MDH Introduction

Post Translational Modification of Malate Dehydrogenase

This document is intended to support faculty who are interested in MDH-related CUREs using mammalian MDH. Each reviewed publication includes a summary and limited commentary. Interpretation of collections of publications are discussed as a mini review on a particular sub-topic. This work is meant to help lower the barrier in implementing a mammalian/human MDH CURE project. A complete description of the Malate Dehydrogenase CURE Community (MCC) was published recently.

Introduction

There are many sites of phosphorylation (predicted and identified experimentally by mass spectrometry) for both MDH isoforms but little has been studied for mammalian MDH phosphorylation. Kinases potentially capable of catalyzing the phosphorylation of specific resides on either MDH isoform have not been identified; yet mass spectrometry experiments show that both isoforms are phosphorylated, confirming that kinases capable of this post-translational modification do exist. MDH1 may be methylated and studies have shown that methylation of either Arg 240 or Arg 248 inhibits the enzyme, presumably by shifting the structure from the active dimer to an inactive monomer form. Acetylation of lysine residues also impacts MDH activity. There are four sites on mitochondrial MDH2 known to be acetylated (K185, 301, 307 and 314) and three sites on MDH1 known to be acetylated (K118, 121, 298). While there is a little disagreement in the literature regarding the number of lysine residues acetylated, the modification appears to activate MDH. Interestingly, GOT, PEPCK and ME1 (all enzymes that share substrates with MDH1) have also been shown to be phosphorylated and methylated. Formation of complexes GOT2-MDH2 and CS-MDH2 may very well be regulated not by phosphorylation but acetylation.

Reviews

Regulation of mitochondrial functions by protein phosphorylation and dephosphorylation, *Lim, et al., Cell Biosci.* (2016) 6:25. While not specific to MDH, this paper provides an extensive review of mitochondrial proteins regulated by phosphorylation, and the kinases and phosphatases that participate in the process. Principles discussed in this review may be relevant to our study of mitochondrial MDH2 and the proteins that interact with it.

Tyrosine phosphorylation of lactate dehydrogenase A is important for NADH/NAD$^+$ redox homeostasis in cancer cells, *Fan et al., Mol. Cell Biol.* (2011) 31:4938. While not a review, the demonstration of tyrosine phosphorylation of LDH is an important paper that may provide information about analogous studies that can be performed using MDH. The paper is discussed in more detail below.

Arginine methylation: The coming of age, *Blanc and Richard, Mol. Cell* (2017) 65:8. This review covers the role of arginine methylation in various forms of disease.

Modulating the modulators: Regulation of protein arginine methyltransferases by post-translational modifications*, Hartley and Lu, Drug Discovery Today* (2020), 25:1735. In this review, each of the nine protein arginine methyltransferases are discussed.

Mechanisms, detection and relevance of protein acetylation in prokaryotes, *Christensen, et al., mBio* (2019) 10:e02708-18. In this review, the authors discuss N^{ε}-lysine acetylation in bacteria and archaea. While acknowledging that acetylation can occur on Cys, Ser and Thr residues, the focus of this review is lysine acetylation.

Post-translational protein acetylation: An elegant mechanism for bacteria to dynamically regulate metabolic function*, Christensen, et al., Front. Microbiol.* (2019) 10:1604. This review largely focuses on N^{ε}-lysine acetylation in bacteria, but there is some information included on mammalian systems as well.

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Post-translational Modification by Phosphorylation

Identification of phosphorylation sites

A search of the Phosphosite.net web site was used to find phosphorylation sites of MDH. This website links to curated studies and databases of protein modifications on MDH1 and MDH2 to identify sites of post-translational modification (PTM), including phosphorylation. The number of references is plotted versus sequence, with the type of post-translational modification shown in color according to the posted legend. The site is interactive and allows the user to choose either "LTP - low throughput papers" which are published records of a phosphorylation not using LCMS; or "HTP – high throughput papers" in which LCMS was used to identify the type of post-translational modification (see Fig. 1). The LTP data for MDH1 shows a single monomethylation PTM; for MDH2, a single ubiquitylation site is shown at K241. The curated databases are large phospho-proteomic studies, none specific for MDH. In each case, tissues or cell lines (mammalian or human) were prepared and positive phosphorylated peptides hits identified.

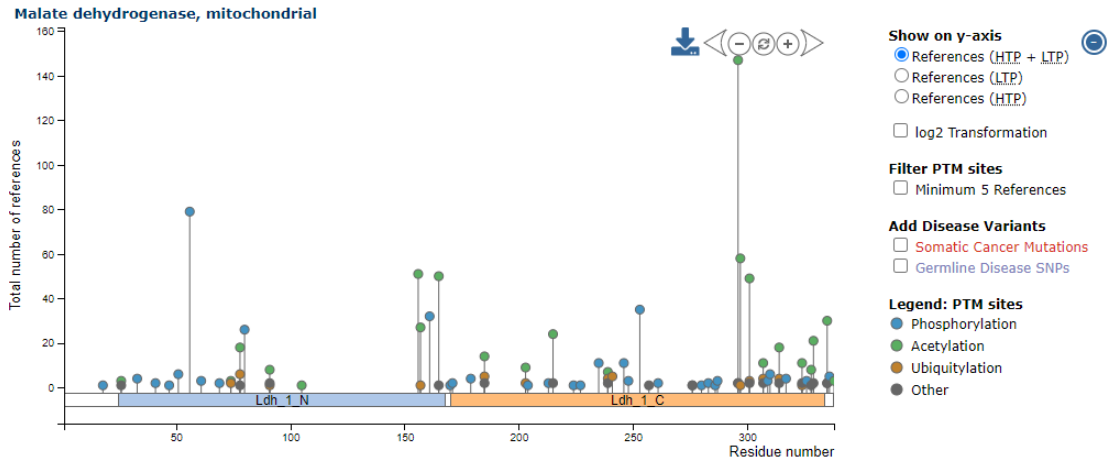

Figure 1: Results generated for MDH2 from PhosphoSite. The number of references is plotted versus residue number, with the type of post-translational modification shown in color according to the legend at right. The radio-button indicated both HTP (high-throughput papers) and LTP (low-throughput papers) was checked.

