Mechanistic insights into the regulation of metabolic enzymes by acetylation

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The activity of metabolic enzymes is controlled by three principle levels: the amount of enzyme, the catalytic activity, and the accessibility of substrates. Reversible lysine acetylation is emerging as a major regulatory mechanism in metabolism that is involved in all three levels of controlling metabolic enzymes and is altered frequently in human diseases. Acetylation rivals other common posttranslational modifications in cell regulation not only in the number of substrates it modifies, but also the variety of regulatory mechanisms it facilitates.

Introduction

Although protein lysine acetylation was first discovered almost 50 years ago on nuclear histones (Phillips, 1963; Allfrey et al., 1964, 1968), the broad involvement of this reversible covalent modification in cell regulation has only been appreciated during the past five years. The discovery that large numbers of cellular proteins are acetylated was made possible by the rapid development of mass spectrometric technology during this period. Using an improved immunopurification scheme to enrich for acetylated peptides, Kim et al. (2006) first identified 388 lysine acetylation sites corresponding to 195 distinct proteins from mouse liver tissue and HeLa cells. Notably, 277 acetylated peptides were derived from 133 proteins located within the mitochondrion, including many intermediary metabolic enzymes. This was a rather surprising finding because previous lysine acetylation studies had primarily identified nuclear proteins. Two subsequent acetylation proteomic studies, using similar methods, significantly expanded the acetylome of mammalian cells and identified an astonishing 1,750 acetylated proteins from three different human cancer cell lines (Choudhary et al., 2009) and 978 acetylated proteins from human liver tissue after excluding nuclear protein (Zhao et al., 2010). Together, these three acetylation studies identified >2,000 acetylated proteins in mammalian cells, making the regulatory scope of acetylation comparable to those by other major posttranslational modifications such as phosphorylation and ubiquitylation (Choudhary and Mann, 2010; Guan and Xiong, 2011).

These acetylated proteins span a wide spectrum of protein classes, ranging from transcription factors to kinases, ubiquitin ligases, ribosomal proteins, structural proteins, and metabolic enzymes, all of which cover a broad range of cellular activities from cell cycle control, DNA damage checkpoints, and cytoskeleton organization to endocytosis and metabolism. Owing to the use of liver, the major metabolic organ in the body, as the tissue source by two of these acetylation proteomic studies, many metabolic enzymes were found to be potentially acetylated. Nearly all enzymes involved in glycolysis, gluconeogenesis, the TCA cycle, fatty acid oxidation, the urea cycle, nitrogen metabolism, and glycogen metabolism are acetylated (Zhao et al., 2010). Enzymes involved in oxidative phosphorylation and amino acid metabolism are abundantly acetylated as well. These findings sparked intense investigation over the past two years into the regulatory mechanisms of the acetylation of metabolic enzymes, which nicely complements the studies on the metabolic regulation by deacetylases in the same period. These investigations raised the notion that acetylation may rival other common posttranslational modifications in cell regulation not only by the number of substrates it modifies, but also the variety of regulatory mechanisms it facilitates.

Metabolism refers to the chemical reactions of both synthesis (anabolism) and breakdown (catabolism) in living organisms and is the essence of life catalyzed by enzymes. The activity of metabolic enzymes is controlled by three principle aspects: the amount of enzyme, the catalytic activity, and the accessibility of substrates. Acetylation has been found to be involved in all three aspects of controlling metabolic enzymes. In this review, we will discuss the mechanistic insights into how acetylation regulates the function of metabolic enzymes. We will focus our discussion on mammalian cells and relate the acetylation of

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Abbreviations used in this paper: CBP, cAMP-response element-binding protein; CMA, chaperone-mediated autophagy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GP, glycogen phosphorylase; KAT, lysine acetyltransferase; OTC, ornithine transcarbamylase; PK, pyruvate kinase; SDHA, succinate dehydrogenase subunit A; TSA, trichostatin A.

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metabolic enzymes to both normal physiology and pathological alteration (Table 1). Investigations of deacetylases, especially mitochondrial localized SIRT3, SIRT4, and SIRT5, and to a less extent lysine acetyltransferases (KATs), have contributed significantly to the physiological significance and genetic support of acetylation in regulation of metabolic enzymes. Several excellent reviews have recently been written on this topic (Finkel et al., 2009; Huang et al., 2010; Albaugh et al., 2011; Chalkiadaki and Guarente, 2012).

Regulating the amount of enzyme

Acetylation-mediated proteasomal degradation. Crosstalk between different posttranslational modifications that occur simultaneously on the same protein provides cells with a means to integrate different pathways and coordinate responses to different physiological conditions. One good example is phosphorylation-targeted protein degradation by the ubiquitin–proteome system to regulate the amount of intracellar protein (Hunter, 2007). Examples are emerging where acetylation plays a similar role in directly regulating the amount of metabolic enzymes through targeting the substrate to ubiquitylation and proteasome-dependent degradation.

