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c-Myc activates multiple metabolic networks to generate substrates for cell cycle entry

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Abstract

Cell proliferation requires the coordinated activity of cytosolic and mitochondrial metabolic pathways to provide ATP and building blocks for DNA, RNA, and protein synthesis. Many metabolic pathway genes are targets of the c-*myc* oncogene and cell cycle regulator. However, the contribution of c-Myc to the activation of cytosolic and mitochondrial metabolic networks during cell cycle entry is unknown. Here, we report the metabolic fates of [U-^{13}C] glucose in serum-stimulated *myc*$^{-/-}$ and *myc*$^{+/+}$ fibroblasts by ^{13}C isotopomer NMR analysis. We demonstrate that endogenous c-*myc* increased ^{13}C-labeling of ribose sugars, purines, and amino acids, indicating partitioning of glucose carbons into C1/folate and pentose phosphate pathways, and increased tricarboxylic acid cycle turnover at the expense of anaplerotic flux. Myc expression also increased global O-linked GlcNAc protein modification, and inhibition of hexosamine biosynthesis selectively reduced growth of Myc-expressing cells, suggesting its importance in Myc-induced proliferation. These data reveal a central organizing role for the Myc oncogene in the metabolism of cycling cells. The pervasive deregulation of this oncogene in human cancers may be explained by its role in directing metabolic networks required for cell proliferation.

Introduction

Cell proliferation is an essential component of normal growth and development, and as such, is highly regulated by hormonal and environmental factors. While our knowledge of cell cycle regulatory genes is advanced, the links between the cell cycle machinery and supply of metabolic intermediates required for cell division is less well understood. Of the known transcription factors exerting cell cycle control, a likely candidate for this regulatory role is the *c-myc* oncogene, due to the diversity of known target genes involved in both cell cycle and metabolic pathways (Zeller *et al.*, 2006). Induction of Myc occurs rapidly in response to growth factors stimulating cell cycle entry, with mRNA levels increasing 20 fold within the first 2 h after serum addition (Dean *et al.*, 1986). Global analysis of downstream targets reveals that Myc potentially regulates up to 15% of human genes through direct binding to target gene sequences (Zeller *et al.*, 2006), and may have even broader effects on gene expression through global chromatin remodeling (Knoepfler *et al.*, 2006).

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Notably, Myc regulates key genes in glycolysis, C1/folate, purine, pyrimidine and lipid metabolism (Dang *et al.*, 2006; Zeller et al., 2006). In addition, Myc targets genes required for mitochondrial DNA replication and transcription (Li *et al.*, 2005). Hence, Myc supports the bi-genomic transcriptional regulation necessary for mitochondrial biogenesis, a key event during cell cycle entry (Mandal *et al.*, 2005; Morrish *et al.*, 2008). In summary, the network of known Myc gene targets suggests that Myc is involved in orchestrating the changes in cell metabolism necessary for cell cycle entry. However, to the best of our knowledge, there have been no studies of central carbon metabolic flux or carbon partitioning associated with physiologic Myc expression. Our goal in this study was to address this question.

Results and Discussion

Myc increases oxidative metabolism of glucose during cell cycle entry

Rat1A fibroblasts with $myc^{-/-}$ and $myc^{+/+}$ genotypes were incubated with [U-^{13}C] labeled glucose starting at 12 h after serum addition to quiescent cells. The metabolic fates of ^{13}C atoms can be assessed in a wide range of metabolites based on the resonant absorption signals of ^{13}C nuclei when placed in an external magnetic field. The positions of ^{13}C tracer in the carbon backbone of metabolites vary according to the proportional fluxes of alternate metabolic pathways, which can be derived by measurements of the relative abundance of the different labeling patterns. NMR spectroscopy was performed on cell extracts after 4 h of labeling to approximate steady state for analysis of ^{13}C-labeled metabolites. This allowed us to compare the metabolic profiles during the G0 to S phase transition of $myc^{+/+}$ cells, which enter S phase at 16 h, and $myc^{-/-}$ cells, which have delayed S phase entry (Schorl and Sedivy, 2003; Tollefsbol et al., 1990).

