

Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation*

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Protein acetylation is a well-studied regulatory mechanism for several cellular processes, ranging from gene expression to metabolism. Recent discoveries of new post-translational modifications, including malonylation, succinylation, and glutarylation, have expanded our understanding of the types of modifications found on proteins. These three acidic lysine modifications are structurally similar but have the potential to regulate different proteins in different pathways. The deacetylase sirtuin 5 (SIRT5) catalyzes the removal of these modifications from a wide range of proteins in different subcellular compartments. Here, we review these new modifications, their regulation by SIRT5, and their emerging role in cellular regulation and diseases. *Molecular & Cellular Proteomics 14: 10.1074/mcp.R114.046664, 2308–2315, 2015.*

Lysine acetylation (Kac)¹ is the addition of acetyl group to the ϵ -amine of a lysine side chain. This chemical modification is a reversible, dynamic, and evolutionarily conserved protein post-translational modification (PTM) (Fig. 1). Early studies on the biology of lysine acetylation focused on nuclear proteins, such as histones and transcriptional factors, which clearly demonstrated an important function of acetylation for chromatin structure and dynamics (1, 2). These studies led to the notion that lysine acetylation was restricted to the nucleus. The discovery of lysine acetylation on tubulin and on mitochondrial proteins demonstrated non-nuclear localization and suggested an important role for acetylation in an expanded array of cellular biology (3–5). Using mass-spectrometry-based proteomics, the cellular acetyl proteomes (a.k.a.

“acetylomes”) have been extensively characterized and have revealed high abundance of lysine acetylation outside the nucleus (6–10). For example, lysine acetylation plays key roles in regulating mitochondrial enzymes and other metabolic and cellular processes in addition to chromatin biology (6, 11–13). Lysine acetylation is abundant on most metabolic pathways, a finding that is conserved from bacteria to mammals (6, 7, 9, 14–21). Dysregulation of lysine acetylation plays a pathogenic role in diverse conditions such as metabolic syndrome, age-associated stem cell dysfunction, cardiac failure, and cancer (22–25).

Beyond protein acetylation, the landscape of protein modifications is rapidly expanding. Using mass spectrometry as a discovery tool, in combination chemical biology and biochemistry as validation tools, we recently identified and validated three new types of lysine modifications: lysine malonylation (Kmal) (26, 27), lysine succinylation (Ksucc) (27, 28), and lysine glutarylation (Kglu) (29), which are collectively referred to as lysine acylation (Fig. 2). Similar to lysine acetylation, emerging evidence suggests that these new lysine acylations are important in regulating cellular metabolism in physiological and pathophysiological states. In this review, we describe these new modifications and their regulation and highlight their emerging role in cellular regulation.

Lysine Malonylation, Succinylation, and Glutarylation Pathways—Recently identified lysine acyl modifications, Kmal (26,27), Ksucc (27,28), and Kglu (29) (Fig. 2), are evolutionarily conserved and dynamic under diverse cellular conditions, such as stressors, metabolic substrates and availability, and genetic mutations (29–31). Since these new modifications have an acidic carboxylic group under physiological pH, they are referred to collectively as acidic lysine acyl modifications; furthermore, the sirtuins are more appropriately referred to as protein deacetylases (Box1) (32).

Short-chain acyl-CoAs, malonyl-CoA, succinyl-CoA, and glutaryl-CoA, are the substrates for the corresponding lysine acylation reactions. These CoAs can possibly be synthesized by their corresponding short-chain acyl salts, malonate, succinate, and glutarate, catalyzed by acyl-CoA synthetases, such as succinyl-CoA synthetase and malonyl-CoA synthetase (33, 34). Additionally, these CoAs can be generated and consumed in the TCA cycle, as well as by metabolism of amino acids and lipids. These CoAs are highly regulated in cells and tissues. For example, dysregulation of malonyl-CoA