Cytosolic phosphoenolpyruvate carboxykinase (PCK1, also known as PEPCK1 or PEPCK-C) catalyzes the first committed, and rate limiting step, of gluconeogenesis by converting oxaloacetate into phosphoenolpyruvate (PEP). PCK1 plays an important role in controlling cellular and organismal glucose homeostasis. Abnormally elevated gluconeogenesis serves as an important marker in the evaluation of type II diabetes (Granner and O'Brien, 1992). Transcriptional control plays a critical role in regulating levels of PCK1, with mRNA levels displaying rapid and robust flux in response to changes in energy signals such as glucagon and insulin (Yang et al., 2009). What has not been adequately appreciated is the control of PCK1 protein stability. Several lysine residues were identified as potential acetylation targets by the acetylation proteomic studies (Kim et al., 2006; Choudhary et al., 2009; Zhao et al., 2010). An early study demonstrated that acetylation of human PEPCK1 is associated with its decreased protein stability in cells fed with high glucose (Zhao et al., 2010). Subsequently, it was found that PCK1 is acetylated by the P300 acetyltransferase (KAT3B) and that this acetylation stimulates the interaction between PCK1 and UBR5, a HECT domain containing E3 ubiquitin ligase, therefore promoting PCK1 ubiquitylation and proteasomal degradation. Conversely, SIRT2 deacetylates and thereby stabilizes PCK1. These observations present an interesting example where acetylation targets a metabolic enzyme to a specific E3 ligase in response to changes in metabolic state (Fig. 1 A; Jiang et al., 2011). Ubiquitylation of PCK1 has previously been observed in C4 plants where PCK1 catalyzes the same reaction and is responsible for the primary fixation of atmospheric CO_2 (Agetsuma et al., 2005). Whether the ubiquitylation of plant PCK1 is linked to acetylation and the identity of its E3 ligases is unknown.

Sphingosine kinase (SPHK1) is a lipid kinase that catalyzes the phosphorylation of the sphingosine to sphingosine-1-phosphate (S1P), a signaling molecule involved in both intracellular and extracellular processes including cell proliferation and survival via G protein–coupled receptor signaling. Candidate approach studies demonstrated that SPHK1 is acetylated by p300 or cAMP-response element-binding protein (CBP) acetyltransferases. Unlike PCK1, acetylation of SPHK1 is associated with protein stabilization. Mutation of two putative acetylation-targeted lysine residues blocked SPHK1 ubiquitylation and degradation, leading to the hypothesis that acetylation and ubiquitylation may compete for the same sites (Yu et al., 2012).

The studies in PCK1 and SPHK1 demonstrate that acetylation can either promote or inhibit proteasome-dependent degradation by either stimulating interaction with an E3 ubiquitin ligase or, perhaps, interfering with the ubiquitylation of lysine residues. Based on bioinformatic analyses, a significant fraction of acetylation-targeted lysine residues can also be modified by ubiquitylation (Weinert et al., 2011). One may speculate that a mutually exclusive competition between acetylation and ubiquitylation regulates protein stability. Indeed, recent studies are consistent with this model in which acetylation and ubiquitination can be mutually exclusive by targeting the same lysine residue (e.g., Grönroos et al., 2002).

Acetylation promotes lysosome-dependent degradation. In addition to regulating proteasome-dependent degradation, acetylation can also regulate the degradation of metabolic enzymes by lysosomes. Once thought to function primarily in the wholesale breaking down of foreign material such as viruses, bacteria, and worn-out cellular organelles (micro- and macroautophagy), lysosomal degradation has been recently recognized for the selective degradation of specific proteins by a process known as chaperone-mediated autophagy (CMA; Mizushima et al., 2008). Pyruvate kinase (PK) catalyzes the transfer of phosphate from PEP to ADP, resulting in the formation of pyruvate and ATP. This is an irreversible and crucial regulatory step in glycolysis. The human genome encodes two distinct *PK* genes, *PKLR* and *PKM2*, that express four PK isoforms: L, R, M1, and M2. The L and R isoforms are expressed specifically in liver and red blood cells, respectively, from the *PKLR* gene through the use of different promoters. M1 and M2 are expressed in almost all adult tissues and during embryogenesis, respectively, from the *PKM2* gene by alternative RNA splicing. Notably, *PKM2* is highly expressed in a variety of tumors (Yamada and Noguchi, 1995; Mazurek et al., 2005). The benefit of expressing the PKM2 isoform in rapidly growing embryonic and tumorigenic cells is believed to result from decreased PK activity, which could lead to accumulation of glycolytic metabolites to drive macromolecular biosynthesis and cell growth. According to this theory, a regulation that decreases or increases PK activity would favor actively dividing or quiescent cells, respectively. Acetylation at K305 is stimulated by high glucose concentrations and promotes the lysosomal-dependent degradation of PKM2 via CMA. This is supported by the findings that K305 acetylation increases PKM2 interaction with HSC70, a chaperone that carries target proteins to lysosomes for CMA, and PKM2 uptake by lysosomes. Replacement of endogenous wild-type PKM2 with acetylation-mimetic K305Q mutant reduced the steady-state level of PKM2, leading