Fig. 1A shows representative ^{1}H-decoupled, ^{13}C NMR spectra from cell extracts. ^{13}C-incorporation was observed for multiple metabolites, with the most prominent peaks corresponding to glutamate and lactate resonance signals. Inspection of the glutamate spectra indicated higher levels of ^{13}C-labeled glutamate and glutamine in $myc^{+/+}$ compared to $myc^{-/-}$ cells (Supplemental Fig. S1). This was confirmed by quantitative mass isotopomer analysis using LC/MS/MS. We detected a 20% increase in the fractional contribution of ^{13}C-labeled glutamate to the total glutamate pool in $myc^{+/+}$ cells, compared to $myc^{-/-}$ cells (Fig. 1C). Glutamate is in equilibrium with alpha-ketoglutarate and can be used to assess TCA cycle flux using the program tcaCALC (Malloy *et al.*, 1990). In our experiments, the multiplet resonances of glutamate were well resolved (Supplemental Fig. S1), and the areas of the glutamate C2, C3 and C4 resonances were used to calculate relative flux of glucose carbons into the TCA cycle via pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). Results of this analysis, illustrated in Fig. 1B, demonstrate a ~7 fold greater PDH flux in $myc^{+/+}$ cells compared to $myc^{-/-}$ cells. We also evaluated the contributions of [U-^{13}C] glucose and endogenous substrates to acetyl CoA entering the TCA cycle (Fig 1B). Analysis by tcaCALC indicated that both [U-^{13}C] pyruvate and endogenous unlabelled substrates each supplied approximately twice as much acetyl CoA in $myc^{+/+}$ cells compared to $myc^{-/-}$ cells.

In contrast, there is higher relative metabolism of pyruvate by the anaplerotic enzyme PC for the $myc^{-/-}$ cells, resulting in a substantially higher PC/PDH flux ratio (4.3 compared to 0.37 for $myc^{+/+}$ cells). In keeping with their higher relative PC flux, $myc^{-/-}$ cells had a 6-fold greater contribution of ^{13}C pyruvate to the total oxaloacetate pool via pyruvate carboxylation. These results identify significant qualitative differences between $myc^{+/+}$ and $myc^{-/-}$ cells in substrate partitioning and pathway flux into the TCA cycle during preparation for S phase entry, consistent with observed differences in serum-induced changes in mitochondrial mass and respiration (Morrish *et al.*, 2008).

The Myc-dependent alterations in TCA cycle and related fluxes can be explained by changes in Myc-regulated gene expression. Induction of Myc (MycER) in $myc^{-/-}$ cells results in temporal repression of PDK2 and PDK4 (Morrish *et al.*, 2008), which would lead to increased pyruvate oxidation by decreasing inhibitory phosphorylation of PDH. Expression of PC is also temporally decreased by Myc induction, reinforcing the allocation of pyruvate to supply the mitochondrial acetyl-CoA pool. These results, together with the effects of Myc on mitochondrial bioenergetics during cell cycle entry, point to a key role for Myc in augmenting mitochondrial capacity and directing substrates to mitochondria during the transition from maintenance energy requirements of quiescent cells to the active biosynthesis of cycling cells (Morrish *et al.*, 2008).

