Chiral Posttranslational Modification to Lysine ε-Amino Groups

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CONSPECTUS. The sophistication of proteomic analysis has revealed that protein lysine residues are posttranslationally modified by a variety of acyl groups. Protein lysine acetylation regulates metabolism, gene expression, and microtubule formation and has been extensively studied; however, the understanding of the biological significance of other acyl posttranslational modifications (PTMs) is still in its infancy. The acylation of lysine residues is either mediated by acyltransferase 'writer' enzymes or through non-enzymatic mechanisms and hydrolase enzymes, termed 'erasers', cleave various acyl PTMs to reverse the modified state. We have studied the human lysine deacylase enzymes, comprising the 11 Zn^{2+}-dependent histone deacetylases (HDACs) and the 7 NAD^{+}-consuming sirtuins (SIRTs), over the last decade. We have thus developed selective inhibitors and molecular probes as well as studied the acyl substrate scope of each enzyme using chemically synthesized peptide substrates and photo-crosslinking probes. Recently, we have turned our attention to lysine PTMs containing a stereogenic center, such as ε-*N*-β-hydroxybutyryllysine (Kbhb) and ε-*N*-lactyllysine (Kla), that each comprise a pair of mirror image stereoisomers. Both modifications are found on histones, where they affect gene transcription in response to specific metabolic states, and they are found

on cytosolic and mitochondrial enzymes involved in fatty acid oxidation (Kbhb) and glycolysis (Kla), respectively. Thus, chiral modifications to lysine side chains give rise to two distinct diastereomeric products, with separate metabolic origins and potentially different activities exhibited by writer and eraser enzymes. Lysine L-lactylation derives from L-lactate, a major energy carrier produced from pyruvate after glycolysis, and it is highly induced by metabolic states such as the Warburg effect. L-Lactate can possibly be activated by acyl-coenzyme A (CoA) synthetases and transferred to lysine residues by histone acetyltransferases such as p300. D-Lactylation, on the other hand, arises primarily from a non-enzymatic reaction with D-lactylglutathione, an intermediate in the glyoxalase pathway. In addition to their distinct origin, we found that both K(L-la) and K(D-la) modifications are erased by HDACs with different catalytic efficiencies. Also, K(L-bhb) and K(D-bhb) arise from different metabolites but depend on interconnected metabolic pathways, while the two stereoisomers of ε-*N*-3-hydroxy-3-methylglutaryllysine (Khmg) derive from a single precursor that may then be regulated differently by eraser enzymes. Distinguishing between the individual stereoisomers of PTMs is therefore of crucial importance. In the present *Account*, we will (1) revisit the long-standing evidence for distinct production and dynamics of enantiomeric forms of chiral metabolites that serve as ε-*N*-acyllysine PTMs and (2) highlight the outstanding questions that arise from the recent literature on chiral lysine PTMs resulting from these metabolites.

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1. INTRODUCTION

Posttranslational modifications (PTMs) to proteins are responsible for a great degree of diversification of the proteome and affect diverse functions in biology.[5] PTMs are important biological signals that affect protein conformation, modulate enzyme activity, mediate interactions with other biomolecules, and lead to protein re-localization, externalization, and degradation. Acyl modifications to lysine residues comprise a diverse series of PTMs and have been studied extensively in the field of epigenetics.[6] The acylation of histone lysine residues affects the interaction of these proteins with DNA and alters chromatin compaction. In addition, ε-*N*-acyllysine PTMs serve as recognition motifs for multiprotein complexes with various functions in gene transcription and DNA repair.[7] Beyond histones, lysine acylation is found in thousands of proteins across all cellular compartments[8-9] and is tightly connected to the metabolic state of the cell, as most ε-*N*-acyllysine PTMs are derived from metabolites.[6-7]

Recent studies have found ε-*N*-acyllysine PTMs derived from two of the most prominent metabolic energy carriers: L-lactate (high glycolytic conditions)[10] and D-β-hydroxybutyrate (ketogenesis).[11] These two molecules contain a chiral center and therefore give rise to a pair of diastereoisomers when conjugated to lysine. Importantly, the enantiomers of each of these metabolites are also present in cells and would generate an opposite diastereomeric adduct with a similar name and structure (Figure 1). These fundamental chemical differences have most often been overlooked in the literature on PTMs, but chirality is essential for understanding mechanisms in biology. For example, human metabolism is fed by D-sugars and not L-sugars, D-peptides are more stable to proteolytic degradation than L-peptides, and the different enantiomers of chiral drug molecules are most often binding with different affinities to the inherently chiral environment of protein pockets. Similarly, each diastereoisomer of a chiral

PTM to lysine is different and may exhibit properties and functions that are specific to its stereochemistry.

