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Molecular Cell, 55(2)
1097-2765
Lewis, CA
Parker, SJ
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et al.
2014-07-17
10.1016/j.molcel.2014.05.008
Peer reviewed
Tracing Compartmentalized NADPH Metabolism in the Cytosol and Mitochondria of Mammalian Cells

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http://dx.doi.org/10.1016/j.molcel.2014.05.008

SUMMARY

Eukaryotic cells compartmentalize biochemical processes in different organelles, often relying on metabolic cycles to shuttle reducing equivalents across intracellular membranes. NADPH serves as the electron carrier for the maintenance of redox homeostasis and reductive biosynthesis, with separate cytosolic and mitochondrial pools providing reducing power in each respective location. This cellular organization is critical for numerous functions but complicates analysis of metabolic pathways using available methods. Here we develop an approach to resolve NADP(H)-dependent pathways present within both the cytosol and the mitochondria. By tracing hydrogen in compartmentalized reactions that use NADPH as a cofactor, including the production of 2-hydroxyglutarate by mutant isocitrate dehydrogenase enzymes, we can observe metabolic pathway activity in these distinct cellular compartments. Using this system we determine the direction of serine/glycine interconversion within the mitochondria and cytosol, highlighting the ability of this approach to resolve compartmentalized reactions in intact cells.

INTRODUCTION

One of the defining characteristics of eukaryotic cell metabolism is the compartmentalization of reactions in different organelles. Although coordination of metabolic flux across organelles is critical for cell physiology, the inability to distinctly observe identical reactions present in more than one subcellular location has been a major barrier to understanding cell metabolism. Many of these compartmentalized reactions are oxidation/reduction (redox) reactions that utilize pyridine nucleotide-based cofactors to transfer electrons between metabolites to support biosynthesis, redox homeostasis, signal transduction, and ATP generation (Pollak et al., 2007a). For instance, reduction of NAD+ to NADH captures energy from catabolic reactions to drive ATP synthesis through mitochondrial oxidative phosphorylation, while NADPH is regenerated via a different set of reactions to maintain reduced glutathione (GSH) pools and support reductive biosynthesis (Lunt and Vander Heiden, 2011). As such, NADPH has been hypothesized to be limiting for proliferation, lipid biosynthesis, and survival in response to cell stress (Diehn et al., 2009; Jeon et al., 2012; Jiang et al., 2013; Schafer et al., 2009). These compartmentalized metabolic processes impact numerous cell and tissue functions; therefore, understanding how biochemical networks function across compartments is necessary to determine how metabolism contributes to disease pathologies.

The pool of NADP(H) in cells is small relative to flux through pathways that utilize this cofactor (Pollak et al., 2007a). Thus, interconversion between the oxidized and reduced states must be coupled across all reactions involving this cofactor, and changes in abundance may not be informative for assessing the use of NADPH in a particular pathway. Neither NAD(H) nor NADP(H) is known to be transported across intracellular membranes (Nikiforov et al., 2011; Pollak et al., 2007b), and multistep shuttles involving compartmentalized redox reactions are used to transfer electrons between the mitochondria and cytosol (Bissett et al., 1976; LaNoue et al., 1974; LaNoue and Schoolwerth, 1979). This organization facilitates the maintenance of different NADPH/NAD+ ratios in each subcellular location and allows for the execution of compartment-specific metabolic processes. Classically, cytosolic NADPH is thought to be regenerated primarily via the oxidative pentose phosphate pathway (PPP) (Lunt and Vander Heiden, 2011; Pollak et al., 2007a). Other potential sources of cytoplasmic NADPH exist in mammalian cells, including reactions catalyzed by specific isozymes of isocitrate dehydrogenase (IDH), malic enzyme (ME), aldehyde dehydrogenase (ALDH), and methylene tetrahydrofolate dehydrogenase (MTHFD) (Pollak et al., 2007a; Tibbetts and Appling, 2010). However, isozymes of several of these enzymes also catalyze identical reactions in the mitochondria and can potentially transfer reducing equivalents between the mitochondria and the cytosol. For example, the reductive carboxylation of alpha-ketoglutarate (zKG) to isocitrate by IDH2 consumes mitochondrial NADPH, with citrate/isocitrate subsequently transported to the cytosol where it can be oxidized by IDH1 to produce cytosolic NADPH (Sazanov and Jackson, 1994; Wise et al., 2011). Theoretically,

Molecular Cell 55, 253–263, July 17, 2014 ©2014 Elsevier Inc. 253
the reverse cycle may be used to produce mitochondrial NADPH. Metabolic cycles such as this utilize compartment-specific enzymes, and existing methods for tracing metabolism rely on breaking apart cells and pooling metabolites from all compartments, making it impossible to reliably distinguish the net reaction flux through each enzyme or pathway.

RESULTS

Tracing NADPH with 2H-Labeled Glucose

Because reaction mechanisms involving pyridine nucleotides transfer electrons as a hydride (H⁻/C⁰⁺) ion, isotope-labeled hydrogen atoms can be used to follow electron movement in these reactions (Katz et al., 1965; Rendina et al., 1984). The transfer of 2H and 3H can also be used to observe redox reactions in central carbon metabolism, an approach that has been used to generate insight into NAD(P)H metabolism in eukaryotic cells (Ben-Yoseph et al., 1994; Rühl et al., 2012). Glucose is the primary carbon source for glycolysis and the oxidative PPP in mammalian cells, with the latter pathway representing an important source of cytosolic NADPH. Nonlabile hydrogen atoms on specific glucose carbons (the 1 and 3 positions, respectively) are transferred to NADPH by the oxidative PPP enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). The hydrogen atom on carbon-3 of glucose (which becomes carbon-1 of dihydroxyacetone phosphate in glycolysis) can exchange with water during isomerization to glyceraldehyde-3-phosphate (GAP) by triose phosphate isomerase (TPI) (Katz et al., 1965). This prevents confounding labeling of downstream metabolites including TCA cycle intermediates, suggesting that tracing this hydrogen atom could provide a means of quantifying the contribution of 6PGD to the cellular NADPH pool (Figure 1A) (Katz et al., 1966; Katz and Rognstad, 1978).