Myc increases glucose carbon flux into branch metabolic pathways of glycolysis

Metabolism of glucose supplies intermediates for amino acid synthesis, C1/folate metabolism, and pentose phosphate and hexosamine biosynthetic pathways, in addition to lactate generation. We found evidence for increased glycolytic flux as ^{13}C-labeling of lactate was greater in $myc^{+/+}$ cells compared to $myc^{-/-}$ cells (Fig. 2A). This result was expected, as multiple glycolytic genes are targets of Myc (Osthus *et al.* 2000), and we have previously shown increased conversion of glucose to pyruvate and lactate in Rat1A $myc^{+/+}$ cells compared to $myc^{-/-}$ cells (Morrish *et al.* 2008). When we evaluated the spectra for additional ^{13}C-enriched intermediary metabolites, we found increased levels of ^{13}C-labeled alanine and glycine, derived from pyruvate and the glycolytic intermediate 3-phosphoglycerate, respectively (Fig. 2B,C). Glycine is a precursor for *de novo* purine synthesis as well as a carbon donor for C1/folate metabolism required for nucleotide biosynthesis. We observed ^{13}C labeling of the C2 position in the adenine base, originating from N^{10}-formyl-tetrahydrofolate, as well as increased incorporation of ^{13}C into the ribose moiety of nucleotides, demonstrated by a 3-fold increase in ribose C4' labeling (Fig 2D). Folate is required for one-carbon transfer reactions in purine and thymidylate biosynthesis, as well as epigenetic modification of DNA and proteins. Expression of ~50% of genes involved in folate metabolism are increased following Myc induction (Morrish *et al.* 2008), and SHMT2 is one of only two Myc target genes results shown to complement growth defects in $myc^{-/-}$ cells (Nikiforov *et al.*, 2002). Treatment with the dihydrofolate reductase inhibitor, methotrexate, inhibited growth of $myc^{+/+}$ cells more effectively than $myc^{-/-}$ cells (Fig. 3C). Similar results were obtained using *myc*−/− cells transfected with inducible Myc (*myc*−/− MycER) (Fig. 3C). These results provide additional evidence of the close relationship between nucleotide synthesis and Myc-dependent cell proliferation (Mannava *et al.*, 2008), and establish direct links between increased glucose metabolism and nucleotide synthesis in Myc-expressing cells.

Myc increases phosphocholine levels

Phosphocholine is an intermediate in phosphatidylcholine synthesis and is required for membrane biosynthesis during cell cycle entry (Jackowski, 1994). Since phosphocholine is difficult to quantitate in ^{13}C spectra due to spectral overlap, we used 1D-^{1}H spectra to evaluate phosphocholine content. We found a 3-fold increase in phosphocholine levels in serum-stimulated $myc^{+/+}$ cells compared to $myc^{-/-}$ cells (Fig. 3A). Myc induction increases choline kinase expression (Morrish *et al.*, 2008), and elevated phosphocholine levels are associated in neuroblastomas with N-Myc amplification (Peet *et al.*, 2007). The rate-limiting enzyme in phosphatidylcholine (PtdCho) synthesis, CTP: phosphocholine cytidylyltransferase (CCT/PCYT1A), is also a Myc target gene (Li *et al.*, 2003), suggesting Myc increases glycerophospholipid synthesis. The increased TCA cycle flux observed in $myc^{+/+}$ cells may also play a role in increased glycerophospholipid synthesis by generating acetyl groups required for *de novo* fatty acid synthesis. In preliminary results, we observed that fatty acid synthesis from [U-^{13}C] glucose is increased in $myc^{+/+}$ cells (Morrish, unpublished). These results may be relevant to the delayed cell cycle entry of $myc^{-/-}$ cells, as net accumulation of

PtdCho near the G1/S boundary is necessary for membrane synthesis (Jackowski, 1994). PtdCho is also a source of lipid second messengers involved in cell cycle progression (Hunt and Postle, 2004).

O-GlcNAc post-translational modification is regulated by Myc

Glycolytic (fructose-6-phosphate) and TCA cycle intermediates (glutamine, acetyl-CoA) are substrates for the hexosamine biosynthesis pathway (HBP). Since the ^{13}C data demonstrated increased glycolytic and TCA cycle flux in serum-stimulated $myc^{+/+}$ cells, we looked for global differences in serum-induced protein modification by O-linked N-acetylglucosamine (O-GlcNAc) in $myc^{+/+}$ and $myc^{-/-}$ cells. Immunoblotting of cell extracts using an O-GlcNAc-specific antibody detected multiple bands, with an overall temporal increase after serum addition in $myc^{+/+}$ cells. Several bands were detected at lower intensity in serum-starved cells, while others appeared with serum stimulation (Fig. 3B). The number of O-GlcNAc-modified proteins detected in $myc^{-/-}$ cells was reduced and the serum response significantly attenuated.

