An Expanding Repertoire of Protein Acylations

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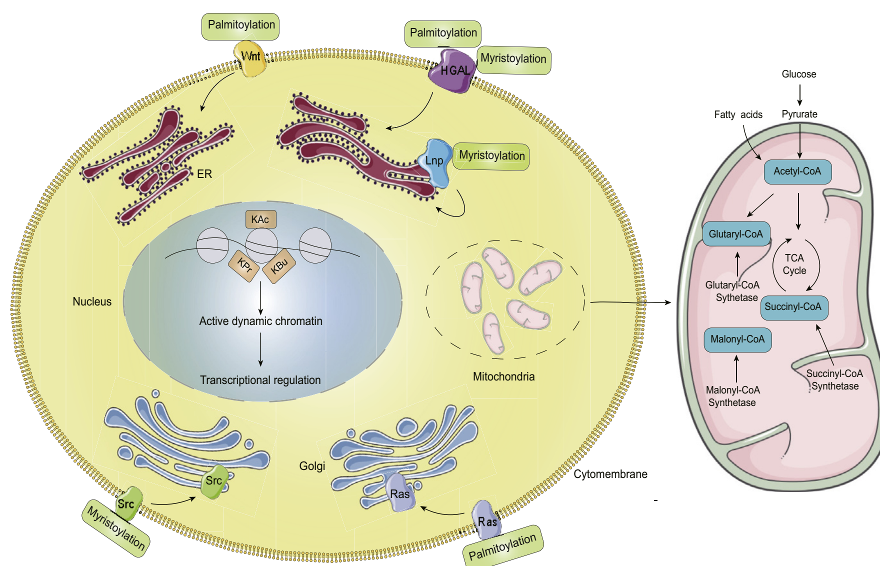
In Brief

In this work, we give a general overview of the 12 main protein acylations, also including novel acylations, such as benzoylation and 2-hydroxyisobutyrylation. We summarize the recent advances in protein acylation, mainly focus on their substrates, enzymes, biological functions, and novel detecting methods and related diseases, especially in cancer. We believe that the review will provide an unprecedented and comprehensive view of protein acylations and bring important reference significance for future research.

Graphical Abstract



Highlights

- Provide a general overview of the 12 main protein acylations.
- Acylation of viral proteins promotes viral integration and infection.
- Hyperacylation of histone has antitumous and neuroprotective effects.
- MS is widely used in the identification of acylation but has its challenges.

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An Expanding Repertoire of Protein Acylations

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Protein post-translational modifications play key roles in multiple cellular processes by allowing rapid reprogramming of individual protein functions. Acylation, one of the most important post-translational modifications, is involved in different physiological activities including cell differentiation and energy metabolism. In recent years, the progression in technologies, especially the antibodies against acylation and the highly sensitive and effective mass spectrometry–based proteomics, as well as optimized functional studies, greatly deepen our understanding of protein acylation. In this review, we give a general overview of the 12 main protein acylations (formylation, acetylation, propionylation, butyrylation, malonylation, succinylation, glutarylation, palmitoylation, myristoylation, benzoylation, crotonylation, and 2-hydroxyisobutyrylation), including their substrates (histones and nonhistone proteins), regulatory enzymes (writers, readers, and erasers), biological functions (transcriptional regulation, metabolic regulation, subcellular targeting, protein–membrane interactions, protein stability, and folding), and related diseases (cancer, diabetes, heart disease, neurodegenerative disease, and viral infection), to present a complete picture of protein acylations and highlight their functional significance in future research.

Protein post-translational modifications (PTMs) regulate fundamental and diverse biological functions in cells, such as cell growth, differentiation, and metabolism (1). With new techniques emerging, especially in the mass spectrometry (MS) field, as well as the discovery and commercialization of antibodies against modification sites of proteins, more than 400 kinds of PTMs of proteins have been uncovered (1, 2), which are involved in whole cellular life circle as a key regulatory mechanism and closely related with many diseases (3, 4).

Acylation is one of the most common cotranslational covalent modifications of proteins in eukaryotes, which play a crucial role in regulation of protein function. The earliest discovered acylation was histone lysine acetylation (Kac) identified as a positive transcriptional regulator (5, 6). In the past few years, proteomics analysis based on high-resolution MS has made a breakthrough in this field, enabling identification of thousands of acetylation sites from a single experiment, thus opening an era of acetylation research (7). A holistic analysis method of acetylome has been established to investigate the role of protein acetylation in biological processes. Kim *et al.* (8) discovered nearly 400 Kac sites in 200 proteins in 2006. Choudhary *et al.* (9) identified more than 3500 acetylated sites among approximately 1700 proteins, which was close to the phosphorylation level in living cells. Further studies also found that Kac has a fundamental impact on cell metabolism, cell growth, and cancer (10, 11). With the improving sensitivity and resolution of the MS, more and more types of acylation have been identified at lysine residues in recent years, including formylation, propionylation, butyrylation, malonylation, succinylation, glutarylation, benzoylation, crotonylation, and 2-hydroxyisobutyrylation (Khib), as well as palmitoylation and myristoylation occurred at cysteine residue and glycine residue, respectively (Fig. 1). As new acylations were discovered, the biological functions of these novel acylations have not been fully understood yet, and a lot of work on disclosing their roles in cellular regulation is still required. In this review, we focused on the 12 main protein acylations and discussed recent advances in substrates, regulatory enzymes, physiology and pathology, and their specific role in life processes.

SUBSTRATES OF ACYLATIONS

The major substrates of acylations are divided into two parts, histone and nonhistone (Table 1). Acylation in histone functions as active marks of chromatin, which can highly promote transcriptional output and affect cellular metabolism by chromatin structure and function alteration (12, 13). Acylation in nonhistone proteins, such as p53 and mitochondrial protein, can also regulate their biological function in different ways (14, 15).

