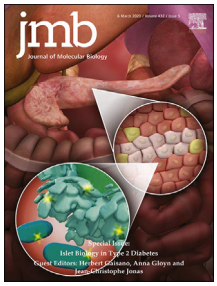

Protein Lysine Acetylation: Grease or Sand in the Gears of β-Cell Mitochondria?

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Abstract

Mitochondria carry out many essential functions in metabolism. A central task is the oxidation of nutrients and the generation of ATP by oxidative phosphorylation. Mitochondrial metabolism needs to be tightly regulated for the cell to respond to changes in ATP demand and nutrient supply. Here, we review how protein lysine acetylation contributes to the regulation of mitochondrial metabolism in insulin target tissues and the insulin-secreting pancreatic β-cell. We summarize recent evidence showing that in pancreatic β-cells, lysine acetylation occurs on a large number of proteins involved in metabolism. Furthermore, we give a brief overview of the molecular mechanism that controls lysine acetylation dynamics. We propose that protein lysine acetylation is an important mechanism for the fine-tuning of mitochondrial activity in β-cells during normal physiology. In contrast, nutrient oversupply, oxidative stress, or inhibition of the mitochondrial deacetylase SIRT3 leads to protein lysine hyperacetylation, which impairs mitochondrial function. By perturbing mitochondrial activity in β-cells and insulin target tissues, protein lysine hyperacetylation may contribute to the development of type 2 diabetes.

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Introduction

Type 2 diabetes (T2D) is the most common metabolic disease. Because of limited exercise and poor energy dense nutrition, an increasing number of individuals develop T2D. The disease is diagnosed because of an elevation of resting blood glucose, elevated HbA1c, or the partial inability to lower blood glucose following a glucose tolerance test. Loss of blood glucose control is due to insufficient insulin secretion in the face of increasing insulin resistance in target tissues. Metabolic changes associated with T2D have severe effects on multiple organ systems leading to complications such as diabetic retinopathy, end-stage renal disease, cardiovascular disease, and diabetic foot disease often requiring limb amputation [1−3].

In healthy individuals, the pancreatic β-cell secretes insulin to stimulate uptake of glucose into large target tissues such as the muscle, liver, and fat. This will rapidly lower blood glucose following a meal. Insulin resistance such as observed in obese individuals prevents the efficient removal of blood glucose. To maintain normal glucose, β-cells oversecrete insulin to compensate for reduced insulin sensitivity [4]. Through such compensation, β-cells may be able to maintain normal glucose homeostasis for many years. Progression to T2D occurs when β-cells fail to compensate for insulin resistance [5,6].

Possible targets to improve metabolic health are mitochondria. Indeed, mitochondrial dysfunction has been proposed as an underlying mechanism impairing both insulin secretion and insulin sensitivity [7−11]. Defective insulin receptor signaling in insulin resistant states lowers mitochondrial function. For example, reduced oxidative capacity and lowered oxidative phosphorylation is observed in skeletal muscle of obese individuals and T2D patients [12−14]. Impaired mitochondrial function in turn interferes with insulin receptor signaling, which promotes the development of insulin resistance.

For example, during aging, ATP synthesis rates are reduced, which explain some of the observed worsening of insulin resistance with age [15].

In β-cells, mitochondria are essential for glucose sensing and insulin secretion. Mitochondrial metabolism of glycolysis-derived pyruvate results in the formation of signals that trigger and amplify insulin secretion [7,10,16–18]. Metabolic stress due to chronic glucose or fatty acid oversupply will lead to β-cell mitochondrial dysfunction lowering glucose-induced insulin secretion. The ability of human islet β-cells from T2D donors to enhance ATP output in response to glucose is poor or absent [19]. Consequently, β-cells in T2D islets fail to respond to glucose despite their continued ability to secrete insulin in response to sulfonylureas [20].

Protein lysine acetylation is an important post-translational modification in the mitochondrial compartment, where it preferentially modifies proteins involved in metabolic processes [21,22]. For a number of proteins, lysine acetylation has been shown to control their function [21,23–25]. Here, we will summarize some earlier findings on protein lysine acetylation in mitochondria of insulin target tissues but focus on recent evidence showing that this posttranslational modification affects β-cell mitochondria. We will discuss how oversupply of nutrients leads to augmented mitochondrial protein lysine acetylation. We propose that excessive mitochondrial protein lysine acetylation contributes to impairment of mitochondrial function in both insulin secretion and action. As a result, protein lysine hyperacetylation may contribute to the development of T2D.

Mitochondria and energy metabolism

Mitochondria are energy-producing organelles [26,27]. They take up macronutrients or derived metabolites to be oxidized in the mitochondrial matrix. Oxidation of nutrients such as glucose-derived pyruvate, fatty acids, amino acids, and ketone bodies reduce nicotinamide adenine dinucleotide (NAD^+) and FAD to NADH and $FADH_2$, which serve as electron donors to complex I and III of the respiratory chain. The acetyl coenzyme A (Ac-CoA) formed during nutrient metabolism is fully oxidized to CO_2 in the tricarboxylic acid cycle (TCA) cycle resulting in additional reducing equivalents for respiration. An additional mechanism to provide reducing equivalents to the respiratory chain is their transfer from the cytosol to the matrix through either the glycerolphosphate or the malate aspartate shuttle [25,28].

Complexes I–IV of the respiratory chain transport electrons derived from NADH and $FADH_2$ to reduce molecular oxygen to water. Electron transport is linked to the export of protons from the mitochondrial matrix to the intermembrane space. In this way, the respiratory chain complexes I, III, and IV establish an electrical potential negatively charged inside and a chemical gradient where the proton concentration in the matrix is low, more alkaline than in the cytosol [29–31]. The electrochemical gradient is the driving force for mitochondrial ATP synthesis. Proton flux through the ATP synthase (complex V of the respiratory chain) is coupled to ATP synthesis from ADP and inorganic phosphate [32]. The newly synthesized ATP is exported from mitochondria in exchange for ADP by adenine nucleotide translocases (ANTs).

Role of mitochondria in β-cell metabolism secretion coupling

Glucose is the primary nutrient secretagogue of the pancreatic β-cell. Rather than activating a plasma membrane receptor, glucose is sensed through its uptake and metabolism [10,16,17]. The link between glucose metabolism and the generation of intracellular signals that stimulate insulin secretion has been termed metabolism-secretion coupling (Fig. 1).