We scanned for all mammalian positive MDH phosphorylation results and cataloged them for each animal. Non-human mammalian positive hits (i.e. mouse, pig, rat) were double-checked against the human sequence and if the consensus sequence was an identical match, this was counted as a "known phosphorylation site" for human MDH. Depending on the tissue, metabolic / hormonal / disease state, it was not surprising to see each putative phosphorylation site show up in one or more study but not all studies. Thus, one hit was enough to recognize the MDH Ser/Thr/Tyr as a "known phosphorylated residue".

There are likely other databases and websites sites worth investigating to obtain a more complete data set. It should be noted that we do not know the fraction of MDH phosphorylated in any one study. The MS result could show a small percentage of the total MDH that are modified, or that all modified, which should be considered when analyzing the results. From this search we found the following: mitochondrial MDH was phosphorylated at 20 unique sites (10 serine, fifteen threonine and five tyrosine residues) and cytosolic MDH is also phosphorylated at twenty sites (twelve Ser, four Thr and four Tyr). In addition to these "known sites", additional putative phosphorylation sites were predicted using the following data bases during Summer 2019: GPS5.0, NetPhos3.1, KinasePhos2.0 and DISPHOS). At this time, there is no indication that the predicted sites are actually phosphorylated. It is likely that there will be several potential kinases predicted for each site depending on the sequence. Each site is identified on their respective alignment maps and a report with both "known" and "predicted" sites and are available to the MCC community.

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Post Translational Modification of Malate Dehydrogenase

Different expressions of cooperativity in the kinetics of two forms of cytoplasmic malic dehydrogenase, *Vetterlein and Cassman, Biochemistry (1974) 13:3243.*

This is the first of only two mammalian publications investigating MDH phosphorylation. Using purified cytosolic MDH isolated from bovine heart, two forms of soluble or "S-MDH" *a* and *b* were examined for their kinetic behavior. The authors note that the amino acid composition of both forms were the same, but that the two forms differed in their phosphate content, with the *a* form having ~2 mol phosphate/mol protein and the *b* form having ~0 mol phosphate/mol protein. These data confirm that the *a* form of S-MDH is phosphorylated. The authors report that the non-phosphorylated MDH *b* form showed cooperativity with NADH and was inhibited by fructose 1,6-bisphosphate (F16BP), while the phosphorylated *a* form MDH was not cooperative with NADH and not inhibited by F16BP. They report the same V_{max} of MDH for NAD^+ for both forms of the enzyme, but in the OAA → malate direction, the phosphorylated *a* MDH was three-fold more active than the non-phosphorylated *b* MDH.

Cytosolic malic dehydrogenase activity is associated with a putative substrate for the transforming gene product of Rous sarcoma virus, *Rübsamen, et al., Proc. Natl. Acad. Sci USA (1982) 79:228.*

The authors used cells transformed by an active Rous sarcoma virus (RSV) and radiolabeled them with [^{32}-P]-orthophosphate. Cells were then lysed and analyzed by SDS-PAGE and autoradiography. A 38,000 Da radiolabeled cytosolic protein was detected. The protein fractions containing this protein also exhibited MDH activity that could not be separated from the radiolabeled protein. The protein was also identified by Western blot. The authors identified the protein as MDH1. No kinetics or comparison to MDH1 isolated from non-transformed cells was performed.

Commentary: There are a very few other papers that investigate phosphorylation. Here are a few from other organisms and a few papers that support (besides the obvious actual phosphorylation data) that MDH is a substrate for kinases.

Glucose-induced degradation of the MDH2 isozyme of malate dehydrogenase in yeast, *Minard and McAlister-Henn, J. Biol. Chem. (1992) 267:17458.*

[Note that there are differences in yeast nomenclature that differ from human; these are outlined in Table 1.] In yeast, it has been observed that cytosolic MDH levels decrease when yeast are grown on glucose. In this study, the authors show that cytosolic protein levels, but not mitochondrial MDH protein levels decrease as cells are grown in glucose-rich media. The authors constructed a shortened version of yeast MDH2 lacking the normal N-terminal extension, which produces a protein analogous to human MDH1v1. They showed that the truncated variant was more stable and not as susceptible to glucose-induced proteolysis as its longer counterpart.

Table 1: Human and yeast MDH nomenclature. For the human and yeast MDH isoforms, the sequences of the N-terminal extensions are shown, with a putative Ser phosphorylation site shown in bold.

Cellular location	Human	Yeast
Cytosol	MDH1 variant 3 (MDH1v3) 18 amino acid N-terminal extension RRC**S**YFPKDVTVFDKDDK	MDH2 12 amino acid N-terminal extension PHSVTPSIEQD**S**LK
Cytosol	MDH1 variant 1 (MDH1v1) No extension	MDH3 No extension
Mitochondrial matrix	MDH2 No extension	MDH1 No extension

***Glucose-induced phosphorylation of the MDH2 isozyme of malate dehydrogenase in* Saccharomyces cerevisiae,** *Minard and McAlister-Henn, Arch. Biochem. Biophys.* (1994) 315:302.

In this study, the authors build on their previous work and show that the cytosolic MDH subject to glucose-induced degradation (see above entry and Table 1) is phosphorylated. They use radiolabeled yeast cultures to show that wild-type cytosolic MDH but not the truncated MDH is phosphorylated. They then constructed S12A and S12D mutants of the cytosolic MDH with the N-terminal extension and showed that the S12A cytosolic MDH mutant enzyme was not subject to glucose-induced phosphorylation and degradation. The S12D cytosolic MDH was subject to phosphorylation and inactivation but not degradation.

Commentary: The yeast N-terminus sequence is 12 amino acids longer than the more stable shorter yeast variant. In human, the extension is 18 amino acids long. The locations of the Ser residues differ (see Table 1). The sequences of the N-terminal extensions differ, so degradation may not be the same for mammalian MDH. However there are several Asp (D) residues adjacent to the phosphorylated S. This suggests that hMDHv3 might have a shorter half-life as does the phosphorylated yeast MDH. The LCMS mammalian database indicates that the human MDH is phosphorylated at the initial serine at position 4. Yeast MDH has a serine at the third residue of the N-terminal extension (see Table 1) but is not phosphorylated. It should be noted the sequence of yeast MDH2's N-terminal extension, with the Ser in position 12, is similar to other degradation sequences.

***Degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events**, Hung, et al., J. Biol. Chem.* (2004) 279:49138.

In this study, the authors show that both F16BPase and MDH2 (cytosolic MDH in yeast; see Table 1) require the N-terminal Pro residue for degradation in both the yeast vacuole (during long term starvation) and the proteasome (short-term starvation). A P1S mutant for both enzymes was not degraded in either circumstance, indicating the requirement for an N-terminal Pro for degradation. The degradation pathway was shown to be cAMP-dependent for vacuole degradation but not proteasome degradation. S3A mutants for both enzymes did not block degradation, arguing against the requirement for phosphorylation of Ser at this position.

Commentary: Because the mammalian MDH is phosphorylated at the N-terminus and the yeast isoform is not, and the N-terminal extension on mammalian MDH does not have a proline; this suggests that the degradation pathways are different in yeast and human. However the N-end rules for mammalian cells and yeast are different and the Arg in the first two positions of the N-terminal indicates the possibility of a short half-life of less than one hour. This would be interesting to test in mammalian cells and not likely to be seen in MDH expressed in *E. coli*. Very little was done with kinetics on the longer yeast isoform. What is the function of the longer tail N-terminal extension besides serving as a potential degradation tag? Does it serve an additional regulatory role?

***Biochemical analysis of the NAD$^+$-dependent malate dehydrogenase, a substrate of several serine/threonine protein kinases of* Mycobacterium tuberculosis,** *Wang, et al., PLoS One* (2015) 10:e0123327.

The authors of this study were interested in the regulation of metabolism of *M. tuberculosis* because this pathogen is able to slow its metabolic rate, shifting from aerobic metabolism to anaerobic metabolism, to enter a latent state which is resistant to drug treatment. *M. tuberculosis* infection is a global health threat, with nearly a third of the world's population harboring an infection. There are eleven eukaryotic-like serine/threonine protein kinases in *Mycobacterium tuberculosis*, named PknA through PknL. In this study, the authors focus on PknD, and the goal was to identify its substrates. They show that MDH is a substrate for PknD (and in fact MDH is the best substrate for PknD and is phosphorylated both in bacterial culture and in vitro), and that MDH is a target for several other protein kinases as well. PknD phosphorylates *Mtb* MDH under physiological conditions at a Thr residue (or perhaps several Thr residues), resulting in a decrease in MDH activity. They do

not identify which Thr residue(s) is(are) phosphorylated. Using a native gel, they show that phosphorylated *Mtb* MDH remains a dimer. Kinetic assays were carried out using a purified recombinant *Mtb* MDH, both non-phosphorylated and phosphorylated by PknD. A graph of rate versus time at pH 9, 140 µM NADH, and 800 µM OAA shows that the phosphorylated MDH was about 40% less active. No kinetic studies were performed on the wild-type enzyme. Phosphorylation enhanced the ability of MDH to interact with other *Mtb* proteins and was observed to increase when the bacteria entered the stationary phase, a phosphate- and oxygen-poor environment. Decreasing the activity of *Mtb* MDH may enable *Mtb* to survive under these conditions.

Commentary: The parameters for the kinetic assays carried out by this research team are far from standard and cannot be used other than to indicate phosphorylation inhibits MDH activity at high OAA levels. The authors confirm Thr residue(s) as the phosphorylation sites but do not identify which. Of note: The consensus sequence recognized by PknD (not discussed in this paper) is XXXXTQX, in which "X" residues are hydrophobic and any residue may precede the T except for basic amino acids. The analogous site on MDH1 and MDH2 has not yet been investigated. There is evidence in the literature of phosphorylation of other dehydrogenases, which are evolutionary similar to MDH (such as LDH). A literature view of other dehydrogenases capable of being regulated by phosphorylation has the potential to inform our work with mammalian MDH.

Activities of glucose metabolic enzymes in human preantral follicles: In vitro modulation by follicle-stimulating hormone, luteinizing hormone, epidermal growth factor, insulin-like growth factor I, and transforming growth factor β1, *Roy and Terada, Biol. Reprod.* (1999) 60:763.

Glycolysis is the main source of ATP in the preantral state of follicle development, in which an oxygen gradient exists. Glucose metabolism is influenced by a variety of hormones, which direct glucose toward ATP- or NADPH-producing pathways, by regulating key enzymes in the pathways. In this study, follicle mitochondria in various states of hormonal activation were isolated and measured for mitochondrial MDH activity. Depending on the class of follicle examined, each agonist treatment increased MDH activity (as measured by NADH oxidation) several fold. Gonadotropins, EGF and IGF-1 were found to stimulate MDH activity in different classes of follicles, to different extents. Stimulation occurred over a 24-hour period, and the total protein expression versus activity was not determined.

β-cell adaptation to insulin resistance, *Liu et al., J. Biol. Chem.* (2002) 277:39163.

Insulin resistance, a risk factor for Type II diabetes, is characterized by the insensitivity of tissues to insulin, which regulates carbohydrate and fat metabolism. Patients whose pancreatic β-cells are able to adapt to insensitivity via a compensatory response involving an increase in insulin secretion generally do not develop diabetes. In this study, the authors performed studies using Zucker fatty (ZF) rats and Zucker lean (ZL) rats. The ZF fats are obese, are hyperlipidemic, but normoglycemic. Pancreatic islet mass and insulin secretion are elevated. Islets isolated from ZF rats had elevated pyruvate carboxylase (PC), malic enzyme (ME) and MDH activities. PDH activity was less than normal. OAA, malate, citrate and pyruvate levels were all elevated, and the release of malate from the mitochondria was doubled. These results are consistent with the interpretation that the malate-pyruvate shuttle has increased activity in β-cells in ZF rats (see Fig. 2).

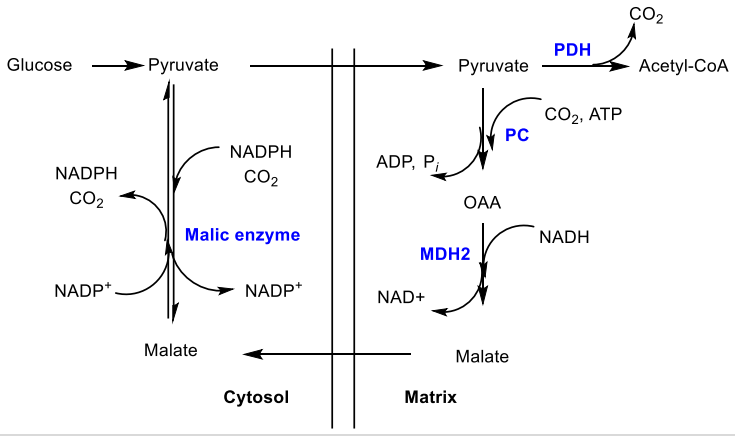

Figure 2: The malate-pyruvate shuttle.