Histone

Histone is a group of basic proteins present in eukaryotic chromatin that are evolutionarily conservative. Histone is usually divided into five types (H1, H2A, H2B, H3, and H4), and H3 and H4 form histone octamers, which are the core components of the nucleosome and account for half of the mass of the nucleosome. Many kinds of acylations have already been

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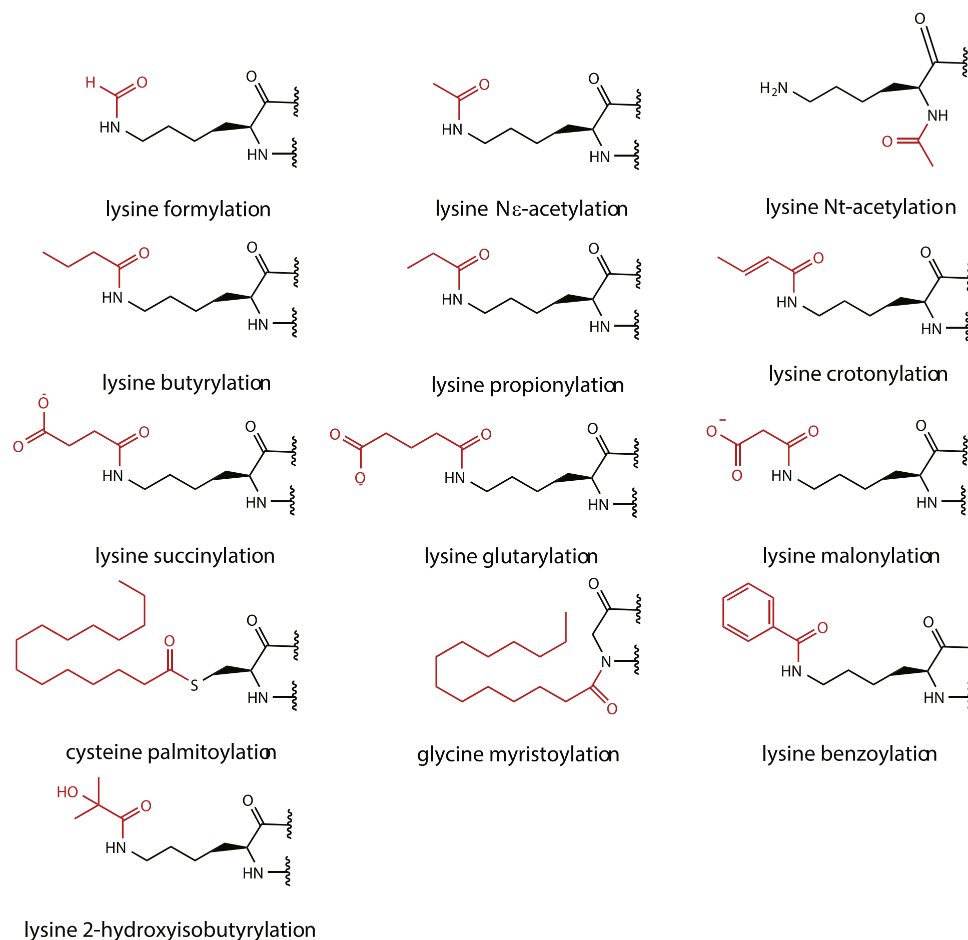

FIG. 1. **Structures of acylations that have been identified are showed.** The acylated parts are colored in *red*.

found on histone yet, especially in the lysine residues. In 1964, reversible Nε-acetylation (ε-amino Kac) of histone was discovered, and the research on histone acetylation has become more and more extensive over the past decades. In 2008, Wisniewski *et al.* (16) identified the formylation of histone H1 with a variance of 0.0364 in relative molecular weight through MS. Up to now, with the latest proteomic analysis technology, histone has been identified as the main target proteins, including H1, H2A, H2B, H3, H4, high mobility group chromosomal protein B1, high mobility group chromosomal protein N2, high mobility group A1, lamin A/C, and calgizzarin (16), modified by formylation usually at lysine residues. Among them, H1 is the most frequently formylated histone and also an important protein that widely regulates gene expression (17). Lysine propionylation and butyrylation were identified in H3 and H4 in 2007 by MS (18). In 2011, Zhao *et al.* (19) first identified histone lysine crotonylation (Kcr) in histone lysine, which is enriched at the transcriptional initiation site of specific genes in testis, indicating that histone crotonylation could be used as a marker for the active transcription of sex chromosome–related genes. After that in 2014, Khib was found as a widely distributed active histone marker and plays an important regulatory role in sperm cell differentiation (20). Recently, succinylation and glutarylation were also disclosed in histone lysine residues and correlated with active gene expression (21, 22). In 2018, benzoylation, a new acylation, was found as the only acylation containing benzene ring in histone lysine (23).

Nonhistone

Nonhistone is all proteins except histone that fundamentally support cell metabolism, signal transduction, and other cellular activities. Almost all the acylations can occur at nonhistones and are essential for regulation of cellular activity. Nonhistone ε-amino Kac was first found in lysine residue in 1985, almost 20 years later after the discovery of histone ε-amino Kac because of the technical limitations (24). After that, interestingly, p53 and HIV transcriptional regulatory protein Tat were reported to be regulated by acetylation (25). Similar to ε-amino Kac, N-terminal acetylation (Nt-acetylation) is also a common acylation estimated to affect approximately 80% of all eukaryotic proteins to a varying extent (26, 27). Nt-acetylation usually occurs in methionine, serine, alanine, threonine, glycine, and valine residues of histone and

TABLE 1
Major substrates and modified amino acid residues of acylations

Acylations	Substrates	Amino acid residues
Formylation	Histone	Lys
ε-amino acetylation	Histone, nonhistone	Lys
Nt-acetylation	80% of all eukaryotic proteins	Met, Ser, Gly, Ala, Thr, Cys, Val
Propionylation	Histone, nonhistone	Lys
Butyrylation	Histone	Lys
Malonylation	Nonhistone (mitochondrial proteins, fibroblast cytoplasm, and nucleus)	Lys
Succinylation	Nonhistone (mitochondrial proteins), histone	Lys
Glutarylation	Mitochondrial protein, histone	Lys
Palmitoylation	Nonhistone (signal transduction proteins)	Cys
Myristoylation	Nonhistone (signal transduction proteins)	Gly
Benzoylation	Histone	Lys
Crotonylation	Histone, nonhistone	Lys
2-hydroxyisobutyrylation	Histone, nonhistone	Lys

nonhistone (28, 29). Lysine propionylation was discovered in p53 and p300 in eukaryotic cells in 2009 (30). In addition, lysine malonylation (Kmal), succinylation, and glutarylation, which are all acidic modifications, tend to be located in mitochondrial proteins, and part of cytoplasm proteins are also found to be malonylated (31–33). Fatty modifications, including palmitoylation and myristoylation, mainly occur at methionine and glycine of signal transduction proteins, respectively, such as programmed cell death ligand 1 and Src tyrosine kinase (34–36). Another nonhistone acylation named nonhistone Kcr was first discovered in 2017 and proved to affect a variety of proteins (37). Recently, in 2018, Khib was found in glycolysis-related enzymes (38).

