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# Mitochondrial Function, Metabolic Regulation, and Human Disease Viewed through the Prism of Sirtuin 4 (SIRT4) Functions

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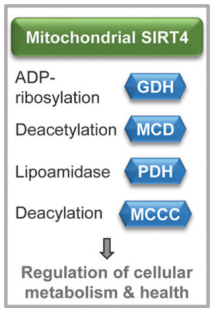
## Abstract

As cellular metabolic hubs, mitochondria are the main energy producers for the cell. These organelles host essential energy producing biochemical processes, including the TCA cycle, fatty acid oxidation, and oxidative phosphorylation. An accumulating body of literature has demonstrated that a majority of mitochondrial proteins are decorated with diverse posttranslational modifications (PTMs). Given the critical roles of these proteins in cellular metabolic pathways and response to environmental stress or pathogens, understanding the role of PTMs in regulating their functions has become an area of intense investigation. A major family of enzymes that regulate PTMs within the mitochondria are sirtuins (SIRTs). Albeit until recently the least understood sirtuin, SIRT4 has emerged as an enzyme capable of removing diverse PTMs from its substrates, thereby modulating their functions. SIRT4 was shown to have ADP-ribosyltransferase, deacetylase, lipoamidase, and deacylase enzymatic activities. As metabolic dysfunction is linked to human disease, SIRT4 levels and activities have been implicated in modulating susceptibility to hyperinsulinemia and diabetes, liver disease, cancer, neurodegeneration, heart disease, aging, and pathogenic infections. Therefore, SIRT4 has emerged as a possible candidate for targeted therapeutics. Here, we discuss the diverse enzymatic activities and substrates of SIRT4 and its roles in human health and disease.

## Graphical Abstract

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**Keywords**

SIRT4; metabolic regulation; mitochondria; PTMs; diabetes; liver disease; cancer; neurodegeneration; cardiovascular disease; aging

## INTRODUCTION

Producing the majority of the chemical energy needed for cells to survive, mitochondria are at the core of cellular homeostasis, as well as the ability of cells to respond to changing environmental conditions or pathogen infections. As such, mitochondrial dysfunction is a characteristic of several human diseases, including cancer, cardiovascular disease, diabetes, viral infection, and neurodegeneration. It has been known for a long time that posttranslational modifications (PTMs) can be essential regulators of mitochondrial protein function, with biotinylation and lipoylation being some of the first ones to be functionally characterized.[1] Methodology and technology advancements in the past decade, including improved PTM enrichment workflows and mass spectrometry instrumentation, have provided a better understanding of the complexity of modifications present on mitochondrial proteins.

Studies investigating protein lysine acetylation have uncovered the prominence of this modification on metabolic enzymes and other mitochondrial proteins,[2] with over 35% of proteins thought to be acetylated within the mitochondria.[3] Less common PTMs, such as ADP-ribosylation and lipoylation, have also been found on mitochondrial enzymes, and shown to have essential roles in their functions.[4,5] Additionally, PTMs derived from reactive acyl-CoA species generated during metabolic processes also decorate mitochondrial enzymes.[6] Altogether, enzymes involved in regulating core metabolic processes including the TCA cycle, fatty acid oxidation, amino acid catabolism, the electron transport chain, as well as proteins involved in regulating mitochondrial morphology and structure have been found to be modified by diverse types of PTMs.

Due to the proton pumping activity of the electron transport chain, the mitochondrial matrix has a high pH that supports enzymatic as well as nonenzymatic addition of modifications to proteins.[7,8] In contrast, removal of lysine PTMs within mitochondria is believed to be predominantly an enzymatic process catalyzed by members of the sirtuin (SIRT) family of proteins. Human SIRTs are a family of seven $NAD^+$-dependent enzymes, three of which reside within the mitochondria (SIRT3–5).[9] Given their different types of enzymatic activities and substrates, the mitochondrial SIRTs are poised as master regulators of mitochondrial structure, health, and function. Specifically, SIRT3 was established as the main mitochondrial deacetylase,[10] while SIRT5 was shown to have demalonylase, desuccinylase, and deglutarylase activities.[11–13] Despite its evolutionary conservation and ubiquitous expression in human tissues, until recent years, SIRT4 remained one of the least understood SIRTs. However, this changed when a significant body of literature discovered the structure and diverse enzymatic activities of SIRT4, establishing it as a core regulator of mitochondrial function. Here, we discuss the multifaceted roles of SIRT4, its implications in human disease, and its promise as a therapeutic target.

## SIRT4 ENZYMATIC ACTIVITIES AND SUBSTRATES

SIRT4 is ubiquitously expressed in human organs, with higher expression levels in the heart, liver, kidney, spleen, prostate, testis, and ovary.[14] Within each tissue, and similar to SIRT3 and SIRT5, SIRT4 is localized to the cellular mitochondrial matrix.[9,15] Although all sirtuins were originally believed to function as lysine deacetylases, further investigation has revealed that several SIRTs, including SIRT4, have little to no deacetylase activity. The catalytic domain of SIRTs, consisting of about 275 amino acids, is highly conserved across prokaryotes and eukaryotes.[14] However, phylogenetic analysis of SIRT proteins revealed that the catalytic domain of SIRT4 is unique among the human SIRTs, while closely resembling those of prokaryotic SIRTs and other metazoan SIRTs.[14,16] In this section, we discuss the studies that uncovered the enzymatic activities of SIRT4, demonstrating its ADP-ribosyltransferase, substrate-specific deacetylase, lipoamidase, and deacylase functions (Table 1).

Following the finding that SIRT4 had little to no detectable deacetylase activity against chemically modified acetylated histone peptides in vitro,[17,18] alternative enzymatic activities for SIRT4 were investigated. SIRT4 was discovered to be able to mono-ADP-ribosylate histone proteins and BSA in vitro, leading to the question of whether it can act as an ADP-ribosyltransferase in vivo.[15,19] Importantly, SIRT4 does not have $NAD^+$ glycohydrolase activity, suggesting that it directly and enzymatically transfers ADP-ribose to substrates.[19] Indeed, SIRT4 was found to transfer an ADP-ribosyl group from $NAD^+$ to the glutamate dehydrogenase enzyme (GDH), repressing the activity of GDH in pancreatic $\beta$-cells of mice.[19] As GDH regulates and links glutamine metabolism to the TCA cycle, SIRT4 functions to inhibit the anaplerotic influx of carbon entering the TCA cycle from glutamine (Figure 1).