Commentary: This is one of the first papers that shows altered and enhanced metabolism through malate and oxaloacetate involving the pyruvate dehydrogenase complex, malic enzyme, while hinting at other shuttles that involve MDH. A follow-up study using knockdown ME isoforms showed that when both isoforms of ME were knocked down, flux of pyruvate through PC decreased, although the pyruvate pool differed for each isozyme. Another uses mathematical modeling to show the involvement of NADH shuttles in NADPH metabolism in support of β-cell islets. And another study investigated shuttles involved in cells in support of insulin release.

Tyrosine phosphorylation of lactate dehydrogenase A is important for NADH/NAD⁺ redox homeostasis in cancer cells, *Fan et al., Mol. Cell Biol.* (2011) 31:4938.

Previous studies have shown that LDH-A activity is stimulated by HIF and Myc in cancer cells as a means to support "aerobic glycolysis" as described by Warburg, in which glucose preferentially undergoes anaerobic fermentation even in the presence of oxygen. The rapid rate of glycolysis requires the continual regeneration of NAD^+ via LDH, which catalyzes the conversion of pyruvate—the end product of glycolysis—to lactate, with concomitant conversion of NADH to NAD^+. In this study, the authors confirm that the tyrosine kinase growth factor fibroblast growth factor receptor 1 (FGFR1) directly phosphorylates LDH-A at two sites, Y10 and Y83, and in so doing, activates the enzyme. FGFR1 may activate additional tyrosine kinases in the cell which subsequently phosphorylate LDH at these sites or others, but here the research team decided to focus on Y10 and Y83, which are phosphorylated in LDHA-A directly by FGFR. Phosphorylation of Y10 activates LDH-A compared to the non-phosphorylated from by enhancing the formation of the more active tetrameric ("dimer of dimers" form) of the enzyme over the less active dimeric form. This was supported by gel filtration chromatography, which showed formation of the tetramer; tetramer formation was not observed in Y10F mutants. Additional experiments showed that the Y10F mutants maintain high levels of glycolysis, but rely on Complex I in the electron transport chain to regenerate NAD+ to support glycolytic production (and not oxidative phosphorylation) of ATP. The research team demonstrated that phosphorylation of Y10 in LDH-A was common in a wide variety of human cancers. Phosphorylation of Y83, which is near the active site, stimulates LDH-A activity by enhancing binding of the enzyme to NADH. This was supported by experiments involving binding of the enzyme to Cibacron Blue (an NADH mimic).

Commentary: This study is one of the early papers investigating the link between LDH and cancer and is now an active area of research. The ideas presented in this paper could be used to investigate similar mechanisms in MDH. For example, phosphorylation of Y83, which is near the active site, stimulates LDH-A activity by enhancing binding of the enzyme to NADH. We see several similar potential phosphorylation sites on human MDH. The second site, Y10, is located in the N-terminus and distal to the substrate binding site. Like MDH, the N-terminus of LDH is involved in dimerization.

Post-translational Modification by Methylation

Post-translational modifications by methylation on lysine and arginine residues are involved in a wide variety of cellular processes. These modifications are involved in an epigenetic role, as they occur on DNA histones and as such participate in gene regulation. There is a growing body of work that indicates that methylation occurs on non-histone proteins as well. In this section, we focus on regulation carried out by proteins that are post-translationally modified on the guanidino side chain of arginine residues. Methylation of arginine residues is catalyzed by protein arginine methyltransferase enzymes (PRMTs). To date, nine different PRMTs have been characterized. In the methylation reaction, the methyl group is provided by *S*-adenosylmethionine (AdoMet). A PRMT enzyme catalyzes the transfer of a methyl group from AdoMet to the Arg residue, producing the methylated Arg side chain and *S*-adenosylhomocysteine (AdoHcy) as a product. In mammals, PRMTs are classified into three types as shown in Table 2.

Table 2: Classification and biological roles of known PRMTs

Type	PRMT	Cellular processes in which Arg modification plays a role
Type I	PRMT1, 2, 3, 4 (CARM1), 6 and 8	Transcription, splicing, repair, signal transduction, ribosomal assembly, cell differentiation
Type II	PRMT5 and 9	Transcription, ribosomal assembly, cell proliferation, repair
Type III	PRMT7	Transcription

PRMTs can be classified as Type I, II, or III, as shown in Figure 3. Arg can be both mono- and di-methylated. The dimethylation can by symmetrical (one each amine group) or asymmetrical (both on the same nitrogen). PRMTs may be involved in the metabolic dysregulation observed during tumorigenesis, which may make them attractive drug targets.

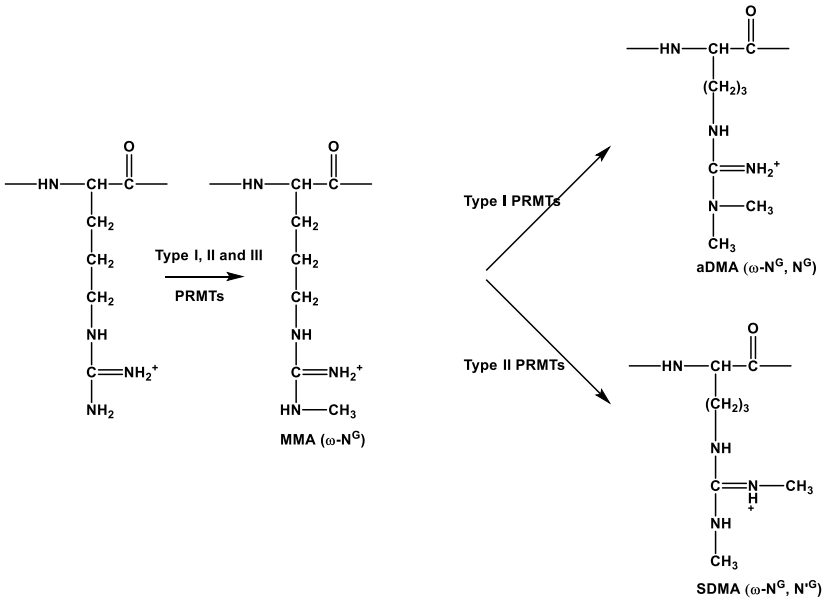

Figure 3: Methylation of arginine residues. Types I, II, and III PRMTs catalyze the mono-methylation of the arginine side chain to produce ω-N^G monomethyl arginine (MMA, or me1). Further methylation by Type I PRMTs produces ω-N^G, N^G asymmetric dimethylarginine (aDMA or me2a); Type II PRMTs produce ω-N^G, N'^G symmetric dimethyl arginine (SDMA or me2s). Not shown is the methyl donor (*S*-adenosylmethionine), and the second product (*S*-adenosylhomocysteine).