REGULATION ENZYMES FOR ACYLATION

Acylation is usually enzymatically regulated by the dynamic balance between writers and erasers (Table 2). Some individual acylations can also occur nonenzymatically, which is strictly dependent on the high acyl-CoA levels and an alkaline pH, and mitochondria is the dominant site for nonenzymatic acylation (39, 40). However, conditions in the nucleus are less favorable to this process (41). In bacteria, Kac can be nonenzymatically acetylated by acetyl phosphate and is known to play an important role in bacterial virulence (42).

Writers

Writers briefly indicate enzymes that catalyze the covalent modification of specific residues, mainly including lysine (K) acetyltransferase (KAT) family, N-terminal acetyltransferase (NAT) family, aspartate–histidine–histidine–cysteine (DHHC) family, and N-terminal myristoyltransferase (NMT) family.

KAT Family—Acetylated lysine residues were first discovered in histone, which is the reason that enzymes catalyzing lysine (K) acetylation were termed histone acetyltransferases (HATs) (43). However, Kac is not limited to histone, and these enzymes have been renamed to KATs (44). Previously characterized HATs were found to have strong acyltransferase activities that can catalyze a variety of acylations other than acetylation. There are three major families of HATs, including p300/cAMP response element–binding protein (CBP), general control nonderepressible 5 (Gcn5)-related *N*-acetyltransferases, MYST (MOZ/MOF, HBO1, and Tip60) (45, 46). Of the three families, p300/CBP is a well-studied enzyme family that has been known as the most promiscuous acyltransferase to date, exceeding its originally described acetyltransferase activity (47, 48). p300/CBP catalyzes histone lysine propionylation (Kpr) (12, 30), lysine butyrylation (Kbu) (18), Kcr (49), lysine glutarylation (Kglu), and Khib (33, 38). Kinetic analysis of the activities of Kac, Kpr, Kbu, and Kcr of p300 showed that the enzyme could catalyze these acylation reactions, and the reaction rate gradually slowed down with the extension of the acyl chain (50). In contrast, Gcn5-related *N*-acetyltransferase family GCN5 and P300/CBP-associated factor as well as MYST family MOZ and MOF can catalyze Kpr more actively (51–53), compared with the catalysis of Kac. GCN5 can also catalyze Kbu (50, 54), with a lower catalytic activity than that of Kpr. In addition, MOF, GCN5, and Tip60 can catalyze Kcr (55), lysine succinylation (Ksucc), and Khib (56, 57), respectively.

NAT Family—About 80 to 90% of proteins in human undergo post-translational acetylation at the N-terminal region of their new polypeptide chain, which is catalyzed in the cytoplasm by the NATs and then produces a neutral charge on the α-amino group (58). There are six NAT subtypes (NatA to NatF), and each of them has unique substrate specificity. Except for NatA and NatD, all others only have acetylation activities of initial methionine, whose catalytic specificity is determined by subsequent amino acids (59). NatD acetylates the N-terminal serine of histone H2A and H4 (60), whereas NatA acetylates serine, alanine, threonine, glycine, and valine at the N-terminal region (produced by initial methionine cleavage) where the subsequent amino acid is not proline (29).

TABLE 2
Summary of enzymes involved in protein acylations

Acylations	Writers		Erasers		Readers
	Family	Members	Family	Members	Members
ε-amino acetylation	KAT	GNAT: GCN5, PCAF CBP/p300 MYST: MOZ, MOF, HBO1, Tip60	KDAC	HDAC1–10 SIRT1–7	BRDs, YEATS, PHD
Formylation	NA	NA	NA	NA	NA
Nt-acetylation	NAT	NatA-F	NA	NA	NA
Propionylation	KAT	p300/CBP, PCAF, GCN5, MOF, HB01, MOZ	NA	NA	BRDs, YEATS
Butyrylation	KAT	p300/CBP, PCAF, GCN5	NA	NA	YEATS
Malonylation	NA	NA	KDAC	SIRT5	NA
Succinylation	NA	NA	KDAC	SIRT5, SIRT7	NA
Glutarylation	NA	NA	KDAC	SIRT5	NA
Palmitoylation	PAT	DHHC1–23	APT	APT1–2	NA
Myristoylation	NMT	NMT1–2	NA	NA	NA
Benzoylation	NA	NA	KDAC	SIRT2	NA
Crotonylation	KAT	P300/CBP, MOF	KDAC	HDAC1–3, SIRT1–3	YEATS, PHD
2-hydroxyisobutyrylation	KAT	P300/CBP, Tip60	KDAC	HDAC2–3	NA

Abbreviations: GNAT, Gcn5-related *N*-acetyltransferase; NA, not available.

DHHC Family—Palmitoylation involves the covalent binding of saturated palmitoyl acid containing 16 carbon atoms to cysteine to form an unstable thioester bond. Palmitoylation is mediated by the protein palmitoyltransferase belonging to DHHC family, which transfers palmitoyl to the palmitoylated protein (61, 62). Palmitoyltransferase is a multiplex transmembrane protein characterized by the presence of a DHHC domain, which has palmitoyltransferase activity and can add palmityl to the sulfhydryl on cysteine. There are 23 and 24 distinct palmitoyltransferases identified in mammals and plants, respectively (63), which mainly locate in the membrane of organelles, such as endoplasmic reticulum, golgi apparatus, and cell membrane (64).

NMT Family—Protein myristoylation at N-terminal glycine residues (N-myristoylation) as a type of lipid modifications of proteins (65) is widespread in eukaryotes. Two NMTs that have been identified with 77% similarity (66), NMT1 and NMT2, also belong to the GCN5 family of acetyltransferases (67).

Erasers

Erasers simply denote enzymes that remove PTMs of proteins, whose function is opposite to writers, mainly including lysine (K) deacetylase (KDAC) family and acyl protein thioesterase (APT) family.