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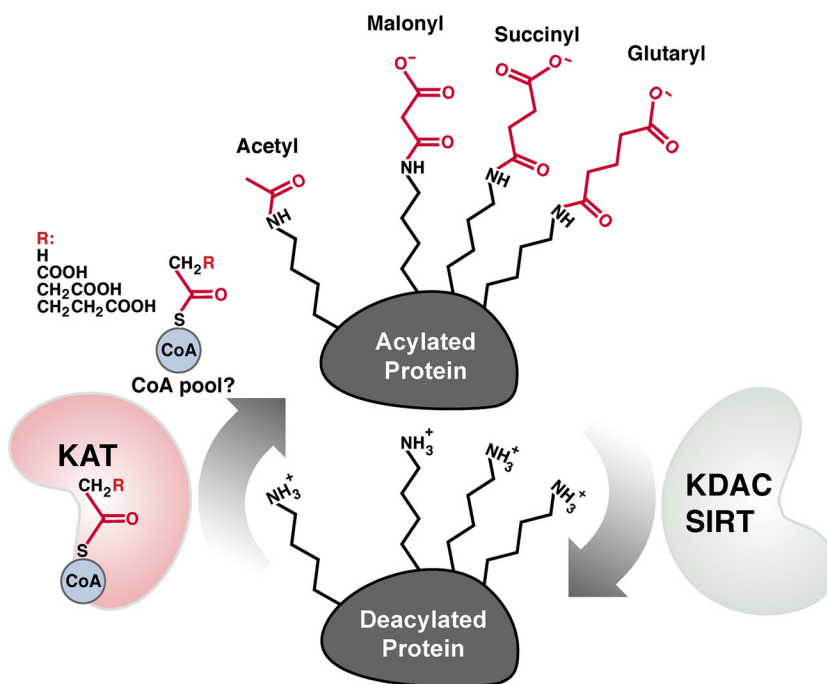
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¹ The abbreviations used are: Kac, Lysine acetylation; PTM, post-translational modification; Kmal, lysine malonylation; Ksucc, lysine succinylation; Kglu, lysine glutarylation; MCD, malonyl-CoA decarboxylase; CPS1, carbamoyl-phosphate synthase 1; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HAT, Histone acetyltransferase; HDAC, Histone deacetylase.

FIG. 1. Protein acylation is balanced by KDACs and KATs. Protein acylation can be enzymatically catalyzed by lysine acyltransferases (KATs) and removed by lysine deacylases (KDACs).



is associated with diseases, such as ischemic heart disease and diabetes (35, 36). Additionally, these short-chain CoAs are thermodynamically favorable for their corresponding lysine acylation reactions (37). Further, these CoAs are structurally similar to acetyl-CoA, leading to apparent enzymatically catalyzed acylation by promiscuous acetyltransferases. For example, the binding pocket of Hat1 has a large enough CoA binding pocket to accommodate CoAs larger than acetyl CoA (38), such as malonyl-CoA, succinyl-CoA, or glutaryl-CoA.

Acidic modifications are chemically different from lysine acylation in two distinct ways. First, they add a bulkier acyl group to lysines compared with acetylation: three, four, or five carbons for malonylation, succinylation, or glutarylation, respectively, compared with two carbons for acetylation. Second, acidic modifications change the charge on lysine from +1 to -1 under physiological conditions (28). Interestingly, protein phosphorylation also generates a -1 charge on proteins. In contrast, lysine acylation changes the charge on lysine from +1 to 0. Because of this difference, it is conceivable that acidic modifications could disrupt any ionic interactions between the positively charged, unmodified lysine side chains with a negatively charged chemical moiety in other molecules, such as nucleotides, proteins, or small molecules. Alternatively, changes in lysine charge could impart structural alterations on proteins, as acidic modifications would be predicted to have a more profound impact on protein structure and function compared with lysine acetylation, if located in the same lysine site. Future studies will address these possibilities and determine how acidic acyl modifications influence protein function.

