Myc-dependent Mitochondrial Generation of Acetyl-CoA Contributes to Fatty Acid Biosynthesis and Histone Acetylation during Cell Cycle Entry

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Cell reprogramming from a quiescent to proliferative state requires coordinate activation of multiple -omic networks. These networks activate histones, increase cellular bioenergetics and the synthesis of macromolecules required for cell proliferation. However, mechanisms that coordinate the regulation of these interconnected networks are not fully understood. The oncogene c-Myc (Myc) activates cellular metabolism and global chromatin remodeling. Here we tested for an interconnection between Myc regulation of metabolism and acetylation of histones. Using [13C6]glucose and a combination of GC/MS and LC/ESI tandem mass spectrometry, we determined the fractional incorporation of 13C-labeled 2-carbon fragments into the fatty acid palmitate, and acetyl-lysines at the N-terminal tail of histone H4 in myc−/− and myc+/+ Rat1A fibroblasts. Our data demonstrate that Myc increases mitochondrial synthesis of acetyl-CoA, as the de novo synthesis of 13C-labeled palmitate was increased 2-fold in Myc-expressing cells. Additionally, Myc induced a forty percent increase in 13C-labeled acetyl-CoA on H4-K16. This is linked to the capacity of Myc to increase mitochondrial production of acetyl-CoA, as we show that mitochondria provide 50% of the acetyl groups on H4-K16. These data point to a key role for Myc in directing the interconnection of -omic networks, and in particular, epigenetic modification of proteins in response to proliferative signals.

Post-translational modification of proteins provides a rapid means of altering cell signaling. Regulation of this process is, therefore, a key requirement for cells to rapidly respond to hormonal and nutritional signals. An example of this is the temporal modification of histones during cell cycle entry (1, 2). Epigenetic modification affecting N-terminal tails of core histones modulate interactions with DNA and other proteins, leading to gene activation or repression (3). The pattern of histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (4). The activity of these enzymes can be modulated by the cellular metabolic state, for example, the sir-tuin family of HDACs require NAD+ as a cofactor (5). All known HATs require acetyl-CoA as substrate. In Saccharomyces cerevisiae, nucleocytoplasmic acetyl-CoA synthase is required for histone acetylation and increases in global transcription (6), while in mammalian cells ATP citrate lyase mediates histone acetylation responses to nutrient availability by exporting mitochondrial acetyl-CoA (7). Hence, robust activation of global chromatin remodeling by HATs may require coordinate regulation of gene expression and cell metabolism. However, cellular factors that control both the expression of HATs and increases in cellular metabolic activity have not been identified.

The oncogene c-Myc (Myc) is a transcription factor with global chromatin remodeling activity. The histone acetyltransferase GCN5 is a direct target of Myc. Quantitative analysis of levels of mono-, di-, tri-, and tetra-acetylation of the N-terminal histone H4 tail demonstrated increased GCN5-dependent acetylation in myc+/+ cells, but not myc−/− cells (8). Gene expression analysis suggests that Myc may also regulate the supply of acetyl-CoA, as metabolic pathways linked to acetyl-CoA synthesis are temporally regulated by Myc (9) and Myc regulates both the biogenesis (10) and function of mitochondria (9), a principal source of acetyl-CoA. Using [13C6]glucose metabolic tracer studies, we have recently shown that Myc regulates central carbon metabolism and the partitioning of glucose carbons in the TCA cycle during cell cycle entry (11). These results raise the possibility that Myc activation of mitochondrial metabolism increases the supply of mitochondrial acetyl-CoA as a substrate for lipid biosynthesis and nuclear histone acetylation. Here we have addressed this question by tracing the fate of [13C6]glucose carbons in myc+/+ and myc−/− cells during cell cycle entry. We formally demonstrate that glucose-derived carbons are utilized for lipid biosynthesis and histone acetylation, and that physiologic levels of Myc increase metabolic flux into these biosynthetic pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were cultured and harvested as described (11) with a modification in the duration of culture to 8 h to allow
incorporation of $^{13}$C into lipids. For serum deprivation studies, cells were grown in 0.2% calf serum for 48 h prior to addition of [1$^{13}$C$_6$]glucose. The concentration of [1$^{13}$C$_6$]glucose was 20 mm as a sole labeled substrate and the dual-labeling concentration for both [1$^{13}$C$_6$]glucose and [1$^{13}$C$_3$]acetate was 10 mm. To allow dynamic labeling of histones with $^{13}$C-labeled acetyl groups we did not treat cells with sodium butyrate.