Increased O-GlcNAc protein modification may result from increased expression of modifying enzyme, O-GlcNAc transferase (OGT), and/or increased hexosamine synthesis. OGT protein expression was reduced as demonstrated by immunoblotting in $myc^{+/+}$ cells compared to $myc^{-/-}$ cells under serum-deprived conditions and an early response to serum was not observed (Fig. 3C). Hence, early Myc-associated increases in protein O-GlcNAcylation are not explained by upregulation of OGT expression.

To examine the role of O-GlcNAcylation in Myc-dependent proliferation, we treated cells with 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutamine:fructose-6-phosphate aminotransferase (GFAT), the first and rate-limiting enzyme of the hexosamine pathway. Both $myc^{+/+}$ and induced $myc^{-/-}$ MycER cells were more sensitive to growth inhibition at 48 h, suggesting an important role for the HBP during proliferation in $myc^{+/+}$ cells (Fig. 3D). O-GlcNAc protein modification is a dynamic process analogous to phosphorylation (Hart *et al.*, 2007). Interference with O-GlcNAc modification has been shown to disrupt cell cycle progression (Slawson *et al.*, 2005). Several HBP genomic loci are bound by Myc, including GNS and NAGPA (Li *et al.*, 2003) and HBP genes are enriched in expression profiling of Myc-induced pancreatic neoplasia (Lawlor *et al.