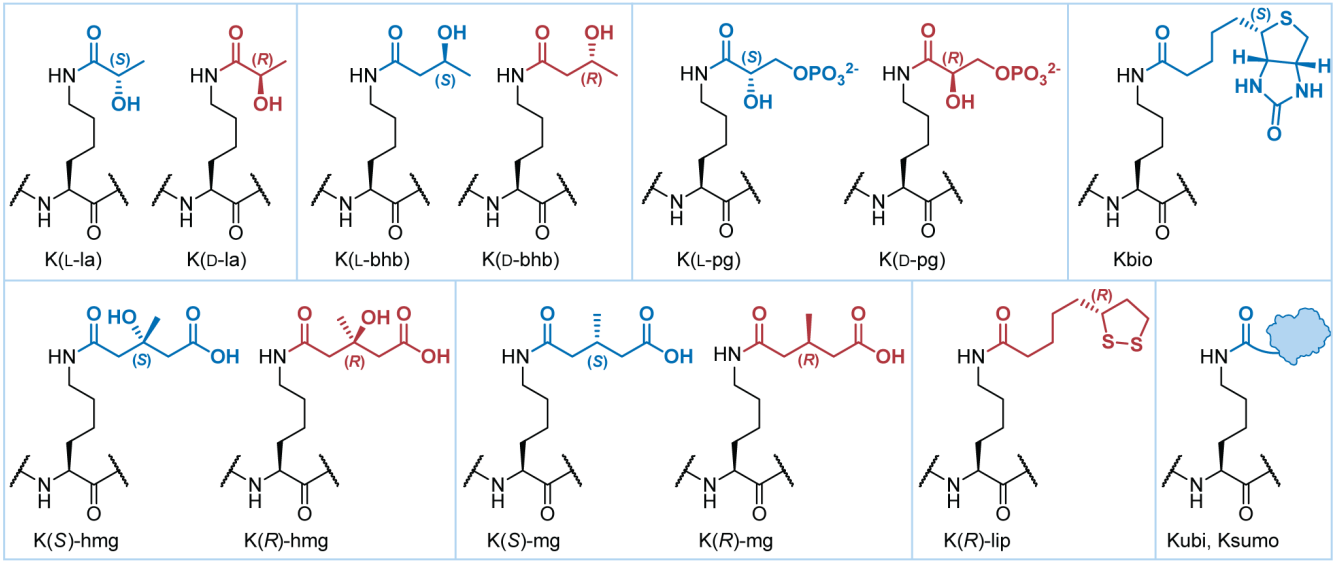

Figure 1. Chiral posttranslational modifications of lysine. K(L/D-la), ε-*N*-L/D-lactyllysine; K(L/D-bhb), ε-*N*-L/D-β-hydroxybutyryllysine; K(L/D-pg), ε-*N*-L/D-3-phosphoglyceryllysine; Kbio, ε-*N*-biotinyllysine; K(*S*/*R*)-hmg, ε-*N*-(*S*/*R*)-3-hydroxy-3-methylglutaryllysine; K(*S*/*R*)-mg, ε-*N*-(*S*/*R*)-3-methylglutaryllysine; K(*R*)-lip, ε-*N*-(*R*)-lipoyllysine; Kubi, ε-*N*-ubiquitylysine; Ksumo, ε-*N*-SUMOyllysine.

The basis of stereospecific recognition in biology of course arises from the intrinsic chirality of biomolecules. Most ε-*N*-acyllysine PTMs are installed by so-called 'writer' enzymes [*e.g.* histone acetyltransferases (HATs)], recognized by 'readers' [*e.g.* bromodomains (BRDs)], and removed by 'erasers' [*e.g.* histone deacetylases (HDACs)].[6] These biomolecules present a degree of promiscuity regarding which PTMs they can accept but are also expected to be sensitive to the stereochemistry of the modification due to their well-defined chiral binding pockets. Our work has focused on the study of the 18 human deacylases – the 11 Zn^{2+}-dependent HDACs and the 7 NAD^{+}-consuming sirtuins (SIRTs). Most of these enzymes regulate the acylation of histones and non-histone proteins in the cell nucleus (HDAC1–3, HDAC8, SIRT1–3, SIRT6, and SIRT7), but they are also efficient deacylases in the cytosol

(HDAC6, HDAC10, and SIRT2), and in the mitochondria (SIRT3–5).[8] We have reported on their substrate specificity,[1-3, 12-13] developed substrate-based photo-affinity probes,[14] and contributed to the collection of selective inhibitors for their investigation in diseased and healthy cells and tissues.[15-16] Recently, we have shown that HDACs are erasers of lactyl and β-hydroxybutyryl PTMs with a preference for one of the two diastereoisomers.[4] This insight supports an inherent difference in regulation, turnover, and biological function for each PTM stereoisomer. Here, we summarize what is known about the source, regulatory mechanism, and enzymatic recognition of each stereoisomer of the chiral acyl PTMs of lysine.

2. PROTEIN LYSINE ε-*N*-LACTYLATION

Lysine lactylation was first described by Zhao and coworkers in 2019.[10] This modification, which is only 30 Da heavier than an acetyl group, is most likely the smallest chiral modification of lysine residues. Its discovery opened up a variety of possible regulatory pathways, as L-lactate is one of the most concentrated metabolites in human serum[17] and both L- and D-lactate enantiomers are produced from glycolytic products in all tissues.

2.1 L-Lactylation

L-Lactate is the most abundant product of glycolysis and a major energy transporter between tissues. Standard plasma concentrations of L-lactate are in the 1–2 mM range[17] and increase over 5 times during intense exercise as well as in pathologies associated with hypoxia or the Warburg effect (switch to glycolytic metabolism).[18] In cancer cells, L-lactate concentration can go up to 20–40 mM.[19] Since L-lactate is produced from cytosolic pyruvate by L-lactate dehydrogenase (L-LDH), L-lactate production is also connected to alanine catabolism and malate recycling (Figure 2A). L-Lactate by itself is not able to modify lysine residues, but the activated lactyl-coenzyme A (lactyl-CoA) species has been detected in mammalian cells in

small amounts (comparable to crotonyl-CoA and ~1000 times lower than acetyl-CoA).[20] This metabolite would presumably consist primarily of L-lactyl-CoA, as a result of the much more concentrated L-lactate enantiomer being processed by an acyl-CoA synthetase. Acyltransferases (e.g. p300), which are known to display promiscuous substrate recognition,[21] have been proposed to catalyze the formation of ε-*N*-L-lactyllysine [K(L-la)] PTMs on cytosolic and nuclear proteins, using L-lactyl-CoA.[10]

K(L-la) modifications are found at multiple lysine residues of the four nucleosome-forming histone isotypes (H2A, H2B, H3, and H4).[10] Histone K(L-la) levels are sensitive to glycolytic flux and oxygen concentration as a result of changes in L-lactate concentration, which connects gene regulatory functions to situations of metabolic stress or reprogramming. Reportedly, a switch to glycolytic metabolism in pro-inflammatory macrophages upon bacterial infection leads to changes in histone K(L-la) levels, which then promote the expression of genes involved in wound healing.[10] Similar epigenetic mechanisms may play an important role in oncogene regulation and hypoxic response.

We found that the Zn^{2+}-dependent HDAC1–3 enzymes are the most efficient K(L-la) erasers *in vitro* using small fluorogenic substrates and histone peptides.[4] Gene manipulation and inhibitor screening corroborated these findings in cells and revealed that HDAC1–3 remove histone K(L-la) PTMs *in cellullo*.[4] HDAC1–3 form multiple regulatory co-repressor complexes that modulate cell division and differentiation[22] and these effects may be related to dynamics in the histone K(L-la) landscape. HDAC1–3 and p300 are also the main regulatory enzymes of histone acetylation and crotonylation, but each modification shows different dynamics and residue distribution.[4, 10, 23] Therefore, K(L-la) modifications likely respond to PTM cross-talk and co-repressor complex recruitment through specific mechanisms that are different from those of

other ε-*N*-acyllysine modifications. A recent study showed that, out of six HDAC1-containing nuclear complexes, only the mitotic deacetylase complex (MiDAC) and the arginine-glutamic acid dipeptide repeats (RERE) could remove K(L-la) marks from histone H2B lysine 11 (H2BK11) on nucleosomes in vitro.[24] Importantly, HDAC1–3 are targets of approved cancer chemotherapy and histone K(L-la) accumulation might therefore be involved in the observed epigenetic effects of HDAC inhibitors.[4, 25]

