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Protein lipoylation: An evolutionarily conserved metabolic regulator of health and disease

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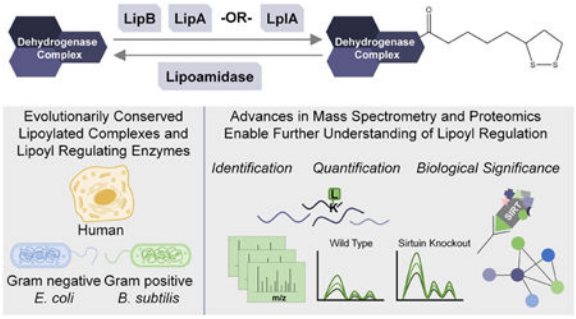
Abstract

Lipoylation is a rare, but highly conserved lysine posttranslational modification. To date, it is known to occur on only four multimeric metabolic enzymes in mammals, yet these proteins are staples in the core metabolic landscape. The dysregulation of these mitochondrial proteins is linked to a range of human metabolic disorders. Perhaps most striking is that lipoylation itself, the proteins that add or remove the modification, as well as the proteins it decorates are all evolutionarily conserved from bacteria to humans, highlighting the importance of this essential cofactor. Here, we discuss the biological significance of protein lipoylation, the importance of understanding its regulation in health and disease states, and the advances in mass spectrometry-based proteomic technologies that can aid these studies.

Graphical abstract



Introduction

Lipoamide is a cofactor central to cellular metabolism [1,2]. Present as a lysine posttranslational modification (PTM) on essential multimeric metabolic complexes, this

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functional group is required for the enzymatic activities of these protein complexes [3,4]. For example, the pyruvate dehydrogenase (PDH) and alpha-ketoglutarate (KDH) complexes regulate distinct carbon entry points into the central metabolic pathway of the TCA cycle. On both complexes, lipoylation is critical for proper enzyme function, and removal of this modification is part of a cellular mechanism to inhibit their activities. The evolutionary conservation of these lipoylated metabolic enzymes in organisms ranging from bacteria to mammals [2,5,6] underlines the critical role of lipoylation in core metabolic pathways. This theme of conservation is seen across the lipoylated complexes, the proteins that add or remove this modification, as well as the function of lipoylation [4-8]. Given this striking evolutionary conservation for this rare modification, it is perhaps not surprising that these lipoylated enzymes are critically linked to maintenance of health and development of disease. PDH dysregulation is known to contribute to numerous human metabolic disorders, cancer, viral infection, and Alzheimer's disease [9-13]. Therefore, advancing the current understanding of the regulation of lipoylation is necessary for defining the underlying molecular causes of these diseases. The low frequency and unique physical characteristics of lipoylation may also offer a therapeutic target for regulating metabolic activities that are disrupted in disease states. Here, we review the function and regulation of protein lipoylation, the importance of understanding its dysregulation, the gap in the knowledge regarding these regulatory mechanisms, and the advanced technologies that can aid these studies. Recent developments in proteomics, such as improvements in quantitative mass spectrometry and ion mobility, promise to provide new ways to investigate lipoylation in different cell types, tissues, and biological contexts.

Biochemical Structure and Function

Lipoylation is a posttranslational modification that involves the covalent attachment of lipoamide to a lysine residue via an amide bond [1,2,5,14-16]. The lipoamide cofactor is an eight-carbon organosulfurous molecule, with C6 and C8 attached to sulfur atoms in a pentatomic ring (Fig 1A). Lipoic acid can have two enantiomeric forms, although only the R(+) form is reactive and produced endogenously [17,18]. Given the large size of lipoyl, for example greater in mass than acetylation or phosphorylation, this modification has the ability to both impact protein structure and provide a “swinging arm” function for enzymatic reactions [19]. The rotational flexibility of this functional group allows it to move between different subunits within the enzyme complex [3,4]. This function facilitates substrate channeling and electron transfer during oxidation-reduction reactions. It has been shown to catalyze reactions including hydrogen transfers, decarboxylation and other acyl group transfers [14,15].