To test this possibility, we cultured H1299 non-small-cell lung cancer cells in the presence of [3-2H]glucose and observed labeling of NADPH using LC/MS-MS (Figure 1B). The rapid turnover of NADPH allows labeling from [3-2H]glucose to reach isotopic steady state within 30 min, as evidenced by the lack of any increased label incorporated into NADPH after culturing cells in the presence of [3-2H]glucose for 24 hr (Figure 1B). NADPH has two hydrogens that can be transferred when it acts as an electron donor. Once labeled, either the labeled or unlabeled hydrogen atom can be transferred depending on the stereospecificity of downstream NADPH-utilizing enzymes. Transfer of the unlabeled hydride from labeled NADPH generates labeled NADP⁺ (see Figure 1C).

Figure 1. Use of 2H Glucose to Label Cytosolic NADPH

(A) Atom-transition map depicting a model of deuterium transfer from [3-2H]glucose through glycolysis and the pentose phosphate pathway. Open large circles represent carbon, and small red circles indicate deuterium label from [3-2H]glucose. Where measured, enrichment of M1 isotopomer (%) for glycolytic intermediates in parental H1299 cells is shown.

(B) Labeling of NADPH from [3-2H]glucose in parental H1299 cells over time.

(C) Saturated fatty acid labeling (myristate; C14:0, palmitate; C16:0 and stearate C18:0) from [3-2H]glucose in parental H1299 cells following incubation for 72 hr.

(D) Cholesterol labeling from [3-2H]glucose in parental H1299 cells cultured for 72 hr.

(E) Enrichment of lipogenic [2H]-NADPH by [3-2H]glucose estimated by a model for saturated fatty acid synthesis (ISA) in parental H1299 and A549 cells following incubation with tracer for 72 hr.

Data plotted in (A)–(D) represent mean ± SD of at least three biological replicates. For (E), data presented are mean ± 95% confidence interval of at least three biological replicates.
The NADPH labeling we observe is a subset of the total cellular pool. To gain insights into compartment-specific redox reactions, we next quantified $^2$H enrichment in specific metabolites downstream of NADPH-dependent reactions. For example, fatty acid and cholesterol synthesis occur specifically in the cytosol and require NADPH. When H1299 cells are cultured with [3-$^2$H]glucose for 72 hr to allow for accumulation of new lipid molecules, we detected significant label on newly synthesized fatty acids, including myristate (C14:0), palmitate (C16:0), and stearate (C18:0), as well as cholesterol (Figures 1C and 1D). Importantly, no label from [3-$^2$H]glucose was detected on citrate (see percent value in Figure 1A), suggesting that isotope enrichment on lipids was from the NADPH pool that was labeled by the oxidative PPP. We also detected labeling of fatty acids from [1-$^2$H]glucose (Figure 1H); however, tracing of GAPD-derived NADPH is complicated by deuterium from [1-$^2$H]glucose being retained on citrate and lipogenic acetyl-CoA (Figure 1D). We next applied isotope ratio mass spectrometry (iMS) to estimate the contribution of [3-$^2$H]glucose to the lipogenic NADPH pool used for palmitate synthesis, as 14 NADP(H) molecules are required during the production of one palmitate molecule (Kharrouri et al., 1992; Metallo et al., 2012). The ISA model includes two parameters representing the deuterium enrichment of the NADPH pool and the percentage of palmitate that was synthesized de novo (Figure S2). Using this method we estimated the enrichment of lipogenic NADPH from [3-$^2$H]glucose ranged from 12%–20% in A549 and H1299 cells (Figure 1E). ISA modeling of other saturated fatty acids (e.g., myristate and stearate) produced similar estimations for the enrichment of lipogenic NADPH from [3-$^2$H]glucose (Figure 1E).

**Use of $^2$H Glucose to Trace NADH Metabolism**

To maintain flux through glycolysis, cytosolic NAD$^+$ pools are regenerated primarily by three enzymes: lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and/or the glycerol phosphate shuttle (Glyc3PDH) (Lunt and Vander Heiden, 2011; Metallo and Vander Heiden, 2013). Distinct hydrogen atoms on glucose are transferred to NAD$^+$ during glycolysis via glyceraldehyde phosphate dehydrogenase (GAPDH). In theory, up to half of the hydrogen transferred to NADH via GAPDH comes from carbon four of glucose; however, exchange with water in the aldolase and TPI reactions decreases the net contribution of this hydrogen atom to NADH (Go et al., 2009) (Figure 2A). Upon culturing A549 and H1299 cells with [4-$^2$H]glucose, significant labeling of lactate, malate, and glycerol 3-phosphate was observed (Figure 2B). Label was detected on GAP in A549 cells; however, the level of GAP in H1299 cells was below the limit of detection. In addition, no label was detected on metabolites in lower glycolysis including PEP, 3PG, and pyruvate. This pattern fits with known reactions using NADH in central carbon metabolism (Figure 2A) and suggests that [4-$^2$H]glucose can be used to label the NADH pool produced by glycolysis in cells. Consistent with these findings, we observed rapid labeling from [4-$^2$H]glucose on NADH (Figure 2C), as well as label on NADPH (Figure 3A) arising from donation of the unlabeled hydride from M1 labeled NADH.

Interestingly, we observed isotope incorporation into fatty acid pools from [4-$^2$H]glucose (Figure 2D), suggesting that some label from NADH transfers to cytosolic NADPH through an unknown mechanism (Figure S3F). While deuterium label from [4-$^2$H]glucose was detected on aspartate, citrate, and isocitrate due to the symmetry of fumarate (Figures S3B–S3D), carbons labeled in this manner do not contribute to lipogenic acetyl-CoA, demonstrating that the observed fatty acid labeling is derived from hydride transferring from NADH to cytosolic NADPH. We also observed some isotope enrichment on NADPH from [4-$^2$H]glucose (Figures 2E and S3A); however, ribose 5-phosphate and ribulose 5-phosphate are also labeled from [4-$^2$H]glucose (Figure S3E). Furthermore, these direct measurements of total cellular NADP(H) cannot distinguish between cytosolic and mitochondrial pools, highlighting the need for methods to elucidate compartment-specific NADP(H) pools.