Sirtuins 1 and 2 are poor delactylases on peptide substrates, with catalytic efficiencies ~1000 times lower than that of HDAC3,[26] but they can remove K(L-la) modifications from histones upon extended incubation times.[4] Thus, we speculate that sirtuins might regulate histone K(L-la) sites complementary to those targeted by HDAC1–3 and function as erasers of potential cytosolic K(L-la) PTMs. Interestingly, recent studies of protein lactylation on the fungal pathogen *Botrytis cinerea* indicate that it may be a modification spread across multiple cellular compartments.[27]

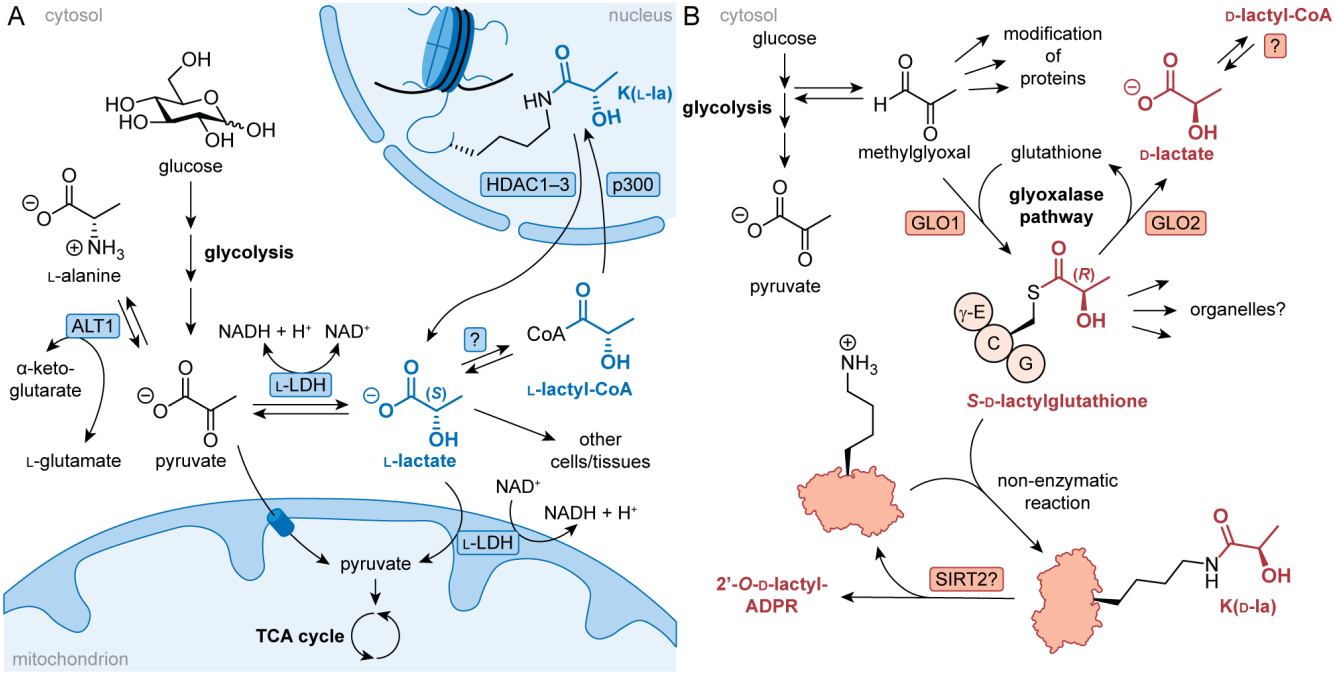

Figure 2. Source and dynamics of (A) K(L-la) and (B) K(D-la) modifications. ADPR, adenosine diphosphate ribose; ALT1, alanine aminotransferase 1; NAD^+, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; GLO, glyoxalase.

2.2 D-Lactylation

Methylglyoxal is a reactive metabolite formed from the glycolysis intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (G3P), among other processes. Methylglyoxal is quenched in the cell through the glyoxalase pathway, where first glyoxalase 1 (GLO1) traps it as *S*-D-lactylglutathione (single diastereomer)[28] and then GLO2 regenerates glutathione by releasing D-lactate (Figure 2B).[29] *S*-D-Lactylglutathione is reactive to nucleophiles and it can transfer the D-lactyl acyl group onto lysine residues affording K(D-la) PTMs. This process was identified by Galligan and coworkers, who reported widespread protein lysine lactylation upon GLO2 ablation.[30] Presumably, this lactylation arises from the accumulation of *S*-D-lactylglutathione, resulting in K(D-la) modification of the identified glycolytic enzymes, which serves as a feedback mechanism to lower the rate of glycolysis.[30]

Our search for lysine delactylases also identified HDAC1–3 as the most efficient erasers of K(D-la) *in vitro*, presenting increased activity against K(D-la) substrates compared to the K(L-la) counterparts (2–10 times higher k_{cat}/K_M).[4] HDAC1–3 are located in the cell nucleus, where they may target a small number of proteins found to display K(D-la) modifications.[30] Cytosolic K(D-la) PTMs, which are more prevalent, were proposed to be cleaved by SIRT2 to afford 2'-*O*-D-lactyl-adenosine 5'-diphosphate ribose (2'-*O*-D-lactyl-ADPR).[26] Even though this enzyme presents poor *in vitro* activity on peptide substrates, the lack of alternative cytosolic delactylases makes it a reasonable candidate to modulate specific K(D-la) protein sites.[4, 26] In addition, SIRT2 has been shown to regulate the activity of glycolytic enzymes,[31] a function that may be mediated by K(D-la) deacylation.

In addition to producing D-lactate as the end-product of the glyoxalase pathway, mammalian cells further acquire it from diet and gut bacteria, which possess D-LDH enzymes. These sources normally afford D-lactate in much lower quantity than the L-lactate pathways with serum levels of D-lactate in the nanomolar range.[32] However, concentrations of D-lactate in plasma of up to 3 mM can be found in rare situations, such as cases of short bowel syndrome, ischemia, and bacterial infection.[32-33] Activation of D-lactate by acyl-CoA synthetases and subsequent acyltransferase action may then occur in analogy with the proposed mechanism for K(L-la) formation. Under these conditions, modification of histones with K(D-la) PTMs might also lead to the lactate epigenetic reprogramming, where the stereospecificity of writer and eraser enzymes would determine the different dynamics and competing effects of K(D-la) versus K(L-la) modifications.