Unlike other posttranslational modifications that are dependent upon local amino acid motifs, substrate lipoylation does not seem to be significantly impacted by mutations of conserved amino acids flanking the modified lysine [20]. Instead, lipoylated domains seem to be better defined by their three-dimensional structure, consisting of around 80 amino acids folded into two anti-parallel beta-sheets that form a flattened beta barrel [21].

Metabolic Roles of Lipoylated Complexes

To date, only four lipoylated protein complexes have been identified in mammals and a fifth in bacteria [4,6] (Fig 1). Despite this rare occurrence, the modified metabolic enzymatic complexes act as linchpins in maintaining proper mitochondrial function. One of the best understood complexes is the PDH, which regulates an important step in glycolysis, the conversion of pyruvate into acetyl-coA prior to its entry in the TCA cycle (Fig 1B). This reaction is a primary entry point for carbohydrates into the citric acid cycle, connecting the anaerobic process of glycolysis to the higher-yield oxidative production of ATP. In mammals, PDH has two lipoylated subunits, with the E2 subunit (DLAT) possessing two lipoylated lysine residues and the binding protein (PDHX) possessing one. A single PDH complex has a core composed of 60 DLAT molecules non-covalently bound to an outer shell formed of PDHX, E1 and E3 subunits [3]. In bacteria, PDH has only the E1, E2 and E3 subunits, and some structural variations exist between types of bacteria. Gram positive bacterial PDH generally contains an icosahedral core of 60 DLAT subunits, each with one lipoylation site. Gram negative bacterial PDH generally contains an octahedral core of 24 DLAT subunits, each with two to three lipoylation sites [4]. In both bacteria and eukaryotes, the lipoyl swinging arms on the PDH E2 core interact with E1 and E3 subunits on the PDH exterior to catalyze the decarboxylation of pyruvate and acyl activation of coenzyme A [1,4,16]. It has long been established that a regulatory mechanism of PDH activity is provided by the phosphorylation of its E1 subunit by the pyruvate dehydrogenase kinase (PDK), which inhibits its activity [22]. This knowledge was more recently expanded by the finding that inhibition via delipoylation provides another point of regulation [7]. A second lipoylated complex with activity related to PDH is the acetoin dehydrogenase complex (AoDH); like PDH, AoDH also produces acetyl-CoA. AoDH is only found in bacteria, and is responsible for the catabolism of the acetoin energy storage molecule into acetyl-CoA and acetylaldehyde [23,24].

In addition to PDH, the lipoylated enzyme KDH regulates the incorporation of an alternative carbon source to glucose into the TCA cycle, catalyzing the decarboxylation of alpha-ketoglutarate to form succinyl-CoA [25]. Similar to PDH, removal of lipoylation from KDH has been shown to inhibit its enzymatic activity in bacteria [8].

A third conserved lipoylated complex in mammals is the branched-chain alpha-ketoacid dehydrogenase complex (BCKDH). Similar in structure to both PDH and KDH, it contains a core of either 24 or 60 E2 lipoylated subunits surrounded by E1 and E3 subunits [26,27]. BCKDH is responsible for the irreversible decarboxylation step in branched-chain amino acid catabolism, and it is known to be inhibited by phosphorylation of its E1 subunit [28]. The impact of delipoylation on its activity remains to be determined.

The fourth known lipoylated enzyme complex in mammals is the glycine cleavage system (GCV), responsible for the reversible decarboxylation of glycine in both bacteria and eukaryotes. It is composed of four subunits, including a lipoylated H protein [4,6]. While it does not produce a direct input into the TCA cycle, this mitochondrial enzyme plays a critical role in glycine catabolism, and can also act in serine catabolism [29]. Together, these

multimeric lipoylated enzymes regulate core cellular metabolic pathways, making the regulation of their functions via lipoylation critical for mitochondrial health.