β-Cells express a member of the glucose transporter family at the plasma membrane [33]. The glucose transporter equilibrates glucose across the plasma membrane. Intracellular glucose is recognized above a threshold set by glucokinase, which phosphorylates glucose to glucose 6-phosphate. Glucokinase activity almost perfectly matches the observed rates of glucose utilization, which are proportional to extracellular glucose between 5 and 10 mM [33–35]. Pyruvate is the end product of glycolysis in β-cells [36]. Pyruvate is taken up by mitochondria through the mitochondrial pyruvate transporter [37]. Inside the mitochondria, pyruvate is either oxidized or carboxylated to the TCA cycle intermediate oxaloacetate [34]. Oxidation is markedly accelerated following glucose stimulation. Up to about 10 mM glucose, a very steep correlation between the extracellular glucose and mitochondrial CO_2 production exists [33,38]. In most cell types, mitochondrial oxidative metabolism is reduced as glycolysis accelerates. In pancreatic β-cells, the fraction of glucose oxidized to CO_2 is further increased as glycolysis is stimulated. These observations show how closely glycolysis and mitochondrial metabolism are linked in pancreatic β-cells (Fig. 1). Accelerated substrate oxidation also results in a pronounced respiratory response [39–42]. Stimulation of respiration also augments the electrochemical gradient across the inner mitochondrial membrane [43,44] to accelerate mitochondrial ATP synthesis.

In β-cells, glucose stimulates a number of energy-consuming processes linked to calcium handling

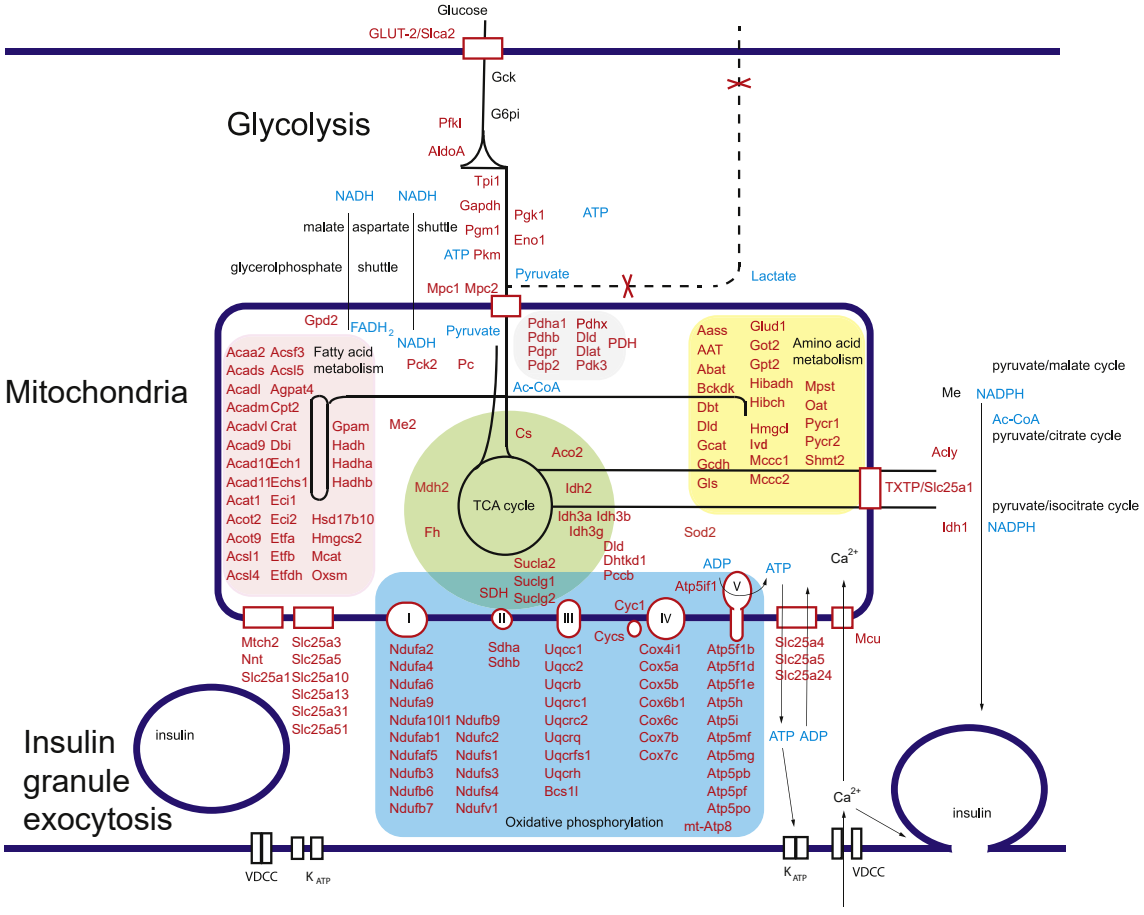

Fig. 1. Metabolism-secretion coupling and mitochondrial lysine-acetylated proteins. Glucose uptake and glycolysis (top) activate mitochondria (center), which generate ATP and other coupling factors stimulating insulin secretion (bottom). Metabolic processes inside mitochondria are highlighted in different colors. TCA cycle (green), oxidative phosphorylation (blue), lipid metabolism (red), and amino acid metabolism (yellow). Only proteins modified by lysine acetylation in insulin-secreting cells or intact islets are listed. In the cytosol and the plasma membrane, only glycolytic enzymes and a few selected proteins involved in metabolism-secretion coupling are shown (proteins displayed with a red font are lysine acetylated). Mitochondrial proteins from other biological processes (mitochondrial ribosomal proteins, protein import machinery, apoptosis regulators, tRNA synthases, protein folding and processing, mitochondrial dynamics, vitamin metabolism, redox biology, nucleotide metabolism, CoA biosynthesis, and proteins of the outer mitochondrial membrane) regulated by lysine acetylation are not shown. For complete data see: [69–71].

and signaling, cytoskeleton dynamics, insulin biosynthesis, and transport [45–47]. Despite the elevated energy demand during glucose stimulation, the cytosolic ATP/ADP ratio increases in response to glucose [48–50]. The ATP/ADP ratio controls the opening probability of the plasma membrane K_{ATP} channel. ATP induces closure, whereas ADP favors opening of the channel [51]. The elevated ATP/ADP ratio following nutrient stimulation causes closure of the K_{ATP} channel resulting in plasma membrane depolarization. Voltage-dependent calcium channels open, and cytosolic calcium transients are initiated. Large calcium rises close to the plasma membrane trigger insulin granule exocytosis [52,53]. The sequence of events linking energy metabolism to the regulation of the K_{ATP} channel and the calcium signals causing insulin secretion is the triggering pathway of insulin secretion. Mitochondria are essential to trigger insulin secretion.