3. PROTEIN LYSINE ε-*N*-β-HYDROXYBUTYRYLATION

The β-hydroxybutyrylation of histones was first reported by Zhao and coworkers in 2016[11] and further studies have identified over 1000 proteins to be β-hydroxybutyrylated across the mammalian cell.[9] This chiral PTM has two potential sources: D-β-hydroxybutyrate (D-bhb) and L-β-hydroxybutyl-CoA (L-bhb-CoA), both of which are produced under standard metabolic conditions and with D-bhb being a major energy transporter during ketogenesis.

3.1 D-β-Hydroxybutyrylation

More than 80% of the stored energy within the human body resides in fatty acids in adipose tissue.[34] Upon scarce availability of glucose, fatty acids are transported to the mitochondria, primarily in the liver, where they are metabolized into acetyl-CoA. During normal conditions, acetyl-CoA would combine with oxaloacetate to produce citrate that drives the tricarboxylic acid

(TCA) cycle. However, glucose shortage leads to oxaloacetate being used for gluconeogenesis and to acetyl-CoA instead being used in ketogenesis (Figure 3A).[35]

Ketogenesis generates three ketone bodies: acetoacetate, acetone, and D-bhb.[35] First, two molecules of acetyl-CoA are converted into acetoacetyl-CoA by 3-ketothiolase.[35] Second, an additional molecule of acetyl-CoA is condensed with acetoacetyl-CoA to form (*S*)-3-hydroxy-3-methylgluraryl-CoA (hmg-CoA). This step is rate-limiting in ketone body formation and is mediated by mitochondrial hmg-CoA synthase (HMGCS2).[36] In the third step, hmg-CoA lyase (HMGCL) forms acetoacetate, the first ketone body that serves as energy transporter. Acetoacetate may also decarboxylate into acetone or be converted to D-bhb in the mitochondria by bhb dehydrogenase (BDH1) – the enzyme that dictates the D-bhb chirality.[34] From the mitochondria, D-bhb is transported to the cytosol and nucleus of the hepatocyte as well as to the blood and from there to extra-hepatic organs.[34] Standard serum levels of D-bhb are in the micromolar range (~77 μM).[17] However, during fasting and intense exercise it quickly reaches concentrations of 1–2 mM and can further rise up to 20 mM in certain pathological states, such as diabetes.[37]

Apart from serving as an energy carrier, D-bhb is posttranslationally installed on lysine side chains of proteins in the nucleus, cytosol, and mitochondria, which provides a further link between metabolism and proteome expansion.[5, 11, 37] Zhao and coworkers hypothesized that D-β-hydroxybutyrylysine [K(D-bhb)] PTMs could be installed on histones by transferases using activated D-bhb-CoA (Figure 3A). Treatment of HEK293 cells with isotopically labeled racemic bhb afforded 28 modified histone peptides as well as a dose-dependent increase of isotopically labeled D/L-bhb-CoA.[11] In a later study, the same group demonstrated that knockdown of the p300 or CBP transferases resulted in decreased levels of K(D/L-bhb) at specific histone sites,

further corroborating their hypothesis, but also highlighting the presence of additional regulatory mechanisms.[9] Histone β-hydroxybutyrylation is induced in mice upon fasting and it was found to be upregulated in a model of diabetes, which suggests that epigenetic pathways are affected by the K(D-bhb) PTM, derived from the D-bhb ketone body.[11, 34] Beyond histones, 891 Kbhb sites were identified across 267 proteins in starved mouse liver,[37] with multiple PTMs being located at unique sites compared to previous studies of lysine acetylation. Thus, K(D-bhb) modifications are proposed to be a widespread metabolic regulatory mechanism with a separate role compared to Kac.[9]

Inspired by the work of Zhao and coworkers, our main deacylase substrate screening campaigns have included a K(D/L-bhb) substrate, which was not recognized by the sirtuins SIRT1–3 and SIRT6[2] but it was cleaved to some extent by HDAC3.[3] More recently, we interrogated the activity of HDAC3 using diastereomerically pure substrates and found a clear preference for K(D-bhb) modifications compared to K(L-bhb) *in vitro* (~12 times higher k_{cat}/K_M).[4] Similar to K(L-la), there are multiple deacylases in the cell that are capable of removing K(D/L-bhb); HDAC1–3 and SIRT1/2 show activity on extracted histones,[9] the MiDAC complex presents activity on nucleosomes bearing H2BK11(D-bhb),[24] and HDAC1–3 are the most efficient de-β-hydroxybutyrylases in the cell.[9] However, it is often unclear to what extent these activities serve as regulatory mechanisms of K(D-bhb) modifications *in vivo*.