Synthesis and Regulation

The addition of lipoyl modifications to substrates has been best characterized in *Escherichia coli*, and shown to occur via two independent pathways [5,6,30] (Fig 1A). The first pathway involves the scavenging and incorporation of exogenous lipoic acid onto proteins through a series of reactions catalyzed by the protein LplA. In these reactions, free lipoic acid is activated by ATP to form lipoyl-AMP, which is then transferred to a lysine side chain [21,31]. An independent pathway entails the biosynthesis of lipoic acid from the eight-carbon fatty acid octanoic acid, donated by the lipoyl/octanoyl-acyl carrier protein [32]. Through this mechanism, the enzyme LipB catalyzes the transfer of octanoic acid to the protein lipoyl domain. Two sulfur atoms are then inserted at C6 and C8 by the enzyme LipA to form a complete lipoamide prosthetic group [33,34].

Eukaryotes, including mammals, possess mitochondrial homologues of LplA, LipA and LipB [35]. Although initially presumed to rely primarily on environmentally-obtained lipoic acid, several studies have demonstrated the essential role of *de novo* lipoic acid synthesis in mammals [36,37]. In addition, lipoylated PDH is conserved in the plastids of plants and fungi [38], raising questions as to whether lipoylated products are produced and then exported from the mitochondria or whether plant and fungi cells might possess multiple sites of lipoylation. The presence of plastid LipA and LipB, but not LplA, homologues suggests that the plastid is another site of *de novo* lipoic acid synthesis [39,40].

While lipoic acid synthesis and protein lipoylation have been examined in multiple kingdoms of life, until recent years only one protein was known as a lipoamidase, i.e., acting to remove lipoyl modifications. This was the protein Lpa in *Enteroccocus faecalis* [41-43]. However, recent studies have highlighted a different family of enzymes as critical regulators of this modification, establishing sirtuins as evolutionary-conserved cellular lipoamidases. This was first discovered for the mitochondrial sirtuin 4 (SIRT4) in mammals [7], and more recently for the sirtuin homologue CobB in *Escherichia coli* [8] (Fig 1B). Suggesting the conservation of this sirtuin function among both Gram positive and negative bacteria, the sirtuin SrtN in *Bacillus subtilus* was also shown as a promising candidate for lipoamidase activity [8] (Fig 1B). The sirtuin-mediated process of lipoyl removal inhibits the activities of PDH and KDH, indicating this is an ancient mechanism of metabolic regulation [7,8].

Role of Lipoylated Complexes in Disease States

Free lipoamide, known as lipoic acid, has received much attention as an oral supplement used to counteract oxidative stress [44,45]. However, perhaps equally important is the role of lipoamide as an enzymatic cofactor. Given their importance in cellular metabolism, lipoylated mitochondrial enzymes, especially PDH, have been implicated in major human diseases. A notable example is the role of PDH in the Warburg Effect, a metabolic profile common in cancer cells in which cells derive most of their energy from the glycolytic process rather than fully oxidizing carbohydrates into carbon dioxide [46,47]. Not

surprisingly, PDH activity in cells displaying the Warburg effect is abnormally low, a phenomenon that has been correlated with increased PDK activity [48,49]. The link between PDH inhibition and cancer has been further strengthened by the observation that the tumor suppressor gene p53 down-regulates PDK transcription [50], and that PDH phosphorylation by distinct tyrosine kinases promotes the Warburg effect [51]. The knowledge that both phosphorylation and lipoylation regulate PDH function raises questions that remain to be answered regarding PTM crosstalk. Characterizing PDH lipoylation status in cancer cells will further the understanding of PDH regulation and may provide additional targets for cancer therapeutics. Indeed, low SIRT4 expression has been observed in certain forms of cancer, already suggesting lipoylation status may act as a disease indicator [12,13].