**A Reporter System to Trace Compartmenalized Sources of NADPH**

The above data demonstrate that we can observe cytosolic production of NADPH and NADH in intact cells. Although we were able to quantify the contribution of oxidative PPP enzymes to the lipogenic NADPH pool, deuterium tracing alone cannot distinguish other compartmentalized sources of NADP(H) production.
NADPH. Therefore, we sought to develop a reporter system that can detect pathway-specific NADPH production in different subcellular compartments. To accomplish this, we took advantage of the neomorphic mutant IDH enzymes that produce \((\text{D})^2\)-hydroxyglutarate (2HG) from \(\alpha\)-KG. This reaction reduces \(\alpha\)-KG by transferring a hydride from NADPH to form 2HG. As 2HG is a xenometabolite that is only present at very low levels in most cells (Matsunaga et al., 2012), it can be used as an endproduct readout. By applying specific metabolic \(2\text{H}\)-tracers to cells and measuring enrichment of 2HG produced by ectopically expressed mutant IDH1 (cytosol) or IDH2 (mitochondria), we reasoned that pathway-specific information on NADPH metabolism in each compartment could be obtained (Figure 3A).

We generated H1299 and A549 cell lines that express epitope-tagged mutant IDH1-R132H (mtIDH1-C) or mutant IDH2-R172K (mtIDH2-M) in a doxycycline-dependent manner (Figure S4A). We employed a weak promoter to minimize effects on endogenous IDH metabolism. Indeed, FLAG-tagged mtIDH1-C was expressed at levels that were not detectable with an antibody recognizing wild-type IDH1 enzyme in these cells (Figure S4A). mtIDH1-C is expected to be expressed in the cytoplasm and mtIDH2-M in the mitochondria, and we confirmed the localization of each using cell fractionation and western blotting (Figure 3B). We also confirmed that the FLAG-tagged mutant IDH enzymes produce 2HG in a doxycycline-dependent manner in both H1299 and A549 cell lines (Figure 3C). Interestingly, mtIDH1-C produced less 2HG than mtIDH2-M in cells, consistent with observations that ectopically expressed IDH2 mutants produce more 2HG than IDH1 mutants due to their mitochondrial localization (Ward et al., 2013). In all cases, 2HG levels were far below those observed in tumor cell lines expressing endogenous mutations in IDH1 (R132C/+), HT1080 or IDH2 (R172S/+, SW1353) (Figure 3D). Introduction of mutant IDH enzymes could impact NADPH or TCA metabolism; however, 2HG production flux observed in cell lines expressing IDH1 mutants at higher levels is small relative to other \(\alpha\)-KG-dependent reaction fluxes, suggesting mutant IDH expression has a minimal direct impact on \(\alpha\)-KG pools (Grassian et al., 2014). Furthermore, no significant change in \([3-\text{2H}]{\text{glucose}}\) contribution to cytosolic NADPH was observed.

Figure 2. Use of \(2\text{H}\) Glucose to Label NADH
(A) Atom-transition map depicting a model of deuterium transfer from \([4-\text{2H}]\)glucose through glycolysis and NAD+-dependent shuttle systems (malate dehydrogenase MDH, glycerol 3-phosphate dehydrogenase Gly3PDH, and lactate dehydrogenase LDH). Open large circles represent carbon, and small green circles indicate deuterium label from \([4-\text{2H}]\)glucose.
(B) Labeling of glycolytic intermediates from \([4-\text{2H}]\)glucose in A549 (left panel) and H1299 (right panel) cells. Glyceraldehyde-3-phosphate (GAP) was below the limit of detection in H1299 cells, indicated by asterisk.
(C) Labeling of NADH from \([4-\text{2H}]\)glucose in parental H1299 cells over time.
(D) Palmitate labeling from \([4-\text{2H}]\)glucose in A549 and H1299 cells following incubation with tracer for 72 hr.
(E) Time course labeling of NADPH from \([4-\text{2H}]\)glucose in parental H1299. Data presented are mean ± SD of at least three biological replicates.
observed in A549 cells following mtIDH1-C or mtIDH2-M expression (Figure S4G).

Although doxycycline can affect the metabolism and proliferation of some mammalian cancer cell lines in culture (Ahler et al., 2013), we saw no doxycycline-dependent changes in the abundance of central carbon metabolites (Figure S4B) or in the proliferation rate (Figure S4C) of A549 or H1299 cells. Importantly, cells expressing doxycycline-inducible GFP also showed no changes in metabolite pool sizes or proliferation rates (Figures S4B and S4C), indicating that when added at this concentration...
doxycycline does not significantly affect metabolism in this system. In addition, we observed no significant differences in pool sizes of NAD⁺, NADH, NADP⁺, or NADPH in H1299 mtIDH1-C and mtIDH2-M cells following the addition of doxycycline for 24 hr, suggesting that doxycycline-dependent production of 2HG was not altering the availability of these cofactors for use in other redox reactions (Figure S4D). This is supported by the reported kcat of mutant IDH1 enzymes being small relative to wild-type IDH1 (Dang et al., 2009) and suggests that any direct effects of these enzymes on cellular redox state are minimal.

**Validation of Compartment-Specific Cofactor Tracing**

To validate the ability of this system to trace compartment-specific NADPH metabolism, we induced expression of the mutant IDH enzymes in cells cultured in the presence of [3-2H]glucose and measured enrichment of ²H in the 2HG pool. In order to ensure that the cells were at or near isotopic steady state prior to induction of mutant IDH expression, the cells were incubated with tracer for 24 hr prior to the addition of doxycycline. Consistent with [3-²H]glucose producing cytosolic NADPH via the oxidative PPP, 2HG was only significantly labeled from [3-²H]glucose in the mtIDH1-C cell lines and not in the mtIDH2-M cell lines (Figure 3E). Importantly, little to no label was observed on xKG under these conditions, ensuring that label on 2HG was a direct result of hydride ion transfer from NADPH by the mutant enzyme. We next asked whether label from [4-²H]glucose was incorporated into 2HG by either mtIDH1-C or mtIDH2-M. Notably, more 2HG was labeled from [4-²H]glucose in mtIDH2-M cells than in mtIDH1-C cells (Figure 3F). These data suggest that transfer of H⁺ from NADH to NADPH occurs through a mitochondrial intermediate (e.g., malate) or via nicotinamide nucleotide transhydrogenase (NNT), and transfer of reducing equivalents from NADH to NADPH mostly supports the mitochondrial NADPH pool. Similar results were observed in cell lines with endogenous, heterozygous IDH1 and IDH2 mutations (Figures S4E and S4F).