Regulatory Enzymes for Lysine Malonylation, Succinylation, and Glutarylation—Two groups of enzymes with opposing activities, lysine acetyltransferases and deacetylases (Box 1), are known to regulate the status of lysine acetylation (Fig. 1) (39). Eighteen known class I, class II, and class IV histone deacetylases (HDAC1–11) and seven class III HDACs (the sirtuin family SIRT1–7) make up the entire HDAC family (Box 1). The HDACs have been a subject of intense study since their discovery in 1996 (40). Indeed, they have important roles in several areas of biology and disease and are increasingly used as targets for drug design. For example, deacetylase inhibitors, such as vorinostat (trade name Zolinza) and romidepsin (trade name Istodax), have been approved by the U.S. Food and Drug Administration for clinical use in cancer therapy (41, 42).

The sirtuin family of HDACs has seven highly conserved NAD⁺-dependent deacetylases, which were discovered in 1999/2000 (43, 44). Sirtuins regulate key biological processes in mammals, including many aspects of metabolism, stress response, and signaling (45). Three mammalian sirtuins (SIRT3, SIRT4, and SIRT5) localize mostly or exclusively to the mitochondrial matrix. SIRT3 has robust deacetylase activity and is the major enzyme in the mitochondria performing this reaction (46). In contrast, neither SIRT4 nor SIRT5 have been shown to regulate global mitochondrial protein acetylation levels (47). Supporting this idea, SIRT4 and SIRT5 have weak or nondetectable deacetylase activity *in vitro*. Low activities of these mitochondrial sirtuins against acetyl-lysine, and the absence of known substrates suggested the possibility they could be removing lysine modifications other than acetylation from proteins.