**Mass Spectrometric Quantification of Acetylation at Specific Lysines within the N-terminal Tail of Histone H4**—Histone proteins were isolated from 1 × 10$^8$ cells and in vitro labeled as previously described (12). We used a protocol previously developed for quantitation of histone acetylation using in vitro isotopic acetylation of unmodified lysines with deuterated acetic anhydride (12). The mass difference between a deuterated acetyl group (45 Da) and a protiated acetyl group (42 Da) is 3 Da, allowing distinction between lysine residues acetylated in vitro and in vivo, respectively. This derivatization step, to fully acetylated H4, ensures that the rates of proteolytic digestion, HPLC elution and losses, electrospray ionization (ESI) efficiencies, and CID are independent of the in vivo acetylation patterns, ultimately allowing relative quantitation by ESI-MS/MS to be performed.

Calculation of the relative enrichment of 1 × 13C acetyl groups from [1$^{13}$C$_6$]acetate and 2 × 13C acetyl groups from [1$^{13}$C$_6$]glucose on histone H4-K16 was determined as follows. Each MS/MS spectrum generated in these experiments contains superimposed copies of the isotopic distribution of the target peptide. These copies are shifted by zero, one, two, or three m/z units depending on the associated label. To determine the contribution of each label we calculated a theoretical isotopic distribution for the unmodified peptide using MS-isotope (13). Shifted copies of this isotopic distribution form a matrix of coefficients in the linear system Ax = b, where A is the coefficient matrix, b is a vector of observed intensities from the centroided MS/MS spectrum, and x is a vector of unknown weights specifying the contribution of each label. We solve for the unknown vector x using the “solve” command in the base package of the R environment for statistical computing (14). The first four elements in the resulting vector x are the contributions of the $^{12}$C, $^{13}$C, $^{13}$C$_2$, and D$_3$ peaks respectively. The sum of the remaining elements of x indicates the residual intensity unexplained by the model. Elements of x are divided by the D$_3$ intensity to normalize and facilitate comparison across spectra. These normalized intensities are used to calculate the percentage of Lys-16 acetylation from any given source (in vitro, acetate and glucose), total acetylation, and unacetylation.

**GC/MS Analysis of Lipids**—For lipid analysis, cell pellets from 3 × 10$^5$ cells were resuspended in methanol/2.5% H$_2$SO$_4$, and incubated for 1 h at 80 °C to convert to fatty acid methyl esters (FAMES), as described (15). The FAMES were analyzed by gas chromatography/mass spectrometry (GC/MS) (Agilent 5975GC/MS). Isotopomers were monitored in a scanning ion mode customized to each fatty acid species of interest; for example, C16:0 MS scans ranged from m/z 265 to m/z 290.

**Data Analysis**—Labeling of palmitate was determined from relative intensities of ions at m/z 270–286. The incorporation of de novo labeled acetyl-CoA into palmitate was calculated using previously described methods that correct for natural abundance. Isotopomer spectral data graphs illustrate the fractional abundance of each isotopomer of palmitate ranging from mass M + 0, palmitate containing only $^{12}$C, to M + 16, palmitate containing all 16 carbons as $^{13}$C (16). The x-axes of these graphs are labeled as M0 to M16 to indicate the numbers of $^{13}$C atoms in palmitate. Fractional contribution of $^{13}$C-labeled carbon sources to fatty acid synthesis, D, and fractional new synthesis of fatty acids during time t, g(t), were estimated from the mass isotopomer distribution of palmitate based on the model of IRA as previously reported (17). All experiments were performed at least three times, and statistical analysis of comparisons between myc$^{-/-}$ and myc$^{+/+}$ samples was conducted using Student’s t test.