Seemingly paradoxically, another metabolic characteristic of cancer cells is increased flux through the TCA cycle, with glutamine-derived intermediates being channeled into biosynthesis of fatty acids and other cellular proliferative materials [52,53]. Some cancers have demonstrated increased expression of TCA cycle enzymes, including KDH [54,55], although a causative link has not been yet established. Characterizing the levels and roles of lipoylation in disease states is expected to help define the underlying mechanisms, as well as indicate whether this modification can be leveraged in disease treatment strategies.

Metabolic trends similar to those observed in cancer cells have also been noted in cells undergoing viral infection. Human cells infected with the widely-spread herpesvirus human cytomegalovirus demonstrate increased flux through glycolysis and an anaplerotic influx of carbon into the TCA cycle via the conversion of glutamate to alpha-ketoglutarate [10,56]. HIV infection demonstrated increased lipid biosynthesis [57], and lipoic acid was found to be an inhibitor of HIV replication [58]. Furthermore, antiviral properties have been observed for the sirtuin lipoamidases SIRT4 in mammals and CobB in *E. coli* [59], perhaps indicating that free lipoic acid contributes towards a mechanism of host defense, while lipoylated complexes may have pro-viral effects. Taken together, these studies underline the importance of lipoamide regulation and its impact on metabolic regulation in disease states.

Detection and Quantification of Lipoylation

As the understanding of the critical contribution of lipoylation in cellular metabolism and metabolic disorders has grown, so has the interest in designing methods for detecting the protein modification status and quantifying site-specific lipoylation. Traditional molecular biology and biochemistry techniques, such as western blotting using antibody against lipoic acid, protein purification, nuclear magnetic resonance spectroscopy, crystallography, SDS-PAGE analysis, and indirect metabolite readings such as NADH production, have all provided valuable insight into the function of lipoylated proteins [4,7,8,16,60-67]. More recently, mass spectrometry (MS) approaches have provided means to accurately investigate the lipoylation status of specific lysine residues in different cell types, tissues, and biological contexts [7,8,68] (Fig 2).

Peptides containing lipoyl-lysine residues can be detected by MS as a 188 Dalton mass shift (addition of $C_8H_{12}OS_2$) relative to unmodified lysines. This method was proven effective for measuring lipoamidase activity *in vitro* after incubation of purified lipoamidases with

synthetically lipoylated peptides [7,8] (Fig 2, center). However, when studying lipoylation in a more complex biological sample (in cells or *in vivo*), it is desirable to convert lipoyl-lysines into a chemical species that cannot undergo further oxidation; this ensures all lipoyl groups are in a uniform oxidation state with the same mass for unbiased detection by MS. This is achieved by treatment with a reducing agent to generate dihydrolipoamide, after which the reduced lipoamide thiols are blocked with N-ethylmaleimide (NEM) [7,8]. This treatment results in a 440 Dalton mass shift relative to unmodified lysine, which can be detected by single stage MS using peptide mass fingerprinting [68] (Fig 2, left).

The precise sites of lipoyl-lysine can be further investigated using tandem MS (MS/MS) [7,8] (Fig 2, left). Analogous to acetylation, lipoylation is considered non-labile upon MS/MS fragmentation, being retained on the lysine residue, in contrast to labile modifications such as phosphorylation. Acquisition of MS/MS spectra for lipoylated peptides also allows the identification of signature fragmentation information that can be used to accurately detect and quantify site-specific lipoylation in different biological samples. These approaches use targeted MS analyses, such as parallel reaction monitoring (PRM), which was shown to be valuable for detecting lipoylated peptides and low abundance enzymes, such as SIRT4 [7,8] (Fig 2, left). In these quantitative studies, the conversion of lipoyl groups into one chemical species via reduction and alkylation is beneficial for facilitating consistent detection by targeted MS. To date, targeted MS has been used to detect all lipoyl-lysine containing peptides in PDH and GCV in bacteria, and PDH in humans [7,8].