Interestingly, mitochondria also play an important role in a second function linking the effects of glucose to insulin secretion. Glucose is able to augment insulin secretion independent of its ability to trigger insulin secretion. This amplifying effect of glucose was initially revealed under experimental conditions where the K_{ATP} channel was maintained in its open state and insulin secretion induced artificially by depolarizing the plasma membrane. Under these conditions, glucose continued to amplify insulin secretion without however affecting

calcium influx [54]. Under physiological conditions, the triggering and amplifying pathways of insulin secretion act in concert. Since the first description of the amplifying pathway, many metabolites, so-called coupling factors, have been proposed to link glucose metabolism to insulin secretion. We refer to reviews dedicated to metabolism-secretion coupling for a better overview on the amplifying pathway of insulin secretion (reviewed in Refs. [16,17,55]). Here, we will only touch on this topic to stress that mitochondrial metabolism is highly relevant also for the amplification pathway of insulin secretion.

The concentration of TCA cycle metabolites increases during glucose stimulation [34,56]. This addition of intermediates to the TCA cycle (anaplerosis) is initiated by pyruvate carboxylase. This enzyme converts pyruvate to the TCA cycle intermediate oxaloacetate. Anaplerosis increases dramatically with increasing glucose with the steepest increase between 5 and 10 mM [34] and is required to amplify insulin secretion [57,58]. This anaplerosis is linked to the efflux of TCA metabolites from β-cell mitochondria. Once exported, they may act as coupling factors in the cytosol [16,59–61]. A particularly interesting hypothesis is that the exported mitochondrial metabolites such as citrate, isocitrate, and malate are metabolized in the cytosol to generate coupling factors as part of pyruvate cycling pathways [16,57]. These reactions also contribute to glucose-induced formation of NADPH in β-cells. Cytosolic NADPH stimulates insulin granules exocytosis [59,61]. Such mechanisms explain how mitochondrial metabolism can be linked to the amplifying pathway of insulin secretion (Fig. 1).

Regulation of mitochondrial metabolism by protein lysine acetylation

Protein lysine acetylation is a covalent but reversible posttranslational modification. It adds ~42 Da per acetyl moiety to the polypeptide and at the same time changes the positively charged epsilon amino group to an acetylated uncharged lysine side chain [62,63]. Protein lysine acetylation occurs on diverse classes of proteins and can have a variety of effects on protein function [21,63,64]. It can stimulate or inhibit enzyme activity and affect protein–protein interactions, protein stability, or localization [21,24,25,65,66].

Earlier studies provided a global view of the lysine acetylome by means of affinity purification and high-resolution high-accuracy mass spectrometry [64,67]. Of the lysine acetylated proteins, 10–20% localize to mitochondria and another 20–25% to the cytosol [64]. Several large mass spectrometry–based proteomic studies demonstrated that in these compartments, enzymes of many metabolic pathways are acetylated [21,22,68].

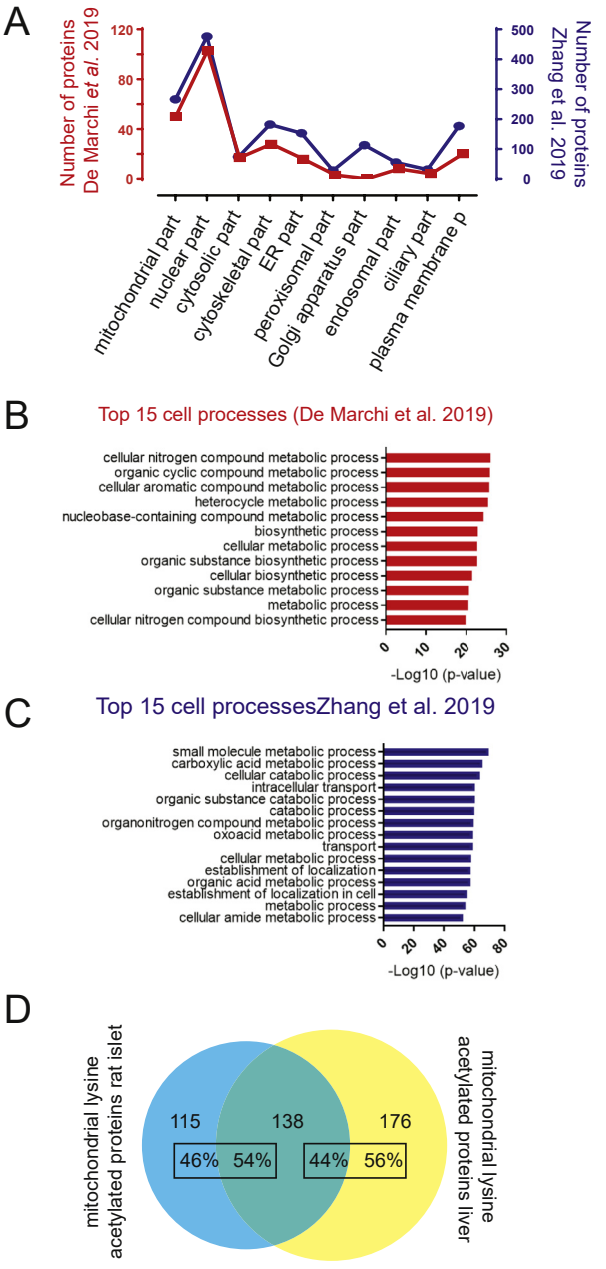

Fig. 2. Acetylome of INS-1E cells and pancreatic islets. (A) Cell component analysis performed with Metacore® (Thomson Reuters). Two independent proteomic studies revealed similar subcellular distribution of acetylated proteins in total lysates from INS-1E cells and rat islets [70,71]. (B and C) Cell process enrichment analysis performed with MetaCore reveals that proteins involved in the cellular metabolism constitutes the main group of acetylated proteins both in INS-1E cells (B) and rat islets (C). (D) Venn diagram displays 50% overlap between mitochondrial lysine-acetylated proteins in rat islets [71] and the liver [68]. Mitochondrial proteins included in the Venn diagram comprise the ones matching with the IMPI database in the category "known to be mitochondrial" (version Q2 2018). http://mitominer.mrc-mbu.cam.ac.uk/release-4.0/impi.do.

Three recent proteomic studies have identified hundreds of lysine-acetylated proteins in insulin-secreting cells and intact rat islets [69–71]. They demonstrated that lysine acetylation in pancreatic β-cells occurs on a large number of proteins with functions in a variety of biological processes similar to findings in insulin target tissues. About 20% of these lysine-acetylated proteins localize to mitochondria (Fig. 2A). Another 20% were found on cytosolic or cytoskeletal proteins, with 30–40% in the nucleus. Proteins-associated with metabolic processes were preferentially acetylated (Fig. 2B and C) similar to observations in other tissues. Among the top 15 enriched pathways, almost all were linked to metabolism. Looking closer into metabolism, lysine acetylation was detected on enzymes of the glycolytic pathway, all enzymes of the TCA cycle, the majority of respiratory chain complex proteins including the ATP synthase, and enzymes involved in amino acid as well as fatty acid metabolism (Fig. 1). In addition, proteins mediating the exchange of metabolites across the inner mitochondrial membrane and several cytosolic enzymes important for the generation of coupling factors amplifying glucose-induced insulin secretion appear to be controlled by lysine acetylation (Fig. 1). This posttranslational modification therefore has the potential to influence metabolism-secretion coupling at multiple steps along both the triggering and amplifying pathway of insulin secretion.