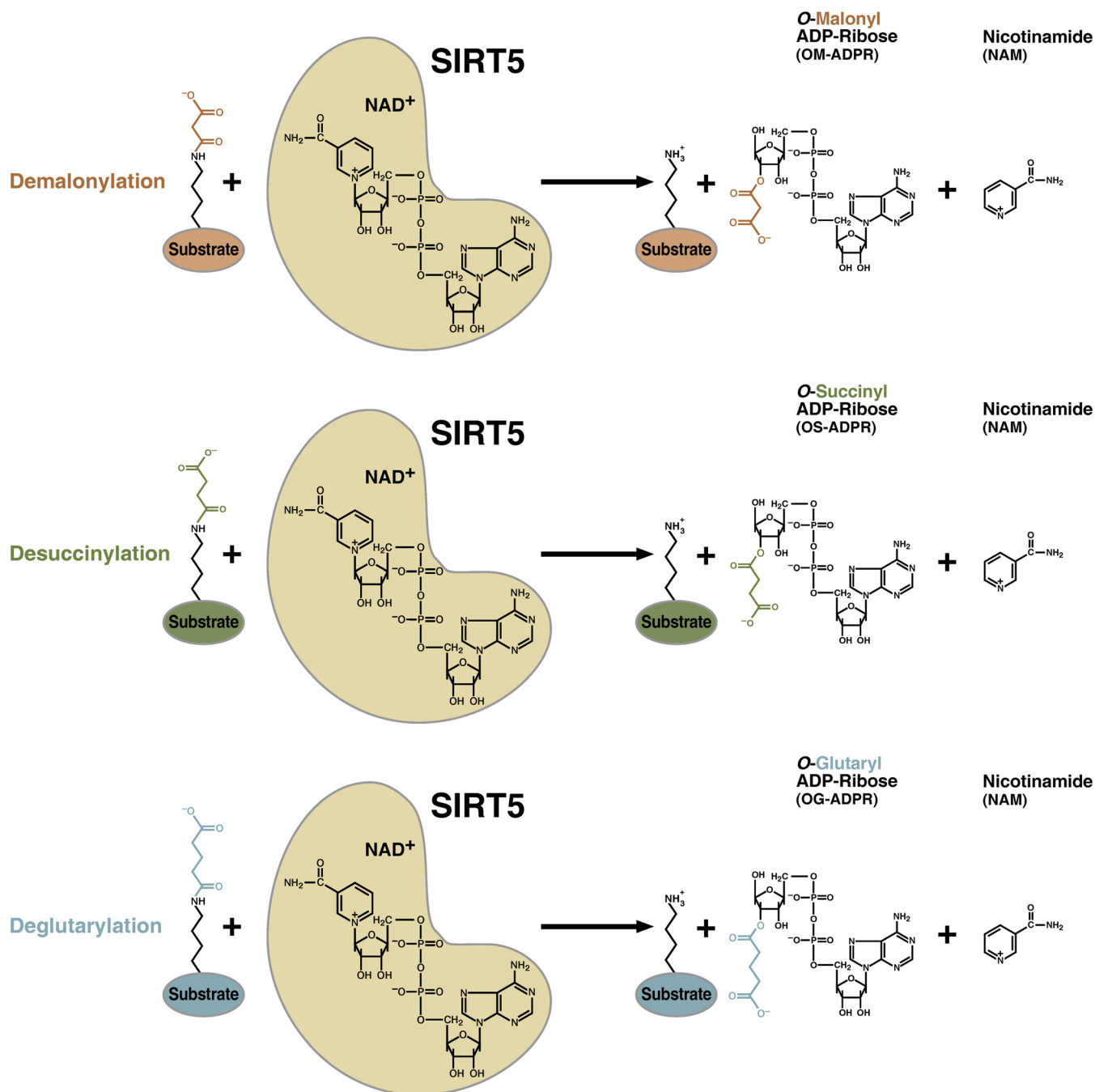


FIG. 2. **Regulation of lysine malonylation, succinylation, and glutarylation.** Lysine malonylation, succinylation, and glutarylation is targeted for removal by the NAD⁺-dependent deacylase SIRT5.

The immediate question after the discovery of acidic acyl modifications was whether they were regulated by HDACs or HDAC-like enzymes. Given the same amide bonds between lysine acetylation and the acidic acyl modifications (Fig. 2), all 18 HDACs were tested for deacetylation activities against malonylation, succinylation, and glutarylation. SIRT5 was shown to have potent demalonylase and desuccinylase activities; this finding was validated using biochemical measurements, as well as quantitative proteomics in cells and tissues, with or

without an expression of SIRT5, both *in vitro* and *in vivo* (26, 48). Importantly, these findings were independently discovered and verified using different, but complementary, methods, including enzymology and structural biology (49). In another recent study using quantitative proteomics, SIRT5 knockout mouse embryonic fibroblasts were shown to have an increase in lysine succinylation but not acetylation. Together, these studies demonstrate that SIRT5 is the major enzyme for lysine desuccinylation in the cells and tissues (48).

Importantly, this result, combined with emerging evidence from other studies, also suggests SIRT5 is unlikely a deacetylase (Box1) *in vivo*. For example, *in vitro* deacetylation activities of SIRT5 is low or undetectable when measured using multiple substrates such as small molecules containing a fluorescent acetylated lysine residue, peptide substrate, and whole cell lysate (26, 48). The current model is for SIRT5 to be a specific mammalian HDAC with potent activity to remove acidic lysine acyl groups. While mammalian SIRT5 seems to have activities restricted to acidic lysine acylation, its bacterial counterpart, CobB, the only deacetylase identified so far in prokaryotes, has both deacetylase and desuccinylase activities (50). Interestingly, CobB mutants can be generated in such a way that the two activities can be differentiated (50), and more work will be required to understand the regulation of protein succinylation in lower organisms.

In addition to lysine malonylation and lysine succinylation, a recent study demonstrated lysine glutarylation as a new lysine acylation and showed that SIRT5 is its deglutarylase (29), consistent with the idea that SIRT5 catalyzes the removal of acidic lysine modifications. Enzymatic studies and structural modeling show that SIRT5 has comparable activities among the three acyl modifications and prefers short-chain carboxylic groups, such as malonyl, succinyl, and glutaryl groups, but not acetyl (shorter) or adipoyl carboxylic groups (with a longer acidic acyl chain, e.g. six carbons or longer) (29). Thus, while SIRT5 is flexible among the three short-chain acidic lysine acylations, it does have selectivity against acidic acyl modifications.