The development of antibodies capable of detecting methylarginines has been a useful tool in the study of this post-translational modification. Mutations in which arginine is converted to a lysine mimics an unmethylated arginine as the positive charge is retained, as it would be if Arg were unmethylated. An Arg → Phe mutation produces a bulky hydrophobic side chain which can mimic a constitutively methylated arginine.

Arginine methylation of MDH1 by CARM1 inhibits glutamine metabolism and suppresses pancreatic cancer, *Wang*, et al., *Mole. Cell* 64, 673-687.

Altered metabolism in cancer cells includes “aerobic glycolysis” described by Warburg, in which tumor cells oxidize glucose anaerobically even in the presence of oxygen; and enhanced uptake of glutamine, which has been shown to perform a dual role in providing nitrogen for amino acid and nucleotide biosynthesis, as well as to contribute to energy production in the tumor cell. In some cancer cells, glutamine is deaminated to form glutamate, which is converted via transamination to α-ketoglutarate, which enters the citric acid cycle. From there, citric acid cycle metabolites can be used as intermediates in biosynthesis. Malate can be exported to the

cytosol where it serves as a substrate for malic enzyme, which provides NADPH for fatty acid and glutathione synthesis.

However, in pancreatic ductal adenocarcinoma (PDAC), an aggressive form of pancreatic cancer with a low survival rate, activation of KRas results in the transformation of Gln to aspartate, which is transported into the cytosol, and then acted upon by GOT1, MDH1, and ME1, which ultimately produces pyruvate and NADPH.

In this study, the authors build on earlier work in which Arg230 in MDH1 in mouse and in HEK cells was methylated. Inhibition of methyltransferases in HEK cells increased the MDH activity by more than 2-fold. Because the mouse MDH1 Arg is conserved, the investigators constructed MDH1 Arg248Lys and Arg248Phe mutants in human MDH1 and showed that Arg248 is the site of methylation in human MDH. Using antibodies against methylated arginines, they show that MDH is both mono- and asymmetrically di-methylated in several cell lines. Modeling using the pig cytosolic MDH structure revealed that the methylation is found at the dimer interface. Using crosslinked MDH from lysates showed that methylation of MDH1 resulted in formation of the monomer. The same result was observed with the MDH1 Arg248Phe mutant. However the Arg248Lys (the substitution of Lys for Arg acts as a non-methylated mimic) failed to dimerize and was much less active. They used the model to hypothesize that some of the hydrogen bonding was lost in the Arg248Lys mutation and thus should not be used in further controls. The PRMT responsible for the methylation is PRMT4 (also known as CARM1), which binds to MDH1 but not MDH2 and inhibits glutamine metabolism. Depletion of ME and GOT1 (cytosolic) did not interfere with redox homeostasis. Furthermore only MDH1 activity is modulated by CARM1 methylation and not GOT or ME. KRas and oxidative stress (ROS) inhibited CARM activity and resulted in a hypomethylated MDH1. As a result glutamine metabolism was fully functional and cells were able to proliferate at a higher level than when MDH1 was methylated. Human tumor samples (pancreatic cancer tissue) had low expression of CARM1 and hypomethylated MDH1 which supports a physiological role for methylation in cancer cell metabolism. In a related study on the same pancreatic cells, a novel compound aspulvinone O inhibits GOT which yields the same metabolic and proliferation results as does methylation of MDH1. Both papers support the cytosolic GOT-MDH pairing to be important for cancer cell metabolism.

Post-translational Modification by Acetylation

Acetylation in mammalian systems has been well documented for histones. Recently, the impact and global involvement of acetylation as a reversible regulating post translational modification has become more and more important. The effect of acetylation is well known in prokaryotes but poorly described in eukaryotes. Acetylated residues can be identified using mass spectrometry. Proteins are acetylated on the ε-amino acid side chain of lysine residues. Acetylation results in an increase in the size and hydrophobicity of the Lys side chain and removes the positive charge. The increased bulk has the potential to change protein structure and involves the loss of ion pairs formation. Like phosphorylation, this is a reversible process. Acetyltransferases (AT) catalyze the acetylation of Lys residues using acetyl-CoA as the acetyl donor. Acetylation can occur either enzymatically or non-enzymatically using acetyl phosphate or acetyl-CoA as acetyl donors. Removal of the acetyl-group is reversed by lysine deacetylases (DACs). Identification of acetylated lysine residues in proteins and the link to function is challenging but some understanding can be gained using mutational analysis. Mutating a Lys → Arg preserves the positive charge but results in a side chain that cannot be acetylated; thus this mutation can serve as a non-acetylated mimic while having minimal impact on protein structure. Lys → Gln mutations remove the positive charge and can be used to mimic an acetylated Lys. MDH has been found to be acetylated, but the impact on function is not currently known and warrants further study. Useful tools for studying acetylation include the use of diacylation inhibitors and antibodies that recognize acetylated lysines. It is likely that acetylation and phosphorylation post-translational modifications may be coordinated.

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Post Translational Modification of Malate Dehydrogenase

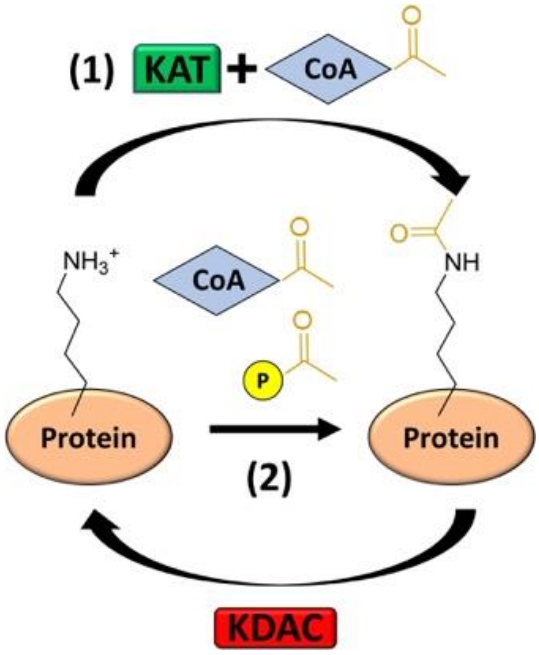

Figure 4: (1) Lysine acetylase (KAT) catalyzes the enzymatic transfer of an acetyl group, using acetyl-CoA as the acetyl donor, to a Lys residue. The process can be reversed by a lysine deacetylase (KDAC). Alternatively, the lysine residue may be nonenzymatically acetylated using acetyl-CoA or acetyl phosphate as the acetyl donor. (© Fronters in Microbiology, 2019. All rights reserved.)