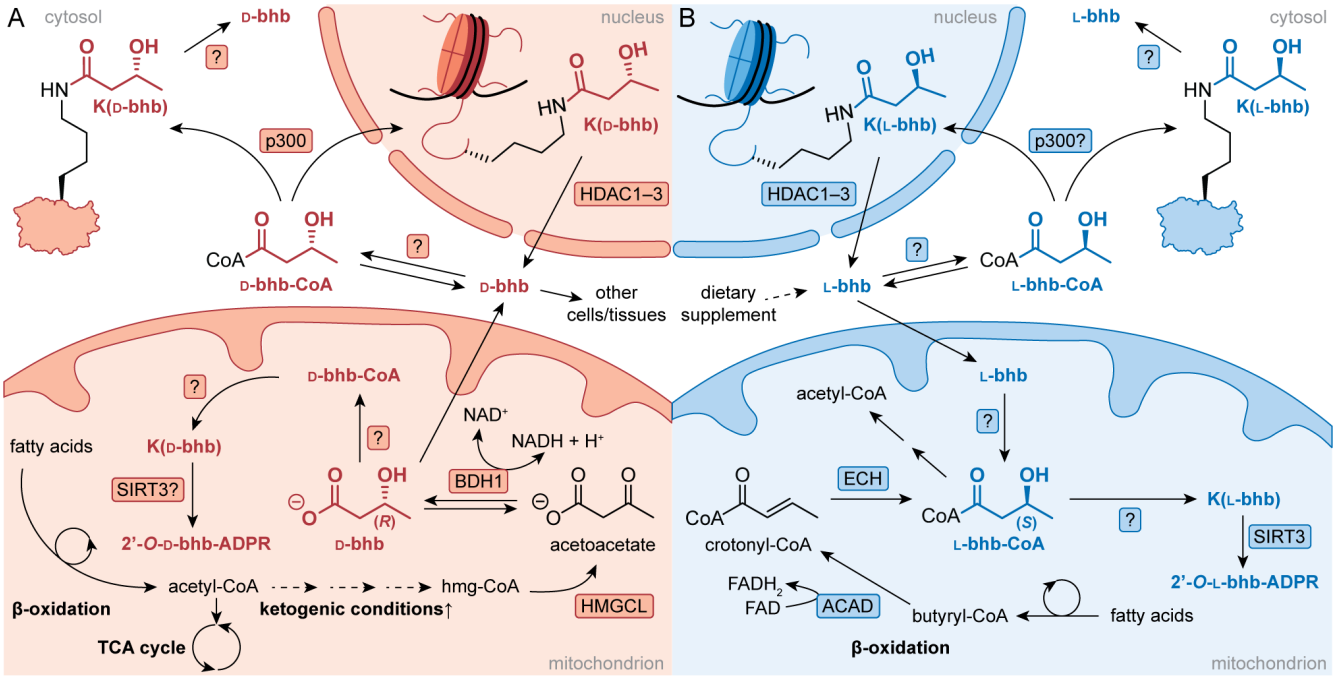

Figure 3. Source and dynamics of (A) K(D-bhb) and (B) K(L-bhb) modifications. Dashed arrows represent pathways that are only upregulated under certain conditions. ACAD, acyl-CoA dehydrogenase; BDH1, β-hydroxybutyrate dehydrogenase; ECH, enoyl-CoA hydratase; HMGCL, hmg-CoA lyase.

3.2 L-β-Hydroxybutyrylation

The hydroxycarboxylic acid L-bhb is not a naturally occurring metabolite in humans but the activated L-bhb-CoA species is formed during the final round of fatty acid β-oxidation. There, butyryl-CoA is oxidized by acyl-CoA dehydrogenase (ACAD) to crotonyl-CoA, which is then transformed to L-bhb-CoA by enoyl-CoA hydratase (ECH).[38-39] β-Oxidation then proceeds to produce acetyl-CoA and the presence of L-bhb-CoA is thus transient under standard conditions (Figure 3B).[34] However, fasting and ketogenesis may boost the mitochondrial concentration of L-bhb-CoA due to the higher rate of β-oxidation and thus promote modification of mitochondrial proteins. Modification of proteins in other cellular comportments, however, would depend on the efficiency of extramitochondrial transportation of L-bhb-CoA.[34]

The role of K(L-bhb) PTMs in mitochondrial and cellular homeostasis has not been studied in detail thus far. Data suggests that, under standard metabolic conditions, K(L-bhb) modifications are scarce and located primarily in the mitochondria. Our studies failed to identify potential mitochondrial K(L-bhb) erasers based on enzyme activities measured *in vitro*[2] but Li and coworkers demonstrated that SIRT3 is a de-β-hydroxybutyrylase *in vitro* and in cells, with a ~2-fold preference for K(L-bhb) over K(D-bhb) peptide substrates.[40] SIRT3 thus shows lower catalytic efficiency than HDAC3 against K(L-bhb) substrates (~23 times lower k_{cat}/K_M) but is likely the main eraser of mitochondrial K(L-bhb) under standard conditions.

Another important aspect of L-bhb dynamics is its presence in racemic dietary ketone supplements proposed to promote ketosis without restricting carbohydrate intake.[41] Through these supplements, L-bhb is introduced as an unnatural metabolite, which can still be used by the mammalian cell although it is metabolized significantly slower than D-bhb.[42-43] L-Bhb is proposed to be metabolized entirely in the mitochondria, presumably through activation as L-bhb-CoA followed by further processing.[44] Activation of D-bhb to D-bhb-CoA might be outcompeted by the longer-lived L-bhb enantiomer, thereby promoting K(L-bhb) modifications across the cell via transferase activity. Since HDAC3 presents lower catalytic efficiency towards K(L-bhb) compared to K(D-bhb),[4] the data may suggest that K(L-bhb) PTMs may persist longer on histones and other nuclear proteins.

3.3 The dilemma of chirality in K(D/L-bhb)

To date, there has been little focus on the chirality of the Kbhb PTM, with most studies assuming a focus on K(D-bhb) due to the inherent configuration of the D-bhb ketone body.[34] Proteomic studies of Kbhb modifications, however, commonly rely on pan- and site-specific Kbhb antibodies, for which the chiral specificity is unknown.[9, 11, 37] In addition, treatment of cells with

bhb to investigate transferases or promote the modification has been performed using racemic D/L-bhb salts. These experiments reportedly afford 3242 Kbhb sites across 1397 proteins in HEK293 cells, where the chirality is uncertain and would depend on the transferase selectivity at each site.[9] So far, only eraser enzymes have been assessed for stereospecific recognition of Kbhb isomers. We reported a 12-fold preference for K(D-bhb) by HDAC3 and SIRT3 was found to display 2-fold selectivity for K(L-bhb).[4, 40] The fact that eraser enzymes exhibit preferences depending on the stereochemistry of the PTM supports the importance of considering the chiral configuration of the Kbhb modification moving forward.

4. PROTEIN LYSINE ε-*N*-3-HYDROXY-3-METHYLGLUTARYLATION

Hmg-CoA is an intermediary metabolite generated in the cytosol and mitochondria, and it is only present in minute amounts under normal metabolic conditions.[45] Cytosolic hmg-CoA is produced stereoselectively from acetoacetyl-CoA and acetyl-CoA by hmg-CoA synthase 1 (HMGCS1),[46-47] as part of the mevalonate pathway towards the synthesis of sterols (Figure 4). The cytosolic hmg-CoA pool is highly sensitive to acetate concentration[45] and has been shown to accumulate upon statin therapy.[48] In the mitochondria, hmg-CoA is produced by the HMGCS2 isotype during ketogenesis[47] as well as by 3-methylglutaconyl-CoA hydratase (AU-specific RNA-binding enoyl-CoA hydratase, AUH) during the catabolism of leucine.[49]

Proteins across multiple cellular compartments are modified as ε-*N*-3-hydroxy-3-methylglutaryllysine (Khmg) *in vivo*, including histones,[50-51] which may be surprising considering the low concentration of hmg-CoA and the lack of known acyltransferases for the modification. The Hirschey lab found that hmg-CoA undergoes intramolecular S→O acyl shift to generate a highly reactive anhydride that non-enzymatically modifies lysine side chains.[50] This mechanism explains its reactivity at cytosolic and nuclear pH and the wider distribution of

the modification compared to the concentration of its acyl-CoA precursor.[50] While the direct non-enzymatic reaction of (*S*)-hmg-CoA with lysine would generate the (*R*)-3-hydroxy-3-methylglutaryllysine modification (i.e., K[(*R*)-hmg]) due to the different Cahn-Ingold-Prelog substituent priority of the thioester and amide moieties, the anhydride intermediate would be expected to react at both carbonyl positions to afford the K[(*R*)-hmg] as well as the K[(*S*)-hmg] modifications (Figure 4, red and blue circles). Thus, contrary to previous PTMs discussed, here a single hmg-CoA diastereomer could give rise to two epimeric modifications. Dedicated studies are required to illuminate this question in detail in cells.