*, 2006). In addition to transcriptional regulation of the HBP, Myc may increase the supply of essential substrates for hexosamine biosynthesis as a consequence of the increased glycolytic and TCA cycle fluxes demonstrated by ^{13}C isotopomer analysis.

In conclusion, our results demonstrate the broad effects of endogenous Myc expression on glucose carbon flux in both catabolic and anabolic pathways, in response to serum stimulation, including glycolysis, the TCA cycle, the pentose phosphate pathway, and C1/folate metabolism (Fig. 4). The obligate role of mitochondria in C1/folate metabolism raises the possibility that Myc-induced mitochondrial biogenesis provides for biosynthesis of key intermediate metabolites in addition to bioenergetic requirements for cell cycle progression. This extensive programming of metabolism may be unique to Myc. For example, HIF-1 alpha directs increased glycolytic metabolism, but at the expense of anabolic synthesis and mitochondrial respiration (Lum et al., 2007). Finally, the observation of Myc effects on O-GlcNAc post-translational protein modification provides an interesting example of the potential intersection of metabolome and transcriptome networks for signal amplification and integration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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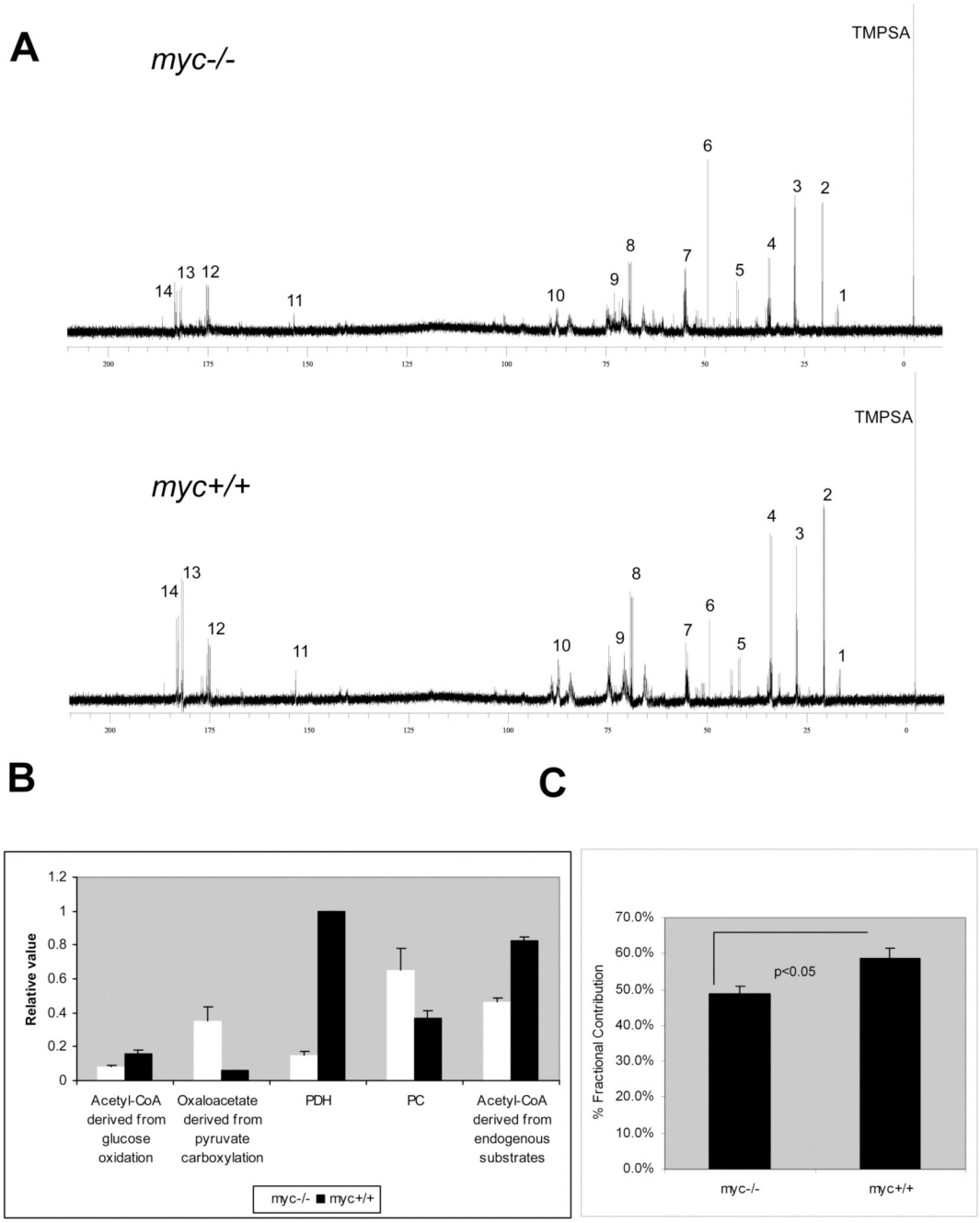