**RESULTS**

Myc Increases Mitochondrial Export of Acetyl Groups during Cell Cycle Entry—Previous studies with myc$^{+/+}$ and myc$^{-/-}$ fibroblasts demonstrated that endogenous levels of Myc are required for increased respiration and mitochondrial oxidation of glucose-derived pyruvate during serum-induced cell cycle entry (9). Acetyl groups generated from pyruvate oxidation in mitochondria can be utilized for de novo fatty acid synthesis in the endoplasmic reticulum via citrate export and cleavage by ATP citrate lyase (18). We used stable isotope labeling to determine if Myc also supported extra-mitochondrial flux of acetyl-CoA for lipid biosynthesis. Fibroblasts arrested by serum deprivation were stimulated to enter cell cycle by addition of serum for 16 h with addition of [1$^{13}$C$_6$]glucose for the last 8 h.

We determined the relative incorporation of $^{13}$C carbon into palmitate by GC/MS measurements of palmitate isotopomers (19). The fractional enrichment of palmitate isotopomers for myc$^{-/-}$ and myc$^{+/+}$ cells are illustrated in Fig. 1A. This analysis indicates that myc$^{+/+}$ cells have a 2-fold increase in palmitate isotopomers, (p < 0.005), with mass units M + 6, M + 14. We used isotopomer spectral analysis (ISA) to quantify lipogenic fluxes (17). The fraction of palmitate resulting from de novo synthesis, g(t), was increased to 11% for myc$^{+/+}$ cells compared with 6% for myc$^{-/-}$ cells. [1$^{13}$C$_6$]glucose metabolism provided 53% of the lipogenic acetyl-CoA pool (D) for myc$^{+/+}$ cells, in contrast to 33% for myc$^{-/-}$ cells. De novo lipid synthesis, and to a lesser extent, fractional contribution of [1$^{13}$C$_6$]glucose to fatty acid synthesis, were up-regulated by serum addition from basal levels in serum-deprived cells (supplemental Fig. S1). Analysis of changes in total fatty acid species on addition of serum demonstrate a significant increase in saturated fatty acids (SFAs) and decrease in polyunsaturated fatty acids (PUFAs) for myc$^{+/+}$ compared with myc$^{-/-}$ cells (Fig. 1B). This shift in fatty acid composition may reflect an increase in de novo fatty acid synthesis as well as increased metabolism of PUFAs. These results demonstrate that Myc-dependent glucose oxidation during cell cycle entry provides substrates for de novo lipid biosynthesis required for cell division.

Acetyl Groups Generated from Mitochondrial Metabolism of Glucose-derived Intermediates Are Substrates for Acetylation of Histone H4—Myc expression correlates with global histone H3 and H4 acetylation, which requires the Myc target GCN5 histone acetyltransferase (8). Histone acetyltransferases utilize
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As illustrated in Fig. 2A, the histone H4 N-terminal tail contains 4 lysines, each of which may be acetylated in vivo to varying degrees. After in vitro acetylation and trypdic digestion the peptides generated will consist of combination of the following: protiated acetyl $^{12}$C, protiated acetyl $^{13}$C, and deuterated acetyl (D) (Fig. 2C). To determine the relative abundance of acetylated histone H4, trypsin-digested peptides were analyzed by LC-ESI MS/MS. The expanded spectral overlay for the doubly charged precursor ion encompassing tryptic peptide residues 4–17 includes all of the isotopic forms of H4(4–17), from fully protiated (HHHH, 4 Ac) to fully deuterated (DDDD, 0 Ac) (Fig. 2A). For each lysine acetylated in vivo, there is an isotopic shift of $3/2 = 1.5$ m/z. Superimposed on this pattern is the $2/2 = 1$ m/z shift associated with $^{13}$C-acetyl groups. Because of signals from naturally occurring $^{12}$C-acetyl groups superimposing on signals from $[^13]$C-glucose-derived acetyl groups, an isotope correction tool was utilized (described under “Experimental Procedures”) to calculate the signals from $^{12}$C, $^{13}$C, and $^2$D-acetyl peaks, which are expressed as relative ratios, with 1 as the total combined unacylated and acetylated histone. Peak height differences are observed for $^{13}$C-labeled singly, doubly, and triply acetylated peptide ions, consistent with increased utilization of $[^13]$C-glucose for histone H4 acetylation in myc$^+/−$ cells (Fig. 2A).