Regulation of cellular metabolism by protein lysine acetylation, *Zhao, et al., Science* (2010), 327:1000, and Supplementary Material.

This research team fractioned human liver tissue into nuclear, mitochondrial, and cytosolic fractions. They subjected proteins in the mitochondrial and cytosolic fractions to trypsin digestion and then purified peptides containing acetylated residues. The purified peptides were analyzed by tandem liquid chromatography-tandem mass spectrometry. They identified more than 1300 acetylated peptides and were able to match most of them to known human proteins. Interestingly, virtually every enzyme involved in pathways of intermediary metabolism—glycolysis, gluconeogenesis, glycogen metabolism, citric acid cycle, fatty acid metabolism and the urea cycle—was acetylated. MDH2 was found to have four acetylated Lys residues: K185, K301, K307 and K314. Depending on conditions, MDH2 may be mono-, di-, tri- or tetra-acetylated. Exposure to glucose enhanced acetylation by 60%, and enzymatic activity increased as well. That acetylation increases enzymatic activity is supported by the observation that activity is also increased in the presence of deacetylation inhibitors. Acetylation was not detected in a mutant protein in which Arg substitutes for Lys at all four of these sites, indicating the specificity of regulation at these four, and only these four, specific Lys residues.

Table 3: Acetylated peptides, in proteins relevant to the MCC project, detected by Zhao, et al. Acetylated Lys residues are identified in red, with asterisks. Residue numbers are indicated when known.

Enzyme	Sequence of acetylated peptides
mMDH (numbering includes transit peptide)	
185	K.VSSFEEK*MISDAIPELK.A
301 and 307	K.GIEK*NLGIGK*VSSFEEK.M
314	R.ANTFVAELK*GLDPAR.V
cPEPCK1	
70 and 71	R.LK*K*YDNCWLALTDPR.D
594	K.EVEDIEK*YLEDQVNADLPCEIER.E
mPEPCK2	R.SYLTEQVNQDLPK*EVLAELEALER.R
mGOT (also known as AAT)	R.VESQLK*ILIR.P
cGOT1 (also known as AAT1)	K.NTPVYVSSPTWENHNAVFSAAGFK*DIR
CS	GMK*GLVYETSVLDPDEGIR

Commentary: Alignment maps shared with the MCC show the acetylation sites for the Lys residues for MDH2. Numbering provided by Zhao, et al. includes the transit peptide which is not shown on the MCC maps. How and which lysine residues are also associated with CS or other proteins remains an interesting story and a potential approach to understanding a possible regulation of MDH-CS interactions. In response to high glucose concentrations, PEPCK was shown to be acetylated and subsequently degraded. These results are supported by those of Latorre-Muro, et al., who show that acetylation of PEPCK shifts the enzyme activity from producing PEP for gluconeogenesis to producing PEP for OAA for anaplerosis. This is a potential link to MDH, and opens an entire avenue of study involving potential interactions between MDH and PEPCK, and how acetylation may potentially affect these interactions and enzyme activity.

Non-histone lysine acetylated proteins in heart failure, *Grillon et al.*, *Biochim. Biophys. Acta* (2012) 1822:607.

In this study, the authors investigated lysine acetylation in two different models of rats: Dahl salt-sensitive (SS) rats and spontaneously hypertensive heart-failure prone (SHHF) rats. They compared the results to control rats (Dahl salt-resistant—DR—rats and W rats). They found that 41 proteins were acetylated in the SS rats and 66 proteins were acetylated in the SHHF rats. Compared to controls, these residues were either less acetylated or not acetylated. Twelve of the acetylated proteins were common to both models of heart failure prone rats, and one of these was MDH2. No impact on protein function was studied. In their paper, the authors reference previous studies showing that MDH2 lysine residues at 165, 185, 301, 314, 329 are acetylated, and GOT is acetylated at residue 154.

Acetylation of malate dehydrogenase 1 promotes adipogenic differentiation via activating its enzymatic activity, *Kim et al., Journal of Lipid Research* (2012) 53:1864***.***

In this study, the authors demonstrate a role for MDH1 in differentiation of adipocytes, which has important implications in obesity. During adipogenesis in preadipocyte cells, factors stimulating differentiation increased cytosolic MDH1 expression by two-fold during late stage adipogenesis and enhanced acetylation of the enzyme six-fold compared to control cells. Increased expression of MDH2 also occurred during differentiation. Transfection and overexpression of MDH1 resulted in an increase in lipid accumulation, indicating a role for MDH1 in adipogenesis. The cellular concentration of NADPH, required for fatty acid synthesis, was observed to increase as well. Lys residues at K118, K121 and K298 were identified as acetylation sites. Single and triple mutants were constructed in which one or all three lysines were either mutated to arginines (a deacetylation mimic) or to glutamines (an acetylation mimic). The MDH1 K118R single mutant and the MDH1 triple mutant (in which all Lys were mutated to Arg) both resulted in decreased fat accumulation and had decreased activity while the other two single mutants had no effect. This supports the conclusion that acetylation on K118 is solely responsible for controlling MDH1 activity during adipogenesis. The authors conclude that acetylation of a specific Lys residue supports the production of NADPH for fatty acid synthesis.

Commentary: The authors report that the K118R and the triple KR mutants have the same activity as wild-type, but they do not show the data to support this. In Figure 5 of their paper it is reported that the wild-type MDH1 is more active after differentiation, but it isn't clear how this is controlled, and the authors attribute the activity difference to acetylation. In the same figure, the authors show that the relative activity of the triple KR mutant is less than half of that of wild-type, and that the activity of a triple KQ mutant is not significantly different from wild-type. This is to be expected as a KQ (Lys → Gln) mutant mimics acetylation in that both an acetylated Lys residue and a Gln residue are uncharged. But in Figure 5b, the data show increased MDH1 activity after eight days of differentiation, yet Figure 5c shows that the MDH triple KQ mutant has the same activity as wild type. Follow-up studies are clearly needed.