K[(*R*)-hmg] and K[(*S*)-hmg] modifications share origin but may still differ in effect and dynamics, as interaction partners and eraser enzymes could be sensitive to the configuration of the chiral center. Current antibodies,[50] peptide and protein substrates,[13, 52] and metabolic probes[51] have been prepared using the symmetric 3-hydroxy-3-methylglutaric acid (hmg) and corresponding anhydride and therefore do not inform on the differences between K[(*R*)-hmg] and K[(*S*)-hmg]. Utilizing these tools, SIRT4 was discovered to be the main mitochondrial eraser of both modifications[13] and high affinity and moderate activity were also demonstrated for the closely related SIRT5 isotype against these modifications.[51-52] It is not currently known whether SIRT4 and SIRT5 display stereoselective turnover of these two modifications, which could have implications in the regulation of metabolic pathways.[13, 50] Future research will shine a light on these differences and the individual roles of each PTM.

Parallel to the discovery of posttranslational Khmg, proteins were found to be modified with chiral 3-methylglutaryl by a similar mechanism,[50] which was found to be deacylated by SIRT4.[13] These PTMs are proposed to derive from the non-specific reduction of (*E*)-3-methylglutaconyl-CoA (mgc-CoA) or its lysine adduct, which generates a mixture of (*S*)- and (*R*)-3-

methylglutaryllysine. Differences in SIRT4 recognition and activity may also influence the dynamics of these two lysine PTMs.

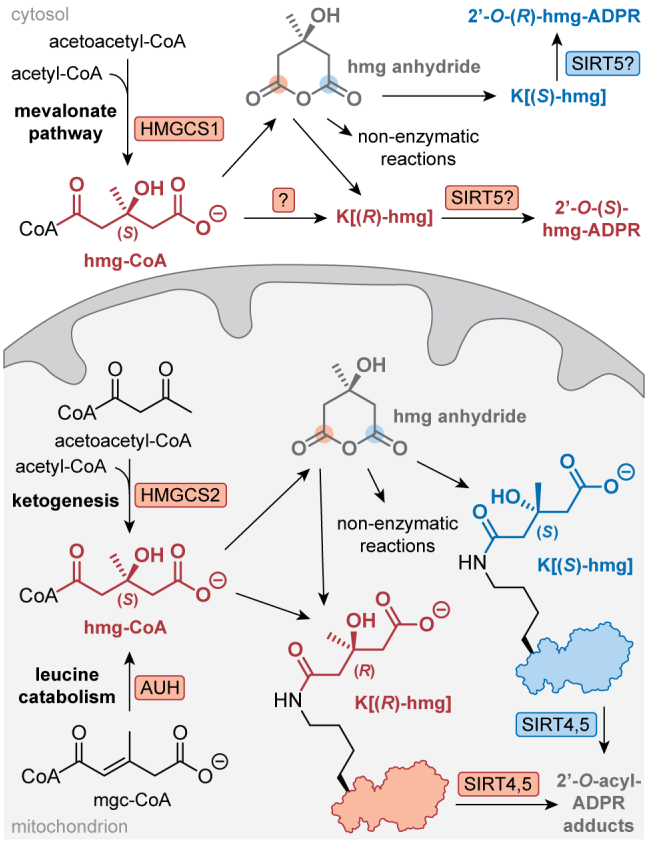

Figure 4. Source and dynamics of K[(*R*)-hmg] and K[(*S*)-hmg] modifications. (*S*)-Hmg-CoA and 2'-*O*-(*S*)-hmg-ADPR share connectivity with K[(*R*)-hmg] and are colored in red accordingly. The change in stereochemical nomenclature is due to the difference in priority between (thio)ester and amide functional groups when compared to carboxylic acids. AUH, AU-specific RNA-binding enoyl-CoA hydratase; HMGCS, HMG-CoA synthase.

5. OTHER CHIRAL MODIFICATIONS TO THE ε-AMINO GROUPS OF LYSINE

In addition to Kla, Kbhb, and Khmg assessed above, lysine residues in the proteome have been found to be modified by biotin, lipoate, and 3-phosphoglycerate, which are also chiral molecules. In the following sections, we focus on these three small chiral PTMs found in the mammalian cell.

5.1 ε-*N*-Biotinylation

Biotin (vitamin B_7) cannot be synthesized in mammalian cells and its absorption relies on dietary uptake from microbial and plant sources. In humans, biotin is mainly known for its role as a coenzyme for carboxylases, but recent reports have also revealed a role in epigenetic reprogramming, with more than 2000 biotin-dependent genes identified in various human tissues.[53] Biotin is therefore a key element in cell signaling and gene regulation, partly through the direct modification of lysine residues in histones as ε-*N*-biotinyllysine (Kbio).

Kbio modifications are observed in all five histone types; although, with lower abundance and at fewer sites identified than for most other known histone lysine PTMs.[54-56] The biological role is not well understood and even though Kbio appears to be implicated in DNA damage signaling, it is still unclear whether Kbio modifications take part in DNA repair or apoptosis.[57-58] The modification of histones with Kbio was originally believed to be catalyzed by biotinidase (BTD, Figure 5A).[54] BTD hydrolyzes the protein degradation product biocytin (free ε-*N*-biotinyllysine) to release biotin, and it was proposed that BTD could also catalyze the reverse reaction.[54] However, further studies showed that increased biotinylation of histones did not rely on BTD activity and concluded that other enzymes are likely to be involved.[55] Later, Gravel and coworkers reported that holocarboxylase synthase (HCS), the enzyme responsible for carboxylase biotinylation, can also biotinylate histones *in vitro* and potentially play a role in epigenetic reprogramming.[59] This hypothesis was supported in *Drosophila melanogaster*, where knockdown of HCS or BTD both led to decreased histone Kbio levels, with the effect of HCS knockdown being most pronounced. While HCS is known to install Kbio modifications, the effects of BTD knockdown may also arise from impaired biotin recycling and biotin deficiency. In this model, the abnormal gene expression patterns resulting from Kbio alteration resulted in a reduction in lifespan.[56]