In vitro steady-state enzyme kinetics experiments have demonstrated that rate constants for reactions involving ²H transfer can be lower compared to studies conducted with unlabeled substrate (Rendina et al., 1984). This phenomenon has been an invaluable tool for elucidating biochemical reaction mechanisms, including many of the reactions responsible for label transfer in our system. However, given the diverse means through which metabolism is regulated and the challenges associated with understanding which enzymatic steps are rate limiting for pathways in intact cells, the relevance of isotope effects to intracellular metabolic fluxes is not clear. To determine the significance of this phenomenon in our system, we cultured cells in different ratios of [3-²H]glucose and unlabeled glucose and measured downstream labeling of lipogenic NADPH and 2HG in H1299 mtIDH1-C cells (Figure 4A). Similarly, we titrated [4-²H]glucose with unlabeled substrate in H1299 mtDH2-M cells and observed whether label transfer to lactate, malate, aspartate, fumarate, citrate, and 2HG was affected by different amounts of labeled substrate (Figure 4B). We reasoned that if reaction rates are affected by the presence of ²H isotopes, the use of unlabeled substrates would be favored and less relative transfer of label would be observed as unlabeled substrate is titrated into the medium. However, in all cases, transfer of label from either tracer decreased linearly as the tracer was diluted with unlabeled substrate, suggesting that kinetic isotope effects minimally impact the results of these experiments (Figures 4A and 4B). A linear decrease in lipogenic NADPH and 2HG labeling was also observed in endogenous mtIDH1 cells (HT1080) that exhibit much higher rates of 2HG production when we titrated [3-²H]glucose, further supporting the notion that isotope effects minimally affect substrate fluxes through the reactions we traced in intact cells (Figure 4C).

**Characterizing Serine/Glycine Metabolism in the Cytosol and Mitochondria**

We next sought to use our reporter system to examine a compartmentalized metabolic cycle. Reactions that make up folate-mediated one-carbon metabolism exist in both the cytosol and the mitochondria, although it is not clear from current literature whether these are linked in a cycle and/or in which direction the reactions proceed (Anderson et al., 2011; Nilsson et al., 2014; Tedeschi et al., 2013; Tibbetts and Appling, 2010). Serine and glycine interconversion via serine hydroxymethyltransferase (SHMT) has been observed in cultured cells (Jain et al., 2012; Levintow and Eagle, 1961; Perry et al., 2007), but ¹³C tracing is unable to ascertain the directionality, compartmentalization, and interconnectivity of this process. Indeed, upon culture with [U-¹³C₃]serine, we observed significant interconversion of serine and glycine in A549 mtIDH1-C and mtIDH2-M cells (Figure 5A). The reactions catalyzed by MTHFD1 (cytosolic) and MTHFD2/MTHFD2L (mitochondrial) utilize NAD(P)H (Figure 5B); therefore, we hypothesized that ²H serine and glycine tracing in combination with our compartment reporter system would enable us to experimentally determine the direction of serine-glycine exchange reactions in the cytosol and mitochondria. Hydrogens on carbon-3 of serine are transferred to 5,10-methylene-THF and subsequently to NADPH via MTHFD1/2 (Figure 5B). Additionally, the glycine cleavage system (GCS) exists in the mitochondria and could transfer hydrogen from carbon two of glycine to 5,10-methylene-THF and generate NADPH (Figure 5B) (Kikuchi et al., 2008).

To study these compartment-specific pathways, we cultured A549 mtIDH1-C and mtIDH2-M cells with either [3,3-²H₂]serine or [2,3,3-²H₃]serine and unlabeled glycine or [2,2-²H₂]glycine and unlabeled serine and measured incorporation of ²H in cytosolic or mitochondrial 2HG, respectively. Strikingly, we detected label from [3,3-²H₃]serine and [2,3,3-²H₃]serine on 2HG only in mtIDH2-M cells, strongly suggesting that serine to glycine conversion occurs primarily in the mitochondria in these cells with the MTHFD2/MTHFD2L reaction operating oxidatively (Figures 5C and S5A–S5C). We did not observe labeling of 2HG from [2,2-²H₂]glycine in cells expressing either mutant IDH (Figure 5C), indicating that either the majority of mitochondrial glycine is generated by SHMT2 (rather than glycine import) or the label is lost in the GCS. Consistent with the lack of label transfer from ²H-labeled serine or glycine to 2HG in mtIDH1-C cells, we detected minimal contribution of these tracers in the lipogenic NADPH pool (Figure 5D). To further confirm the direction of MTHFD1, we cultured A549 mtIDH1-C and mtIDH2-M cells with [3-²H]glucose, which specifically labels cytosolic NADPH.
We observed transfer of $^2$H from $[3-^{2}H]$glucose onto serine, suggesting that cytosolic MTHFD1 can operate in the reductive direction in these cells (Figure 5E). The lack of glycine labeling from $[3-^{2}H]$glucose confirms label transfer to serine was obtained from 5,10-methyleneTHF (Figures 5E and S5D). The lack of labeling from either $[2,3,3-^{2}H_3]$serine or $[3,3-^{2}H_2]$serine on either fatty acids or 2HG produced by mtIDH1-C is also consistent with minimal contribution of MTHFD1 to the cytoplasmic NADPH pool in these cells, although channeling to other reactions cannot be ruled out by these methods. Shuttling of serine labeled by $[3-^{2}H]$glucose by MTHFD1/SHMT1 into the mitochondria for catabolism by MTHFD2/SHMT2 may account for the small amount of label (<1%) we observe on mitochondrial 2HG in A549 mtIDH2-M cells cultured with $[3-^{2}H]$glucose (Figure 3E). Collectively, these data provide direct evidence that serine metabolism can contribute to regenerating mitochondrial NADPH in cells.