Acceleration of adipogenic differentiation via acetylation of malate dehydrogenase 2*, Kim et al. Beachem. Biophys. Res. Comm* (2013) 441:77.

This study builds on the previous work of this research team. Here they investigate the role of MDH2 in adipocyte differentiation. As seen with cMDH, overexpression of mMDH2 increased differentiation into fat cells,

glucose stimulates acetylation levels of mMDH and the activity of mMDH is increased. Lys → Arg mutations (deacetylation mimics) at positions 301, 307 and 314 showed slower rates of differentiation and decreased activity indicating that acetylation of MDH2 is critical for the process.

Structural, kinetic and proteomic characterization of acetyl phosphate-dependent bacterial protein acetylation*, Kuhn, M. L., et al., PLoS ONE (2014) 9:394816.*

In this extensive study, the authors provide mass spectrometry, biochemical and structural evidence for N^{ε}-lysine acetylation in *E. coli*. Bacteria were grown, harvested, lysed and digested with trypsin. The results show that thousands of Lys resides are acetylated on hundreds of proteins. They demonstrate that acetylation using acetyl phosphate as the acetyl donor occurs both non-enzymatically (independent of KAT) and specifically. This is supported by their observation that proteins with binding sites for phosphate can easily accommodate acetyl phosphate and indicates that proteins able to do so may be regulated by acetyl-phosphate non-enzymatic acetylation. In addition to the non-enzymatic acetylation, the authors also show that KAT-dependent acetylation can occur using acetyl CoA as the acetyl donor (see Fig. 4). Lysine acetylation in bacteria is shown to be important in regulating cellular processes such as transcription, translation and metabolism.

Commentary: In this paper, there is interesting information regarding how the authors did the screen, the types of pathways involved in bacterial acetylation in response to glucose, and some detailed examples. The supplementary materials, which are extensive, can be searched to find relevant acetylation sites on MDH, GOT, PEPCK and CS. Non-enzymatic acetylation by acetyl phosphate and acetyl-CoA are also known to occur in mammalian cells but if the site and motif is homologous to that found in bacteria, it has not yet defined for MDH or other proteins. Misty Kuhn, the first author on this paper who is also involved with the MCC, reports (via personal communication) that there is still debate about the levels of acetylation in bacteria. She indicates that while they know proteins are being acetylated, the stoichiometry of the modification under various conditions is unclear.

SIRT3-dependent GOT2 acetylation status affects the malate-aspartate NADH shuttle activity and pancreatic tumor growth, *Yang et al., EMBO J. (2015), 34:1110.*

This is a particularly interesting study looking at mitochondrial GOT-MDH interactions controlled by acetylation. In this study, the authors show GOT2 is acetylated at three lysine residues—K159, K185 and K404, and that acetylation on these three residues enhances the association between GOT2 and MDH2 in pancreatic tumor cells. This stimulates the malate-aspartate shuttle activity, which brings NADH into the mitochondrial matrix and regenerates NAD^+ in the cytosol to support the high rates of glycolysis observed in cancer cells. In addition, acetylation of GOT2 also resulted in an increase in NADPH production, which protects the tumor cell from damage by reactive oxygen species (ROS). Increased acetylation of GOT2 and GOT2-MDH2 association are stimulated by high concentrations of glucose and glutamine, which are important carbon sources for the tumor cell. SIRT3 is a deacetylase, and one of its targets is GOT2, so these results are consistent with the observation that SIRT3 has reduced expression on human pancreatic tumor cells. Additional experiments showed that GOT acetylation supports the mitochondrial redox state, NADPH metabolism, ATP production and shuttling with LDH activity.

They investigated interactions using epitope tagged GOT2 and MDH1 (different tag for each) and expressed in HEK cells. It is unclear whether or not these constructs included the mitochondrial transit peptide. In this system they showed that GOT2 was acetylated and that the interactions between GOT and MDH were weak until a deacylase inhibitor was used. Then GOT and MDH were easily isolated in a pull-down experiment. An MDH2 quadruple mutant in which Lys185, Lys301, Lys 307 and Lys314 were all mutated to Arg residues (Lys → Arg preserves the positive charge and acts as a deacetylation mimic) was constructed. The results showed that either the quadruple mutant, in which acetylation is blocked, and wild type MDH2, do not significantly interact with GOT2. But when GOT2, and not MDH, was acetylated, interactions were observed. This indicates that the MDH2-GOT2 complex relies on the acetylation of GOT2 and not MDH2.

Mutational analysis experiments revealed that acetylation of all three Lys residues at positions 159, 185 and 404 are all required for binding of GOT2 to MDH2; experiments using single or double Lys → Arg

mutations either decreased or abolished binding. Using a unique approach to incorporate acetylated lysines into recombinant GOT expressed in bacteria, they found that GOT2 ac159 had the same activity as wild-type GOT2. They also measured activities of HEK-expressed wild-type GOT, a Lys → Arg triple mutant (which preserves the positive change and acts as a deacetylation mimic) and a Lys → Gln triple mutant (which abolishes the positive charge and acts as an acetylation mimic) and each had the same relative activity.

Commentary: Could an acetylated MDH2 dissociate from CS, which would then allow the MDH2 to interact with GOT2, replacing the MDH2-CS interaction with an MDH2-GOT2 interaction? That might indicate a unique binding site for GOT2-MDH2 that differs CS-MDH2, which could easily be observed using competition assays. Does this mean that when we look at protein-protein interactions of these different partners we should include deacetylase inhibitors (as the authors of this study did) to increase the chance of forming a complex?

Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation, Overmyer et al., *Cell Metab.* (2015), 21: 468.

This is an interesting study that looks at metabolic shifts in highly exercised rats. Rats with a high running capacity (HCR) have a healthier metabolism and live longer than rats with a low capacity for exercise (low running capacity rats, or LCR). HCR preferentially use fats and branched chain amino acids for fuel during exercise, sparing carbohydrates, whereas the LCR preferentially use carbohydrates and are less able to make the switch to using lipids and amino acids as fuel. Mitochondrial proteins in the HCR are hypo-acetylated when the rats are at rest, and acetylation levels drop further upon endurance exercise. By contrast, protein acetylation levels in the LCR did not change. In particular, the K239 residue (a known target of the deacetylase SIRT3) in MDH2 is under-acetylated in HCR compared to LCR, indicating a possible role of SIRT3 in exercise that was not explored here.