To evaluate the fractional enrichment of $^{13}$C$_2$-acetyl groups at specific lysines in H4(4–17), we analyzed CID-produced fragment ions by MS/MS. By determining the intensity of signals from endogenous acetylation (m/z 530), $[^13]$C$_6$glucose-derived acetylation (m/z 532) and deuterium-labeled acetylation (m/z 533), both the fractional contribution of $^{13}$C$_2$-derived glucose carbons to acetylation of H4-K16 and the total (combined $^{12}$C and $^{13}$C) levels of acetylation are calculated (Fig. 2C). As illustrated in Fig. 2B, the expression of Myc increased the level of $^{13}$C$_2$-acetyl present on histone H4-K16, contributing to the higher total acetylation present in myc$^+/+$ cells compared with myc$^−$/− cells (Fig. 3A). Comparing levels of total acetylation with $^{13}$C$_2$-acetyl groups, it is evident that acetyl groups derived from $[^13]$C-glucose contributed ~50% of acetylated H4-K16 in myc$^+/+$ cells (Fig. 3A).

The calculation of both total acetylation and levels of $^{13}$C-acetylation provide insights into two Myc-regulated events that control levels of histone acetylation. The first (Fig. 4A) is HAT activity, demonstrated by a log$_2$ ratio for total H4-K16-acetylation of 0.35 ($p < 0.015$) in myc$^+/+$ cells compared with myc$^−$/− cells. The second (Fig. 4B) is the pool of nucleocytoplasmic $^{13}$C-acetyl groups from the mitochondria, shown by a log$_2$ ratio for $^{13}$C-acetylation at H4-K16 of 0.5 ($p = 0.035$) for myc$^+/+$ compared with myc$^−$/− cells. Taken together, the data suggest Myc effects on substrate supply contribute substantially to overall HAT activity when fed glucose.

Histone Acetylation with Mixed Substrates—To further investigate the importance of mitochondrial acetyl-CoA supply
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A

![Graph showing relative abundance and m/z values for Acetyl-CoA with Myc genotypes: myc+/+ and myc-/-]

B

![Graph showing relative abundance and m/z values for Myc genotypes: myc-/- and myc+/+]

C

| Acetylation Form                  | Mass Increase | Observed y<sub>5</sub> m/z |
|-----------------------------------|--------------|--------------------------|
| Endogenous <sup>12</sup>C<sub>2</sub>H<sub>5</sub>O | 0            | 530                      |
| [<sup>13</sup>C-U] glucose <sup>12</sup>C<sub>2</sub>H<sub>5</sub>O | +2           | 532                      |
| Chemical <sup>12</sup>C<sub>2</sub>D<sub>3</sub>O       | +3           | 533                      |

100% Occupancy
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tate and glucose maintain an increased dependence on glucose substrate for histone acetylation (Fig. 4D). The increased utilization of acetate by myc−/− cells is highlighted by the significant negative log2 ratio for acetate-derived 13C-acetyl groups when compared with myc+/+ cells (Fig. 4D). As a result the difference between myc+/+ and myc−/− cells in total acetyl-H4-K16 is reduced (Fig. 4C).