MS-based workflows that integrate immunoaffinity purifications have also been used to investigate lipoamidase-substrate interactions or protein associations with lipoylated complexes [7,8], providing information about the regulation of lipoylation (Fig 2, right). However, several available and powerful MS workflows are yet to be applied to studying aspects of protein lipoylation *in vitro* or *in vivo*. For example, while PTM enrichment is commonly used with MS analysis for studying different modifications, including acetylation and phosphorylation [69-71], it remains to be determined whether it can be used to study protein lipoylation, perhaps using anti-lipoic acid antibody. This method could provide a better understanding of the complete landscape of lipoylated proteins in different organisms. Although western blot analyses and searches for lipoylated motif patterns have not provided evidence for additional lipoylated proteins in humans, future mass spectrometry analyses can help determine whether the few known lipoylated protein complexes are indeed the only ones modified.

Given the co-existence of lipoylation- and phosphorylation-driven regulation of metabolic complex enzymatic activities, MS methods that allow examining intact proteins or protein complexes can also prove valuable when studying the coordinated functions of lipoylation. One such method that has gained significant recognition is ion mobility MS, which has been elegantly applied to studying intact protein complexes [72], protein structure and conformational changes [73,74], and protein-ligand interactions [75]. During recent years, this methodology has undergone optimizations for improved mass accuracy and sensitivity [76], and has been also applied to studying metabolites [77-79]. These methods can provide important insights into how lipoylation may act as both a facilitator of enzymatic reactions

and a structural support of protein complexes. For example, the lipoylated PDHX subunit of PDH is known to be necessary for the structural integrity and function of the complex [64,80]. However, how the addition or removal of lipoylation on PDHX impacts the complex structure remains to be fully understood. Another example is provided by LplA, the enzyme that adds the lipoyl modification, which was shown to undergo distinct conformational changes during the multistep process of lipoylation [66]. Ion mobility MS can help determine whether lipoylation-dependent conformations are linked to metabolic disorders and related diseases. In addition to ion mobility, cross-linking MS has proven to be valuable for gaining insights into the conformations and interactions of protein complexes [81-85]. Directly applicable to the study of lipoylation, cross-linking MS methods have been applied to (1) uncovering the mitochondrial interactome, where all four lipoylated complexes reside [86], and (3) investigating PTM crosstalk mechanisms [87]. The ability to analyze intact multimeric protein complexes, like PDH and KDH, and their modification status in conjunction with metabolite analyses, has the promise to place the function of lipoylation within its metabolic environment in healthy and disease states.

As quantitative MS techniques have now become routine for defining the proteome and metabolome landscapes in biologically diverse systems [88-92], such methods offer the means to better understand diverse aspects of the regulation of lipoylation. These methods include the use of labeling with multiplexed isobaric tags, such as tandem mass tags (TMT) and isobaric tags for relative and absolute quantitation (iTRAQ), which have been applied to studying temporal proteome and PTM changes associated with disease states, such as cancer and viral infection [89,93-95]. Metabolic labeling methods have also been used to uncover proteome and metabolome regulation during a biological process. For example, liquid chromatography-tandem MS in combination with ^{13}C metabolic labelling has enabled quantitative profiling of metabolic flux [56], which is relevant when evaluating the impact of lipoylation on metabolic regulation. As MS technologies are constantly being improved, coordinated metabolic and proteomic monitoring during a dynamic biological process starts to become feasible, and will enable to further understand the function of lipoamide as a locus of cellular metabolic control.

Concluding remarks

This is an exciting time in the research area of cellular metabolism regulation, and characterizing the function of lipoylation allows access to another important facet. As we discuss in this review, advancements have been made in the detection of lipoylation and in understanding its enzymatic regulation and functions. Sensitive MS methods are now available for identifying and quantifying protein lipoylation events, even when present at low abundance. Targeted MS provides the opportunity to accurately track changes in these modifications during a biological process. With the recent progress in MS technologies for studying intact protein complexes and direct protein interactions, such as ion mobility and cross-linking workflows, the impact of lipoylation on protein complex structure and associations can be interrogated in greater detail.