Commentary: As we have seen in the adipocyte differentiation paper and others, acetylation of MDH2 is associated with a lower activity of MDH2 in the OAA → malate direction. This was observed as a trend in this study and no proper kinetic studies were conducted.

Studying the lysine acetylation of malate dehydrogenase, *Venkat et al., J. Mol. Biol.* (2017) 429:1396.

Using the information from several publications including Kuhn et al., two residues in *E. coli* MDH (here, *E. coli* MDH is referred to as eMDH) were reported as acetylated in five studies: K99 and K140. They compared these two bacterial sites with the four acetylation sites on the human MDH2 (at locations 185, 301, 307 and 314) and noted that the human sites are located at the C-terminus whereas the eMDH sites are internally located (they used PDB codes 1EMD for the eMDH and 2DFD for human MDH2 when comparing structures; they also note that MDH2 was chosen because it is more homologous to eMDH than is MDH1. The human MDH2 K185 corresponds eMDH 162 and was used as a control. Using a genetic code expansion technique in which N^{ε}-acetyl Lys residues are incorporated into recombinant proteins, they expressed eMDH with acetylated lysines at positions 99, 140 and 162 as well as human MDH2 at positions 185, 307 and 314. Using an MTT-linked kit, purified enzymes were assayed for activity in the OAA → malate direction. Neither the study authors nor the manufacturer indicate the OAA and NADH concentrations or pH for the assay. The results show that acetylation of eMDH at K99 and K140 but not K162 activated eMDH over wild-type 2-3 fold. Only the K307 mutation activated the human mitochondrial MHD2 over wild-type enzyme. This is different from the Zhao and Kim papers which indicate that all four mMDH lysines are required, although all studies including this one, show that at least one or more lysines activate MDH2 activity.

The authors report K_M and k_{cat}/K_M values as shown in Table 1 of the paper (below). The acetylated variants for both bacterial and human MDH had similar K_M values as their wild-type counterparts (indicating that substrate binding was not affected), but the k_{cat} and k_{cat}/K_M values were significantly greater, indicating an improvement in turnover.

MDH Introduction

Post Translational Modification of Malate Dehydrogenase

Table 1. Kinetic analyses of MDHs and acetylated variants

	k_{cat} (s^{-1})	$K_{M,\ NAD}^{+}$ (mM)	$K_{M,\ malate}$ (mM)	$k_{cat}/K_{M,\ NAD}^{+}$ (S^{-1} mM^{-1})	$k_{cat}/K_{M,\ malate}$ (S^{-1} mM^{-1})
eMDH WT	20.2 ± 0.4	0.23 ± 0.02	2.61 ± 0.20	87.8	7.74
eMDH 99AcK	51.2 ± 1.3	0.25 ± 0.07	2.32 ± 0.32	204.8	22.07
eMDH 140AcK	74.3 ± 1.1	0.26 ± 0.06	2.54 ± 0.64	285.8	29.25
hMDH2 WT	69.6 ± 2.9	0.16 ± 0.01	0.24 ± 0.08	435.0	290.0
hMDH2 307AcK	178.9 ± 3.1	0.12 ± 0.04	0.20 ± 0.12	1490.8	894.5

Commentary: In this study the authors used a BL21(DE3) strain for protein overexpression and not a K12-derived strain in order to avoid bacterial-induced acetylation. BL21 (a B strain bacterium) has low acetate concentration at high glucose levels, so it is reasonable to assume that these conditions should minimize non-enzymatic acetylation. However, this assumption should be supported with clear evidence and should be a consideration when conducting acetylation studies. Note that in Table 1, V_{max} data were not reported. It is also interesting that authors do not explain why Lys 301 on MDH2 was not investigated. No comparisons of the data in this report with previously published reports were made.

Dynamic regulation of ME1 phosphorylation and acetylation affects lipid metabolism and colorectal tumorigenesis, *Zhu et al., Mole. Cell* 77 (2020) 77:138.

In this study, the authors show that a Ser/Thr phosphatase dephosphorylates ME1 at S336, which subsequently results in the acetylation of K337. This promotes the formation of an ME1 dimer with increased activity. These results are supported by the observation that using a kinase to phosphorylate S336 blocks dimerization and acetylation of K337. Phosphorylation and acetylation are mutually exclusive. Increased ME1 activity results in increased levels of NADPH production and increased lipogenesis.

Commentary*:* This is a potentially exciting as both phosphorylation and acetylation are studied on one protein. What happens after ME1 is dephosphorylated and acetylated? These studies can be extended to our work on MDH.

Appendix

Table A1: Cellular enzymes important in the study of MDH1 and MDH2

Enzyme name	Abbreviation	Alternative names or abbreviations	Cellular location	UniProt ID	UniProt accession number
NADP dependent malic enzyme	ME1		Cytosol	MAOX_HUMAN	P48163
Glutamate oxaloacetate transaminase	GOT1	aspartate transaminase (cAST, AST1) aspartate aminotransferase (cAAT, AAT1)		AATC_HUMAN	P17174
ATP citrate lyase	ACL		Cytosol	ACLY_HUMAN	P53396
Phosphoenol-pyruvate carboxykinase	PCK1, cPEPCK		Cytosol	PCKGC_HUMAN	P35558
Citrate synthase	CS		Mitochondrion	CISY_HUMAN	O75390
Glutamate oxidase transaminase	GOT2	aspartate transaminase (mAST, AST2) aspartate aminotransferase (mAAT and AAT2)	Mitochondrion	AATM_HUMAN	P00505
NAD dependent malic enzyme	ME2		Mitochondrion	MAOM_HUMAN	P23368
NADP dependent malic enzyme	ME3		Mitochondrion	MAON_HUMAN	Q16798
Fumarase	FH		Mitochondrion	FUMH_HUMAN	P07954
Aconitase	ACO2		Mitochondrion	ACON_HUMAN	Q99798
Phosphoenol-pyruvate carboxykinase	PCK2, mPEPCK		Mitochondrion	PCKGM_HUMAN	Q16822