A large overlap between mitochondrial lysine-acetylated proteins in β-cells and liver can be observed (Fig. 2D). Of the mitochondrial lysine-acetylated proteins identified in rat islets, 54% are also acetylated in the liver (Fig. 2D). Even when looking at lysines acetylated in individual proteins, there is a clear overlap between the modified sites identified with mass spectrometry in the two tissues. Among the limited number of acetylation sites with published regulatory function, many were also acetylated in total lysates from INS-1E cells or whole islets (for examples, see Fig. 3). Many lessons learnt on the role of mitochondrial protein lysine acetylation in insulin target tissues may therefore be relevant for the regulation of mitochondrial metabolism in the pancreatic β-cell.

The list of lysine-acetylated proteins in β-cells is rapidly growing benefiting from developments in

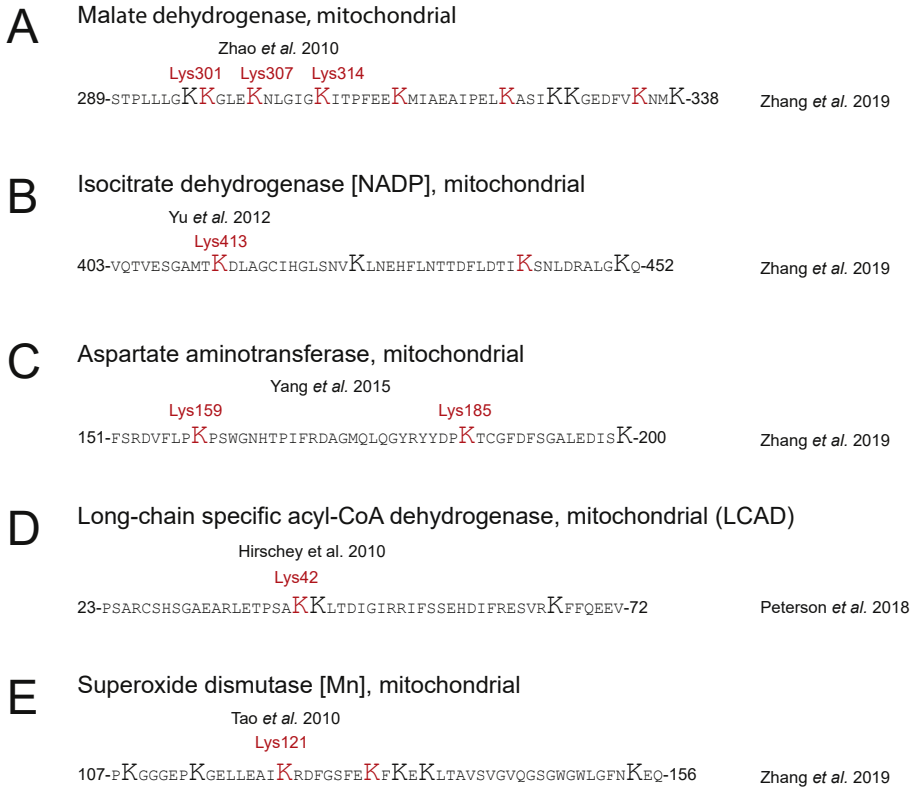

Fig. 3. Control of protein function by lysine acetylation. Part of the amino acid sequence with regulatory lysine acetylation sites from (A) malate dehydrogenase, (B) isocitrate dehydrogenase, (C) aspartate aminotransferase, (D) long-chain acyl-CoA dehydrogenase, and (E) superoxide dismutase (Mn). A larger font is used for lysine residues (K). In red are those K-residues found to be acetylated in INS-1E cells [69] or rat islets [71]. Acetylation of the corresponding lysine residues on top of the sequence have been shown to be regulatory by Refs. [21,23–25,99].

proteomic technologies [72]. It should be kept in mind, however, that for the majority of the mitochondrial proteins lysine acetylation sites, regulatory function has not been established. In particular, for many lysines, acetylation stoichiometry may be low. If only a small sub-fraction of a specific lysine is acetylated (i.e., <10%), then a regulatory role of this particular site is less likely. More information needs to be gathered on the role of individual acetylation sites and their impact on protein function before we understand the effects of lysine acetylation on (mitochondrial) metabolism in pancreatic β-cells.

Mechanisms of lysine acetylation in mitochondria

Lysine acetylation sites undergo cycles of acetylation and deacetylation that regulate protein function such as enzymatic activity. Advancement in the understanding of dynamic mitochondrial protein lysine acetylation has been hampered by the relative lack of knowledge regarding mitochondrial lysine acetyltransferases.

In 2012, GCN5L1/BLOC-1 subunit 1 was identified as the first potential mitochondrial lysine acetyltransferase [75]. GCN5L1 has a dual cytosolic and mitochondrial localization. GCN5L1 displays sequence homologies with the nuclear lysine acetyltransferase GCN5. When overexpressed, GCN5L1 increased lysine acetylation of two OXPHOS subunits. Knockdown of GCN5L1 specifically lowered lysine acetylation in mitochondria and stimulated oxygen consumption [75]. In transgenic mice overexpressing GCN5L1 in the liver, hyperacetylation of a number of mitochondrial proteins was observed most notably on several proteins involved in fatty acid oxidation [76]. Liver cells lacking GCN5L1 displayed increased rates of palmitate oxidation, which *in vivo* protected them from hepatic lipid accumulation after exposure to a high-fat diet. Regulation is in part mediated by acetylation of the α subunit of the trifunctional enzyme in the fatty acid oxidation complex. These data suggest that GCN5L1 is a mitochondrial lysine acetyl transferase important for the regulation of nutrient oxidation and oxidative phosphorylation. GCN5L1 has not been studied in pancreatic β-cells, but it is expressed at the RNA level in human β-cells [77].