Table 1. **Regulation of metabolic enzymes by acetylation**

Name	Organism	Acetylation site	Acetylase	Deacetylase	Effect on enzyme	Mechanism	Nutrient signal	References
Acyl-Coenzyme A dehydrogenase, long-chain (Acadl)	Mouse	K42	Unknown	SIRT3	Down-regulation	Unknown	Inhibited by fasting	Hirschey et al., 2010
Aldehyde dehydrogenase 2 (Aldh2)	Mouse	Unknown	Unknown	SIRT3	Up-regulation	Deacetylation increases acetaminophen toxic-metabolite binding	Inhibited by fasting	Lu et al., 2011
Acyl-CoA synthetase short-chain family member 1 (ACSS1)	Human	Unknown	Unknown	SIRT1	Down-regulation	Unknown	Unknown	Hallows et al., 2006
Acyl-CoA synthetase short-chain family member 1 (ACSS2)	Human	K642	Unknown	SIRT3	Down-regulation	Active site interference	Unknown	Hallows et al., 2006; Schwer et al., 2006
Argininosuccinate lyase (ASL)	Human	K288	Unknown	Unknown	Down-regulation	Active site interference	Stimulated by high glucose and inhibited by high amino acid	Zhao et al., 2010
Carbamoyl phosphate synthetase 1(CPS1)	Human	Unknown	Unknown	SIRT5	Down-regulation	Unknown	Inhibited by starvation, high protein diet and calorie restriction	Nakagawa et al., 2009
Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH)	Human	K165,K171, K346, K586	Unknown	Unknown	Up-regulation	Unknown	Stimulated by high fatty acid	Zhao et al., 2010
GAPDH	Human	K117, K227, K251	PCAF	Unknown	Nuclear translocation	Promoting Siah binding and nuclear translocation	Stimulated by apoptotic stresses	Ventura et al., 2010
Glutamate dehydrogenase (GDH)	Mouse	Unknown	Unknown	SIRT3	Unknown	Unknown	Unknown	Lombard et al., 2007
GP	Human	K470	Unknown	Unknown	Down-regulation	Promoting GP dephosphorylation and inactivation	Stimulated by glucose, insulin and inhibited by glucagon	Zhang et al., 2012
3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2)	Human	K310, K447, K473	Unknown	SIRT3	Down-regulation	Causing conformational changes	Inhibited by fasting	Shimazu et al., 2010
Isocitrate dehydrogenase 2 (IDH2)	Mouse	Unknown	Unknown	SIRT3	Down-regulation	Unknown	Inhibited by caloric restriction	Someya et al., 2010
Malate dehydrogenase (MDH)	Human	K185, K301, K307, K314	Unknown	Unknown	Up-regulation	Unknown	Stimulated by high glucose	Zhao et al., 2010
Ornithine carbamoyltransferase (OTC)	Human	K88	Unknown	SIRT3	Down-regulation	Active site interference	Stimulated by high glucose and high amino acid	Yu et al., 2009; Hallows et al., 2011b
Phosphoenolpyruvate carboxykinase 1 (PCK1)	Human	K70, K71, K594	P300	SIRT2	Down-regulation	Promoting degradation via proteasome	Stimulated by high glucose	Jiang et al., 2011
Phosphoglycerate mutase 1 (PGAM1)	Human	K251, K253, K254	Unknown	SIRT1	Up-regulation	Allowing efficient phosphotransfer	Stimulated by high glucose	Hallows et al., 2011a
PK, muscle (PKM2)	Human	K305	PCAF	Unknown	Down-regulation	Targeting to lysosomal degradation	Stimulated by high glucose	Lv et al., 2011
Succinate dehydrogenase complex, subunit A (Sdha)	Mouse	K179, K485, K498, K538	Unknown	SIRT3	Down-regulation	Acetylation controls the substrate entry	Unknown	Cimen et al., 2010
Superoxide dismutase 2 (SOD2)	Human	K53, K68 K89, K122	Unknown	SIRT3	Down-regulation	Unknown	Inhibited by nutrient starvation	Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011
Sphingosine kinase 1 (SPHK1)	Human	K27, K29	P300/CBP	Unknown	Up-regulation	Inhibiting degradation via proteasome	Unknown	Yu et al., 2012

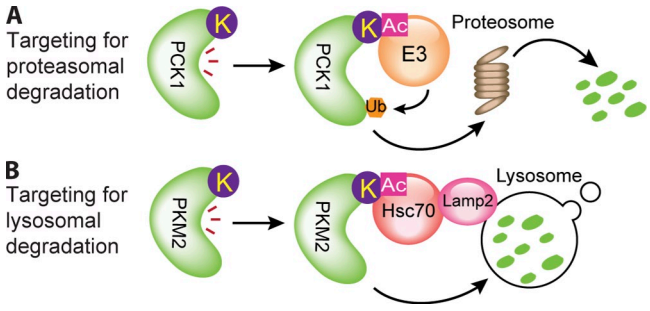

Figure 1. **Acetylation regulates the amount of metabolic enzymes.** Acetylation can regulate the steady-state levels of metabolic enzymes by promoting their degradation through either the ubiquitin–proteasomal system in the case of phosphoenolpyruvate carboxykinase (PCK1; A) or CMA in the case of PK M2 isoform (PKM2; B). Metabolic enzymes and acetylated lysine residues (K) are colored in light green and purple, respectively. Active sites are indicated by three red radial dashes.

to the accumulation of several glycolytic intermediates and promotion of cell proliferation and growth (Lv et al., 2011). These results not only reveal a novel regulation of PK, but also provide one of the first examples of acetylation-targeted protein degradation via CMA (Fig. 1 B).