A study investigating the ADP-ribosyltransferase activity of several SIRTs did not detect ADP-ribosylation of GDH (or histone proteins) when SIRT4 was incubated with an $NAD^+$ analogue.[20] The rate constant of SIRT ADP-ribosyltransferase activity was estimated to be approximately 500 times weaker than the deacetylase activity of SIRTs, and over 5000-fold lower than bacterial ADP-ribosyltransferases, calling into question the physiological relevance of SIRT ADP-ribosyltransferase activity.[20] However, for many years SIRT4 has been difficult to purify and, due to this difficulty, the authors were unable to directly measure the efficiency of SIRT4 ADP-ribosyltransferase activity.[20] It is therefore possible that SIRT4 is a more efficient ADP-ribosyltransferase than the other SIRTs and has this activity in vivo.

Although SIRT4 was found not to catalyze deacetylation of acetylated histone peptides in vitro, a follow-up acetylome peptide microarray analysis revealed that SIRT4 had low, but reproducible and substrate-specific, deacetylase activity.[21] Specifically, SIRT4 was found to deacetylate mitochondrial Hsp60, Stress-70, and NAD(P) transhydrogenase (NNT), the latter of which was also confirmed in vitro.[21] SIRT4 was also discovered to bind to, deacetylate, and inhibit the activity of malonyl-CoA decarboxylase (MCD) in skeletal muscle and white adipose tissue[22] (Figure 1). The substrate of MCD, malonyl-CoA, is the chain-elongating unit for fatty acid biosynthesis. Malonyl-CoA also allosterically inhibits CPT1, an enzyme involved in the transfer of fatty acids from the cytosol into the mitochondria for degradation.[22] Therefore, SIRT4 functions to regulate the balance between

fatty acid synthesis and oxidation by inhibiting fatty acid oxidation in skeletal muscle and white adipose tissue. Typically, deacetylation increases the enzymatic activity of substrates; however, the acetylated residue of MCD is near the malonyl-CoA entry point, suggesting that acetylation may gate the enzymatic pocket.[22] Most recently, SIRT4 was discovered to deacetylate the mitochondrial trifunctional protein $\alpha$-subunit (MTP$\alpha$) in hepatocytes.[23] MTP$\alpha$ catalyzes the second and third steps of fatty acid oxidation and was found to be acetylated or ubiquitinated on the same three lysine residues.[23] SIRT4-mediated deacetylation of MTP$\alpha$ induces destabilization of the enzyme by promoting its ubiquitination and degradation.[23] In this way, SIRT4 also functions to inhibit fatty acid oxidation in the liver.

Another study using histone peptides containing different modifications indicated that mammalian SIRTs also possess long-chain deacylation activity in vitro.[24] A separate study performed steady-state kinetic assays to measure the in vitro catalytic activities of SIRT4, demonstrating that SIRT4 more efficiently hydrolyzed lipoyl-and biotinyl-lysine modifications compared to acetyl-lysine modifications.[25] Lipoylation is thought to be an exceedingly rare PTM, as there are only four known lipoylated multicomponent enzymatic complexes in mammals: pyruvate dehydrogenase complex (PDH), $\alpha$-ketoglutarate dehydrogenase complex (KDH), branched-chain $\alpha$-ketoacid dehydrogenase complex (BCKDH), and the glycine cleavage system (GCV).[5] Lipoyl modifications are essential for the enzymatic activities of these complexes and function as “swinging arms”, capturing and presenting substrate intermediates between the active sites of the multicomponent enzymes.[5]

Following the investigation of potential cellular substrates of SIRT4, it was discovered that SIRT4 interacts with subunits from all four lipoylated multicomponent enzymatic complexes, as well as biotin-dependent carboxylases in humans.[25] SIRT4 was shown to remove lipoyl groups from the PDH subunit DLAT, with steady-state kinetic assays estimating that SIRT4 is approximately 1270-fold more efficient at hydrolyzing lipoyl from DLAT than acetyl from MCD.[25] The lipoamidase activity of SIRT4 was further confirmed in cell culture and in mice in vivo, indicating that removal of lipoylation inhibits the activity of PDH[25] (Figure 1). Therefore, SIRT4 is positioned at an important regulatory point between glycolysis and the TCA cycle. SIRT4 regulation of KDH, GCV, and BCKDH, the other lipoylated complexes in humans, remains to be examined. The ability of SIRTs to regulate substrate lipoylation appears to be evolutionarily conserved, as CobB, the SIRT homologue in *E. coli,* was discovered to delipoylate and inhibit the activity of both PDH and KDH.[26] Interestingly, SIRT4 and its C. *elegans* orthologs, SIR-2.2 and SIR-2.3, were found to associate with the biotin-dependent carboxylases PC, PCC, and MCCC.[27] Given the finding that human SIRT4 can hydrolyze biotinyl-lysine modifications in vitro,[25] it remains to be seen whether this enzyme may also remove biotinyl from these carboxylases in vivo.