KDAC Family—Unlike Nt-acetylation, which is considered irreversible, ε-amino Kac of lysine is reversible and strictly regulated. Several families of KDACs have antagonistic effects against KATs. KATs and KDACs have developed a balanced and complex relationship in many cellular functions. The NAD^+-dependent sirtuin (SIRT1–7) family and the Zn^{2+}-dependent histone deacetylase (HDAC1–10) family are two main families of KDACs (68). All seven SIRTs (SIRT1–7) were initially annotated as HDACs, whereas there has been increasing evidence confirming that SIRTs have a broader deacylation activity. Shortly after the first discovery of Kpr and Kbu, several SIRT enzymes, including SIRT1/2/3 (69, 70), were also proved to have lower depropionylase, debutyrylase, and decrotonylase activities in addition to their deacetylase activities. In addition, SIRT2 has histone lysine debenzoylase activity (23), SIRT5 has high desuccinylase (71), demalonylase, and deglutarylase activities (15, 33), but low deacetylase activity. SIRT7 showed no high activity (72) and was identified as a histone desuccinylase with function in the DNA damage reactions (73). However, the role of HDAC in histone deacylation is relatively unknown (74). Based on one study that profiled the activity of HDACs for substrates including Kcr and Ksucc, HDACs showed no activity for Ksucc, whereas only HDAC1–3 showed activity for Kcr (75) that significantly lower than deacetylase. Recently, de2-hydroxyisobutyrylase activity has been found in HDAC2/HDAC3 (20, 57). It is worth mentioning that HDAC inhibitors (HDACi) could inhibit the proliferation of tumor cells and induce cell differentiation or apoptosis by increasing the acetylation levels of histone in cells and have become a new hotspot of targeted tumor therapy. There are several HDACis, mainly including hydroxamic acid–based cyclic tetra/depsipeptides, aminobenzamide–based short-chain fatty acid–derived inhibitors, and hydrazide-based HDACis (76, 77).

APT Family—APTs are thioesterases of palmitoyl protein, which are necessary for deacylation of proteins modified by palmitoylation and can lead to the reversible palmitoylation at specific sites (61, 78). APT1 and APT2 have been identified so far (79); however, like DHHC, APT proteins may be more diverse than previously appreciated, and further researches are needed.

Readers

Readers refer to a kind of proteins that recognize the appropriate modification site of proteins, usually with a

conserved domain that could be recognized by the writer or eraser to catalyze the reaction, mainly including bromodomains, YEATS (Yaf9, ENL, AF9, Taf14, and Sas5), and double plant homeodomain (PHD) finger proteins.

Bromodomains—The discovery of acylations has led to the study of whether specific protein domains existed that can recognize their unique and diverse structures. Bromodomain is a typical "reader" of histone acetylation; however, a research showed it can also bind Kpr and Kbu, with lower affinity than that of Kac (80). Another study identifying non-acetylated acylation in the 49 bromodomains showed that the bromodomains could bind Kac and Kpr, but the binding to Kbu, Kcr, or acidic acylation was significantly reduced (81). Although bromodomains are known to bind to Kac, their ability to bind to nonacetylated acylation remains to be investigated.

YEATS—The AF9 YEATS domain was identified as a new type of histone acetylation reader in human in 2014 (82) and later corroborated by studies of Taf14 (83). YEATS domain that is highly conservative mainly exists in proteins associated with transcriptional regulation (84). Recent studies showed that YEATS has higher affinity for Kpr, Kbu, and Kcr than Kac, compared with the bromodomain. The cocrystal structures of AF9 YEATS domain with H3K9cr showed that Kcr was indeed located in the combination pocket of YEATS and was bound in a direction similar to Kac (85–87).

PHD—PHD and double plant homeodomain (double plant homeodomain finger 2 [DPF]) were identified to bind Kac in 2008 (88); however, PHD was initially found as a lysine methylation reader (89, 90). Five DPF domains, including MOZ and MORF (MYST family members), DPF1, DPF2, and DPF3, have been identified as Kac readers (91–93). A recent study showed that DPF domains also have stronger affinity for Kcr than Kac (94), which is because a distinct mechanism of hydrophobic encapsulation and coordinated hydrogen bonding (94).

BIOLOGICAL FUNCTION OF ACYLATIONS

Transcriptional Regulation

Acylation in histone usually acts as a marker of chromatin activity and promotes high transcriptional output (12, 13). The cell controls gene expression by regulating the acetylation of DNA-binding proteins, transcription factors, or transcription-related proteins. Histone Kac is closely related to transcriptional activity, and highly acetylated histone is specifically clustered in active chromatin areas. After the inhibition of deacetylase by trichostatin A, histone H4 Kac was accelerated, which resulted in increased production of tissue plasminogen activator in human umbilical vein endothelial cells (95). The use of MS and chromatin immunoprecipitation experiments also confirmed the existence of different histone acylations of regulatory elements related to transcriptional activity. Formylation can affect the assembly of nucleosomes by altering certain functions of histone and further affect gene expression through epigenetic mechanisms (96). The dynamic competing histone Kpr, Kbu, and Kac in promoter region can improve the transcriptional activity of genes (12, 97) (Fig. 2). A recent research found that an increase in acylation/acetylation ratio would loosen BRD4 from its chromatin binding sites and make BRD4 more mobile and usable for gene expression regulation, suggesting that acetylation and acylation alternately co-occur and should be considered as a ratio (98). Ksucc and Kglu of acidic acylations can also affect the acylation of histone and thus regulate chromatin activity (21, 22). Notably, Kcr and Khib are located in the transcription start sites of the sex chromosome–associated genes (19, 20, 49). Benzoylation also has localization in histone and dynamically modulating chromatin structures and functions (23). These studies showed that different acylations in histone are located at specific sites with different proportions, which regulate the transcriptional activity of related genes.

Metabolic Regulation

Early biological studies of Kac focused on nuclear proteins, such as histone and transcriptional factors, showing that acetylation played an important role in chromatin structure maintenance (46, 99). Despite in chromatin activity, Kac also plays a key role in regulating mitochondrial and other cellular metabolism (100, 101). Most cellular ATP comes from acetyl-coenzyme A (Ac-CoA), which can be produced in the following three ways: glycolysis, β-oxidation of fatty acids, and citric acid cycle, with the involvement of mitochondria. Ac-CoA synthetases are feedback regulated by reversible inhibitory acetylation. Most metabolism-related enzymes are located in mitochondria, where their activities can be modulated by acetylation, and indeed, proteins in mitochondria are widely acetylated (9, 102). Enzymes related to metabolic pathways also exist mainly in the form of acetylation, whose acetylation level increases with the increased Ac-CoA concentration. Enzyme activity can be increased or decreased by acetylation. The acetylation of malate dehydrogenase in citric acid cycle increased its metabolic activity (103), whereas the acetylation of carbamoyl phosphate synthetase I decreased its catalytic activity (104). Similar to Kac, studies have shown that some novel lysine acylations, including Kmal, Ksucc, and Kglu, are also important in regulating cell metabolism (Fig. 2). Malonyl-CoA, succinyl-CoA, and glutaryl-CoA, the substrates for the corresponding lysine acylation reactions, can be generated through corresponding synthetic reaction or citric acid cycle (105, 106). When lysine is modified with these three acidic modifications, its original positive charge is changed to negative charge, which may have a greater effect on protein structure and function than acetylation. Proteomic studies on Kmal, Ksucc, and Kglu have shown that, similar to Kac, there are enrichment of these three modifications in metabolism-