Acetylation affects enzyme catalytic activity

Acetylation neutralizes the lysine residue in the active site. Lysine has a positively charged ϵ-amino group due to protonation at physiological pH. Acetylation of the ϵ-amino group prevents protonation and thus abolishes the positive charge on the lysine side chain. Lysine residues are frequently used by enzymes to bind negatively charged substrates. Consequently, an acetylated lysine residue has reduced affinity to negatively charged groups with which it may interact. Acetylation of a lysine residue that participates in an enzyme's catalytic reaction would therefore likely impair the enzyme activity. An example of this mechanism is provided by the study of ornithine transcarbamylase (OTC), a urea cycle enzyme that catalyzes the condensation of ornithine and carbamoyl phosphate into citrulline. Ornithine is the deamination product of arginine, whereas carbamoyl phosphate is the condensation product of ammonium generated by amino acid deamination and carbon dioxide. Because there is no alternative way of urea synthesis, inhibition of any one of the six urea cycle enzymes would result in devastating health consequences, with OTC deficiency being the most common urea cycle disorder (Scaglia et al., 2002). A deficiency of OTC usually results in severe central nervous system dysfunction, hyperammonemia, irreversible brain damage, and death in newborn infants (Hauser et al., 1990). Acetylation proteomic studies have identified several acetylated lysine residues in OTC, including the highly conserved Lys88 (K88), which is mutated in OTC-deficient patients and situated in the active site involved in substrate binding (Shi et al., 2001). Both the treatment of cells with deacetylase inhibitors—nicotinamide (NAM) and trichostatin A (TSA)—and substitution to an acetylmimetic glutamine residue (K88Q) were found to decrease the affinity for carbamoyl phosphate and the maximum velocity, thereby leading to the inhibition of OTC activity (Yu et al., 2009) . A simple model explaining these results, as for the Lys88-to-Asn (K88N) mutation seen in human OTC deficiency (Arranz et al., 2007) or chemical modification of a lysine residue in dolphin OTC homologous to K88 of human OTC (Valentini et al., 1996), would be that acetylation neutralizes the positive charge of K88 and reduces the substrate binding to OTC (Fig. 2 A). Mitochondrial SIRT3 has subsequently been demonstrated to directly deacetylate OTC at K88 and stimulate OTC activity, which is consistent with the observation that OTC acetylation is decreased and activity is increased in wild-type but not *Sirt3*$^{-/-}$ mice under caloric restriction (Hallows et al., 2011b).

Before the acetylation proteomic studies, earlier studies had already identified, via the candidate approach, mammalian cytoplasmic acetyl-CoA-synthetase (ACSS1, also known as AceCS1) and mitochondrial ACSS2 acetylation at specific residues (K661 in ACSS1 and K642 in ACSS2), and deacetylation by cytoplasmic SIRT1 and mitochondrial SIRT3, respectively (Hallows et al., 2006; Schwer et al., 2006). ACSS enzymes catalyze ATP-dependent ligations of acetate and CoA to produce acetyl-CoA. Acetylation of both ACSS1 and ACSS2 negatively regulates their activity, which can be reactivated by incubation with or overexpression of the respective SIRT deacetylases. Both K661 and K642 are highly conserved and located within the active site of ACSS's to function in the ATP-dependent adenylation of acetate during the initial catalysis, which suggests that acetylation may impair the catalytic activity by neutralizing the positive charge of lysine residues and its interaction with either ATP or acetate.

Acetylation antagonizes allosteric activation. Glycogen phosphorylase (GP) catalyzes the phosphorolytic cleavage of glycogen to produce glucose-1-phosphate for glucose-dependent tissues when serum glucose is low, usually due to demand such as during exercise. Defects in glycogen synthesis and breakdown in liver, muscle, and other glucose-dependent tissues cause glycogen storage diseases (Stegelmeier et al., 1995). McArdle's disease is a prototypical glycogen storage disorder that is caused by mutations in muscle GP and characterized by pain and fatigue after exercise (Tang et al., 2003; Andreu et al., 2007). Extensive investigations have been performed on this historic enzyme that lead to the discoveries of two principles in enzyme regulation: allosteric regulation by Carl and Gerty Cori during the 1930s and 1940s (Cori and Cori, 1936) and reversible phosphorylation by Edmond Fischer and Edwin Krebs during the 1950s (Fischer and Krebs, 1955). These discoveries exemplify how the regulation of enzyme activity is linked to the levels of intracellular metabolites such as AMP, and extracellular nutrients such as glucose. When glucose concentration is low, glucagon triggers a signal transduction cascade leading to the activation of phosphorylase kinase (PhK), which, in turn, activates GP by phosphorylating serine-15, leading to increased glycogen breakdown and ultimately higher glucose levels. Conversely, under high serum glucose conditions, release of insulin indirectly activates protein phosphatase-1 (PP1), which dephosphorylates serine-15 and converts the active form of GP to its unphosphorylated, inactive form, leading to the inhibition of

glycogen breakdown (Browner and Fletterick, 1992). Acetylation was recently found to negatively regulate human GP activity, in part by promoting dephosphorylation (Zhang et al., 2012). Acetylation of K470 enhances GP's interaction with the PP1 substrate–targeting subunit, G_L (official name PPP1R3A), and thus PP1, thereby promoting GP dephosphorylation and inactivation. GP acetylation is stimulated by insulin and high glucose, and inhibited by glucagon, thereby placing acetylation into the network of GP regulation by both nutrients and hormones (Fig. 2 B). As much as 50% of GP was acetylated at K470, and inhibition of deacetylases resulted in an increase in the ratio of acetylated K470 versus unacetylated K470 from roughly 1:1 to 2:1, which indicates dynamic regulation of K470 acetylation in cells. It is pleasantly surprising to see that a new regulatory mechanism can still be discovered on such a historically and extensively investigated metabolic enzyme, making one wondering how much more we can learn from studying these almost forgotten metabolic enzymes.