While HDACs have been systematically tested for their activities for demalonylation, desuccinylation, and deglutarylation, such screens have not yet been reported in the literature for HATs (Box1). We showed that p300 has enzymatic activity for lysine glutarylation *in vitro* (29). More recently, Garcia and his colleagues showed that p300/CBP can catalyze lysine succinylation *in vitro* (51). These two lines of evidence suggest a possibility that enzyme-catalyzed lysine acylation may exist in cells. In addition, identification of lysine succinylation on N-terminal regions of histones also supports the notion that nuclear acetyltransferases could catalyze lysine succinylation.

Substrates of Lysine Malonylation, Succinylation, and Glutarylation—Identification of individual protein with acidic acyl lysines is a critical next step to understand the role of these modifications, an idea well illustrated by the history of protein acetylation. Progress in our understanding of lysine acetylation in each cellular compartment is largely synchronized with identification of modified proteins. Technological advances in mass-spectrometry-based PTM proteomics, such as improved mass spectrometry sensitivity, high-quality antibodies against PTMs of interest, and peptide fractionation procedure, make it possible to quickly identify sites of acylation in proteins, and primary substrates targeted by the HDACs/sirtuins.

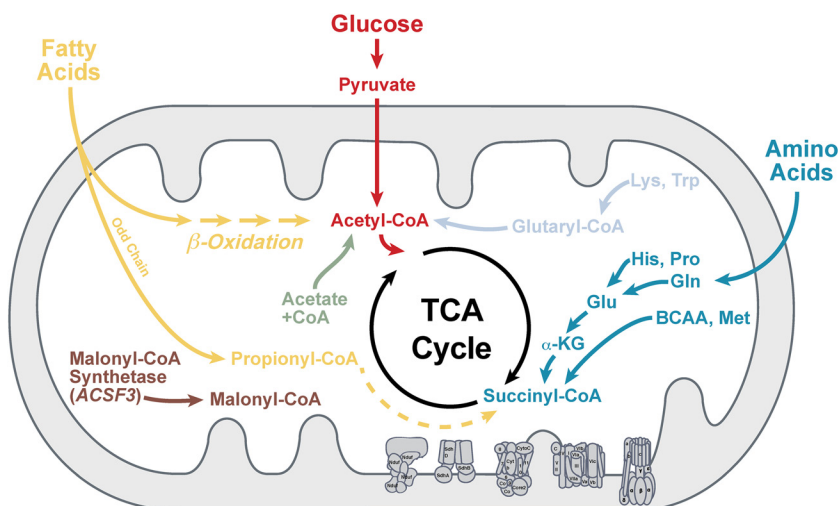
Efficient affinity enrichment of the peptides containing modifications using sequence-independent anti-acyl-lysine antibodies followed by HPLC/MS/MS analysis and protein sequence alignment enables proteome-wide analysis of PTM substrates. The acylated lysine is relatively stable, and its peptide can lead to a good peptide-bond fragmentation signature in a mass spectrometer. Thus, the acylation sites can be accurately mapped in a similar fashion as those for lysine acetylation.

While succinyl-lysine was fully established few years ago, proteomic screenings have only recently been carried out to identify the proteins that contain this modification. Using a quantitative proteomic approach, a total of 2,565 succinylated lysine sites from 779 proteins in mouse cells were identified by comparing the difference of succinyl-lysine levels in MEFs with or without SIRT5 (48). Similarly, profiling of lysine succinylation has also been performed in bacteria, yeast, HeLa cells (52), and mouse liver mitochondria (48, 52, 53).