Figure 1. ^{13}C NMR analysis of ^{13}C glucose metabolism in myc$^{-/-}$ and myc$^{+/+}$ cells during cell cycle entry

(A) ^{1}H decoupled ^{13}C spectra. Key. (1) C3 alanine, (2) C3 lactate, (3) C3 glutamate, (4) C4 glutamate, (5) C2 glycine, (6) C5 proline, (7) C2 glutamate, (8) C2 lactate, (9) ribose and sugar moieties, (10) C4‘ NXP, (11) C2 Adenine, (12) C1 glutamate, (13) C5 glutamate, (14) C1 lactate. TMPSA is internal standard. NXP refers to the mono- di- or triphosphate form of any nucleotide. (B) tcaCALC-derived relative fluxes. (C) Percentage contribution of ^{13}C labeled glutamate to total glutamate by LC/MS/MS mass isotopomer analysis.

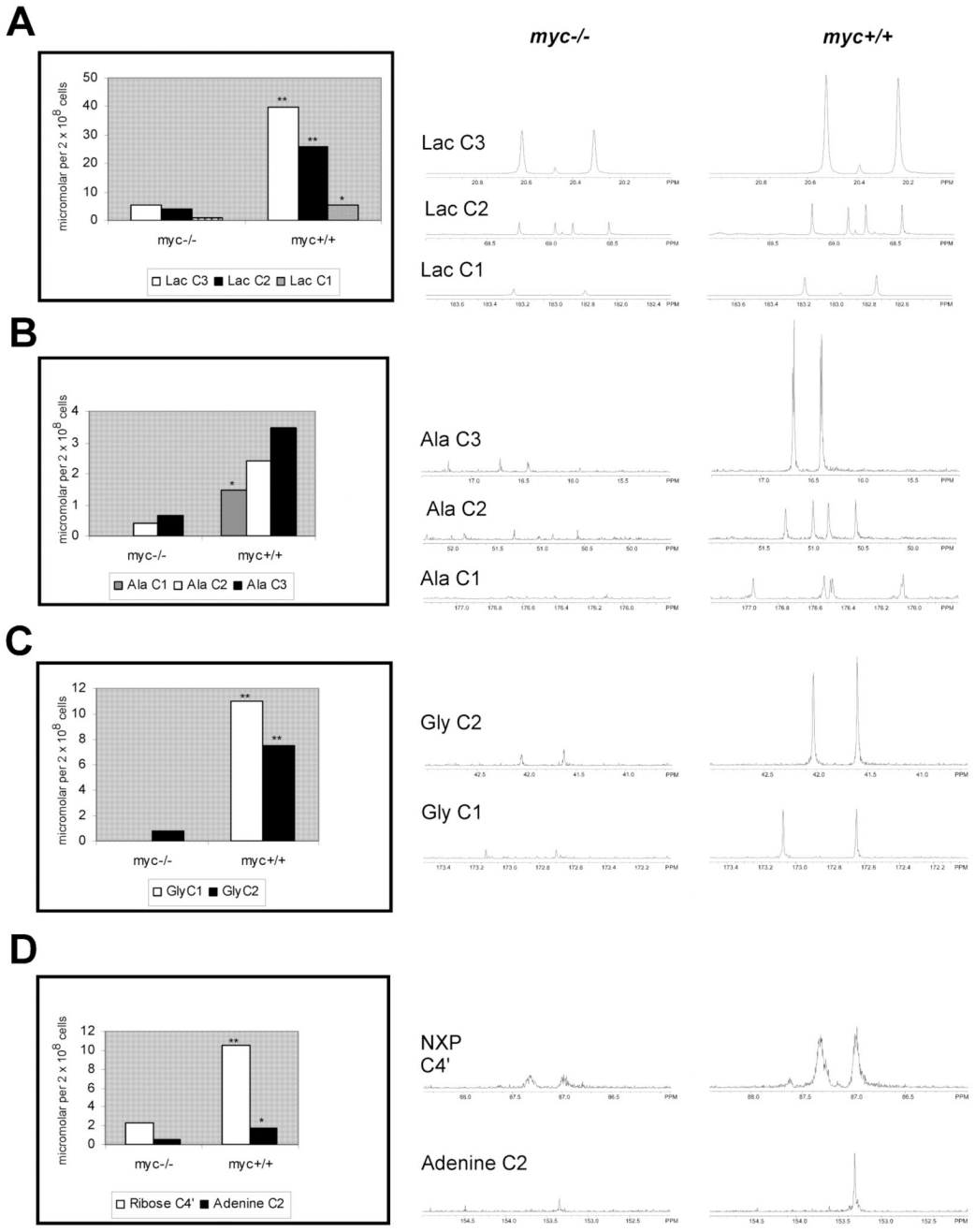

Figure 2. ^{13}C labeling of selected metabolites in myc$^{-/-}$ and myc$^{+/+}$ cells

^{13}C enrichment at individual carbons (left). Data is calculated from integrated peak areas relative to internal standard. Mean of 3 experiments. Significant differences (*=p<0.05, **=p<0.001) assessed by Student's t test. Corresponding NMR spectra (right) for (A) lactate, (B) alanine, (C) glycine, (D) NXP ribose C4' and adenine C2.