Acetylation causes conformational changes in the active site. In fasting and diabetic animals, many cells cease carbohydrate utilization and fatty acid synthesis, and switch their metabolic programs to fatty acid oxidation (degradation), with the concomitant formation of ketone bodies (ketogenesis) in the liver that can be transported to other tissues such as brain to supply energy. A key enzyme in ketogenesis is mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase, HMGCS2) which, like cytoplasmically localized HMGCS1, catalyzes the rate-limiting conversion of acetoacetyl-CoA and acetyl-CoA into HMG-CoA, an intermediate in the formation of ketone bodies. As expected, the function of HMGCS2 is subjected to regulation by multiple signals and mechanisms, including transcriptional activation of HMGCS2 gene expression by glucagon and cAMP (Hegardt, 1999), and competitive inhibition at the active site by succinyl-CoA (Quant et al., 1990). In a search for the substrates of SIRT3 deacetylase, Shimazu et al. (2010) subjected total extracts derived from wild-type and *Sirt3*$^{-/-}$ liver mitochondria to mass spectrometric analysis and identified HMGCS2 as a substrate of SIRT3. Fasting up-regulated Sirt3 levels and reduced HMGCS2 acetylation by 58% in wild-type mice liver, but had no effect on the hypermethylation of HMGCS2 in *Sirt3*-deficient liver. Deacetylation by SIRT3 or mutation of three acetylation sites enhanced HMGCS2 enzymatic activity, a finding that is consistent with the decrease of β-hydroxybutyrate levels during fasting in *Sirt3*-deficient mice. Molecular modeling suggests that acetylation at these three lysine residues could cause a significant conformational change around in the acetyl-CoA binding site, thereby affecting the positioning of several catalytic residues (Fig. 2 C).

Mammalian phosphoglycerate mutase 1 (PGAM1) catalyzes the reversible reaction of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA) in glycolysis. Acetylation of PGAM1 stimulates its enzymatic activity by 30–40%, and is decreased by glucose deprivation, presumably by the increase of SIRT1 deacetylase, which can deacetylate PGAM1 in vitro (Hallows et al., 2011a). Substitutions of a cluster of three adjacent lysine residues in the C-terminal region of PGAM1—K251, K253, and K254—reduced PGAM1 acetylation by 90% and

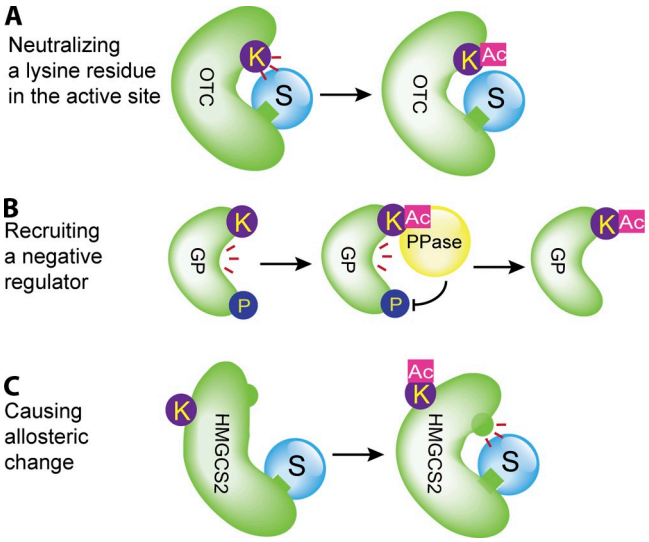

Figure 2. **Acetylation regulates the catalytic activity of metabolic enzymes.** Acetylation can regulate the catalytic activity of metabolic enzymes through directly neutralizing the positive charge of lysine residues in the active site of OTC (A), recruiting a negative regulator such as phosphatase (PPase) to inhibit GP (B), or causing allosteric changes in 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2; C). Enzymes, acetylated lysine residues (K), and active sites are labeled as in Fig. 1. S, substrate.

increased the catalytic activity (k_{cat}) by almost 50%. The C-terminal region of PGAM1, whose removal is associated with loss of mutase activity, was previously proposed to act as a so-called "dynamic cap" to maintain the enzyme in its active, phosphorylated form by positioning the substrate for catalysis (Walter et al., 1999). This result suggests that acetylation of the three lysine residues in this region may impact the catalytic activity by optimizing the position of the cap to allow efficient phosphate transfer from the 3 to the 2 position in glycerate.

Acetylation of lysine residues near the active site has also been implicated in the inhibition of manganese superoxide dismutase (SOD2; Qiu et al., 2010), a major antioxidant enzyme whose deficiency is associated with various human diseases such as idiopathic cardiomyopathy, sporadic motor neuron disease, aging, and cancer (Miao and St Clair, 2009). SOD scavenges reactive oxygen species (ROS) by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide, which is then converted to oxygen and water by catalase. Three separate studies have demonstrated that acetylation of SOD2 inhibits its enzymatic activity, and that oxidative stress stimulates SIRT3 to deacetylate SOD2, leading to SOD2 activation and ROS reduction (Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011). The precise mechanism by which acetylation negatively regulates SOD2 activity, however, remains uncertain, as these three studies each identified a different lysine as the major site of acetylation in SOD2.

Acetylation regulates substrate accessibility

Acetylation blocks substrate binding to the enzyme. Direct immunoblotting of mouse liver mitochondrial lysates with anti-acetyl antibody revealed two proteins that are noticeably acetylated to a greater degree in *Sirt3*$^{-/-}$ mice than

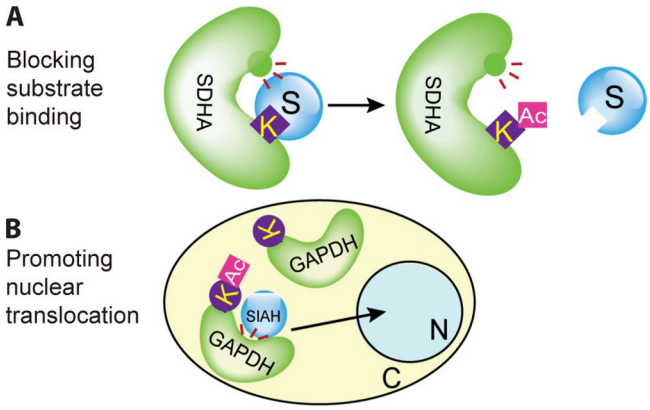

Figure 3. **Acetylation regulates the substrate accessibility to metabolic enzymes.** (A) Acetylation can regulate the substrate accessibility to metabolic enzymes by modifying the conserved lysine residues located on the hydrophilic surface of SDHA to hinder the entry of substrate (S) into the active site. (B) Acetylation can also alter the access of cytoplasmic substrates to GAPDH by promoting nuclear accumulation of GAPDH. Enzymes, acetylated lysine residues (K), and active sites are labeled as in Fig. 1. N, nucleus; C, cytoplasm.

