, and the overall rate of biosynthetic activity, since it is controlling (with PKA) cellular access to large amounts of stored carbohydrate. We believe CDK regulation of carbohydrate is important for co-ordinating the use of scarce carbohydrate in the final cell division with the need to store some carbohydrate, perhaps for stress resistance, perhaps for mating, perhaps for sporulation, perhaps for re-entering the cell cycle. Cells that cannot manage this co-ordination either divide too many times, and fail to store carbohydrate, or divide too few times, and do not generate the optimum number of progeny.

Although we have focused on the CDK-induced liquidation of carbohydrate via Nth1 and Gph1, liquidation cannot fully explain the large effects of CDK activity on carbohydrate stores (Figures 1–3). High CDK activity may also repress synthesis.

NTH1 has a paralog, *NTH2*. The Nth2 protein does not conserve the CDK site at S66, SPFK, but instead has the sequence SDFK. That is, at the site where we believe Nth1 is phosphorylated to generate negative charge and activate the enzyme, Nth2 already has an acidic residue. Possibly an acidic residue is ancestral, and a phosphorylatable residue has evolved to allow control in Nth1 (Pearlman et al., 2011).

The CHO- mutant is reminiscent of *whi2*, the second discovered cell size mutant of *S. cerevisiae* (Sudbery et al., 1980). Like the CHO- mutant, *whi2* has normal-sized cells in exponential growth, but small cells in stationary phase, because it undertakes an extra division, and like the CHO- mutant, *whi2* is sensitive to heat shock. Whi2 is associated with the Psr1 plasma membrane phosphatase, and together they help regulate the phosphorylation state and activity of Msn2 (Kaida et al., 2002), a stress-related transcription factor. Conceivably they also regulate phosphorylation of enzymes involved in glycogen and trehalose synthesis and liquidation.

Gph1 and Nth1 seem to be activated by both PKA and CDK. It is noteworthy that the CDK sites are nestled close to the PKA sites (Figure 2A); both kinds of phosphorylations may activate by the same mechanism and may do so synergistically, perhaps via 14-3-3 proteins (Veisova et al., 2012). We have not explored the relationship between PKA activation and CDK activation, but this is potentially very interesting. We note that Bcy1, the regulatory subunit of PKA, was found as a CDK substrate in multiple studies (Holt et al., 2009; Ubersax et al., 2003) and that the three G1 cyclins, Cln1, Cln2, and Cln3, all have multiple consensus PKA phosphorylation sites (data not shown) and that (direct or indirect) regulation of Cln-Cdc28 activity by PKA has previously been shown (Tokiwa et al., 1994). Müller et al. (2003) found a peak of PKA activity at the same time as Start, when there is high CDK activity. Thus, in addition to interacting on substrates, the two protein kinases could regulate each other.

Although we have focused on carbohydrate storage, our overriding point is that the sphere of influence of CDK is not limited to cell-cycle-specific events. Instead, CDK activity also modulates metabolism, and the regulation of Gph1 and Nth1 are just examples of this, perhaps the tip of the iceberg. The validation of Gph1 and Nth1 as relevant CDK substrates implies that many of the other 126 "metabolic" substrates of Table S1 may also be real and relevant. Appreciating the full extent of CDK influence on metabolism will require careful experiments specifically looking for such effects.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth

Yeast strains are listed in Table S3. GZ strains 238, 239, 240, and 241 are derived from a cross between DBY12032 (D. Botstein) and UCC8376 (Dimitrov et al., 2009), both in the S288C background. These GZ strains are "HSCMM," meaning that they carry the fully WT *HAP1* allele of DBY12032 (i.e., not the 3′ interrupted version of *HAP1* in S288c) (Gaisne et al., 1999), and the mitochondria-stabilizing *SAL1 CAT5-91M MIP1-661T MKT1-30G* alleles of UCC8376. Consequently, these strains and their descendants have well-regulated respiration and stable mitochondria.

Standard molecular biology protocols were used. Cell number and cell size distributions were measured with a Z2 Beckman-Coulter Counter. Gene manipulation was performed either by PCR-based homologous recombination cassettes (Longtine et al., 1998) or by a modified CRISPR-Cas9 system (G.Z. and B.F., unpublished data). Yeasts were grown in YEP (1% yeast extract, 2% peptone) or standard Synthetic Complete (SC) and Synthetic Minimal (SM) media, supplemented with various carbon sources (ethanol, glycerol, glucose, galactose) as described.

Elutriation

Small G1 cells were obtained by centrifugal elutriation (Supplemental Experimental Procedures).

Glycogen and Trehalose Measurement

Methods were based on Becker (1978) and Parrou and François (1997), as described in detail in Supplemental Experimental Procedures.

Protein Overexpression and In Vitro Phosphorylation

Nth1 and Gph1 were expressed in pET21b or pET28a, respectively, in *E. coli* BL21(DE3) cells. Protein was purified by Ni-NTA agarose chromatography. 5 μg of purified Nth1 or Gph1 protein was mixed with 50 U of human CDK1-Cyclin B (New England Biolabs) in 1× NEBuffer for Protein Kinases supplement with 200 μM ATP and incubated at 30°C for 1 hr. The protein was heat denatured in 1× SDS loading buffer and resolved in a 10% SDS-PAGE gel containing 50 μM of Mn^{2+}Phos-tag (Wako). The mobility-shifted bands corresponding to phosphorylated proteins were sliced from the gel and submitted for mass spectrometry analysis at the Proteomic Center at Stony Brook University. Further detail is given in Supplemental Experimental Procedures.

Fluctuation Tests

Fluctuation tests were done by standard methods using 20 independent cultures for each measurement (see Supplemental Experimental Procedures).

Single-Cell Measurements and RNA-Seq

Single cell measurements and RNA-seq experiments are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.04.026.

AUTHOR CONTRIBUTIONS

The study was proposed by B.F. Experiments were performed by G.Z., Y.C., L.C., and B.F. The paper was written by G.Z. and B.F.

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