Using molecular dynamic simulations with a predicted model of the SIRT4 catalytic pocket, it was further discovered that SIRT4 has high affinity for negatively charged acyl-lysine modifications.[28] Specifically, SIRT4 was found to catalyze removal of 3-hydroxy-3-methylglutaryl (HMG), 3-methylglutaryl (MG), 3-methylglutaconyl (MGc), and glutaryl (G) from lysine residues of substrates both in vitro and in vivo[28] (Figure 1). Using a $^{32}$P-NAD$^{+}$-consumption assay and a fluorogenic assay, SIRT4 was shown to more efficiently remove G-

and MG-lysine modifications, followed by HMG-lysine and MGc-lysine.[28] MGc-CoA and HMG-CoA are generated as metabolic intermediates during leucine catabolism, while MG-CoA is believed to be formed via reduction of MGc-CoA.[29] SIRT4 was discovered to interact with proteins involved in branched chain amino acid (BCAA) catabolism, and specifically with components of the above-mentioned methylcrotonyl-CoA carboxylase complex (MCCC), an enzymatic complex that functions in leucine metabolism and catalyzes the conversion of 3-methylcrotonyl-CoA (MC) into MGc.[25,28] Therefore, the authors further investigated whether SIRT4 deacylates and regulates MCCC activity in vivo. SIRT4 KO increased MCCC acylation in mice livers, resulting in decreased MCCC activity.[28] MCCC forms a heterododecamer enzymatic complex and acylation sites were discovered to cluster near intramolecular interfaces within the complex.[28] Increased MCCC acylation following SIRT4 KO destabilized the complex, reducing leucine flux through the BCAA catabolic pathway.[28] SIRT4 KO also resulted in reduced flux through BCAA catabolism in mitochondria isolated from heart tissue, suggesting that SIRT4 also regulates BCAA catabolism in other tissues.[28]

## SIRT4 Structure Underlying its Enzymatic Activities

Importantly, the above-mentioned enzymatic activities of SIRT4 for removing acylations, lipoylation, biotinylation, and acetylation have recently been verified upon solving the crystal structure of SIRT4 (Figure 2).[30] SIRTs are characterized by a conserved catalytic domain consisting of about 275 amino acids, SIRT-specific N-and C-terminal domains that determine localization and regulation, a Rossmann-fold domain where $NAD^+$ docks, and a Zn binding domain.[14,30] SIRT4 has an N-terminal domain that contains the mitochondrial localization signal, and does not have a C-terminal domain.[30] A conserved His residue within the catalytic pocket, H161 in the case of human SIRT4, is also essential for the enzymatic activity of other SIRTs.[14]

Analysis of the SIRT4 crystal structure revealed that several features are specific to SIRT4 compared to other SIRTs (Figure 2A).[30] A flexible loop lines the catalytic pocket of SIRT4, which may contribute to substrate binding or regulate active site dynamics.[30] An additional active site entry channel was also discovered, which branches off the main active site and leads to the surface of SIRT4.[30] The channel is predominantly hydrophobic, with a small positively charged region at the outer surface.[30] This suggests that the channel may accommodate longer acyl groups, such as lipoyl, or bind to regulatory factors, such as fatty acids. In fact, the model of SIRT4 in complex with lipoyl places the lipoyl group at the bottom of the channel, supporting this hypothesis.[30] Additionally, it was discovered that SIRT4 prefers polar residues around acetyl-lysine modifications, which contributes to the substrate specificity of SIRT4 catalyzed deacetylation reactions.[30] Strikingly, SIRT4 was found to have similar $k_{cat}$ values for HMG-, lipoyl-, and acetyl-modifications in an in vitro deacylation assay, but different $K_M$ values.[30] This suggests that, while turnover rates for different modifications are similar, SIRT4 has higher affinity for HMG-and lipoyl-modifications compared to acetyl groups.[30]

## SIRT4 Nonenzymatic or Unknown Enzymatic Functions

In addition to the previously described enzymatic activities and substrates of SIRT4, several studies have implicated SIRT4 in regulating additional mitochondrial processes via indirect, nonenzymatic or unknown enzymatic mechanisms. Overexpression of SIRT4 was discovered to repress PPAR$\alpha$ activity in mice liver, kidney, fibroblast, and hepatoma cells.[31] PPAR$\alpha$ is a transcription factor that promotes expression of mitochondrial fatty acid oxidation genes. Hepatocytes from SIRT4 KO mice exhibited higher rates of fatty acid oxidation, which was dependent on functional SIRT1.[31] SIRT1 had previously been shown to activate PPAR$\alpha$, suggesting that SIRT4 functions in a retrograde signaling pathway from the mitochondria to the nucleus to decrease SIRT1 activity, thereby resulting in decreased PPAR$\alpha$ activity.[31]

SIRT4 was also discovered to inhibit ANT2, resulting in increased ATP levels in several mouse tissues.[32] ANT2 is an uncoupling protein, which regulates the link between the electron transport chain and ATP synthesis. SIRT4-mediated inhibition of ANT2 and the resulting increase in ATP levels leads to reduced AMPK activity, which reduces fatty acid oxidation.[32] Although the mechanism of SIRT4-mediated ANT2 inhibition remains to be determined, acylated ANT2 has been discovered to act as an uncoupling protein, suggesting that SIRT4 could inhibit ANT2 through deacylation.[32]

Additionally, SIRT4 was discovered to compete with MnSOD for binding to SIRT3.[33] SIRT3 typically deacetylates and activates MnSOD, an antioxidant enzyme.[33] Therefore, sequestration of SIRT3 from MnSOD results in increased mitochondrial reactive oxygen species (ROS) generation. However, this SIRT4 function was discovered to be nonenzymatic, as SIRT4 H161Y, a catalytically inactive mutant of SIRT4, still interacted with and sequestered SIRT3 from MnSOD.[33]

Most recently, SIRT4 was found to interact physically with OPA1, the mitochondrial fusion protein.[34] The binding of SIRT4 to OPA1 was dependent on the enzymatic activity of SIRT4.[34] SIRT4 overexpression resulted in increased mitochondrial mass and fusion, supporting the finding that SIRT4 perturbs OPA1 activity.[34] It was suggested that SIRT4 could stabilize OPA1 or protect it from proteolytic processing.[34]

As described, these enzymatic activities and substrates of SIRT4 have revealed its essential function in regulating diverse cellular processes, including carbon entry into the TCA cycle, fatty acid metabolism, branched-chain amino acid catabolism, electron transport chain function, regulation of reactive oxygen species generation, and mitochondrial morphology. Therefore, SIRT4 is positioned as a critical regulator of mitochondrial function and health.