Recent work has revealed that the p300/CBP-associated factor (PCAF) works as a lysine acetyl transferase inside mitochondria as well. PCAF has a mitochondrial targeting sequence and is localized to both the nucleus and mitochondria [78]. In the nucleus, PCAF is a coregulator of transcription through the lysine acetylation of histones and transcription factors. In the mitochondrial matrix, PCAF acetylates isocitrate dehydrogenase 2 (NADP) (IDH2) on lysine residue 180, which interferes with the catalytic mechanisms of isocitrate binding and oxidation (Fig. 3B). In this way, PCAF-mediated lysine acetylation may affect intermediary metabolism. Acetylation of lysine 180 in IDH2 was observed in both INS-1 cells [69,70] and intact rat islets [71] suggesting PCAF is not only expressed [77] but also active in β-cell mitochondria. Inhibition of IDH2 in β-cells may slow down TCA cycle activity and oxidative phosphorylation, therefore lowering insulin secretion. Alternatively, because the upstream metabolites citrate and isocitrate can be transported out of mitochondria as part of the pyruvate/citrate or pyruvate/isocitrate cycle [16], IDH2 inhibition may stimulate the amplifying pathway of insulin secretion. In this model, inhibition of IDH2 inhibition would permit oxidative metabolism and at the same time stimulate the formation of cytosolic coupling factors potentiating insulin secretion. How regulation of IDH2 by acetylation affects insulin secretion remains to be studied.

Given the large number of metabolic enzymes modified by lysine acetylation in the mitochondrial matrix space, it was proposed that the majority of protein lysine acetylation in mitochondria is due to nonenzymatic modification of protein lysine residues [79,80]. These acetylations would occur as a result of enzyme-independent reactions between the epsilon amino group of lysine side chains on proteins with Ac-CoA. However, the epsilon amino group of lysine is not a very good nucleophile at physiological pH as a very large fraction is in its protonated state. Interestingly, the mitochondrial matrix space is more alkaline than the cytosolic pH, which may therefore favor spontaneous lysine acetylation inside mitochondria. The presence of high Ac-CoA concentrations in the mitochondrial matrix space should also favor spontaneous lysine acetylation. This mechanism is therefore directly dependent on nutrient metabolism such as fatty acid oxidation leading to the formation of Ac-CoA [74].

Mitochondrial export of Ac-CoA can be promoted by carnitine acetyltransferase (CrAT) [80], which catalyzes the exchange of the acetyl group on CoA to carnitine. In skeletal muscle of CrAT knockout (KO) mice, tissue Ac-CoA concentrations were elevated and the acetylation of many mitochondrial proteins increased. These results suggest excessive lysine acetylation in the mitochondrial matrix can be prevented by lowering the matrix Ac-CoA concentration.

Nonenzymatic protein lysine acetylation is an attractive concept as it may explain how chronic nutrient oversupply can cause mitochondrial lysine hyperacetylation.

Lysine deacetylation and other posttranslational modifications controlled by sirtuins

Sirtuins are a family of protein that use the oxidized form of NAD^+ as a cofactor of their catalytic function [62,81–83]. NAD^+ is consumed during protein deacylation or ADP-ribosylation.

Several sirtuins deacetylate lysine residues by catalyzing the transfer of the acetyl group onto the ADP-ribose moiety of NAD^+ forming 2′-O-acetyl-ADP-ribose and free nicotinamide [62]. The availability of NAD^+ for this deacetylation reaction depends on the redox state of NADH/NAD^+. Based on their use of NAD^+ as a cofactor, sirtuins have been proposed to act as sensors of NAD^+ and energy metabolism [81,86,89,90]. In the following, we will focus on SIRT3 and mitochondrial deacetylation in β-cell nutrient sensing. Nevertheless, it should be mentioned that several other sirtuins are important for β-cell secretory function and health. This was for instance clearly demonstrated for the lysine deacetylase SIRT1. Overexpressing SIRT1 in β-cells improves glucose tolerance in mice because of augmented glucose-induced insulin secretion [91]. SIRT6 also plays an essential role in β-cells. SIRT6 deacetylates FoxO1 to maintain a β-cells transcriptional program inducing, for example, the expression of the β-cell transcription factor Pdx1. In mice lacking SIRT6 expression in β-cells, glucose-induced insulin secretion is disrupted, and the animals become severely glucose intolerant [92]. The role of SIRT1 and SIRT6 as well as other sirtuins in β-cell function has been discussed in several recent reviews [93–96].

Three members of the sirtuin family SIRT3, SIRT4, and SIRT5 localize to the mitochondrial matrix [84,85]. SIRT4 is a deacylase removing methylglutaryl, hydroxymethylglutaryl, and 3-methylglutaconyl groups from lysine residues [87]. SIRT5 has weak deacetylase activity, but its main function is to remove malonyl or succinyl groups from lysines carrying these posttranslational modifications [88]. SIRT3 is the main lysine deacetylase in the mitochondrial matrix [84,85]. In cells or tissue lacking SIRT3, a large number of mitochondrial proteins, in particular, proteins of oxidative metabolism and energy production are hyperacetylated [22,68,73]. For example, hyperacetylation of complex I, complex II, and ATP synthase subunits in mice lacking SIRT3 impaired mitochondrial energy metabolism [97,98]. Consequently, basal ATP levels were reduced. In addition, fatty acid oxidation was slowed in mice lacking SIRT3. Hyperacetylation of long-chain acyl-CoA dehydrogenase (LCAD) inhibits enzyme activity and can be restored following deacetylation by SIRT3 [99] (Fig. 3D). By deacetylating subunits of the respiratory chain or fatty acid oxidation complex subunits, SIRT3 activity improves cellular energy homeostasis.

SIRT3 is expressed in INS-1 insulinoma cells [69,70,100] and pancreatic islets [71,100,101]. In β-cells lacking SIRT3, hyperacetylation was observed in enzymes of the TCA cycle, the electron transport chain, the ATP synthase, ANTs, fatty acid oxidation, amino acid metabolism, elimination of reactive oxygen species (ROS), and protein quality control [69,70]. Manipulation of SIRT3 expression in INS-1 insulinoma cells caused differential lysine acetylation of only a subfraction (e.g., 16% in De Marchi et al., 2019 and 22% in Peterson et al., 2018) of all the lysine-acetylated peptides quantified in control and SIRT3 KO cells. These findings are similar to lysine acetylome analysis in several tissues of SIRT3 knockout mice. Only 10–30% of the quantified lysine residues were hyperacetylated after disruption of the *SIRT3* gene [22,68]. Therefore, SIRT3 deacetylates only a specific subset of acetylated lysine residues in mitochondria. It is likely that additional mechanisms contribute to lysine deacetylation inside mitochondria.

In conclusion, mitochondrial lysine acetyl transferases and deacetylases control lysine acetylation dynamics. Interestingly, these enzymes are influenced by the matrix Ac-CoA concentration or the mitochondrial NADH/NAD^+ redox state, respectively, generating interesting links between metabolism and this posttranslational modification. Poor control of the balance between lysine acetylation and deacetylation results in mitochondrial dysfunction.