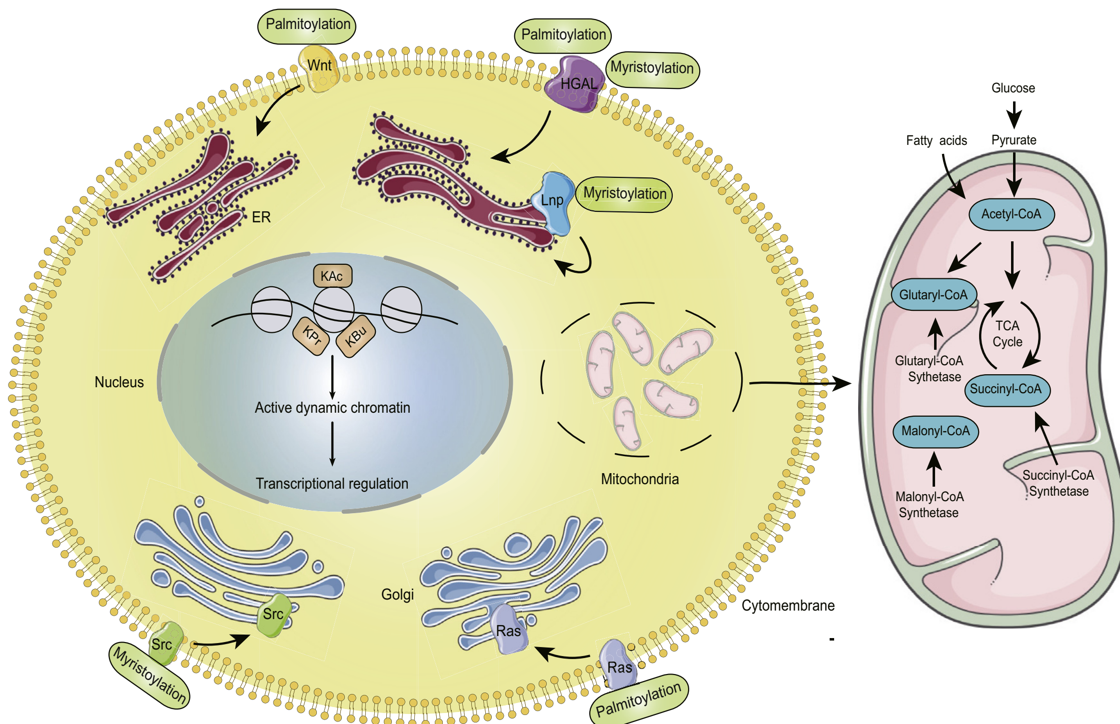

Fig. 2. **Functions of main acylations, including transcriptional regulation (acetylation, propionylation and butyrylation), metabolic regulation (acetylation, malonylation, succinylation and glutarylation), and protein–membrane interactions (palmitoylation and myristoylation)**.

related proteins. Gene Ontology analyses on the acylated proteins also demonstrated that the proteins were mostly enriched in metabolism-related pathways (33, 71). In recent years, researchers found that p300-mediated Khib could also regulate glycolysis (38).

Regulation for Subcellular Targeting and Protein–Membrane Interactions

Lipid modifications of proteins in eukaryotic cells, which occur at membranes, cytoplasm, and organelles, mainly including palmitoylation and myristoylation, are also important acylations (107) (Fig. 2). When proteins undergo lipid modification, their hydrophobicity can be increased, which is conducive to the reversible binding of proteins to membranes and plays an important role in regulating subcellular localization and protein–membrane interactions (108–110). Fluorescence microscopy tracking of palmitoylated proteins showed that the palmitoylated proteins were distributed throughout the cell and tended to flow from the Golgi apparatus to the plasma membrane rather than an equilibrium distribution of palmitoylated proteins at all membranes (111). Ras signaling, which is important in controlling a variety of biological functions including cell growth and differentiation, is also mediated by membrane association. It was found that the Ras signaling pathway is regulated by palmitoylation (112), which can increase the affinity for biofilms and is essential for intracellular transportation. Prior to the advent of large-scale proteomic analysis, myristoylation has been found in a few important proteins, such as Src kinase and 26S proteasome regulatory subunits (113). The myristoylated transmembrane protein Lunapark is involved in endoplasmic reticulum network formation, and morphological changes of endoplasmic reticulum would be inhibited when the Lunapark myristoylation motif was mutated (114).

Regulation for Protein Stability and Folding

Kac is also involved in the regulation of protein stability (115). The ribonuclease RNase R is essential for the survival of bacteria. The expression of RNase R was induced by various adversities, and acetylation could promote the binding of transfer-messenger RNA and small protein B complex, change the structure of RNase R, and lead to its degradation by protease (116). Under adverse conditions, the acetylation level of RNase R was reduced, and the stability was maintained. Parkinsonism and Alzheimer's disease (AD) are characterized by hyperphosphorylation of Tau protein, and lowering Tau levels can improve patients' symptoms. Lysine Nt-acetylation catalyzed by NAT family also plays a similar role in the regulation of protein stability and protein folding. Nt-acetylation catalyzed by NatA promotes protein degradation (29), whereas that catalyzed by NatE regulates protein folding (117, 118). In addition, Nt-acetylation catalyzed by NatB, NatC, and NatD is also important in protein interaction (119), cytosolic sorting, and membrane interaction, respectively (117).