DISCUSSION

We have developed a system that can distinguish compartmentalized pools of NADPH, demonstrating the directionality and interconnectivity of serine/glycine metabolism in the cytosol and mitochondria of intact cells. In the cells studied, conversion of serine to glycine occurs primarily in the mitochondria with the reaction catalyzed by MTHFD2/MTHFD2L contributing to NADPH production in this compartment. Interestingly, label transfer from both $[2,3,3-^{2}H_3]$serine and $[3,3-^{2}H_2]$serine to mitochondrial 2HG was observed, suggesting that serine metabolism by SHMT2 is a contributor to the mitochondrial NADPH and glycine pools. These data also provide direct experimental support for the hypothesis that the cytoplasmic source of formate used for purine synthesis can be mitochondrially derived in some cells. Previous efforts to ascertain directionality of folate-mediated one-carbon metabolism have been unable to distinguish between compartments, relying on expression data (Nilsson et al., 2014), mathematical modeling (Scotti et al., 2013; Tedeschi et al., 2013), or isolated mitochondrial preparations (Barlowe and Appling, 1988). The importance of distinguishing compartmentalized redox pathways is highlighted by the large number of potential pathways that have been implicated in the shuttling of reducing equivalents between the cytosol and mitochondria. For instance, compartment-specific metabolic cycling through citrate/aKG (Sazanov and Jackson, 1994; Ward et al., 2010), malate/pyruvate (Jiang et al., 2013; Son et al., 2013), proline (Hagedorn and Phang, 1983; Nilsson et al., 2014), and serine (Tibbetts and Appling, 2010) have been suggested to be important for mammalian cell physiology. Although carbon tracing is increasingly combined with genetic approaches to implicate a role for compartment-specific...
isozymes in such processes, adaptation to genetic depletion strategies that break these cycles can confound interpretation. The reporter system described here circumvents these issues for reactions involving NADPH by providing direct visualization of compartmentalized reaction activity and direction in intact cells.

Figure 5. Characterizing Serine/Glycine Metabolism in the Cytosol/Mitochondria
(A) Serine (left panel) and glycine (right panel) labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [U-13C3]serine. Cells were incubated with [U-13C3]serine for 24 hr prior to dox-induction (0.1 μg/mL) for an additional 48 hr. Middle panel demonstrates interconversion of serine and glycine by SHMT. Data plotted represent mean ± SD for three biological replicates.

(B) A schematic of folate-mediated one-carbon metabolism in cytosolic and mitochondrial compartments catalyzed via SHMT and MTHFD. Deuterium transfer from [3,3-2H2]serine is shown for pathways containing SHMT and MTHFD and is indicated by small red or blue circles for cytosolic and mitochondrial isozymes, respectively. The extra deuterium on [2,3,3-2H3]serine is indicated by an orange (cytosolic) or a turquoise (mitochondrial) circle. Deuterium transfer from [2,2-2H2]glycine is shown for the glycine cleavage system (GCS) pathway indicated by small green circles.

(C) 2HG labeling from [3,3-2H2]serine, [2,3,3-2H3]serine or [2,2-2H2]glycine in A549 mtIDH1-C and mtIDH2-M cells. Cells were incubated with either tracer for 24 hr prior to dox induction (0.1 μg/mL) for an additional 48 hr. No label was detected on 2HG in mtIDH1-C cells from either [3,3-2H2]serine or [2,3,3-2H3]serine, nor was label detected on 2HG from [2,2-2H2]glycine in mtIDH2-M cells (indicated by asterisk).

(D) Fatty acid labeling from A549 mtIDH1-C and mtIDH2-M cells cultured with either [3,3-2H2]serine, [2,3,3-2H3]serine, or [2,2-2H2]glycine. Cells were incubated with tracer for 24 hr prior to dox induction (0.1 μg/mL) for an additional 48 hr.

(E) Serine and glycine labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [3-2H]glucose. Cells were incubated with tracer for 24 hr prior to dox induction (0.1 μg/mL) for 48 hr. Data represent mean ± SEM of at least three biological replicates.
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Resolving Compartmentalized NADPH Metabolism

Other subcellular compartments also have distinct metabolic needs in eukaryotic cells and could be probed with an analogous approach by engineering the localization of mutant IDH enzymes and/or monitoring \(^{2}H\) transfer between other metabolites. The endoplasmic reticulum (ER) is an important site for protein folding, disulfide bond formation, long chain fatty acid extension, and sterol reduction; as such, the NADP\(^+/\)NADPH ratio within the ER can influence diverse cellular and physiological processes (Bánhegyi et al., 2009; Kardon et al., 2008; Száráz et al., 2010). This approach may also be adapted to quantify the NADP\(^+/\)NADPH ratio in particular organelles and the turnover rate of NADPH in specific compartments. Altering the cofactor selectivity of the mutant IDH enzyme, or relying on NADH-dependent production of another xenometabolite could similarly be used to visualize compartmentalized reactions that utilize NADH. Finally, these data may be integrated with compartmentalized \(^{13}C\) metabolic flux analysis (MFA) models to better understand the role of cofactor metabolism in metabolic engineering applications or disease models (Rühl et al., 2012). Thus, this approach opens up new avenues to observe metabolic processes in complex cells and improve our understanding of metabolism in normal and disease states.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Isotopic Labeling**

All cell lines were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, and 4 mM L-glutamine. The pSLIK-mtDH cell lines (see below) were maintained as above, but FBS was substituted for Tet-free FBS (Clontech). Cell number was determined using an automated cell counter (Nexcelom) or by haemocytometer. For isotopic labeling experiments in the tracer medium was prepared from custom phenol red-, glucose-, sodium bicarbonate, and DMEM-levels of L-arginine, L-cystine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine prepared as a 100 mM stock for both L-phenylalanine and L-valine (Cambridge Isotope Laboratories, Inc.). Cells were cultured in the presence of tracer medium for 24 hr prior to doxycycline induction and accumulation 2HG. Isotope-labeled glycine and serine were cultivated in 6-well or 12-well plates, and volumes of tracer media and extraction buffers were adjusted accordingly. Derivatization of both polar metabolites and fatty acids has been described previously (Metallo et al., 2012). Briefly, polar metabolites were derivatized to form methoxime-tBDMs derivatives by incubation with 2% methoxylamine hydrochloride (MP Biomedicals) in pyridine (or MOX reagent (Thermo Scientific) followed by addition of N-tert-butylmethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with 1% tert-butylmethylichlorosilane (t-BDMS) (Regis Technologies). Nonpolar fractions, including triacylglycerides and phospholipids, were saponified to free fatty acids and esterified to form fatty acid methyl esters by incubation with 2% H\(_2\)SO\(_4\) in methanol. Derivatized samples were analyzed by GC-MS using a DB-35MS column (30 m x 0.25 mm i.d. x 0.25 μm, Agilent J&W Scientific) in an Agilent 7890A gas chromatograph (GC) interfaced with an Agilent 5975C mass spectrometer (MS). Mass isotopomer distributions were determined by integrating metabolite ion fragments (Table S1) and corrected for natural abundance using in-house algorithms adapted from Fernandez et al. (1996).