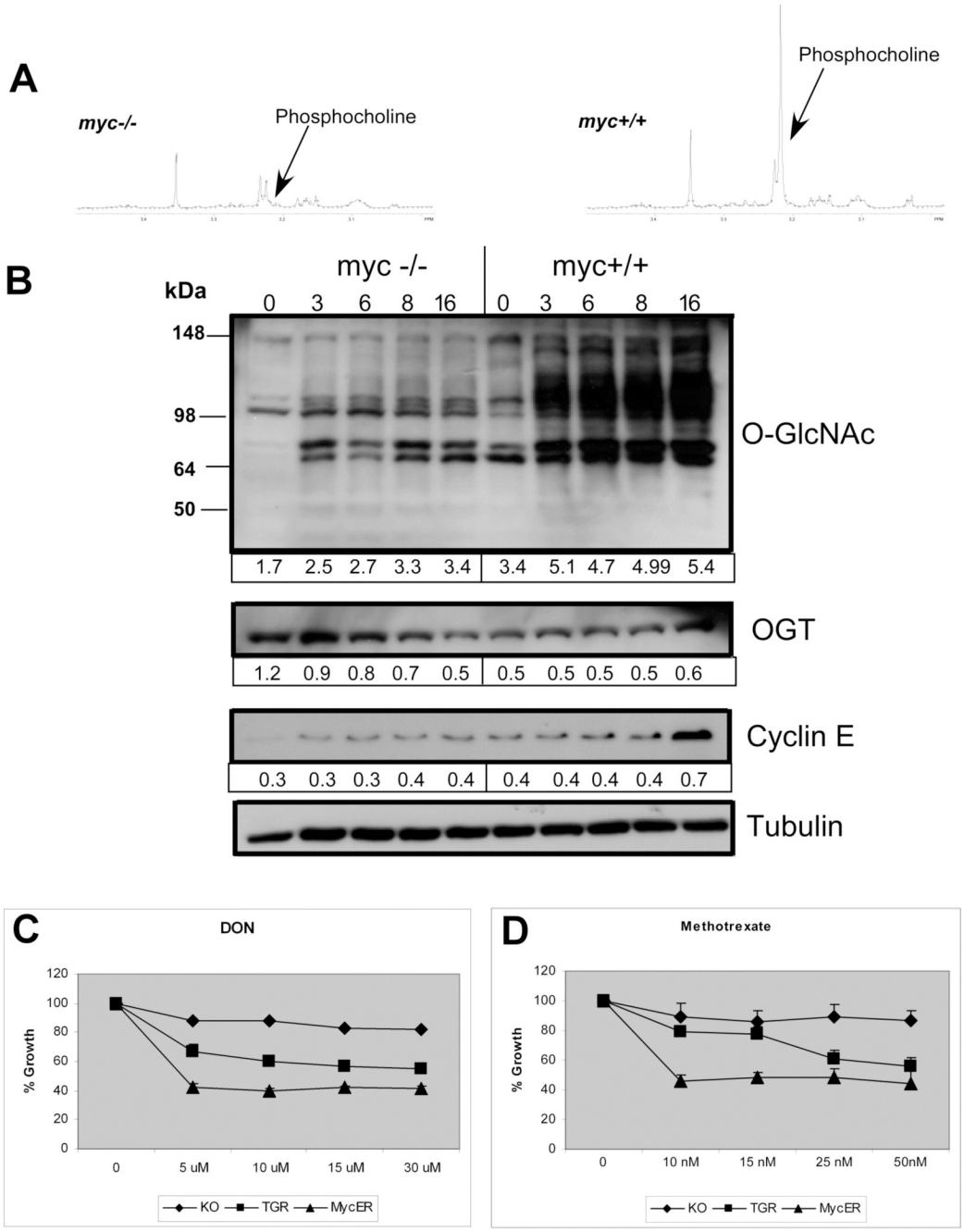

Figure 3. Metabolic pathways required for cell cycle progression in myc$^{+/+}$ cells

(A) Proton spectra for phosphocholine (3.2 ppm). (B) Immunoblots showing O-GlcNAcylated proteins and OGT expression with serum stimulation. Cyclin E and tubulin are positive and loading controls. Densitometry values indicated below each lane (ImageQuant). (C) Dose-response assay of cell growth by HO33342 staining for methotrexate and DON. TGR represents myc+/+ cells. Relative growth rates of untreated cell lines: myc−/− 0.69, mycER 0.83. Representative of 3 experiments in triplicate.