Acetylation of H4-K16 serves as a switch for altering chromatin from a repressed to a transcriptionally active state in yeast and humans (20), subject to dynamic modification during cell cycle entry (1), and an epigenetic signature of cell proliferation (21). The increased 13C-acetylation of H4(4–17) observed with Myc expression indicates an important role for Myc in substrate supply in addition to increased GCN5 expression affecting histone acetylation rates. These data confirm that mitochondria provide a source of acetyl-CoA for histone acetylation and demonstrate how Myc activation of mitochondrial function can amplify its ability to induce global chromatin remodeling by providing substrates required for histone acetylation.

**DISCUSSION**

This study demonstrates that the oncogene c-Myc increases the supply of acetyl groups from glucose required for histone H4-K16-acetylation, during serum-induced cell cycle entry (1). Our analysis using [13C6]glucose as a tracer demonstrates that these acetyl groups are derived from mitochondrially oxidized glucose, consistent with the recent report that ATP citrate lyase is required for nutrient-dependent regulation of histone acetylation (7). By conducting these studies with 13C-labeled glucose, we were able to trace the de novo production and addition of acetyl groups to histones during cell cycle entry (Fig. 3D). We additionally addressed the relative prioritization of substrates for histone acetylation, and the impact of alternative carbon sources, we incubated cells in [13C6]glucose in combination with [13C1]acetate. This dual-label stable isotope protocol allows tracking of the source of the acetyl groups on histones, as 13C1-Ac derived from acetate or 13C2-Ac derived from glucose (Fig. 3D). As illustrated in Fig. 3, A and B, the addition of acetate increased the total acetylation of histones. Both myc−/− and myc+/+ cells utilized acetate in addition to glucose as substrates for H4 acetylation (Fig. 3B), but myc−/− cells have a significantly greater contribution from acetate compared with myc+/+ cells (Fig. 3C). The myc+/+ cells, in contrast, have equivalent contributions from both acetate and glucose, and continue to utilize glucose at higher rates than myc−/− cells (Fig. 3C).

When the contributions of substrate supply to HAT activity are compared, myc−/− cells incubated with labeled acetyl groups are compared, myc−/− cells incubated with labeled acetyl donor used by HATs. The single 13C1 label present in 2 carbon acetyls derived from acetate by acetyl-CoA synthase or from citrate derived from glucose oxidation by ATP citrate lyase provides the contribution of both mitochondrial and nuclear pools of acetyl-CoA to histone acetylation. Production of acetyl-CoA from acetate by acetyl-CoA synthase or from citrate derived from glucose oxidation by ATP citrate lyase provides the acetyl donor used by HATs. The single 13C1 label present in 2 carbon acetyls derived from acetate or 13C2-Ac derived from glucose (Fig. 3B, C). The myc+/+ cells, in contrast, have equivalent contributions from both acetate and glucose, and continue to utilize glucose at higher rates than myc−/− cells (Fig. 3C).

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acetylation by provision of both $^{13}\text{C}_1$-acetate and $^{13}\text{C}_6$glucose.

**Myc Mobilizes Glucose-derived Acetyl-CoA for de Novo Synthesis of Fatty Acids**—We show that endogenous levels of Myc increase the export and utilization of mitochondrially derived acetyl groups for synthesis of the fatty acid palmitate during cell cycle entry. During a labeling period restricted to 8 h during G1 progression, 11% of total palmitate was synthesized *de novo* from a cytoplasmic acetyl-CoA pool, 53% of which is derived from the $^{13}\text{C}$glucose tracer. The remainder of the cytoplasmic acetyl-CoA pool is likely derived from fatty acids or endogenous glycogen stores.

Higher glucose concentrations in the media increase cytoplasmic acetyl-CoA pools and lipid synthesis by increasing glycolytic flux (7, 29). In the current study, comparing *myc*+/−/− cells entering the cell cycle, the mechanisms responsible for Myc-induced increases in the contribution of glucose to cytosolic acetyl-CoA likely include Myc activation of mitochondrial biogenesis (9), increased glycolytic flux and entry of glucose carbons into the TCA cycle during cell cycle entry (11).
A comparison of fatty acid profiles for these two cell lines shows increased levels of saturated fatty acids in myc\(^{+/+}\) cells. The reduced levels of polyunsaturated fatty acids in myc\(^{+/+}\) cells may reflect turnover or increased generation of bioactive lipids.