Several interesting findings emerge from these early proteomic studies. First, SIRT5 is the major enzyme for lysine desuccinylation in mammalian cells, which is supported by the findings that *Sirt5* ablation (SIRT5KO) in mice potently increases global lysine succinylation levels (26), and almost all succinylated sites are increased in SIRT5KO MEFs (48). A second finding from lysine succinylation proteomic studies is that succinylation is a widespread protein post-translational modification in diverse model organisms (48, 50, 52, 53). Third, succinylation is abundant on mitochondrial proteins and is estimated to have high stoichiometry. Specifically, quantitative proteomics of both protein expression and succinyl-lysine show that 32% of the succinylated sites had stoichiometries greater than 10% in SIRT5 WT cells, whereas this figure increased to 56% in SIRT5 KO cells (48). However, these are only estimates of stoichiometry, and further work needs to be done to further test this finding. Fourth, succinyl-lysine can be more dynamic than acetyl-lysine; for example, in *Escherichia coli*, high glucose concentration induce a more significant change in protein succinylation than acetylation (50). This observation suggests that under some cellular conditions, succinylation may play a different role in cellular regulation than acetylation. Global lysine succinylation is dynamically changed in response growth conditions and genetic mutations that are associated succinyl-CoA homeostasis (48, 50).

In addition to succinylation, proteomic screening was carried out on lysine glutarylation in mouse liver. This study identified 683 lysine glutarylated sites in 191 proteins (29). A comparison between glutarylated and succinylated sites in mouse liver tissues show that 459 of the 683 glutarylated sites (about 67%) are overlapping with succinylated lysines, suggesting that the two modifications could be influencing the same proteins and by the same mechanism. Interestingly, carbamoyl-phosphate synthase 1 (CPS1), a protein known to

FIG. 3. Metabolic regulation of malonyl-, succinyl-, and glutaryl-CoAs. Lipid, glucose, and amino acid metabolism all lead to the generation of acidic acyl-CoA species.



be both acetylated and succinylated, was also found to be glutarylated and regulated by SIRT5 (29).

Efforts have been taken to identify substrates for lysine malonylation. Early experiments on lysine malonylation led to the identification of 17 lysine malonylated proteins in HeLa cells (26). Recent large-scale proteomic screening to characterize mammalian malonylome identified 4,042 malonylation sites on 1,426 substrate proteins in mouse liver and 4,943 malonylation sites in 1,822 substrate proteins in human fibroblasts, with or without expression malonyl-CoA decarboxylase (MCD) (31). Four hundred sixty-one malonylation sites on 339 substrate proteins showed a two-fold increase or more in MCD^{-/-} cells relative to MCD^{+/+} cells, and 1,452 malonylation sites on 822 proteins were only detected in MCD^{-/-} cells. Lysine malonylation regulates mitochondrial function and fatty acid oxidation in MCD-deficient cells. Interestingly, MCD^{-/-} cells have higher level of malonyl-CoA than MCD^{+/+} cells. This result indicates a possibility that MCD activity modulate cellular malonyl-CoA concentration that in turns impacts cellular lysine malonylation.

Physiological Role of Acylation—The expanding landscape of protein acylation coupled with the important role of the corresponding acyl-CoAs and the sirtuins in metabolic regulation have led to the idea that these new acyl-based lysine modifications are important regulators of metabolism (Fig. 3). Indeed, proteomic studies on lysine malonylation, succinylation, and glutarylation have shown that, like acetylation, metabolic proteins are over-represented (29, 48, 50, 52, 53). Gene ontology analyses on the proteins identified as acylated consistently find mitochondrial and metabolic pathways. While less is known about malonylation, the top pathways identified as having succinylated and glutarylated lysines are largely overlapping and include oxidoreductase activity, fatty acid metabolism, and amino acid metabolism (29, 48, 53).

As predicted, pathway analyses showed proteins containing lysine succinylation and glutarylation are largely found within mitochondria. Succinylation is enriched in the mito-

chondria, but non-mitochondrial proteins are also succinylated, including ribosomes and nuclear proteins. Follow-up studies showed several sites of both succinylation and malonylation on histones (27), suggesting these modifications could be an underappreciated part of the histone code. Glutarylation was also found in both mitochondrial and extra-mitochondrial compartments (29). The small subset of proteins known to be malonylated are largely found in the cytoplasm, but whether this snapshot is representative of the overall landscape is unknown.