## Methods for Investigating SIRT4 Enzymatic Activities and Substrates

A variety of techniques have been used to investigate the substrates and activities of SIRT4, including fluorometric activity assays, $^{32}$P-NAD$^{+}$ consumption assays, and mass spectrometry (MS)-based techniques. The Fluor-de-Lys assay has been primarily used to study deacetylation events by HDACs, including SIRTs.[19,21,28,30,35] The Fluor-de-Lys assay is a fluorometric activity assay in which deacetylation of a substrate peptide sensitizes it to proteolytic digestion, generating a fluorophore that can be quantified. The Fluor-de-Lys

assay can also be modified to measure other deacylation events in addition to deacetylation. As with any in vitro assay using synthetic peptide substrates, the disadvantage to using the Fluor-de-Lys assay is that it removes the protein substrate landscape from the analysis, making it difficult to detect substrate-specific deacylation events. Another assay frequently used in SIRT biology is the $^{32}$P-NAD$^+$ consumption assay.[11,20,24,25] In this technique, SIRTs are incubated with substrate and $^{32}$P-NAD$^+$ and the resulting radioactive 2'-O-acyl-ADPR reaction product is visualized using thin-layer chromatography (TLC). The main drawback to this technique is its qualitative nature. However, it is possible to purify and quantify the 2'-O-acyl-ADPR reaction product using MS, as a quantitative alternative to the $^{32}$P-NAD$^+$ consumption assay.

Due to their accuracy and versatility, mass spectrometry (MS)-based techniques are being increasingly applied to investigating diverse SIRT enzymatic activities and substrates. To gain insight into cellular processes regulated by SIRT4 and to guide follow-up analyses, several studies employed immunoaffinity purification-mass spectrometry (IP-MS) to investigate SIRT4 interacting proteins.[15,25] Many of the enzymatic activities and substrates of SIRT4 were first revealed using this method, including its lipoamidase activity and regulation of PDH.[25] HPLC-based steady-state enzyme kinetic assays have also been developed to determine the $k_{cat}$ and $K_M$ values for the various SIRT4 enzymatic activities.[11,25,30] In this assay, substrate (acylated peptide) and product (deacylated peptide) are separated using HPLC, and the product concentration as the deacylation reaction progresses is measured using UV absorption. $k_{cat}$ and $K_M$ can be quantified by curve-fitting the resulting $V_i/[E]$ versus [$S$] plot. Targeted MS/MS, such as parallel reaction monitoring (PRM), can be used for site-specific quantification of substrate modification status, which is advantageous in that it can be used to investigate changes in PTM status in response to perturbations performed in cell culture or in vivo. However, the peptide sequence and modification location must be specified beforehand, so this technique cannot be used to discover novel PTMs. Recently, top-down MS was used to investigate the acylation status of intact protein in the presence or absence of SIRT4.[30] Although this technique is currently limited in its ability to investigate proteins with high molecular mass or low abundance, future developments in top-down proteomics promise to make important contributions to defining proteo-forms and to characterizing sirtuin enzymatic activities and substrates.

## THE ROLE OF SIRT4 IN HUMAN DISEASE

Due to its role in regulating metabolic processes and mitochondrial functions, SIRT4 expression and activities have been implicated in the emergence and pathogenesis of various human diseases, including hyperinsulinemia and diabetes, liver disease, cancer, neurodegeneration, heart disease, aging, and pathogenic infections (Figure 3).

### Hyperinsulinemia and Diabetes

Insulin secretion in pancreatic $\beta$-cells can be stimulated by glucose or amino acids.[36] In amino acid-stimulated insulin secretion, amino acids are catabolized to TCA cycle intermediates and are used to generate ATP, which triggers insulin release. The involvement of SIRT4 in regulating glutamine metabolism through ADP-ribosylation of GDH suggested

it may play a role in regulating amino acid-stimulated insulin secretion.[19] Indeed, SIRT4 knockout mice were discovered to have increased glutamine-and glucose-stimulated insulin levels, as well as increased leucine-stimulated secretion.[19] The finding that SIRT4 knockdown resulted in increased glucose-stimulated insulin secretion was recapitulated in a separate study, supporting the role of SIRT4 in regulating insulin secretion.[15] It is likely that regulation of leucine catabolism and ANT2 activity by SIRT4 also contribute to SIRT4 regulation of insulin secretion. Since leucine is an allosteric activator of GDH, SIRT4 promotion of leucine catabolism via MCCC activation would result in decreased glutamine-stimulated insulin secretion by decreasing the concentration of leucine and preventing GDH activation. However, it is possible that SIRT4 also promotes insulin secretion by increasing ATP levels through inhibition of ANT2.[32,37] Therefore, several SIRT4 activities likely contribute to the regulation of insulin secretion in pancreatic $\beta$-cells.

Due to its involvement in regulating insulin secretion, SIRT4 has been implicated in the emergence of hyperinsulinemia and type II diabetes. Early in the development of type II diabetes mellitus, insulin resistance occurs along with a reduction in SIRT4 expression.[38] Moreover, SIRT4 knockout mice develop hyperinsulinemia, glucose intolerance, and insulin resistance, supporting the role of SIRT4 in type II diabetes.[28] SIRT4 has also been found to protect against some of the complications associated with diabetes. Diabetic nephropathy is a complication associated with increased ROS, apoptosis, and inflammation in the kidneys as a result of hyperglycemia due to diabetes. SIRT4 was found to protect podocytes against apoptosis and ROS accumulation under hyperglycemic conditions, suggesting that it has a protective role against diabetic nephropathy.[39]

## Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a risk factor for coronary artery disease and is characterized by decreased fatty acid oxidation and the accumulation of lipids in the liver.[40] Due to its role in regulating fatty acid oxidation, as well as the finding that SIRT4 expression was increased in the livers of patients with NAFLD, SIRT4 was implicated in functioning in NAFLD pathogenesis.[23,41] It was discovered that decreased SIRT4 expression in mouse liver protected against NAFLD by inhibiting MTP$\alpha$ deacetylation.[23] This suggested that upregulated levels of SIRT4 in NAFLD patients contributes to the development of the disease by decreasing MTP$\alpha$-catalyzed fatty acid oxidation, thereby promoting lipid accumulation. Additionally, circulating SIRT4 protein levels were discovered to be decreased in the serum of obese patients with NAFLD.[40] It was suggested that circulating SIRT4 levels are decreased in response to high calorie diet in order to increase fatty acid oxidation and protect against NAFLD.[40]

## Cancer

SIRT4 expression was found to be reduced in several human cancers, including liver,[42,43] endometrial,[44,45] lung,[43] ovarian,[43] pancreatic,[43] bladder,[43] prostate,[43] renal,[43] esophageal,[46] breast,[47] colorectal,[48,49] throat,[50] and stomach cancers,[51] lymphoma,[52] and leukemia.[53] Lower SIRT4 expression in tumor cells is often correlated with poorer prognosis and survival in cancer patients, and SIRT4 knockout mice were found to spontaneously develop tumors, suggesting that SIRT4 typically functions as a tumor suppressor.[43,44,46–52,54,55]

Glutamine metabolism plays an important role in the proliferation of cancer cells by replenishing TCA cycle intermediates to support increased growth and by producing ammonia, which neutralizes the acidic metabolites frequently produced as a result of increased glycolysis in cancer cells. Therefore, SIRT4 inhibition of glutamine metabolism via repression of GDH activity is thought to contribute to its function as a tumor suppressor.

Several recent studies support the function of SIRT4 as a tumor suppressor and provide mechanistic insights into the SIRT4 dysregulation during cancer progression. c-Myc is a transcription factor that is involved in driving increased glutamine metabolism in many cancer cells. SIRT4 was shown to repress the proliferation of Myc-induced Burkitt lymphoma by inhibiting GDH activity, thereby preventing increased glutamine metabolism.[52] The transcriptional regulator CtBP is highly expressed in breast cancer patients and was discovered to promote glutamine metabolism and cancer progression by directly repressing the expression of SIRT4.[56] mTORC1 also has increased activity in many cancer tissues and enhances the proliferation of cancer cells, partially by contributing to the increase in glutamine metabolism. mTORC1 was discovered to repress SIRT4 mRNA expression by promoting degradation of the transcription factor CREB2, resulting in GDH activation and increased glutamine metabolism in cancer cells.[57] CtBP-and/or mTORCl-mediated repression of SIRT4 could explain the decreased SIRT4 expression in cancer tissues. Additionally, leucine increases mTORC1 activity, which suggests that regulation of leucine catabolism by SIRT4 might also contribute to its function as a tumor suppressor.[58]

In addition to its role in regulating glutamine metabolism, SIRT4 has also been found to regulate several other processes involved in cancer, including cell cycle progression, tumor growth, metastasis, and apoptosis. SIRT4 has been implicated in preventing cell cycle progression following DNA damage.[54] In colorectal cancer cells, SIRT4 overexpression delayed mitosis, which increased the sensitivity of the cancer cells to a chemotherapeutic drug.[48] Mitochondrial fission, which is known to enhance cancer cell growth and prevent apoptosis, is also inhibited in lung cancer cells by SIRT4.[43] SIRT4 reduced MEK and ERK activity in lung cells, which prevented the downstream phosphorylation and activation of the mitochondrial fission factor Drp1, resulting in decreased mitochondrial fission and reduced invasion ability of cancer cells.[43] SIRT4 may also regulate the epithelial-mesenchymal transition of cancer cells by upregulating expression of E-cadherin, which promotes cell-cell adhesion and prevents tumor invasion and metastasis.[49] It was suggested that upregulation of E-cadherin was related to SIRT4 inhibition of glutamine metabolism, as addition of $\alpha$-ketoglutarate decreased E-cadherin expression.[49] SIRT4 is also believed to induce glycolysis and respiration, as well as promote increased proton leak in response to excess glucose in cancer cells.[59] Proton leak protects against ROS generation and prevents excess energy generation, thereby restricting ROS-mediated DNA damage and proliferation of tumor cells.[59]

## Neurodegeneration

Excess glutamate at synapses in the brain prevents efficient neurotransmission and results in excitotoxicity, which causes cell death and has been implicated in neurodegenerative disease.[60] For this reason, glutamate transport within the brain is essential and functions to

maintain low extracellular levels of glutamate at synapses. SIRT4 protein levels were discovered to be upregulated in response to excitotoxins, and SIRT4 knockout mice were more sensitive to excitotoxicity.[60] In SIRT4 KO mice, the expression of the glutamate transporter was decreased at the cell surface of neurons, resulting in decreased glutamate uptake.[60] This suggests that SIRT4 is neuroprotective in the brains of mice. Additionally, SIRT4 was discovered to regulate glial development in the brain.[61] In cell culture, SIRT4 and GDH were found to have opposing effects on gliogenesis, where SIRT4 overexpression prevented, while GDH overexpression promoted the differentiation of radial glia into astrocytes.[61] Patients with congenital hyperinsulinism-hyperammonemia syndrome, a form of hyperinsulinism, frequently experience seizures, hypoglycemia, and developmental defects. It was suggested that increased GDH activity, potentially resulting from decreased SIRT4 ADP-ribosylation, could cause defects in glial development in the brain, resulting in the neurological disorders observed in patients with congenital hyperinsulinism-hyperammonemia syndrome.[61]