**Extraction of NAD+, NADH, NADP+, and NADPH and Analysis by LC-MS/MS**

The extraction protocol for NADPH was based on one previously described by Emdt et al. (2013) and was optimized for analysis by LC-MS/MS. Briefly, cells were cultured in 6-well plates over the course of 30 min, washed once in ice-cold water, and immediately quenched in liquid nitrogen. A total of 200 μl ice-cold extraction buffer (40:40:20 acetonitrile/methanol/200 mM NaCl, 10 mM Tris-HCl (pH 9.2)) was added directly to the cells. Cells were scraped on ice and cleared by centrifugation at 4°C. A total of 50 μl of supernatant was transferred to a polypropylene vial, and samples were analyzed using a Q Exactive Benchtop LC-MS/MS (Thermo Fisher Scientific).

For measurement of NAD(P)H at 24 hr, cells were cultured in 10 cm plates. After 24 hr incubation with tracer, approximately 1 x 10\(^7\) cells were washed in ice-cold 0.9% saline and immediately quenched in 1 ml of 80% methanol at ~80°C. Cells were scraped on ice and cleared by centrifugation at 4°C. Cleared supernatant was transferred to Eppendorf tube, dried under vacuum using a Centriflup (Labconco), resuspended in water, and immediately loaded onto a XSELECT HSS X5 150 mm x 2.1 mm x 2.5 μm (Waters, Milford, MA).
MA with an UFLC XR HPLC (Shimadzu, Columbia, MD) coupled to an AB SCIEX Qtrap 5500 mass spectrometer (AB SCIEX, Framingham, MA) operating in negative ion mode. Mass isotopomer distributions were corrected for natural abundance using in-house software adapted from Fernandez et al. (1996). Additional information regarding chromatographic separation, mass spectrometry, and data acquisition can be found in the Supplemental Experimental Procedures.

Isotopomer Spectral Analysis

The ISA method compares a measured palmitate mass isotopomer distribution to one that is simulated using a reaction network for palmitate synthesis whereby 14 NADPH molecules are consumed to form one palmitate molecule. Models were also generated for myristate and stearate synthesis whereby 12 or 16 NADPH molecules are consumed to form one myristate or stearate molecule, respectively. Parameters for the relative enrichment of the lipogenic NADPH pool from a given [3H] tracer and the percentage of fatty acids that are de novo synthesized are extracted from a best-fit model using the INCA MFA software package (Figure S2) (Young, 2014). The 95% confidence intervals for both parameters were estimated by evaluating the sensitivity of the sum of squared residuals between measured and simulated palmitate mass isotopomer distributions to small flux variations (Antoniewicz et al., 2006).

Cell Proliferation Assays

On day -1, 1/10 of a confluent 10 cm dish of cells was seeded in six 6-wells of a 6-well plate. Twenty-four hours later, cells were counted on a automated cell counter (Nexcelom), and this time point was considered T0. At T0, all other time points were media changed to ± doxycycline media (0.1 mg/mL doxycycline hydrochloride [Sigma] in water). Cells were counted every 24 hr in technical triplicate. Media was changed every 48 hr to prevent degradation of doxycycline in the media.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article at [http://dx.doi.org/10.1016/j.molcel.2014.05.008].

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank Michael Pacold, Elizaveta Freinkman, David Sabatini, and Bernhard Palsson for providing access to equipment. We are grateful to Patrick Ward and Craig B. Thompson for providing the cDNA for IDH1-R132H and IDH2-R172K. Eric L. Bell and Sarah-Maria Fendt provided advice and reagents. This work was supported in part by NIH grants P30CA147882, US4-CA121852-09, and R01CA168653, as well as by the Koch Institute/DHFCC Bridge Project, the Koch Institute Frontier Research and Kathy and Curt Marble Cancer Research Fund, the Burroughs Wellcome Fund, the Damon Runyon Cancer Research Foundation, American Cancer Society grant IRG #70-002, DOD grant W81XWH-13-1-0105, a University of California Cancer Research Coordinating Committee grant, and a Searle Scholar Award.

Received: April 5, 2014
Revised: April 30, 2014
Accepted: May 6, 2014
Published: May 29, 2014

REFERENCES


Supplemental Information

Tracing Compartmentalized NADPH Metabolism in the Cytosol and Mitochondria of Mammalian Cells

Caroline A. Lewis, Seth J. Parker, Brian P. Fiske, Douglas McCloskey, Dan Y. Gui, Courtney R. Green, Natalie I. Vokes, Adam M. Feist, Matthew G. Vander Heiden, and Christian M. Metallo
Figure S1

A. NADP+ labeling from [3-2H]glucose (%).

B. Ru5P and RuSP labeling from [3-2H]glucose (%).

C. NAD+ labeling from [3-2H]glucose (%).

D. Glucose metabolism diagram showing labeling from [1-2H]glucose at various time points.

E. NADP+ and NADPH labeling from [1-2H]glucose (%).

F. Ru5P and RuSP labeling from [1-2H]glucose (%).

G. Glucose 6-phosphate labeling from [1-2H]glucose and [3-2H]glucose (%).

H. Fatty acid labeling from [1-2H]glucose (%).
Figure S2

[3-2H]Glucose

G6P

G6PD

G6P

6PG

1-D

NADPH

Ru5P

Cytosolic NADPH pool

de novo synthesized palmitate (some 2H label)

Pre-existing palmitate (no 2H label)

Measured palmitate mass isotopomer

1-g(t)