The presence of lipoamide regulation at multiple carbon entry points into the TCA cycle, such as the incorporation of acetyl-coA and alpha-ketoglutarate, indicates its essential role in

maintaining homeostasis and metabolic flexibility. Given the ability of lipoylation to modulate multiple central cellular metabolic pathways, as well as the evolutionary conservation of lipoylated substrates from bacteria to mammals, it is expected that future studies will be informative in diverse fields of research, including cancer research, bacteriology, microbiome studies, and virology. These insights can further illuminate fundamental aspects of dynamic metabolic response to environmental and nutritional conditions, as well as provide opportunities for therapeutic interventions. For example, lipoylation provides a so far insufficiently explored tool for examining PDH deficiencies in cancer, heart disease, Alzheimer's disease and autism [9,11,96]. We hope that this review will provide food for thought and will stimulate future studies in this important growing research area.

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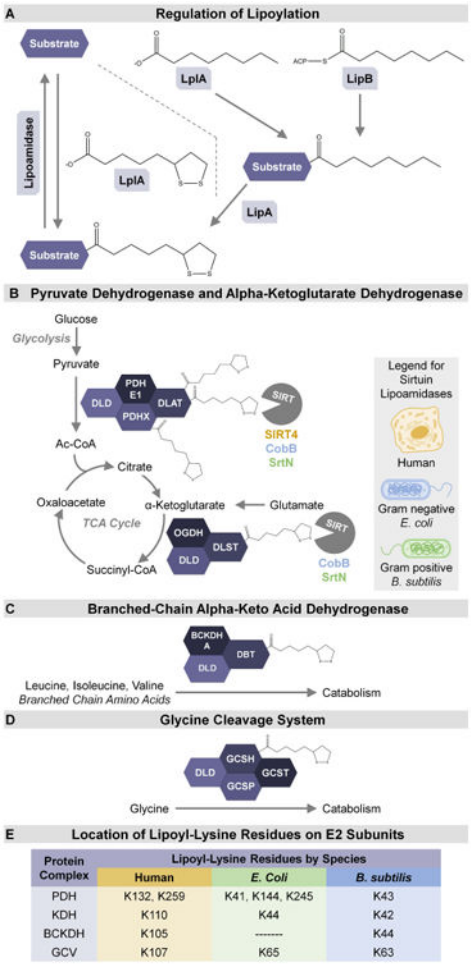
- Lipoylation is a rare, but highly conserved lysine posttranslational modification
- Lipoylated metabolic complexes are evolutionarily conserved from bacteria to humans
- Dysregulation of lipoylated complexes is linked to a wide range of human diseases
- The regulation and biological significance of lipoylation is not fully understood
- Advances in mass spectrometry-based proteomics provide new avenues of investigation



Protein Complex	Lipoyl-Lysine Residues by Species		
	Human	*E. Coli*	*B. subtilis*
PDH	K132, K259	K41, K144, K245	K43
KDH	K110	K44	K42
BCKDH	K105	-------	K44
GCV	K107	K65	K63

Figure 1. The evolutionarily conserved metabolic role of lipoylation

(A) Regulation of lipoylation on substrates. Lipoic acid can be added directly by LplA, or in a stepwise manner using LipB or LplA, followed by LipA. Delipoylation is mediated by lipoamidases (ACP: acyl-carrier protein). (B) Pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (KDH) complexes regulate two points of carbon entry into the TCA cycle. Inhibition of these two complexes has been demonstrated in humans and bacteria by the sirtuin (SIRT)-mediated lipoamidase activity. Font color of each SIRT represents the name of the lipoamidase sirtuin for that species (yellow: human, blue: *E. coli*, green: *B. subtilis*). (C) Lipoylated branched-chain alpha-keto acid dehydrogenase (BCKDH) catabolizes branched chain amino acids: leucine, isoleucine, and valine. (D) Glycine is degraded via the lipoylated glycine cleavage system (GCV). (E) The location of each lipoyl-lysine residue is provided for each E2 subunit of the conserved lipoylated protein complexes (--- indicates not conserved in *E. coli*).