We have shown that nuclear HDACs and sirtuins are not able to efficiently hydrolyze Kbio modifications *in vitro*[3, 12] and only the mitochondrial enzyme SIRT4 is reported to present debiotinylase activity.[60] Instead, Zempleni and coworkers found that, in addition to its biocytin hydrolase activity, BTD is also a protein debiotinylase that can remove Kbio from histones.[61] The regulatory factors, which determine whether BTD acts as a Kbio writer or eraser remain largely unknown and may depend on the chromatin microenvironment or specific BTD splice variants.[61]

5.2 ε-*N*-3-Phospho-D-glycerylation

1,3-Bisphospho-D-glycerate (1,3-bpg) is a primary metabolite produced in the sixth step of glycolysis from glyceraldehyde 3-phosphate (G3P), mediated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Figure 5B).[62] Since energy metabolism is restricted to D-sugars in eukaryotes, only the D-enantiomer of 1,3-bpg is produced endogenously in complex organisms. 1,3-Bpg is a reactive species prone to rearrange or react with nucleophiles due to the highly electrophilic acyl-phosphate group and it has been shown to acylate lysine residues. Therefore, ε-*N*-D-3-phosphoglyceryllysine [K(D-pg)] is presumably the PTM formed by the reaction of lysine side chains with 1,3-bpg in a non-enzymatic fashion.[62] Albeit, the stereochemical configuration of the resulting PTM has not been investigated.

Likewise, K(D-pg) modifications are found on regulatory lysine residues in the active sites of glycolytic enzymes; especially, those that transform three-carbon substrates with the only exceptions of phosphoglycerate kinase and bisphosphoglycerate mutase.[62] GAPDH and enolase 1 (ENO1) have both been found to contain K(D-pg) PTMs in their active sites in response to increased glucose concentration, while K(D-pg) sites outside of the active sites remained unchanged under these conditions.[62] The active site modifications appear to lower

the affinity of the putative substrates of GAPDH and ENO1.[62] Thus, K(D-pg) PTMs are proposed to serve as an intrinsic feedback mechanism that regulates product distribution across glycolysis in response to changes in glucose uptake and metabolism.

So far, no enzyme has been reported to remove K(D-pg) modifications, leaving a gap in the knowledge of the regulatory mechanisms of this PTM.

5.3 ε-*N*-(*R*)-Lipoylation

Lipoic acid is a crucial cofactor in cellular metabolism. This organosulfur compound can exist in two enantiomeric forms; however, only the (*R*)-enantiomer is naturally occurring.[63]

ε-*N*-(*R*)-Lipoyllysine [K(*R*)-lip] is a rare PTM in eukaryotes that has been identified in four multimeric enzymatic complexes in mammalian mitochondria.[64] The first two complexes play major roles in mitochondrial homeostasis. Pyruvate dehydrogenase (PDH) transforms pyruvate into acetyl-CoA as the primary entry point of glycolytic products into the TCA cycle and α-ketoglutarate dehydrogenase forms succinyl-CoA as an alternative carbon source for the TCA cycle.[64] The remaining two K(*R*)-lip modified proteins are the branched-chain α-ketoacid dehydrogenase complex and the glycine cleavage system, which are responsible for decarboxylation steps in the catabolism of branched-chain amino acids and glycine, respectively.[64] Given their importance in cellular metabolism, malfunction or dysregulation of these metabolic complexes, especially PDH, can lead to numerous metabolic disorders as well as cancer and Alzheimer's disease.[64]

The enzymatic addition of lipoic acid to lysine residues may occur via two independent pathways. On one hand, lipoic acid is taken up through diet and transferred to lysine residues by a mechanism that is not fully understood in mammals. Alternatively, K(*R*)-lip prosthetic

groups are synthesized *de novo* starting from ε-*N*-octanoyllysine (Koct), through the action of lipoyl synthase (LIAS, Figure 5C).[63]

The removal of K(*R*)-lip modifications has been studied through different techniques. The Denu laboratory screened the *in vitro* deacylation activity of human sirtuins against several substrates, including K(*R*)-lip histone peptides, and found indication of delipoylation activity by SIRT1–4.[65] Cristea and coworkers showed that human SIRT4 can hydrolyze K(*R*)-lip PTMs *in vitro* and can modulate the activity of PDH through delipoylation in living cells.[60] These activities were not recapitulated using small fluorogenic substrates, suggesting that removal of this modification is more sensitive to the surrounding environment of the modified lysine residue within the substrate.[12] More recently, an affinity-based probe was used to explore potential interaction partners of K(*R*)-lip. This chemoproteomic approach did not detect SIRT4 but instead found SIRT2 as a major interaction partner of the modification. Further investigations revealed that SIRT2 presents ~400-fold higher catalytic efficiency compared to that of SIRT4 on a putative lipoylated peptide and overexpression/silencing experiments indicated that SIRT2 modulates K(*R*)-lip levels as well as the activity of endogenous PDH.[66]