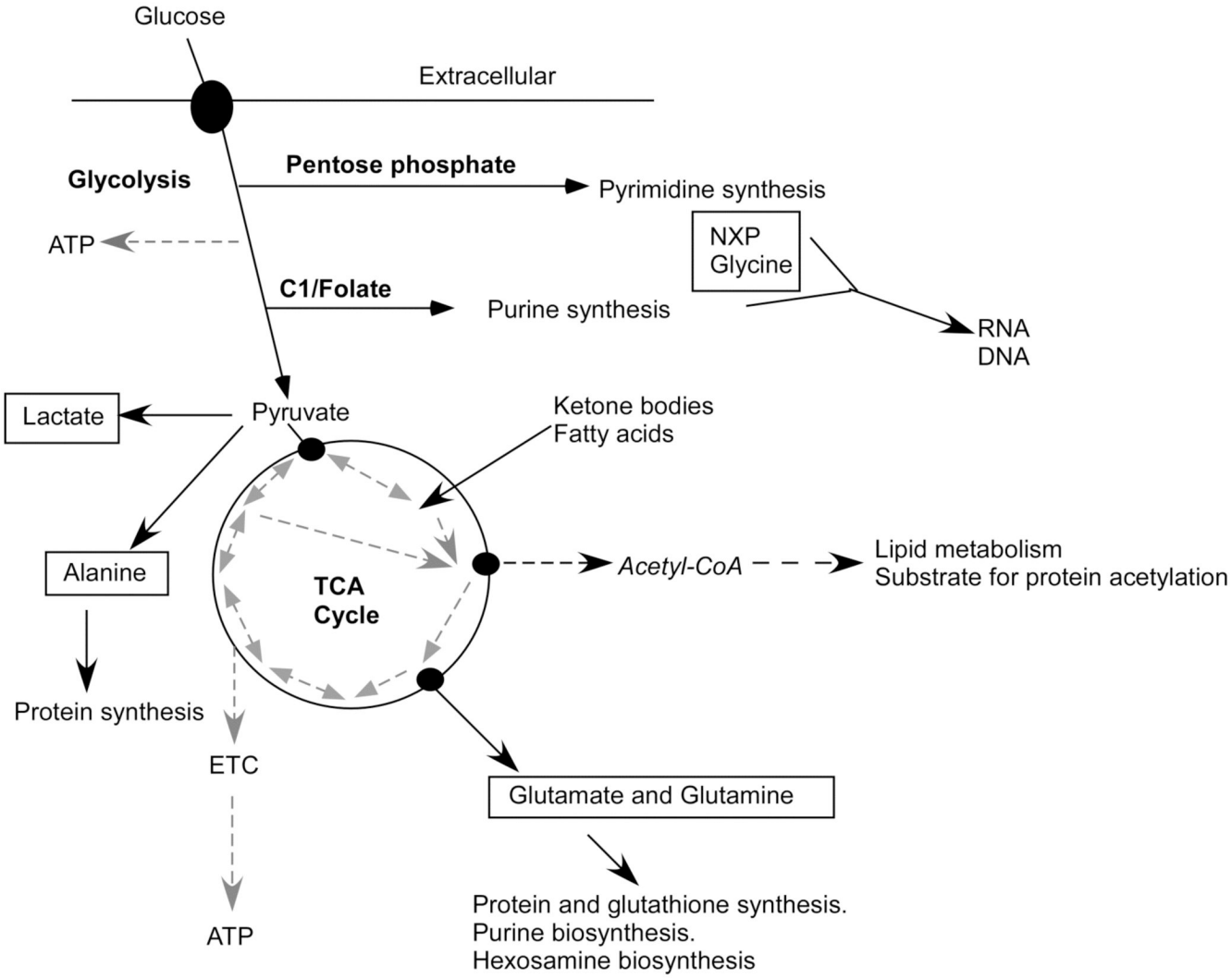

Figure 4.

Myc regulation of glucose metabolism may provide key intermediates, energy and reducing power for cell proliferation. 13C-labeled metabolites from this study are boxed. Dashed arrows link mitochondrial metabolites with reversible (double-ended arrows) or irreversible (single arrows) reactions. Both glycolysis and the mitochondrial TCA cycle generate metabolic intermediates, in addition to ATP, providing building blocks for protein, lipid and nucleic acid synthesis. Post-translational protein modification may be subject to substrate-level control, including extra-mitochondrial acetyl-CoA, derived from intra-mitochondrial pyruvate and fatty acid metabolism, and glucosamine-6-phosphate, derived from fructose-6-phosphate and glutamine.