Number of isotopes per molecule

Palmitate labeling from [3-2H]glucose (%)
**Figure S4**

**A**

H1299  A549

mtIDH1-C-FLAG

mtIDH2-M-FLAG

mtIDH1-C

mtIDH2-M

Hsp70

Dox

**B**

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**C**

H1299-GFP

H1299-mtIDH1-C

H1299-mtIDH2-M

A549-GFP

A549-mtIDH1-C

A549-mtIDH2-M

**D**

mtIDH1-C

mtIDH2-M

**E**

% M1 from [3-2H]-glucose

2HG

aKG

**F**

% M1 from [4-2H]-glucose

2HG

aKG

**G**

[3-2H]glucose contribution to lipogenic NADPH (%)

GFP

mtIDH1-C

mtIDH2-M

A549, +Dox
Figure S5

A

B

C

D

Glycine Serine

5,10-methylene-THF

THF

SHMT1

[2H]-NADPH

NADP+

MTHFD1

%m1 Ser from [2-2H]glucose

Ser_C1C2 Ser_C2C3

5,10-methylene-THF

NADPH

mtDH1-C

mtDH2-M
Supplemental Figure Legends

Figure S1, related to Figure 1: [1-2H]glucose and [3-2H]glucose label cytosolic NADPH via G6PD and 6PGD.

A) NADP+ labeling in parental H1299 cells incubated for 24 hours with [3-2H]glucose measured via LC-MS/MS. B) Labeling of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P) from [3-2H]glucose measured via LC-MS/MS in parental H1299 cells after 24 hour incubation. C) NAD+ (top panel) and NADH (bottom panel) labeling in parental H1299 cells incubated over a course of 30 minutes and for 24 hours with [3-2H]glucose measured via LC-MS/MS. D) Atom-transition map depicting a model of deuterium transfer from [1-2H]glucose through glycolysis and the pentose phosphate pathway. Open large circles represent carbon and small blue circles indicate deuterium label from [1-2H]glucose. Enrichments of M1 isotopomer (%) for glycolytic intermediates from [1-2H]glucose in parental H1299 cells measured via GC-MS are indicated below specific metabolites. E) Labeling of NADP+ and NADPH in parental H1299 cells incubated for 24 hours with [1-2H]glucose measured via LC-MS/MS. F) Labeling of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P) in parental H1299 cells incubated for 24 hours with [1-2H]glucose measured via LC-MS/MS. G) Glucose 6-phosphate (G6P) labeling in parental H1299 cells incubated for 24 hours with either [1-2H]glucose or [3-2H]glucose measured via LC-MS/MS. Lower G6P labeling from [1-2H]glucose is due to deuterium exchange with hydrogen from water from phosphoglucone isomerase (PGI) reversibility. H) Saturated fatty acid labeling (myristate, palmitate, and stearate) in parental H1299 cells incubated for 72 hours with [1-2H]glucose. Due to label from [1-2H]glucose on citrate, some fatty acid labeling arises from deuterium on lipogenic acetyl-CoA. Data shown represent mean ± SD of at least three biological replicates.
**Figure S2, related to Figure 1: Overview of $[^2\text{H}]$glucose Isotopomer Spectral Analysis (ISA).**

The ISA method applied to palmitate synthesis provides estimates for the relative enrichment of lipogenic NADPH from a particular source (e.g. $[3^{-2}\text{H}]$glucose labels NADPH via 6PGD), the D parameter, and the fraction of the fatty acid pool that was synthesized *de novo*, the $g(t)$ parameter. Parameters are estimated by comparing simulated to measured palmitate mass isotopomer distributions, and the 95% confidence interval is determined by sensitivity analysis.

**Figure S3, related to Figure 2: $[4^{-2}\text{H}]$glucose labels NADH via GAPDH.**

A) NAD$^+$ (left panel) and NADP$^+$ (right panel) labeling over 24 hours in parental H1299 cells incubated with $[4^{-2}\text{H}]$glucose. B) Atom-transition map depicting a model of label transfer from $^2\text{H}$-labeled NADH to malate, via malate dehydrogenase, and subsequent labeling of TCA intermediates. Malate labeling enters the TCA cycle via the malate-aspartate shuttle (small black circles) and labeling is scrambled due to the symmetry of fumarate and reversibility of fumarase (small green circles). Open large circles represent carbon and small coloured circles (black/green) indicated deuterium label from $^2\text{H}$-labeled NADH. No deuterium labels lipogenic acetyl-CoA. C) M1 and M2 labeling of malate (Mal), fumarate (Fum), and succinate (Succ) in parental A549 (left panel) and H1299 (right panel) cells from $[4^{-2}\text{H}]$glucose. D) M1 labeling of aspartate (Asp), citrate (Cit), isocitrate (ICT), and alpha-ketoglutarate (aKG) in parental A549 (left panel) and H1299 (right panel) cells from $[4^{-2}\text{H}]$glucose. E) Labeling of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P) in parental H1299 cells incubated for 24 hours with $[4^{-2}\text{H}]$glucose. Data represent mean ± SD of three biological replicates. F) Possible mechanisms for $[4^{-2}\text{H}]$glucose labeled NADH transferring to cytosolic and mitochondrial NADPH. Labeling of
malate and TCA intermediates are described in Figure S3B. Labeled malate can label cytosolic NADPH through malic enzyme 1 (ME1) or label mitochondrial NAD(P)H through either ME2 or ME3. Mitochondrial NADH can transfer H⁺ to mitochondrial NADPH via transhydrogenase (NNT). Due to fumarate symmetry, label from [4-²H]glucose can transfer to citrate, as described in Figure S3B, and label mitochondrial NAD(P)H via isocitrate dehydrogenase 2 or 3 (IDH2/3) or label cytosolic NADPH via IDH1.