SIRT3 function in glucose-induced insulin secretion

Much of what we know about the regulatory role of protein lysine acetylation on mitochondrial metabolism has been obtained indirectly through the study and manipulation of expression of the lysine deacetylase SIRT3 [22,97,99,102]. Loss of SIRT3 function in β-cells causes dysregulation of insulin secretion [70,71,100,101]. Knocking down SIRT3 expression lowered the expression of PDX-1 and MafA, two essential transcription factors in pancreatic β-cells. As a result, expression of *Ins1* and *Slc2a2* mRNAs encoding insulin and GLUT2, respectively, was reduced [100,101]. Lowering SIRT3 expression in insulin-secreting cells therefore strongly decreased their insulin content [70]. Because the insulin content is strongly reduced, both basal and glucose-induced insulin secretion when expressed per content are actually higher in INS-1E cells lacking SIRT3. Importantly, the fold nutrient-induced insulin secretion is reduced, demonstrating a role for SIRT3 in metabolism-secretion coupling [70,100,101]. Another carefully executed study observed robust glucose-induced insulin secretion in INS-1 832/13 SIRT3ko cells [69]. Overexpression of SIRT3 in these KO cells

corrected mitochondrial lysine hyperacetylation but without improving insulin secretion. These findings are hard to reconcile with the strong phenotype described by Caton et al., 2013, Zhou et al., 2017, and De Marchi et al., 2019. A possible explanation is that both insulin expression and glucose-induced insulin secretion are particularly robust in the INS-1 832/13 cells. In this INS-1 clone, inhibitory mechanisms such as lysine hyperacetylation may go undetected, as mechanisms promoting insulin expression and secretion are particularly strong.

Glucose-induced insulin secretion is also about 2-fold reduced in SIRT3 KO mouse islets [101]. During a glucose tolerance test in SIRT3 KO mice, fasting insulin concentrations were lower, and the glucose-induced increase was markedly reduced [101]. In vitro, glucose-induced insulin secretion was impaired in SIRT3 KO islets and restored by adenovirus driven expression of SIRT3. In a different mouse strain, glucose-induced insulin secretion was normal in SIRT3 mice when fed a standard diet. A phenotype was only revealed when animals were fed a high-fat high-sucrose diet [69].

Despite these strain-specific differences regarding the role of SIRT3 in β-cell function, most of the current work demonstrates that SIRT3 dysfunction and the associated lysine hyperacetylation impair glucose-induced insulin secretion.

SIRT3 regulation of β-cell energy metabolism and calcium signaling

In β-cells, many proteins of the respiratory chain are lysine acetylated similar to findings in insulin target tissues [97,98,103]. In particular, several subunits of the ATP synthase were found to be hyperacetylated in insulin secreting cells [69,70] and rat islets lacking SIRT3 [71]. In addition, we found inhibitory factor 1 (IF1) as the most hyperacetylated protein in our INS-1E SIRT3 KO cell line [70]. IF1 is a regulator of the ATP synthase [104,105]. Interaction between IF1 and ATP synthase may be regulated by lysine acetylation, which would add another mechanism of how lysine acetylation modulates oxidative phosphorylation. Two main respiratory phenotypes were observed in INS-1E cells lacking SIRT3. First, basal respiration was elevated. Second, glucose-induced acceleration of respiration was impaired [70]. After glucose stimulation, ATP synthase–dependent respiration in SIRT3 KO cell was 50% lower than in control INS-1E cells. Comparable energetic defects have been described in a number of tissues from SIRT3 KO mice [97,98].

Impaired respiration in MIN6 and INS-1E cells strongly reduced the glucose-dependent ATP increase [70,101]. The glucose-dependent ATP increase was also blunted in SIRT3 KO islets and rescued by SIRT3 overexpression [101]. In SIRT3 KO INS-1E cells, glucose increased ATP transiently but failed to maintain ATP elevated beyond the first few minutes after stimulation [70]. This energetic phenotype suggests lysine hyperacetylation of respiratory chain complex subunits lowers the ability of β-cells to respond to glucose. Such impaired glucose-dependent ATP synthesis likely contributes to the observed metabolism-secretion coupling defects in SIRT3 KO β-cells.

A surprising phenotype of the SIRT3 KO INS-1E cells is strongly elevated cytosolic calcium transients under resting glucose conditions [70]. Overactivation of mitochondria as suggested by the elevated respiratory rates under resting glucose conditions may increase the ATP/ADP ratio sufficiently to cause closure of the K_{ATP} channel and the triggering of insulin secretion. In response to glucose, cytosolic calcium signaling was very poor. Furthermore, glucose-induced mitochondrial calcium rises were blunted in SIRT3 KO INS-1E cells. Impaired mitochondrial calcium signaling likely worsened the ability of SIRT3 KO cells to maintain an elevated ATP/ADP ratio during sustained glucose stimulation. Reexpression of SIRT3 rescued calcium signaling in the SIRT3 KO cells confirming a close link between SIRT3 function and metabolism-secretion coupling.

In summary, mitochondria of SIRT3 KO insulin secreting cells present an energetic defect. From a mitochondrial energetics point of view, lysine acetylation therefore only seems to play an inhibitory role. The physiological benefit of such an exclusively inhibitor mechanism is not obvious. We speculate that mitochondrial protein lysine acetylation of respiratory chain complex subunits may be a way to add an additional level of regulation. In the beta cell, for instance, maximal oxygen consumption is not desirable as the ATP output is linked to insulin secretion. As insulin secretion needs to be adjusted to the short- and long-term physiological needs, mitochondrial lysine acetylation may be a mechanism to fine-tune ATP production. This type of regulation is partially lost in insulin-secreting cells lacking SIRT3 function. The consequence is basal hypersecretion and poor glucose-induced mitochondrial ATP synthesis and insulin secretion.

Mitochondrial lysine acetylation and acute glucose–induced insulin secretion

During elevated ATP demand, such as in exercising muscle, NADH consumption is enhanced, and NAD^+ levels increase (low NADH/NAD^+ ratio) [89]. This provides more NAD^+ as a cofactor for SIRT3-dependent protein deacetylation. By deacetylating, for example, subunits of the respiratory chain, SIRT3

may stimulate respiration to balance the high energy demand during exercise.

In contrast to muscle, changes in the NADH/NAD^+ in pancreatic β-cells are nutrient driven. Acute glucose stimulation rapidly increases the NADH/NAD^+ ratio over the time course of about a minute [50,106,107]. In the continuous presence of stimulatory glucose, the NADH/NAD^+ ratio remains elevated. Continued glucose stimulation therefore lowers matrix NAD^+ and may inhibit SIRT3 activity causing an increase in lysine acetylation. Inconsistent with this possibility, we did not observe any increase in protein lysine acetylation in mitochondrial after 30 min of glucose stimulation [70]. Enhanced metabolic flux and acceleration of respiration known to occur following glucose stimulation of insulin-secreting cells are therefore not accompanied by regulation of the involved metabolic enzymes by lysine acetylation. The observed net changes in NADH/NAD^+ ratio do not affect SIRT3 activity sufficiently to cause acute glucose-induced changes in mitochondrial protein lysine acetylation. Extending glucose stimulation (16.7 mM) to 6 h as shown by Zhang et al. was not sufficient to promote consistent hyperacetylation of mitochondrial proteins either [71]. We conclude a short-lasting glucose rise, for example, following a meal does not alter the acetylation status of mitochondrial proteins in β-cells.