ASBMB

Connection Between Acylations and Other PTMs

PTM is an important mechanism in regulating protein function. Acylations can interact and coordinate with other PTMs to regulate the entire cellular signaling pathway. Formylation, acetylation, and methylation are common types of PTMs of histone and nucleoproteins, usually occurring at lysine residues. Studies have shown that these modifications may compete with each other for these modification sites (120). Ubiquitination is a widespread PTM of proteins, which is involved in protein stability and regulation of various protein functions. Acetylation is closely related to ubiquitination, and there is mutual regulation between them. For example, acetylation of splicing factor serine/arginine-rich splicing factor 5 antagonized its ubiquitination and promoted glucose metabolism by selective splicing of cell division cycle and apoptosis regulator 1S protein (121). Beyond ubiquitination, protein acetylation could also intercoordinate with its phosphorylation in the heart, and HDACi regulates acetylation–phosphorylation crossovers to regulate cardiac function. HDACi inhibits the expression of phosphatase genes under stress conditions, leading to dephosphorylation of extracellular signal–regulated kinases 1/2 and reducing stress cardiac hypertrophy (122). Kac, Kpr, and Kbu could regulate the transcription of related genes together (12, 97). In metabolic regulation, Kac in mitochondrial proteins interacts with Ksucc to jointly regulate cell metabolism (123). In addition, Ksucc, Kmal, and Kglu could also interact with each other. Comparing the lysine glutarylated and succinylated sites in mouse liver showed that about 67% of the modification sites are overlapped. In the mitochondria of mouse liver regulated by SIRT, the researchers found that 56% of the malonylated sites overlapped with the succinylated sites (15, 33). Two lipid modifications, palmitoylation and myristoylation, which are related to membrane binding, also coordinate to provide stable membrane anchoring for protein transportation and subcellular localization in cells (36, 124).

PATHOLOGICAL MARKERS AND DISEASES

Cancer

Studies have elucidated that Kac, Kbu, Ksucc, palmitoylation, and myristylation, with different mechanisms, all have important effects on the tumorigenesis and development of cancer. Several KDACs had been proved abnormally expressed in different tumor tissues, which was a typical characteristic of cancer cells (125). Immunohistochemical studies of multiple cancer tissues showed a link between low levels of histone acetylation and tumor malignancy, and lower levels of acetylation associated with poorer clinical prognosis, including increased risk of tumor recurrence and reduced survival (126), which may be caused by abnormal expression of genes related to cell growth and differentiation. HDACs that have been proved to play a crucial role in the development of breast cancer could serve as potential targets for breast cancer therapy. Multivariate analysis demonstrated that HDAC-1 is an independent prognostic marker, thus HDAC-1 expression analysis might be clinically useful to facilitate an individual, risk-directed, and adjuvant systemic therapy in breast cancer patients (127). RUNX3/p300/HDAC5/miR-125a-5p loop network that modulates HDAC5 levels was identified when HDACis were used to treat breast cancer cells. HDACi influences tumorigenesis and apoptosis *via* downregulation of miR-125a-5p expression, providing clinical implications in breast cancer chemotherapy (128). Pretreatment of breast cancer with HDAC6i induced critical changes in the tumor microenvironment, resulting in improved effectiveness of immune checkpoint blockade and preventing dissemination of cancer cells to secondary niches by noncanonical mechanisms that are unrelated to the previously cytotoxic properties attributed to HDACi (129). In addition, the expression of fructose-1,6 diphosphatase 1 (FBP1) in the tumor tissues of hepatocellular carcinoma (HCC) patients was downregulated, which was related to the poor prognosis of HCC patients. After using HDACi, the expression of FBP1 could be restored, and the proliferation of HCC cells could be inhibited. It is concluded that HDAC-mediated inhibition of FBP1 expression was associated with reduced acetylation (H3K27Ac) of histone H3 lysine in FBP1 enhancers (130). A comparative analysis of histone markers in two types of esophageal squamous cell carcinoma was performed using MS. The comprehensive spectrum of histones H3 and H4, including lysine methylation, acetylation, and butyrylation, was obtained, suggesting the synergistic effects of multiple modifications in tumor development (131). Palmitoylation and myristoylation play roles in the regulation of signaling pathways, such as Ras and Src signaling pathways, which are closely related to tumor progression. Palmitoylation of Ras at the Golgi ensures the delivery of Ras to the plasma membrane (132). *In vivo*, Ras-driven leukemia was dependent on its own palmitoylation. Mice transplanted with nonpalmitoylated-activated Ras protein could survive for 2 years, whereas those transplanted with bone marrow cells expressing palmitoylated-activated Ras protein died of leukemia within 3 months (133). Src kinase family is a driver gene for a variety of cancers including prostate cancer, and Src kinase activity can be regulated by myristoylation. Studies have shown that the expression of NMT in prostate cancer was positively correlated with Src kinase activity (134). It is known that Ksucc is associated with a variety of cancer, such as liver cancer, colon cancer, head and neck cancer, and breast cancer, and SIRT5 has been shown to exhibit tumor-suppressing activity as well as tumor promoter functions under different cellular conditions. SIRT5 expression was significantly decreased in endometrial carcinoma and head and neck squamous cell carcinoma, suggesting that the increased level of Ksucc could promote the occurrence of these tumors. On the contrary, elevated SIRT5 expression was detected in nonsmall cell lung cancer, breast

cancer, HCC, and colon cancer, suggesting that Ksucc may inhibit the development and progression of these tumors (135).

Diabetes

Kac, Kmal, Ksucc, and Kglu may be involved in the development of diabetes by regulating blood glucose and the activity of metabolic enzymes or the expression of related genes. Once insulin binds to the insulin receptor substrate 1 (IRS-1), which is phosphorylated and then activates PI3K and AKT, ultimately activating glucose transporter 4 that moved from intracellular vesicles to the plasma membrane and promoted glucose absorption. Several studies have shown that KDACs could regulate insulin signaling. HDAC2 could bind to IRS-1, reducing acetylation and insulin receptor–mediated tyrosine phosphorylation of IRS-1, and elevating blood glucose (136). In addition, nine succinylation, two benzoylation, and seven malonylation sites were identified in diabetic mice (137), and glutarylation of mitochondrial proteins significantly increased in the glutarate mouse model (33). Compared with wildtype mice, 573 malonylation sites were identified among 268 proteins in liver tissue of diabetic mice, and enrichment analysis showed that proteins modified by malonylation were mainly related to metabolism (138).