## Heart Disease

SIRT4 has also been implicated in the pathogenesis of heart disease. Interestingly, SIRT4 has been found to promote cardiac hypertrophy but play an ameliorative role during ischemic heart injury.[33,62,63] Cardiac hypertrophy refers to abnormal enlargement of the heart muscle and is associated with heart failure. SIRT4 was discovered to promote cardiac hypertrophy by sequestering SIRT3 from its substrate, the antioxidant enzyme MnSOD.[33] Sequestration of SIRT3 from MnSOD prevents SIRT3-mediated MnSOD activation and therefore increases ROS levels and oxidative stress, which are linked to enhanced risk for development of cardiac hyper-tophy.[33] Additionally, the level of microRNA miR-497 was shown to decrease following cardiac hypertrophy in mouse hearts, whereas SIRT4 protein levels increased.[62] Since SIRT4 is a direct target of miR-497 and overexpression of miR-497 inhibits myocardial hypertrophy, it is possible that miR-497 suppresses cardiac hypertrophy by targeting SIRT4.[62] In contrast to its role in cardiac hypertrophy, SIRT4 has been found to play a protective role during ischemic heart injury.[63] As adult cardiomyocyte cells are not capable of proliferation, apoptosis plays a significant role in the pathogenesis of ischemic heart injury by causing cardiomyocyte loss.[63] SIRT4 was discovered to protect against hypoxia-induced apoptosis by preventing translocation of the pro-apoptotic protein Bax to the mitochondria, thereby increasing cell viability and protecting against the pathogenesis of ischemic heart injury.[63]

## Aging

Following the discovery that SIRT proteins in yeast increase replicative lifespan, there has been interest in determining whether SIRT4 and the other human SIRTs are antiaging factors. Aging is characterized by the accumulation of senescent cells, and SIRT4 was shown to be upregulated in various cell lines following senescence or during aging.[64–69] Further supporting the role of SIRT4 in aging, its transcript levels were decreased in mouse spermatogonial stem cells following treatment with a lifespan-enhancing drug.[68] SIRT4 transcript levels were also increased with increasing age in the preoptic area of the brain, which regulates the release of hormones that are important for reproduction.[66] This suggests

that SIRT4 expression might regulate the decrease in serotonin and testosterone levels that occurs with age.

ROS generation following photodamage has also been implicated in aging-related phenotypes seen in skin.[65,67,69] UV radiation was found to disrupt the temporal cycle of SIRT4 expression in epidermal cells.[69] SIRT4 and SIRT3 expression were inversely correlated and followed a temporal cycle in nonirradiated cells.[69] Following UV radiation, SIRT4 mRNA levels were increased relative to SIRT3 levels and the temporal cycle of expression was lost.[69] Since SIRT4 has been shown to prevent SIRT3 from activating MnSOD,[33] dysregulated SIRT4 and SIRT3 temporal expression following UV damage could contribute to aging-related phenotypes by promoting increased ROS generation. SIRT4 mRNA and protein levels also increased in irradiated human dermal fibroblast cells.[65] This was attributed to the activity of miR-15b, which targets the SIRT4 gene.[65] In vivo, SIRT4 mRNA levels were upregulated and miR-15b levels were downregulated in photoaged skin compared to nonphotoaged human skin. Inhibition of miR-15b increased aging-related phenotypes, further supporting a function for SIRT4 in aging.[65]

### Pathogenic Infection

SIRT4 has also been implicated in protecting cells against bacterial and viral pathogens. The bacterial lipopolysaccharide (LPS) inhibits testicular steroidogenesis and induces apoptosis in male accessory glands. LPS was discovered to activate the JNK/MAPK signal transduction pathway, which resulted in suppression of SIRT4 expression.[70] SIRT4 overexpression in LPS-treated cells increased steroidogenesis and decreased apoptosis, suggesting that SIRT4 protects cells against LPS-induced pathogenesis during bacterial infection.[70] Additionally, all seven SIRTs have been shown to have antiviral properties against several DNA and RNA viruses.[71] siRNA-mediated knockdown of SIRT4 was discovered to increase the replication of the herpesviruses human cytomegalovirus (HCMV) and herpes simplex virus 1 (HSV-1), as well as adenovirus and influenza virus.[71] Interestingly, in a separate study investigating canine coronavirus type II (CCoV-II), SIRT4 mRNA and protein levels were found to increase in infected cells, suggesting that SIRT4 may be involved in the pathogenesis of CCoV-II.[72] Additionally, CobB was shown to protect *E. coli* against bacteriophage infection, suggesting that the ability of SIRTs to protect cells against viral pathogens is evolutionarily conserved.[71]

## PERSPECTIVE AND FUTURE DIRECTIONS

SIRT4 remained the least understood sirtuin protein until recent years, when a significant body of literature elucidated several of its enzymatic activities and substrates (Table 1). Given that there is still limited understanding of the roles of SIRT4 in regulating mitochondrial processes, we expect that this knowledge of its enzymatic activities will propel numerous future investigations in different biological and clinical contexts. It has become evident that several SIRT4 functions and substrates are tissue-specific. For instance, SIRT4 knockout did not impact lipid synthesis or malonyl-CoA levels in mouse liver, suggesting that SIRT4 regulation of MCD is specific to skeletal muscle and white adipose tissue.[22] Furthermore, although skeletal muscle is an important tissue for branched-chain

amino acid catabolism, SIRT4 knockout did not result in reduced leucine catabolism in mouse skeletal muscle, suggesting that SIRT4 regulation of MCCC might also be tissue-specific.[28] Additionally, SIRT4 was found to ADP-ribosylate and inhibit GDH activity in pancreatic $\beta$-cells;[19] however, it remains to be determined whether SIRT4 has this activity in other tissues. Correlative evidence regarding the role of SIRT4 in regulating glutamine metabolism in cancer cells[49,52,54,56] and the finding that GDH activity is regulated via ADP-ribosylation in brain and liver[19] suggests that SIRT4 inhibits GDH activity in other tissue types, however causative evidence and mechanisms for this activity need to be established.

It is also likely that SIRT4 has additional enzymatic activities and substrates that have yet to be identified. Steady-state kinetic assays demonstrated that SIRT4 possesses the ability to hydrolyze biotinyl groups from proteins.[25] Although the presence of biotinylated proteins in the mitochondria has been known for some time,[1] the ability of SIRT4 to remove biotinyl groups from substrates in vivo has not yet been investigated. In addition to PDH, three other lipoylated complexes exist in the mitochondria.[5] It is possible that SIRT4 also delipoylates and regulates KDH, GCV, and BCKDH activity in cells. Following the discovery that mitochondrial proteins can be nonenzymatically modified with reactive acyl species, there has been a push to establish the mitochondrial acylome. As new acylations and targets are being identified, SIRT4 may contribute to the removal of additional, and possibly yet unidentified, acyl groups from mitochondrial proteins, thereby expanding its regulation of metabolic pathways within the mitochondria.