Effect of the high-fat diet on mitochondrial protein lysine acetylation

The high-fat diet promotes protein lysine hyperacetylation. In the liver, for example, mitochondrial protein acetylation is markedly increased when mice are challenged with a high-fat diet [73,108,109]. Similarly, in skeletal muscle, chronic high-fat feeding elevates mitochondrial protein lysine acetylation [80,110]. The number of lysine acetylated proteins identified after high-fat feeding almost doubled in this tissue. Because hyperacetylation of LCAD and hydroxyacyl-CoA dehydrogenase trifunctional enzyme subunit α (HADHA) inhibits fatty acid oxidation [99], pushing the balance towards more lysine acetylation likely contributes to liver fat accumulation [73,76]. Lowering mitochondrial lysine acetylation should therefore be beneficial. Consistent with this conclusion, mice lacking the lysine acetyl transferase GCNL1 are protected from enhanced fat deposition in the liver [111].

High fat-induced hyperacetylation seems less pronounced in pancreatic islets *in vivo*. Islet proteins from mice on a high-fat high-sucrose diet showed no clear difference in the abundance of lysine-acetylated proteins. Nevertheless, the high-caloric diet seemed to stimulate acetylation also in this cell type. In islets from SIRT3 KO animals, lysine acetylation was more pronounced in animals eating the high-fat high-sucrose diet [69]. Together, these results suggest that SIRT3 activity in wild-type β-cells is able to keep lysine acetylation under control despite nutrient-induced acceleration of the acetylation reaction.

Characterization of the whole body SIRT3 KO mice has provided strong evidence for the link between overnutrition, hyperacetylation, and mitochondrial dysfunction. SIRT3 KO animals that fed a normal diet do not have a striking metabolic phenotype. They display normal body weight and composition. Physical activity, oxygen consumption, and the respiratory quotient were not different between control and SIRT3 KO animals, although hyperacetylation of mitochondrial proteins was apparent [85]. On a high-fat diet, the KO mice showed accelerated weight gain, developed insulin resistance, and associated glucose intolerance [73]. They also had dyslipidemia and developed a fatty liver. The results suggest that metabolically SIRT3 KO mice are able to compensate despite early mitochondrial impairment because of lysine hyperacetylation. A high-fat diet pushes them over the edge with regard to the development of insulin resistance and the development of the metabolic syndrome.

Assessment of similar SIRT3 KO mice showed that the high-fat diet also worsens β-cell function. Peterson et al. showed that their SIRT3 KO mice display normal glucose-induced insulin secretion when fed a standard diet [69]. In vitro, islets from these animals showed normal biphasic insulin secretion. In contrast, when the animals were fed a high-fat high sucrose diet, basal insulin secretion and insulin secretion during an intraperitoneal glucose tolerance test were strongly reduced. In islets isolated from high-fat-fed SIRT3 KO animals, first- and second-phase insulin secretion were reduced [69]. The high-fat diet negatively influences insulin secretion and action when SIRT3 function is impaired.

Diet-induced hyperacetylation can be mimicked *in vitro* [21,25,74,101,112]. Several mitochondrial enzymes were shown to be hyperacetylated after culture in medium containing either high palmitate or high glucose. These nutrient conditions are frequently used in β-cell research to induce gluco- or lipotoxic stress. Following several days of culture in elevated glucose concentrations, beta cells loose part of their glucose responsiveness [113]. Whether lysine hyperacetylation of mitochondrial proteins plays a role in chronic glucose-induced β-cell dysfunction is not known. Prolonged exposure to the long-chain fatty acid palmitate also impairs β-cell function increasing basal insulin secretion and blunting the glucose-stimulated response [71,112]. Chronic exposure to palmitate markedly increased protein lysine acetylation in INS-1E cells and human islets [112]. SIRT3 overexpression decreased

palmitate-induced basal hypersecretion of insulin in rat islets [71]. Lysine hyperacetylation of β-cell proteins may therefore be one of the several mechanisms contributing to palmitate-induced β-cell dysfunction.

Under metabolically challenging conditions such as chronic exposure to a high-caloric diet, the ability to efficiently deacetylate proteins becomes critical. To make things worse, the high-fat diet lowers NAD^+ concentrations in several metabolically important organs [114–116]. Because sirtuin deacetylases depend on NAD^+ as a cofactor, their enzymatic activity is reduced when mice are on a high-fat diet. From a mitochondrial perspective, lowered NAD^+ concentrations with impaired sirtuin function will cause hyperacetylation and impaired energy production. The importance of NAD^+ biosynthesis for metabolic health has been demonstrated, for example, in mice with reduced nicotinamide phosphoribosyltransferase (Nampt) activity [117]. Nampt synthesizes nicotinamide mononucleotide (NMN), a precursor of NAD^+. Female $Nampt^{+/-}$ mice have reduced plasma NMN concentrations, have impaired glucose-induced insulin secretion, and are moderately glucose intolerant. Interestingly, the mice were fully rescued following NMN injection. Similarly, NMN caused a marked improvement of glucose tolerance in high-fat diet–induced diabetic mice [114]. In addition, NMN dramatically improved β-cell function in islets from mice fed a fructose-rich diet [118]. SIRT1 is one of the mediators of the metabolic health benefit of NMN [91,114,118]. In the β-cell, some of the health benefits of NMN may also be mediated by SIRT3 as knockdown of SIRT3 prevented the protective effects of NMN to inflammatory cytokine exposure [100]. Restoring NAD^+ levels in beta cells and insulin target tissues using NMN or nicotinamide riboside is being pursued as a strategy to maintain metabolic health [115,116]. An alternative strategy is the direct activation of sirtuins to regain a normal balance between protein acetylation and deacetylation [119,120].

Diet-dependent SIRT3 expression

Diet-dependent expression of lysine acetyl transferases and deacetylases is an additional mode of regulation of lysine acetylation. In the mouse liver, a chronic high-fat diet lowers SIRT3 expression [73,108,109] and causes a marked induction of GCNL1 expression [111]. The net effect of these expression changes is consistent with the observed lysine hyperacetylation of liver mitochondrial proteins in mice on a high-fat diet.