These observations lead to the question about the subcellular localization of SIRT5, the only known regulator of these acidic modifications. An early study on SIRT5 identified two distinct human isoforms with only minor differences in the C-terminus (54). This study proposed a model where both isoforms of SIRT5 are targeted to the mitochondria for N-terminal processing, which can translocate out of the mitochondria to the cytoplasm and have varying degrees of protein stability (54). A recent study supported this finding, showing SIRT5 was present in mitochondrial, cytoplasmic, and nuclear compartments (48). They further showed that dynamic sites of succinylation can be found outside the mitochondria (48), suggesting that SIRT5 regulates both mitochondrial and extra-mitochondrial lysine succinylation. This idea could also be extended to suggest that SIRT5 regulates sites of malonylation and glutarylation outside the mitochondria, but further studies are required to test these ideas and validate putative sites of regulation.

Indeed, a major and ongoing effort in the field is to validate sites of malonylation, succinylation, and glutarylation identified in proteomic studies and to understand how acylation affects the activity of its targets. Succinylation has been shown to regulate CPS1 (49), pyruvate dehydrogenase (PDH) (48), succinate dehydrogenase (SDH) (48), and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) (53). Additionally, several members of the fatty acid oxidation pathway have succinylated lysines, and this pathway is repressed in mice or

cells lacking SIRT5 (53). Glutarylation has also been shown to regulate CPS1 activity and is the only target known to be regulated by this new modification (29).

No clear picture has emerged on the regulatory role of malonylation, succinylation, and glutarylation. Some proteins show reduced activity in the acylated state, either by *ex vivo* measurements or *in vitro* mimetic strategies. For example, CPS1 and HMGCS2 are suppressed, whereas PDH and SDH were shown to have increased activity in the acylated state. How these modifications regulate biological pathways, are regulated by SIRT5, and coordinate overall cellular metabolism are major questions being addressed in the field. Nonetheless, acyl-lysine modifications are emerging as important players in regulating metabolism and cellular physiology.

Pathophysiological Role of Acylation—As an extension of their role in regulating physiological processes, lysine malonylation, succinylation, and glutarylation are also associated with pathophysiological processes and disease. For example, mitochondrial protein glutarylation is significantly elevated in a mouse model of glutaric acidemia (29). Glutaric acidemia is an inborn error of metabolism (OMIM 231670) caused by a deleterious mutation in glutaryl-CoA dehydrogenase, which is a mitochondrial enzyme that degrades glutaryl-CoA. Additionally, elevated protein malonylation was recently observed in human fibroblasts of patients with MCD deficiency (OMIM 606761) (30). Supporting this idea, malonyl-CoA concentrations are elevated in MCD^{-/-} cells compared to MCD^{+/+} cells, which correlates well with elevated levels of global lysine malonylation (31). Similar elevations in protein acylation were observed in inborn errors of metabolism for other modifications, including protein butyrylation and protein propionylation, which suggests that protein acylation could be a common biochemical feature of mutations in acyl-CoA-handling enzymes (30). Genetic mutations in ketoglutarate dehydrogenase and succinyl-CoA synthetase, two key enzymes for metabolism of succinyl-CoA, are associated with metabolic diseases (55, 56). It is therefore highly possible that succinyl-CoA concentration is dysregulated in the patients' cells that in turn modulates lysine succinylation. While the role of acyl-lysine modifications in inborn errors of metabolism is not known, alterations in enzymatic activity (either elevations or reductions) of key metabolic proteins could contribute to the pathogenesis of these diseases.