As the ability of SIRT4 to regulate diverse cellular processes and PTMs is becoming increasingly apparent, important questions regarding SIRT4 biology remain to be addressed. Given its multifaceted roles within the mitochondria, understanding how SIRT4 is regulated in response to fluctuating cellular conditions remains a major area of investigation. The recently solved structure of SIRT4 revealed unique structural features, namely, the SIRT4-loop and the SIRT4-specific channel, which could shed light on SIRT4 regulatory mechanisms.[30] The SIRT4-loop is flexible and must rearrange for $NAD^+$ to bind in the catalytic pocket, suggesting that SIRT4 can assume an inactive state.[30] The transition of SIRT4 into an active configuration could be controlled by a yet undiscovered mechanism, possibly a PTM or a small-molecule activator such as a free fatty acid, as is the case with SIRT6.[24] Indeed, SIRT4 has predicted phosphorylation sites that could potentially regulate its activity. Discovery of the SIRT4-specific channel leading to the surface of the enzyme also calls for further investigation into its function.[30] It is tempting to speculate that physiological metabolites enter the SIRT4 channel from outside the enzyme, eliciting a conformational change in the catalytic pocket that could alter the substrate affinity or enzymatic activity of SIRT4. This hypothesis is supported in part by the finding that lipoyl groups can enter the SIRT4 channel from either outside or inside the enzyme.[30] It has also been suggested that SIRT4 may be embedded within the multimeric enzymatic complexes of its substrates, such as the PDH complex.[25] If this is indeed the case, it would support that SIRT4 functions are actively regulated instead of resulting from diffusion of the enzyme through the mitochondrial matrix and random interaction with its substrates.

Looking ahead, a better mechanistic understanding of how SIRT4 contributes to human disease is certainly necessary. SIRT4 has been implicated in hyperinsulinemia and diabetes,

liver disease, cancer, neurodegeneration, heart disease, aging, and pathogenic infections. However, most of the findings regarding the role of SIRT4 in human disease have been correlative in nature. Additionally, due to the diverse enzymatic activities and substrates of SIRT4, it is likely that SIRT4 regulates disease pathogenesis through multiple mechanisms. For instance, although cancer studies have predominantly focused on the role of SIRT4 in regulating glutamine metabolism, glycolytic flux also increases in many cancer tissues. As a key regulator of the link between glycolysis and the TCA cycle,[25] SIRT4 may also contribute to the emergence and progression of cancer by regulating PDH activity. Elucidating the mechanisms that drive the role of SIRT4 in human disease is necessary in order to better evaluate the potential of SIRT4 as a therapeutic target. We hope that this perspective manuscript will inspire future investigations into the diverse functions of this critical mitochondrial enzyme.

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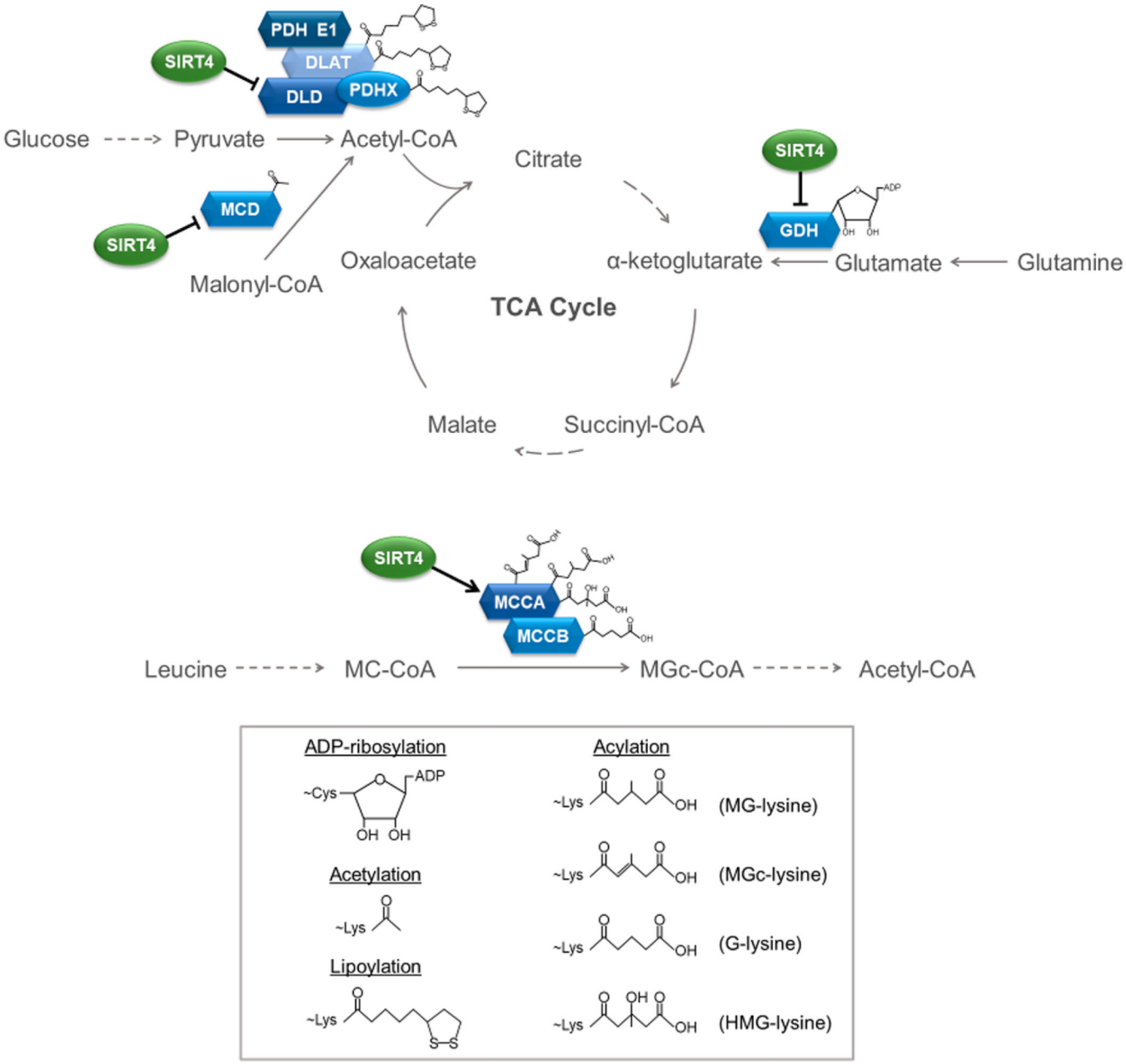