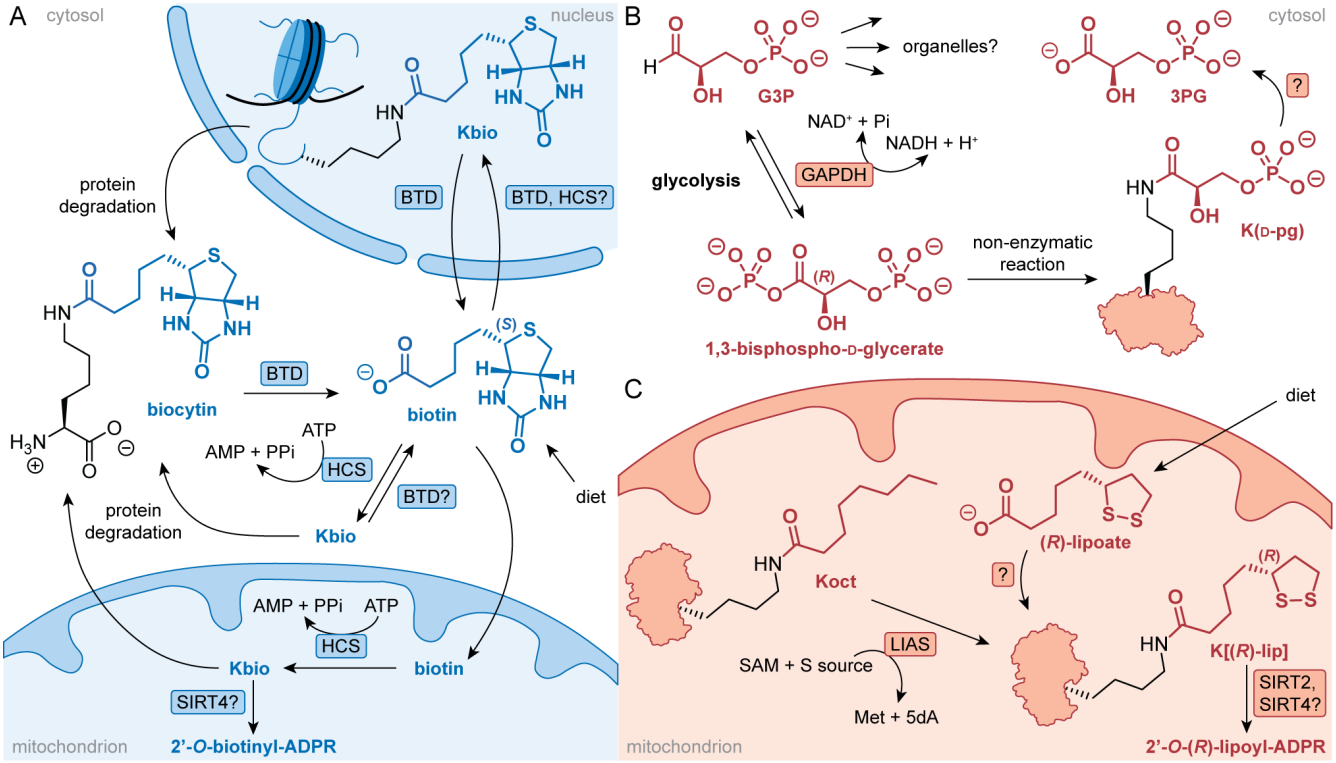

Figure 5. Source and dynamics of additional chiral modifications. (**A**) Proposed cellular formation of ε-*N*-biotinyllysine (Kbio). BTD, biotinidase; HCS, holocarboxylase synthetase. (**B**) Proposed cellular formation of ε-*N*-3-phospho-D-glyceryllysine [K(D-pg)]. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**C**) Proposed cellular formation of ε-*N*-(*R*)-lipoyllysine (K[(*R*)-lip)]). LIAS, lipoyl synthase; SAM, *S*-adenosyl-L-methionine.

6. FUTURE PERSPECTIVES

Over the last 10 years, we have studied the erasers of lysine PTMs and developed chemical tools to elucidate their activity and function. It was therefore exciting to witness the discovery that the chiral carboxylic acids, lactic acid and β-hydroxybutyric acid gives rise to the protein lysine modifications, Kla and Kbhb.[10-11] Especially, because these modifications potentially constitute two separate PTMs each, K(D/L-la) and K(D/L-bhb), the introduction of which could all be rationalized metabolically, giving isomers with distinct metabolic origins and possibly differences in regulation. Taking advantage of the precision of *in vitro* enzymology, we revealed that HDAC1–3 are efficient erasers of lactyl and β-hydroxybutyryl modifications, with a distinct

preference for the K(D-la) and K(D-bhb) isomers, respectively.[4] Sirtuins also display selectivity with SIRT2 more efficiently cleaving K(L-la) than K(D-la) PTMs[26] and SIRT3 showing higher activity against K(L-bhb) than K(D-bhb) modifications.[40] These preferences appear less pronounced for sirtuins than for the Zn^{2+}-dependent HDACs but may still dictate differences in PTM half-lives and metabolite recycling kinetics. Furthermore, the localization of each enzyme to different cellular compartments (*e.g.* HDAC1–3 to the nucleus vs. SIRT3 to the mitochondria) may indicate that the regulatory mechanisms for each PTM across cellular locations are diverse.

Further elements that may dictate differences in diastereomeric PTM regulation are the localization of each isomeric metabolite, and the selectivity of writer enzymes and protein reader domains. Here, dedicated research efforts will be crucial for the elucidation of specific regulatory pathways and potential therapeutic avenues.

The proteomic approaches commonly used to identify lysine PTMs do not distinguish between chiral PTMs.[11] It is our hope that more future studies will begin to address the complexity of chiral PTMs more rigorously using techniques that can distinguish between diastereoisomers. *In vitro* biophysical and biochemical systems are highly controllable through the chemical synthesis of their components, as demonstrated for studying eraser enzymes.[3-4, 26, 40] Similar experiments could help address whether acyltransferase and reader proteins also present diastereomeric selectivity. To extend these detailed studies to the regulation and recognition in cells, it would be highly important to control PTM stereochemistry from the source (*e.g.* metabolite feeding) and/or the detection point of view (*e.g.* specific antibodies). The stereochemistry of exogenous metabolites is easy to control but could lead to ambiguous results due to the interconnection of metabolic pathways, such as those of the D-bhb and L-bhb

species.[34] Thus, specific antibodies with validated specificity using stereopure modified peptide or protein standards would be highly valuable in these investigations. For example, dot-blot experiments have revealed that two commonly used Kla antibodies are highly selective towards the K(L-la) PTM.[4]

Two additional techniques for the discrimination of chiral PTMs in cells are chemoproteomics and site-selective protein modification. (1) Metabolic and activity-/affinity-based probes allow for stereoisomeric control through careful chemical synthesis.[67] Metabolic labeling was employed by Li and coworkers to study K[(*R*/*S*)-hmg] modifications[51] and by Galligan and coworkers to investigate methylglyoxal-derived PTMs,[30] although neither study addressed stereochemistry. In addition, we and others have applied affinity-based probes to identify erasers and readers of lysine PTMs, a platform that is amenable to the incorporation of most chiral and non-chiral modifications known to date.[14, 66, 68] (2) Proteins can be chemically-modified in cells through genetic code expansion,[69] intein-mediated semi-synthesis,[70] or targeted acyl transfer.[71] These technologies enable not only the control of stereochemistry but also of the PTM site, thereby providing further detail of enzyme and reader recognition and potentially of the different stereoisomeric PTM dynamics.

Proteome diversification through posttranslational modification is strongly intertwined with metabolism and in addition to the still outstanding questions related to already identified chiral PTMs, additional metabolites that could result in chiral lysine PTMs can be envisioned. For example, isoleucine catabolism generates the chiral (*S*)-2-methylbutyryl-CoA, (2*S*,3*S*)-3-hydroxy-2-methylbutyryl-CoA, and (*S*)-methylacetoacetyl-CoA species. It is therefore our hope that this Account will provide impetus for studies that carefully distinguish stereoisomers in the investigation of chiral PTMs in the future.

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