Similarly, SIRT3 expression in islets from mice consuming a high-fat diet is strongly reduced. Differences with the control were visible after 2 weeks and pronounced after 8 weeks on the high-fat diet [101]. These effects of the high-fat diet are mimicked *in vitro* by culturing the islets in the presence of palmitate. Within 72 h, SIRT3 expression in mouse islets was markedly lower when palmitate was included in the culture medium [101]. In human islets, incubation for 24 h in the presence of elevated palmitate concentrations was sufficient to increase the acetylation status of a subset of lysine acetylated proteins in total lysates [112].

Several other signals causing β-cell stress are known to lower SIRT3 expression [101]. For instance, the proinflammatory cytokines TNFα and IL1β reduce SIRT3 mRNA and protein in INS-1E cells and mouse islets [100]. Cytokines are part of the inflammatory response observed in islets during T2D progression [121]. Importantly, SIRT3 mRNA levels were strongly reduced (−60%) in islets from individuals with severe T2D [100]. There appears to be a strong correlation between reduced SIRT3 expression and β-cell dysfunction.

Fasting, caloric restriction, or exercise training induces SIRT3 expression in insulin target tissues [89,99,122,123]. These interventions promote mitochondrial lysine deacetylation and mitochondrial energy metabolism. As a result, SIRT3 may improve conditions linked to metabolic stress and dysfunction such as obesity and T2D (reviewed in Refs. [83,90]).

SIRT3 mRNA expression in islets from 24-h-fasted mice is reduced [100], and glucose- and leucine-stimulated insulin secretion are lowered [100]. This is consistent with early findings showing fasting-induced suppression of insulin secretion in rat islets [124]. SIRT3 expression levels are faithfully correlated to the ability of β-cells to respond to nutrients. Augmented lysine acetylation in beta-cell mitochondria during fasting may be a mechanism to lower their energy metabolism to prevent the triggering of insulin secretion that otherwise would be harmful as blood glucose levels are already low.

SIRT3 protects β-cell function and viability

β-Cell dysfunction in islets lacking SIRT3 function is aggravated under nutrient stress such as the high-fat diet. Hyperacetylation following SIRT3 disruption also affects β-cell health. Lowering SIRT3 expression in insulin-secreting cells strongly increased the formation of cytokine-induced caspase-3 activation and cell death [100]. SIRT3 gene disruption also sensitizes mouse β-cells to undergo apoptosis in a diet-dependent manner. β-Cell death was only modestly increased in islets from SIRT3 KO mice on a standard diet. In islets from high-fat-fed animals, β-cell apoptosis was dramatically increased and further doubled in islets from SIRT3 KO animals [101]. These findings demonstrated that loss of SIRT3 function renders the β-cells more sensitive to the nutrient stress. However, disruption of the SIRT3

gene alone is not sufficient to induce β-cell death. The reduction of SIRT3 expression following chronic palmitate treatment is one of several mechanisms leading to lipotoxicity-induced β-cell death.

On the other hand, overexpression of SIRT3 is protective. Overexpression of SIRT3 in insulin-secreting cells lowered the level of lysine-acetylated proteins and reduced palmitate-induced caspase-3 activity. Impaired insulin secretion from rat islets after palmitate treatment was also restored when SIRT3 was overexpressed [125]. SIRT3 may improve β-cell dysfunction by interfering with ROS formation and ROS signaling [101]. This is important as excessive ROS formation is one of the stressors leading to β-cell dysfunction and loss.

Oxidative stress induced by H_2O_2, cytokines, or palmitate was more pronounced in insulin-secreting cells lacking SIRT3. For instance, oxidative stress markers were strongly increased in islets from SIRT3 KO mice. ROS activate a retrograde signaling pathway from the mitochondria to the nucleus via phosphorylation of the c-Jun N-terminal kinase (JNK). Through the stimulation of JNK, oxidative stress inactivates PDX-1. This signaling pathway is overstimulated in β-cells lacking SIRT3. SIRT3 overexpression protected β-cells reducing JNK signaling and preventing cleavage of caspase-3 [100,101,125].

SIRT3 exerts its protective role in part through the direct inhibition of mitochondrial ROS formation. SIRT3 deacetylates and thereby activates the mitochondrial manganese-dependent superoxide (MnSOD/SOD2) dismutase [23,65]. Acetylation of lysine residue 122 on MnSOD has been shown to inhibit enzyme activity in the liver [23](Fig. 3E). Acetylation of lysine 122 has also been observed in INS-1E cells [69] and rat islets [71](Fig. 3E). Knockdown of SIRT3 significantly increased MnSOD acetylation [101]. This regulatory mechanism is shared between the liver and pancreatic β-cells. Chronic palmitate stress caused a pronounced hyperacetylation of MnSOD in insulin-secreting cells. SIRT3 overexpression reversed hyperacetylation of the enzyme. The findings suggest SIRT3 lowers ROS through the deacetylation of MnSOD.

Together, these studies highlight the importance of SIRT3 in the maintenance of β-cell function. MnSOD is just one example to illustrate the beneficial effect of lysine deacetylation in β-cells. The many other acetylation sites regulated by SIRT3 are likely equally important to control mitochondrial function.

Conclusion

Today, we still know relatively little about the physiological role of mitochondrial protein lysine acetylation in the regulation of metabolism. As we start to learn more about the regulation of individual enzymes by lysine acetylation, we will likely get a better understanding of the big picture as well. Lysine acetylation is a way of controlling enzyme activity. Its role may be to control the activity of the existing enzyme pool rapidly without the need for protein synthesis or degradation. For example, the control of mitochondrial MnSOD is such that ROS are controlled at concentrations that are compatible with their signaling function without reaching levels that are toxic for the cell. Protein lysine acetylation also affects protein complexes. Many subunits of the respiratory chain undergo lysine acetylation. Dynamic acetylation of these complexes may allow mitochondria to adjust their respiratory rate to either ATP demand or nutrient supply. In this sense, protein lysine acetylation may be the grease in the gear of mitochondria that allow them to accelerate or slowdown in response to physiological nutrient changes.

In contrast, chronic nutrient stress may lock proteins in a state of hyperacetylation. Under these conditions, lysine acetylation will act like sand in the gears of mitochondria. Hyperacetylation of mitochondrial proteins such as in animals on a high-fat diet impairs mitochondrial function. This will have negative consequences on β-cell function and insulin target tissues.

We propose that protein lysine hyperacetylation should be added to the list of molecular mechanisms that can perturb mitochondrial function with the potential to accelerate the development of T2D. Restoring lysine acetylation dynamics in mitochondria would be a strategy to improve pancreatic β-cell health and function as well as metabolic health in target tissues.

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Abbreviations used:
Ac-CoA, acetyl coenzyme A; ANT, adenine nucleotide translocase; CrAT, carnitine acetyltransferase; IF1, inhibitory factor 1; JNK, c-Jun N-terminal kinase; Nampt,

nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; PCAF, p300/CBP-associated factor; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; T2D, Type 2 diabetes.

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