Heart Disease

Increased utilization of fatty acids by the heart is a typical characteristic of obesity and diabetes, and the associated increase in circulating free fatty acids and triglycerides can promote lipid accumulation and dysfunction of cardiac myocytes. The acetylation levels of mitochondrial fatty acid oxidase and pyruvate oxidase in the heart of mice were significantly increased after feeding a long-term high-fat diet, which was related to the increase of the abundance of mitochondrial GCN5-like protein 1. Knockdown of GCN5-like protein 1 in H9C2 cells could reduce the acetylation level of mitochondrial fatty acid oxidase and subsequently reduced the intracellular fatty acid oxidation (139). Moreover, Ksucc plays an important role in cardiac physiology, regulating a variety of cardiac energy metabolism (135). Succinyl-CoA is the most abundant acyl-CoA molecule in the heart. When SIRT5 was absent, succinyl-modified lysine accumulated in the heart, as a result, cardiac weight increased abnormally and the ejection fraction decreased, leading to the occurrence of hypertrophic cardiomyopathy (140), suggesting that Ksucc may promote the occurrence of hypertrophic cardiomyopathy. When myocardial ischemia occurred, the significant downregulation of SIRT5 exacerbated heart ischemia-reperfusion injury (141), revealing that Ksucc is a risk factor for heart ischemia-reperfusion injury.

Neurodegenerative Disease

In neurological diseases, Parkinsonism and AD are characterized by hyperphosphorylation of Tau protein, and lowering Tau levels can reduce patients' symptoms. Klein *et al.* (142) found that P300 and SIRT1 could regulate the acetylation of Tau, and deacetylation and phosphorylation of Tau could be promoted and reduced respectively through inhibiting P300 to promote the degradation of Tau. Histone acetylation is greatly reduced in neurodegenerative disease. Increased HDAC2 expression and reduced histone Kac have also been associated with cognitive decline in typical AD (143). Therefore, hyperacetylation of histone produced by inhibition of HDACs has neuroprotective effects, showing a great potential target for neurodegenerative disease treatment. The pathophysiology of Huntington's disease is closely related to deficiency of brain-derived neurotrophic factor and heat shock protein 70 in brain, whose expression could be promoted by HDACi *via* acetylation (144, 145). A study in AD models showed chronic treatment of TG2576 mice with CM-695 (a compound that selectively inhibits HDAC6) led to increase in heat shock protein 70 and glucose-regulated protein 78, bringing therapeutic benefits in AD models (146). The degeneration of dopamine neurons in Parkinson's patients with SIRT5 deletion was more severe (147), and more sensitive to paraquat-induced dopaminergic degeneration in the substantia nigra striatum (148), indicating that Ksucc may be a risk factor for Parkinson's disease. The succinyltransferase α-ketoglutarate dehydrogenase complex was significantly decreased in the mitochondria of AD patients (149), and SIRT5 expression was upregulated when the disease developed (150), suggesting that Ksucc may be a protective factor for AD. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of H1299 cell line showed that nonhistone Kcr was involved in multiple metabolic and Parkinson's disease signaling pathways (151).

Viral Infection

Acylations of proteins are also important for viral infection. Replication analysis of HIV-1 clones carrying substitutions at the HIV-1 integrase lysines (K264, K266, and K273) acetylated by both GCN5 and p300 demonstrated that these residues are required for efficient viral integration (152). Acetylation of K28 of HIV Tat by P300/CBP-associated factor enhances the ability of Tat to recruit the positive transcription elongation factor b complex, whereas modification of K50 by p300/CBP and GCN5 leads to Tat dissociation from transactivation response RNA, which results in an increased transactivation activity on the HIV long terminal repeat promoter and promoting viral integration (152, 153). On the other hand, the host could also modify its proteins to enhance the antiviral effect. For example, virus infection induces p53 acetylation at K379, which is absolutely required for p53-mediated control of virus replication, and for p53-dependent transcriptional transactivation of both proapoptotic and interferon-stimulated genes induced by virus infection (154). Except for acetylation, palmitoylation and myristoylation of virus proteins could

also contribute to the virus growth by enhancing virulence, infectivity, cell fusion, viral protein localization, and virion assembly during the infection (155, 156).

Other Diseases

Acylations also play a role in other diseases, such as liver disease, kidney disease, spermatogenesis, though studies about these diseases are relatively rare. Cheng *et al.* (157) found that Ksucc was enriched in mitochondrial proteins of nonalcoholic fatty liver of mouse, and Kcr levels were higher in mice with acute kidney injury induced by folic acid or cisplatin than that in controls (158). Furthermore, Kcr has also been found involved in sperm formation and differentiation as well as Khip (20, 56).

METHODOLOGIES FOR ACYLATION

MS

MS, based on the determination of the mass of protein molecules and the PTM of protein groups, is powerful in the research on protein identification, protein PTM, and protein interaction. In recent years, protein histochemistry based on LC–MS has developed rapidly, providing a powerful research tool for PTM of proteins. PTM analysis of proteins is generally divided into the following four steps: sample preparation, preseparation and enrichment of modified polypeptide samples, LC–MS analysis, and bioinformatics analysis (Fig. 3).

Sample Preparation—Sample complex needs to be reduced in order to enhance the yield of affinity enrichment before PTM peptides are enriched. Protein lysates are first prepared from cultured cells or tissues and then digested by proteolysis. In some cases, organelles or protein complexes are required before hydrolysis and digestion by using organelle separation, electrophoresis, or multidimensional chromatography (159).

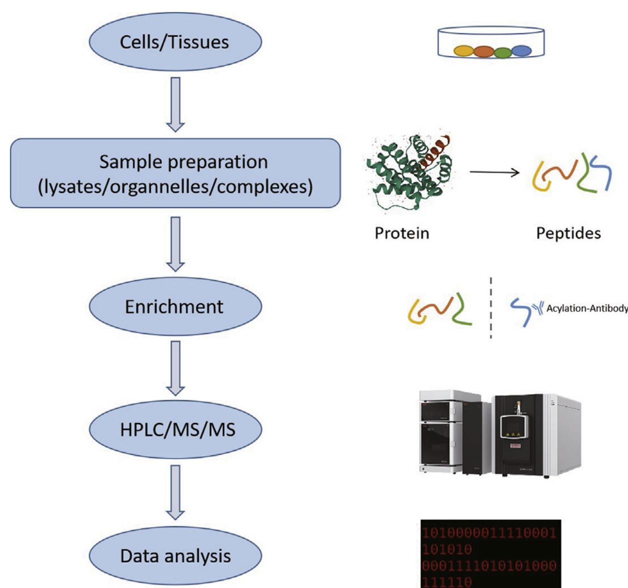

Fig. 3. **The MS workflows for PTM proteomics.** MS, mass spectrometry; PTM, post-translational modification.