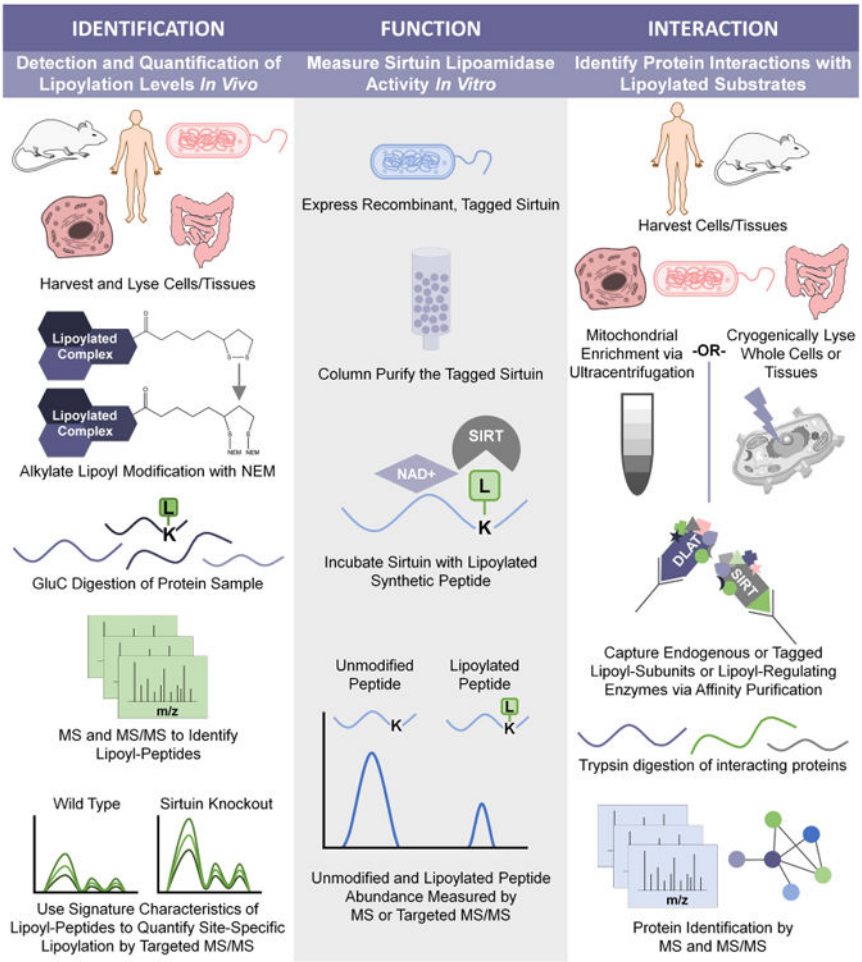


Figure 2. Mass spectrometry-based proteomic workflows that have been applied to studying lipoylation

(Left) Common steps for identifying lipoyl peptides across various organisms *in vivo* or in cell culture. Comparison of wild type and enzyme knockout backgrounds allows for the relative quantification of lipoylation by targeted MS/MS (NEM: N-ethylmaleimide, L: lipoylation, MS: mass spectrometry, *m/z*: mass to charge ratio). (Center) *In vitro* analysis of lipoamidase activity. Purified sirtuins, SIRT4 and CobB, remove lipoylation from synthetic peptides in an NAD^+-dependent manner, as measured by MS or targeted MS/MS. (Right) Identification of interactions with lipoylated proteins and lipoyl-regulating enzymes. Two methods for preparing harvested cell/tissue samples are depicted; low abundance targets usually benefit from mitochondrial enrichment prior to affinity purification, while high abundance targets can be directly isolated from whole cell lysates. Endogenous or tagged proteins of interest and their interaction partners are captured via immunoaffinity purification. Subsequent MS and MS/MS analyses provide insight into functional interaction networks associated with lipoylated complexes.