**Figure S4, related to Figure 3: Characterization of cell lines expressing inducible mtIDH1-C and mtIDH2-M.**

A) Flag-tagged exogenous mutant IDH proteins (mtIDH1-C and mtIDH2-M) induced by doxycycline are not overexpressed when compared to endogenous IDH levels. B) Doxycycline does not affect pool sizes of central carbon metabolites as measured by GCMS and compared to vehicle (water) treated (No Dox) H1299 (upper panel) and A549 (lower panel) cells. C) Doxycycline does not affect proliferation rates of H1299 (upper panels) or A549 (lower panels) cell lines. D) Doxycycline addition and 2HG production does not affect pool sizes of NAD⁺, NADH, NADP⁺ or NADPH in H1299 mtIDH1-C cells (upper panel) or mtIDH2-M cells (lower panel). E) NADPH production from the pentose phosphate pathway (as shown by [3-²H]glucose tracing) occurs in cells harboring endogenous IDH1 mutations (HT1080) but not endogenous IDH2 mutations (SW1353). F) [4-²H]glucose labels mostly 2HG in SW1353 cells (IDH2 R172S/+ ) and not in HT1080 (IDH1 R132C/+ ) cells. Data shown are mean ± SEM of three biological replicates (B-D). E-F: data shown represent mean ± SD of three biological replicates. G) Enrichment of lipogenic NADPH from [3-²H]glucose in A549 cells expressing either GFP, mtIDH1-C, or mtIDH2-M following incubation with tracer for 24 hours prior to dox-induction.
(0.1 µg/mL) for 48 hours. Data plotted as mean ± 95% confidence interval of at least three biological replicates.

**Figure S5, related to Figure 5: Directionality of folate-mediated serine and glycine metabolism.**

A) Serine and glycine labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [3,3-²H₂]serine for 24 hours plus 48 hours mtIDH induction (0.1 µg/mL dox). B) Serine and glycine labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [2,3,3-²H₃]serine for 24 hours plus 48 hours mtIDH induction (0.1 µg/mL dox). C) Serine and glycine labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [2,2-²H₂]glycine for 24 hours prior to 48 hours mtIDH induction (0.1 µg/mL dox). Data shown represent mean ± SEM of three biological replicates. D) Serine fragment (C1C2 or C2C3) labeling in A549 mtIDH1-C and mtIDH2-M cells cultured with [3-²H]glucose for 24 hours plus 48 hours mtIDH induction (0.1 µg/mL dox). Presence of label on the C2C3 fragment but not the C1C2 fragment of serine reveals that label arises from transfer of H⁻ from NADPH to 5,10-methylene-THF. Data shown represent mean ± SEM of at least three biological replicates.
Table S1: Metabolite fragment ions used for GCMS and LCMS analysis.

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Supplemental Experimental Procedures

LC-MS/MS Analysis of NAD(H) and NADP(H)

For analysis of NAD(H) and NADP(H) using a Q Exactive Benchtop LC-MS/MS (Thermo Fisher Scientific), chromatographic separation was achieved by injecting 2uL of sample on a SeQuant ZIC-pHILIC Polymeric column (2.1x150mm 5uM, EMD Millipore). Flow rate was set to 100uL/min, column compartment was set to 25°C, and autosampler sample tray was set to 4°C. Mobile Phase A consisted of 20mM Ammonium Carbonate, 0.1% Ammonium Hydroxide in 100% Water. Mobile Phase B was 100% Acetonitrile. The mobile phase gradient (%B) was as follows: 0min 80%, 5min 80%, 30min 20%, 31min 80%, 42min 80%. All mobile phase was introduced into the Ion Max source equipped with a HESI II probe set with the following parameters: Sheath Gas = 40, Aux Gas = 15, Sweep Gas = 1, Spray Voltage = 3.1kV, Capillary Temperature = 275°C, S-lens RF level = 40, Heater Temp = 350°C. NAD+, NADP+, NADH, and NADPH were monitored in negative mode using a targeted selected ion monitoring (tSIM) method with the quadropole centred on the M-H ion m+2 mass. Isolation window was 5amu, resolution was set to 140,000, and AGC target was set to 1e5 ions. Data were acquired and analysed using Xcalibur v2.2 software (Thermo Fisher Scientific). Raw counts were corrected for quadropole bias by measuring the quadropole bias experimentally in a set of adjacent runs of samples at natural abundance. Quadropole bias was measured for all species by monitoring the measured vs. theoretical m1/m0 ratio at natural abundance of all species with m-1, m0, m1, and m2 centred scans. Quadropole bias-corrected counts were additionally corrected for natural abundance to obtain the final mass isotopomer distribution for each compound in each sample.
Chromatographic separation of NAD(H) and NADP(H) using a XSELECT HSS XP (Waters®, Milford, MA) with an UFLC XR HPLC (Shimadzu, Columbia, MD) was operated as follows. Mobile phase A was composed of 10 mM tributylamine (TBA), 10 mM acetic acid (pH 6.86), 5% methanol, and 2% 2-propanol; mobile phase B was 2-propanol. Oven temperature was 40°C. The chromatographic conditions are as follows: 0, 0, 0.4; 5, 0, 0.4; 9, 2, 0.4; 9.5, 6, 0.4; 11.5, 6, 0.4; 12, 11, 0.4; 13.5, 11, 0.4; 15.5, 28, 0.4; 16.5, 53, 0.15; 22.5, 53, 0.15; 23, 0, 0.15; 27, 0, 0.4; 33, 0, 0.4; (Total time [min], Eluent B [vol.%], Flow rate [mL*min⁻¹]). The autosampler temperature was 10°C and the injection volume was 10 uL with full loop injection. An AB SCIEX Qtrap® 5500 mass spectrometer (AB SCIEX, Framingham, MA) was operated in negative mode. Electrospray ionization parameters were optimized for 0.4 mL/min flow rate, and are as follows: electrospray voltage of -4500 V, temperature of 500 °C, curtain gas of 40, CAD gas of 12, and gas 1 and 2 of 50 and 50 psi, respectively. Analyser parameters were optimized for each compound using manual tuning. The instrument was mass calibrated with a mixture of polypropylene glycol (PPG) standards. Samples were acquired using the scheduled MRM pro algorithm in Analyst® 1.6.2. The acquisition method consisted of a multiple reaction monitoring (MRM) survey scan coupled to an information dependent acquisition (IDA) consisting of an enhanced resolution (ER) and an enhanced product ion (EPI) scan for compound isotopomer distribution and compound identity confirmation. MRMs were processed using Multiquant® 2.1.1. Enhanced production ion (EPI) scans and enhanced resolution scans were processed using Analyst®. The identity of each compound was previously determined running pure standards. Linearity of each compound was determined by running calibration curves that spanned the upper and lower limits of detection. Quality controls and carry-over checks were included with each batch.