in wild-type mice (Cimen et al., 2010). These two bands were identified by mass spectrometry to be succinate dehydrogenase subunit A (SDHA) and glutamate dehydrogenase (GDH). SDH is a unique enzyme that participates in both the TCA cycle, to catalyze the oxidation of succinate to fumarate, and in oxidative phosphorylation as a component of the electron transport chain (complex II), where it catalyzes the reduction of ubiquinone to ubiquinol. Human SDH is composed of four subunits encoded by four distinct genes, *SDHA*, *SDHB*, *SDHC*, and *SDHD*, and is activated by a newly discovered assembly factor, SDHAF2 (also known as SDH5). In addition to its critical physiological function, SDH regulation also bears important pathological significance, as mutations in all five *SDH* genes have been linked to tumor development (Bardella et al., 2011). Acetylation of SDHA through chemical inhibition of cellular SIRTs and deacetylation of SDHA by the overexpression of SIRT3 resulted in decreased and increased complex II activity, respectively, which indicates an inhibitory role of acetylation toward SDHA (Cimen et al., 2010). Four lysine residues were identified by mass spectrometry to be acetylated. They are highly conserved during the evolution from bacteria to human, and are located on the hydrophilic surface of SDHA, which suggests that acetylation at these sites may hinder the entry of substrate into the active site (Fig. 3 A).

Acetylation modulates enzyme subcellular localization. Besides directly regulating the accessibility of substrate, acetylation can indirectly regulate substrate accessibility by affecting subcellular localization of the metabolic enzyme. One such an example is acetylation-mediated nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ventura et al., 2010). GAPDH, long considered to be a housekeeping gene that is widely used as a protein loading control because of its relatively constant levels, catalyzes the NAD^+-dependent conversion of glyceraldehyde-3-phosphate (G3P) to 1,3 bisphosphoglycerate (1,3BPG). Besides its conventional role in catalyzing glycolysis within the cytoplasm, GAPDH also participates in other cellular processes including transcriptional regulation, DNA repair, and telomere maintenance in the nucleus (Zheng et al., 2003). Translocation of GAPDH into the nucleus can be influenced by cellular growth conditions such as cell cycle and apoptosis (Tristan et al., 2011). Three different types of posttranslational modifications, *O*-GlcNAcylation (Park et al., 2009), S-nitrosylation (Hara et al., 2005), and acetylation (Ventura et al., 2010), have been linked to GAPDH nuclear translocation. Overexpression of PCAF acetyltransferase (p300/CBP-associated factor, KAT2B), which binds to and acetylates GAPDH, and treatment of cells with a deacetylase inhibitor, TSA, increases nuclear accumulation of ectopically expressed GAPDH (Ventura et al., 2010). Substitution of two putative PCAF acetylation residues with arginine in GAPDH, but not the acetyl-mimetic glutamine, blocked TSA-mediated nuclear accumulation in GAPDH, suggesting that acetylation promotes GAPDH nuclear accumulation (Fig. 3 B). It remains to be elucidated how acetylation promotes GAPDH nuclear translocation, whether by changing the conformation to inhibit tetramer formation like *O*-GlcNAcylation (Park et al., 2009), or by promoting its binding to and getting a ride with nuclear-localized proteins such as SIAH1 (Sen et al., 2008).

Acetylation blocks the binding of metabolites. In addition to impacting substrate binding, acetylation has also been found to affect the binding of allosteric regulating metabolites. In a study aimed at unraveling the paradoxical roles of mitochondrial-enriched SIRT3 in fasting and calorie restriction, Lu et al., (2011) investigated the role of acetylation in both protecting against redox stress and exacerbating redox-dependent toxicity of the pain relief agent acetaminophen (e.g., Tylenol). Acetaminophen causes a potentially fatal hepatic necrosis when taken in overdose, resulting from the production by cytochrome P450 enzymes of a reactive metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI), that binds to hepatic cysteine residues as well as (unmodified) lysine residues (James et al., 2003). Protein acetylation, like *N*-acetylcysteine therapy, may block the NAPQI binding. Conversely, deacetylation, such as loss of function in SIRT3, may exacerbate NAPQI binding and acetaminophen hepatotoxicity. Using two-dimensional gel and mass spectrometric of differentially acetylated spots, Lu et al. (2011) identified 17 liver mitochondrial proteins whose acetylation is enhanced in *Sirt3*$^{-/-}$ mice, including aldehyde dehydrogenase (ALDH2). ALDH2 is a key enzyme in alcohol metabolism because of its high affinity for its substrate acetaldehyde, which it reduces to acetate. ALDH2 is a known target of NAPQI, and is inactivated by it. Substitution of a single lysine residue, K377, with an acetyl-mimetic glutamine nearly completely abolished NAPQI binding (Lu et al., 2011), which demonstrates the importance of acetylation at this lysine residue in antagonizing the binding of NAPQI. This provides an intriguing example in which acetylation can affect enzyme function by interfering with the binding of allosterically regulating molecules.