These data are in contrast to results from inducible MycER expression in MEFs, in which Myc promotes preferential utilization of glutamine for phospholipid synthesis (22). However, cells with oncogenic levels of Myc are glutamine-dependent, unlike cells with physiologic expression of Myc. The metabolic effects of oncogenic Myc were analyzed in actively dividing cells, with no differences in cell proliferation reported with Myc induction (22). Our recent data indicates that physiologic expression of Myc also increases the synthesis of lipids during cell cycle entry when cells are provided with glutamine, acetate, or acetocetate as a sole carbon source. A role for Myc in activation of de novo lipid synthesis required for cell division is further substantiated by the temporal activation of multiple genes involved in lipid synthesis including acetocetate-CoA synthetase (Aacs) and ATP citrate lyase (Acly) (9). Of note, labeling with \(^{13}\)C\(_6\) was carried out in the presence of fetal calf serum dialyzed to remove competing fuels with the exception of protein-bound lipids. Thus, endogenous levels of Myc promote de novo fatty acid synthesis even when exogenous fatty acids are available.

**Mitochondria Provide Acetyl Groups for de Novo Acetylation of H4(4–17)**—Changes in histone acetylation occur during cell cycle entry (1). Using metabolic tracer techniques, we determined that up to ~50% of acetyl groups detected on the N-terminal tail of histone H4 are derived from oxidation of glucose. As cells were only labeled for the second of two 8-h periods after serum addition, this value is likely an underestimate of de novo labeling during the G1/S transition. Two carbon fragments are only generated from hexose substrates by mitochondrial metabolism. This significant contribution documents the importance of mitochondria in the provision of substrates for post-translational modification of proteins in mammalian cells which, unlike S. cerevisiae, contain ATP citrate lyase and, as a result, access to a mitochondrial supply of acetyl groups (6, 23). These experiments were conducted with \(^{13}\)C-labeled glucose, however, exogenous acetate can also supply acetyl-CoA for histone acetyltransferases in mammalian cells (7).

Our analysis of the contribution of exogenous \(^{13}\)C\(_{1}\)acetate to acetylation of histones demonstrates that acetyl-CoA synthetase is active in both cell lines. Glucose-derived acetylation is diminished by the additional substrate, but less prominently in myc\(^{+/+}\) cells, perhaps as a consequence of Myc-dependent glucose metabolism. Total acetylation increases in both cell lines, reflecting the increased availability of nuclear acetyl-CoA. In addition, at least one HAT, P/CAF, can be activated and translocated to the nucleus by autoacetylation (24).

Acetyl groups generated by peroxisomal fatty acid oxidation (25) or ketone body metabolism (26) also provide a source of cytoplasmic acetyl groups for histone acetylation. The methods we have developed here provide a way to evaluate the contribution of different substrates to histone acetylation, and other recently identified acetylated proteins (27), in different physiological contexts.

We have previously shown that myc\(^{−/−}\) cells have reduced mitochondrial function and treatment of myc\(^{+/+}\) cells with rotenone recapitulates the slow growth phenotype of myc\(^{−/−}\) cells (9). As illustrated in Fig. 4, up-regulation of histone acetyltransferase activities, such as GCN5, would not explain the differences in \(^{13}\)C-acetyl histone labeling between myc\(^{+/+}\) and myc\(^{−/−}\) cells. This increased labeling requires an increase in the \(^{13}\)C-acetyl-CoA pool, which in these experiments is derived from mitochondrial glucose oxidation. Together, these data suggest that Myc ability to increase mitochondrial function and the flux of glucose carbons through PDH play a significant role in the ability of Myc to increase histone acetylation required for global chromatin remodeling. Overall these results add to the accumulating evidence for Myc involvement as a nutritional sensor (28) and regulator of the coordination between proteomic and metabolomic networks required for post-translational modification of proteins (11).

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3 F. Morrish, unpublished data.

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