Future Studies—A major question remaining in the field is the mechanism by which lysine acylation occurs (for a recent review, see ref (37)). No acyltransferases have been shown to catalyze the transfer of malonyl-, succinyl-, or glutaryl-modifications to proteins *in vivo*, although this can be performed using a truncated p300 acetyltransferase *in vitro* (29). Interestingly, 35 histone lysine succinylation sites were detected in histones (27), whose succinylation status is regulated by SIRT5 in MEFs (48). Different from the lysine acetylation sites, which primarily exist at the N-terminal of histones, nearly all the lysine succinylation sites localize to the C-terminal glob-

ular domains (27). Detection of lysine succinylation in histones, but not other highly abundant nuclear proteins, such as lamin-B (48), suggests substrate specificity of lysine succinylation. This further implies the possibility of succinyltransferase-catalyzed lysine succinylation reactions in nuclei and the net histone succinylation balanced by succinylation catalyzed by succinyltransferase and desuccinylation catalyzed by the SIRT5. Given the structural similarity among three acidic modifications with a difference of only one or two methylene groups (Fig. 1), all three could share a putative acyltransferase in a similar fashion as their deacylase.

In principle, lysine acylation could be controlled by a balance between concentrations of acyl-CoAs and activities of acyltransferases and deacylases. While structurally similar, malonyl-CoA, succinyl-CoA, glutaryl-CoA, and their corresponding short-chain acyl salts (malonate, succinate, and glutarate) are generated in different metabolic pathways (26, 28, 29) (Fig. 3). As a consequence, these three PTMs might not be redundant and have very different dynamics depending on fluctuation of metabolites in response to changes in cellular physiology and stress. It is also possible that the deacylase SIRT5 may have different activities and subcellular localization.

While enzymatic mechanisms of transfer in these conditions cannot be excluded in these studies, acyl-CoA accumulation in the setting of genetic mutations demonstrates a strong relationship between the levels of acyl-CoAs and the levels of acyl-lysines (29, 30). This relationship is further strengthened by physiological studies showing that protein succinylation is dependent upon succinyl-CoA generation (52) and that protein glutarylation fluctuates as a function of glutaryl-CoA generation (29). However, in the absence of known acyltransferases and the permissivity of these reactions *in vitro* (57), the idea of nonenzymatic mechanisms contributing to the global acylation landscape is gaining traction, at least in mitochondrion, where pH is higher than cytosol and nuclei. Like acetylation, both chemical and enzyme-catalyzed reactions could be occurring in cells, depending on cellular pH and chemical properties of CoAs, and likely contributes to the global acyl proteome. Additional studies on the mechanisms of acylation will lead to a deeper understanding of the role of acylation in biology.

CONCLUSION

The rapidly increasing number of protein modifications expands the known landscape of lysine acylation. Current thinking positions these modifications to be regulatory. Indeed, complementary techniques to manipulate acylation lead to reproducible changes in protein function. However, on any given protein, some acylation events will be regulated (by SIRT5, for example) and some not; furthermore, some acylation events will have a functional consequence and some not, similar to phosphorylation and acetylation. While these findings increase the complexity of protein regulation, continued

research in this exciting area will clarify this important aspect of the regulatory balance between acylation and deacylation.

Boxes—

Box 1: HAT, HDAC, sirtuins, acyltransferase, deacylase, lysine acylation—Histone acetyltransferase (HAT, also called lysine acetyltransferase (KAT)): an enzyme that catalyze the transferring an acetyl group from acetyl CoA to the side chain amine to form ϵ -N-acetyllysine.

Histone deacetylase (HDAC, also called lysine deacetylases (KDAC)): an enzyme that removes acetyl group from ϵ -N-acetyllysine to form unmodified lysine.

Sirtuins (SIRT1–7): annotated as a subfamily of HDACs based on their protein sequence. Their enzymatic activities require NAD⁺ as a cofactor/cosubstrate.

Lysine acylation: introduction of an acyl group from a donor, such as acyl CoA, onto lysine side chains to form ϵ -N-acyl-lysine.

Lysine acyltransferase (KAT): an enzyme that catalyze the transferring an acyl group from acyl-CoA to the side chain amine to form ϵ -N-acyl-lysine.

Lysine deacylase (KDAC): an enzyme that removes acyl group from ϵ -N-acyl-lysine to form unmodified lysine.

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