Major questions in the field

In addition to the examples discussed above, there are several metabolic enzymes whose acetylation has been firmly

established and linked to the cellular response to specific nutrient and growth conditions (Table 1). For example, the activity of mitochondrial isocitrate dehydrogenase 2 (IDH2), a TCA enzyme that catalyzes the decarboxylation of isocitrate to α-ketoglutarate, is inhibited by acetylation and is stimulated by SIRT3-mediated deacetylation in response to caloric restriction, leading to increased production of NAPDH and reduced oxidative damage (Someya et al., 2010). The acetylation site for IDH2 has not been determined, and the mechanism of acetylation regulation of IDH2 therefore remains to be elucidated. Long chain acyl coenzyme A dehydrogenase 2 (ACADC, also known as LCAD) catalyzes the initial step in each cycle of fatty acid β-oxidation in the mitochondria, and is acetylated in fed mice but deacetylated by SIRT3 during fasting (Hirschey et al., 2010). One specific lysine residue, K42, was identified to be its major acetylation site, which, when mutated, significantly increased the activity of ACADC. How acetylation at K42 mechanistically impairs the activity of ACADC is presently unknown, as the location of K42 does not suggest an obvious impact from its acetylation.

Major questions in the field

1. How to functionally validate the acetylation identified by proteomics?
More than 2,000 putative acetylated proteins have already been identified by just a few proteomic studies, and there are almost certainly more that have yet to be discovered. Generation and use of anti-acetylation site antibodies remains the major technique to validate and elucidate the function of acetylation on a given lysine residue.

2. What fraction of a given metabolic enzyme is acetylated?
In most studies thus far on the regulation of metabolic enzymes by acetylation, there is a lack of quantification of what fraction is acetylated under specific physiological conditions. The rapid progress of quantitative mass spectrometry is significantly facilitating the quantification of acetylation, and may soon become a standard in the field.

3. How do a few modifying enzymes control so many substrates?
More than 2,000 proteins have been identified by proteomic studies to be potentially acetylated, but there are only 22 acetyltransferases and 18 deacetylases that have been identified in human cells. Three different mechanisms can be envisioned on how so few modifying enzymes control the acetylation of so many substrates: the existence of a novel class of acetyltransferases and/or deacetylases, the existence of a novel class of substrate receptors, and nonenzymatic covalent conjugation of an acetyl group to a lysine residue.

4. How does acetylation affect many metabolic enzymes in a coordinated manner?
Nearly all of the enzymes involved in glycolysis, the TCA cycle, the urea cycle, fatty acid oxidation, and nitrogen metabolism are potentially acetylated. A unique feature of acetylation is that the acetyl group donor for all acetyltransferases, Ac-CoA, and the electron acceptor (or coenzyme) of SIRT family of deacetylases, NAD^+, are both key intermediate metabolites produced and consumed by many metabolic reactions. This feature suggests that acetylation of multiple enzymes on a pathway could be coordinated in part by the global change in the levels of intracellular Ac-CoA and NAD^+.

We can anticipate many new findings on the regulation of metabolic enzymes by acetylation, including novel mechanistic insights, to be made in the near future. Many outstanding and critical issues have emerged. Among them are four that directly relate to the regulation of metabolic enzymes and metabolism, and bear broad implications for the acetylation regulation of proteins in other cellular processes (see Text Box).

How to functionally validate the acetylation identified by proteomics? More than 2,000 putative acetylated proteins have already been identified by just a few proteomic studies, and there are almost certainly more that have yet to be identified. Functional validation of these studies is rapidly becoming a critical issue. A commonly used method to study the function of a putative acetylation site is to determine the effect of substituting a given lysine with glutamine. Although both acetyl-lysine and glutamine may be most similar among all amino acids, and there are many good examples where the K-to-Q mutation mimics acetylation, glutamine is structurally different from N^{ϵ}-acetyl-lysine because of a significantly shorter carbon chain and the carbonyl group of the amide being at a different position. Of larger concern is that lysine is known to be modified by many additional types of post-translational modifications such as ubiquitylation, methylation, and hydroxylation. In particularly, two new types of lysine modifications, succinylation and malonylation, were recently described, and SIRT5 has been identified to be the enzyme that removes both modifying groups (desuccinylase and demolanylase; Du et al., 2011; Peng et al., 2011). Preliminary identification of succinylation and malonylation substrates includes many metabolic enzymes, immediately implicating both modifications in metabolic regulation. Substitution of a lysine with a glutamine would effectively eliminate all these modifications on lysine, and therefore potentially create a complicated network of unforeseen metabolic consequences. An effective way to address this issue is to use anti-acetylation site antibodies for functional studies, which can also help to determine the in vivo change of acetylation on a given site in response to a change in cellular conditions.

What fraction of a given metabolic enzyme is acetylated? In most studies thus far on the acetylation regulation of metabolic enzymes, there is a lack of quantification of what fraction is acetylated under specific physiological conditions. Acetylation of a small fraction of steady-state proteins may be sufficient to regulate enzymatic function if acetylation promotes enzyme degradation. Similarly, a substoichiometric acetylation may also play a significant role in physiological regulation if acetylation results in a gain of function or alters the subcellular localization. Quantification of acetylation is, however, critically important for many metabolic enzymes, as acetylation often targets evolutionary conserved and functionally important lysine residues (Weinert et al., 2011), and is therefore expected to cause loss of function changes. For example, inhibitory acetylation of 10% of a given enzyme would leave cells with 90% enzyme activity, a change that is unlikely to have a significant impact on cell metabolism. In several studies where acetylation on a specific site or an enzyme has been determined, however, it appears that a significant fraction of the enzyme is acetylated. For example, 27% and 67% of ectopically expressed MDH2 was acetylated in the absence or presence of deacetylase inhibitors, respectively, as determined by Fourier transform ion cyclotron resonance (FTICR) mass spectrometry (Zhao et al., 2010). Using a different