Enrichment Technology—Except for palmitoylation, myristoylation, and acetylation, almost all other acylations are hydrophilic. In principle, hydrophilic antibody can be used for specific enrichment, which is easy to operate. Yingming Zhao's team at the University of Chicago first developed an antibody against acetylated proteins, the first systematic level analysis of protein acetylation sites, and identified a total of 388 acetylation sites (8). Mann *et al.* (9) used the same antibody and combined with advanced orbital mass spectrometer to achieve the largest identification of acetylation sites and identified about 3600 Kac sites from human acute myeloid leukemia organelles. The high efficiency of specific antibodies in the enrichment of low-abundance modified polypeptides and the importance of advanced MS for large-scale proteomics analysis are fully demonstrated. In addition, highly specific propionylated antibody, butyrylated antibody, malonylated antibody, succinylated antibody, glutarylated antibody, benzoylated antibody, and 2-hydroxyisobutyrylated antibody have all been developed. However, the two lipid modifications, palmitoylation and myristoylation, are hydrophobic and lack enrichment antibodies with high affinity. Moreover, they usually occur at proteins with low and medium abundance, which will be affected by nonlipid-modified proteins/peptides during MS analysis, resulting in severe signal inhibition. In order to overcome the shortcomings of existing enrichment methods, Lu's team at Fudan University reported the selective enrichment of palmitoylated modified proteome by magnetic nanoparticles (Fe_3O_4/SiO_2–SSpy microspheres) based on the functionalization of dithiodipyridine for the first time (160).

Multidimensional Liquid Chromatography—Protein histochemical samples are extremely complex, so it is necessary to isolate them efficiently to improve the efficiency of MS identification. In terms of acylation, effective preseparation of enzymatic peptides is often required before or after selective enrichment of modified peptides. HPLC is a standardized separation method in proteomics strategies, mainly because of its extremely high capacity of enzymatic hydrolysis of polypeptides and its good on-line combination with mass spectrometers.

Biomass Spectrum Identification—MS data analysis might be the most challenging aspect of the MS workflows, mainly needs to accomplish the following tasks: (1) identification of the modified proteins; (2) identify the modified amino acid sites and the chemical structure of the modified groups; and (3) quantitative analysis of the proportion of modified proteins in the total protein expression. Protein sequence alignment is performed on the MS–MS dataset generated by HPLC/MS–MS analysis of the enriched PTM peptides to determine the PTM peptide sequence and PTM site for quantitative analysis. Despite previous efforts to improve the accuracy of protein sequence database searching, the false identification of PTM

peptides and their sites continues to be a problem, especially in the context of large-scale PTM analysis, or when multiple PTMs are included in the protein sequence database search (159). To reduce the false discovery rate, PTM sites should be precisely mapped, and all the major peaks in MS–MS spectra of PTM peptides should be assigned; several bioinformatics tools are developed to perform this process, such as Mascot and SEQUEST (161). Moreover, manual verification is necessary to precisely locate the PTM sites with high confidence. However, most large-scale proteomics reports of PTMs, although providing information about the computational constraints applied to data interpretation, do not manually validate spectral interpretation (159, 161).

Click Chemistry

The specific reaction of azide alkynyl catalyzed by univalent copper is called "click chemistry," which can realize simple and efficient connection between specific small molecular groups at the molecular level and has been widely used in proteomics analysis, including the identification of newly generated proteins and the identification of various post-translational modified proteins (162). Acetylene and azide fatty acid probes are usually effective in labeling adipoylated proteins in cells. Cell proteins are added with the metabolin of palmitic acid or myristic acid analogies containing alkynyl groups, biotin with azide groups is then chemically attached to proteins labeled with acetylene probes, at last, agar gel is used to enrich and identify the palmitoylated and myristoylated proteins.

CONCLUSION

With the development of proteomics technologies, more and more acylations have been confirmed, expanding the original list of acylations. Similarities on the substrates, catalytic enzymes, biological function, and related disease among acylations were disclosed through studies. For example, histone can be regulated by a variety of acylations, including acetylation, propionylation, and butyrylation. SIRT5 can catalyze the occurrence of kinds of acylations, such as acetylation, succinylation, and malonylation. However, some acylations, such as palmitoylation and myristoylation, have been identified with specific catalytic enzymes, substrates, and biological functions. In addition, acylations can also interact with other PTMs to regulate protein function, which remains to be further explored.

Most of the early work of proteomics focused on the changes of protein expression level under the influence of different factors upon cells. However, many important life processes are not only controlled by the relative abundance of proteins but also by the specific distribution of PTMs of proteins. The studies on PTM of proteins provide a new perspective for the determination of protein structure, modification processing, transport location, and protein interaction. Acylation, as an important part of PTM, plays a crucial role in regulation of protein function. In recent years, the continuous optimization and development of the technologies on affinity enrichment, multidimensional separation, and bioMS provide an important opportunity for acylation omics, and identification of new acylations has especially been the hot spot of the protein PTM-omics. In this review, we summarize the characteristics of diverse acylations and their special effects on proteins. However, some novel acylations still remain unclear, such as benzoylation and Khib, and require further studies on their biological functions. The previous findings in this area, as well as future studies, could greatly deepen our understanding on the complexity of protein regulation and PTM.

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Abbreviations—The abbreviations used are: Ac-CoA, acetyl-coenzyme A; AD, Alzheimer's disease; APT, acyl protein thioesterase; CBP, cAMP response element–binding protein; DHHC, aspartate–histidine–histidine–cysteine; DPF, double plant homeodomain finger 2; FBP1, fructose-1,6 diphosphatase 1; Gcn5, general control nonderepressible 5; HAT, histone acetyltransferase; HDACi, HDAC inhibitor; IRS-1, insulin receptor substrate 1; Kac, lysine acetylation; KAT, lysine (K) acetyltransferase; Kbu, lysine butyrylation; Kcr, lysine crotonylation; KDAC, lysine (K) deacetylase; Kglu, lysine glutarylation; Khib, lysine 2-hydroxyisobutyrylation; Kmal, lysine malonylation; Kpr, histone lysine propionylation; Ksucc, lysine succinylation; MS, mass spectrometry; MYST, MOZ/MOF, HBO1, and Tip60; Nε-acetylation, ε-amino acetylation; NAT, N-terminal acetyltransferase; NMT, N-terminal myristoyltransferase; Nt-acetylation, N-terminal acetylation; PHD, plant homeodomain; PTM, post-translational modification; SIRT, sirtuin; YEATS, Yaf9, ENL, AF9, Taf14, and Sas5.

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