technique, known as isobaric tags for relative and absolute quantification (iTRAQ), it was determined that as much as 50% of GP was acetylated at K470 (Zhang et al., 2012); and 44% and 47% of enoyl-CoA, hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH), an essential enzyme that metabolizes fatty acids to produce actyl CoA and release energy, is acetylated at K171 and K346, respectively (Zhao et al., 2010). Both FTICR and iTRAQ techniques have some obvious limitations: they have low throughput, and are labor intensive and costly. Given the rapid progress of quantitative mass spectrometry, such as stable isotope labeling by amino acids (SILAC) or label-free, high-throughput quantification, one can expect that determining the quantification of acetylation will soon become a standard in the field.

How do a few modifying enzymes control so many substrates? The identification of such a large number of acetylated proteins by proteomic studies, already >2,000, raises an acute conundrum: how do so few enzymes, 22 acetyltransferases (KATs; Allis et al., 2007) and 18 deacetylases (11 HDACs [Riccio, 2010] and 7 SIRTs [Schwer and Verdin, 2008]) in human cells, control the acetylation of so many substrates? Making the puzzle even more challenging is the fact that only three (SIRT1, -2, and -3) have clearly demonstrated deacetylase activity. Distinct from the others, SIRT5 has been found to be a lysine desuccinylase and demalonylase (Du et al., 2011; Peng et al., 2011). Phylogenetic analysis suggests that the remaining three (SIRT4, -6, and -7) may have novel enzymatic activity in removing different acyl modifications (Hirschey, 2011). For perspective, more than 500 protein kinases and an equally large numbers of phosphatases (the exact number is less certain because of the complex combinations of different subunits), and more than an estimated 700 E3 ubiquitin ligases with more than 100 deubiquitinases in human cells are found to control reversible protein phosphorylation and ubiquitylation, respectively. There are no obvious clues, much less answers, to how so few modifying enzymes regulate so many substrates. Three different mechanisms can be envisioned though. First, there may exist a novel class of acetyltransferases and/or deacetylases yet to be discovered. A reminder of this scenario is the history of E3 ubiquitin ligase research, where HECT domain–containing proteins (homologous to E6-AP carboxyl terminus) were the major type of E3 ligases until the discovery of RING-type E3 ligases. Human cells contain 28 HECT E3 ligases, but more than 300 RING finger proteins and an estimated 400 additional RING-type E3 ligases were assembled by the Cullin family proteins through binding, in trans, with a small RING finger protein. Second, an individual acetyltransferase and deacetylase could control multiple substrates. This certainly is happening in the cell to some extent. Analyses of acetylation levels of several metabolic enzymes in mouse organs deficient for individual SIRTs, such as SIRT3 (e.g., Table 1), or in vitro deacetylation assays, can be seen as consistent with this model. However, this model poses a serious question: how can acetylation of different proteins be specifically regulated under different physiological conditions? One possibility is that each acetyltransferase or deacetylase may have additional binding partners that regulate substrate binding or enzyme activity. Therefore, the limited numbers of catalytic subunits of the modifying enzymes may be assembled with binding partners into a large family that can meet the need for specificity in vivo. Lastly, and more radically, the covalent conjugation of an acetyl group to a lysine residue, or removal of an acetyl group from acetyl-lysine, could occur nonenzymatically. One such precedent is protein succination, in which a mitochondrial metabolic intermediate, fumarate, reacts spontaneously with cysteine sulfhydryl group in a Michael reaction (nucleophilic addition of a carbanion) to form a stable S-(2-succinyl) cysteine (Alderson et al., 2006).

How does acetylation affect many metabolic enzymes in a coordinated manner? Many metabolic enzymes were identified by the acetylation proteomic studies. This is particularly obvious for enzymes involved in glycolysis, TCA, urea cycle, fatty acid oxidation, and nitrogen metabolism, where most enzymes are potentially acetylated. Such far-reaching regulation of a specific cellular process, metabolism, by one specific type of modification is reminiscent of the regulation of signal transduction by phosphorylation, and the cell cycle by ubiquitylation. It raises the question as to how cells coordinate the acetylation of multiple enzymes involved in a single pathway. This question becomes even more profound when considering two additional issues: only a few acetyltransferases and deacetylases are known to be involved, and most metabolic pathways are not linear; rather, they form a network with many branches from each pathway sharing common intermediates.

The answer may come in part from a unique feature of acetylation: the fact that the acetyl group donor for all acetyltransferases, Ac-CoA, and the electron acceptor (or coenzyme) of SIRT family of deacetylases, NAD^+, are both key intermediate metabolites produced and consumed by many metabolic reactions. Support for this notion comes from a study showing that nuclear histone acetylation in mammalian cells is reduced by the knocking down of ATP-citrate lyase (ACL), the enzyme that converts glucose-derived citrate into acetyl-CoA (Wellen et al., 2009), which suggests that acetylation of substrate proteins can be influenced by the global change in the levels of intracellular Ac-CoA. One implication of this finding would be to allow cells to rapidly sense the change of concentration of acetyl-CoA and NAD^+ and to globally influence the acetylation level and activity of metabolic enzymes in response.

Lysine acetylation has emerged as a major posttranslational modification in the regulation of metabolism and many other similar cellular processes. Given the wide range of regulatory mechanisms it impacts and the high degree of evolutionary conservation, acetylation regulation of metabolism seems poised to only grow in significance as we continue to discover its functions and mechanisms.

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