**Figure 1.**

The biological function of SIRT4 substrates. SIRT4 substrates are core regulators of cellular metabolism. PDH links glycolysis to the TCA cycle by catalyzing the conversion of pyruvate to acetyl-CoA, while GDH replenishes TCA cycle intermediates by catalyzing the conversion of glutamate to $\alpha$-ketoglutarate. MCD catalyzes the production of acetyl-CoA from malonyl-CoA and is a key enzyme in lipid catabolism. MCCC regulates branched-chain amino acid catabolism in cells, specifically functioning in leucine catabolism. The posttranslational modifications regulated by SIRT4 on these substrates are indicated.

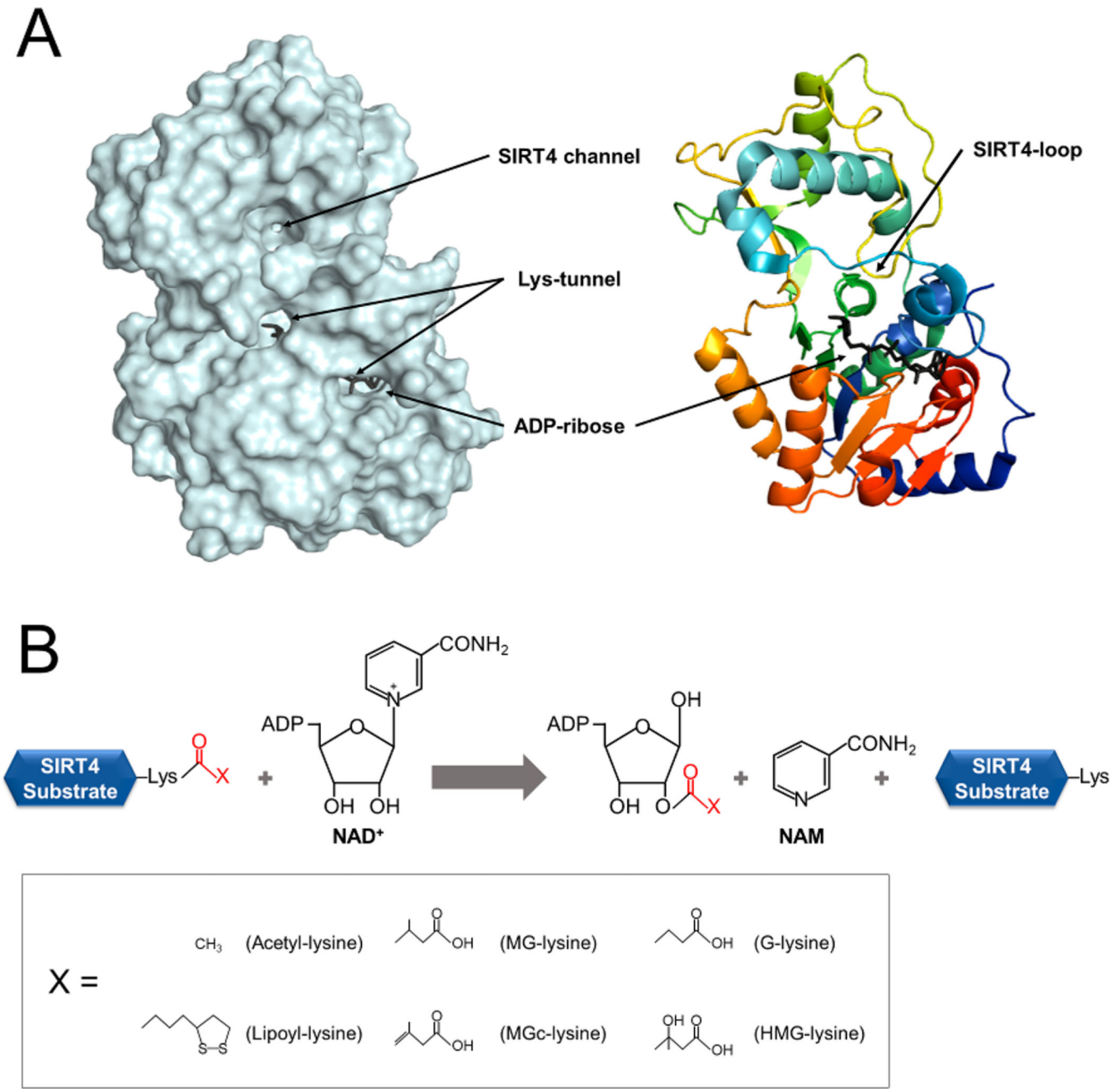


**Figure 2.**

SIRT4 structure and enzymatic mechanism. (A) The structure of SIRT4, as recently solved by Pannek et al. (2017). The structure revealed a SIRT4-specific channel (left) and SIRT4-loop (right, shown in yellow). (B) SIRT4 catalyzes the deacylation of its substrates using $NAD^+$ as a cofactor, releasing nicotinamide (NAM) and resulting in the formation of an 2'-O-acyl-ADPR molecule and deacylated substrate.

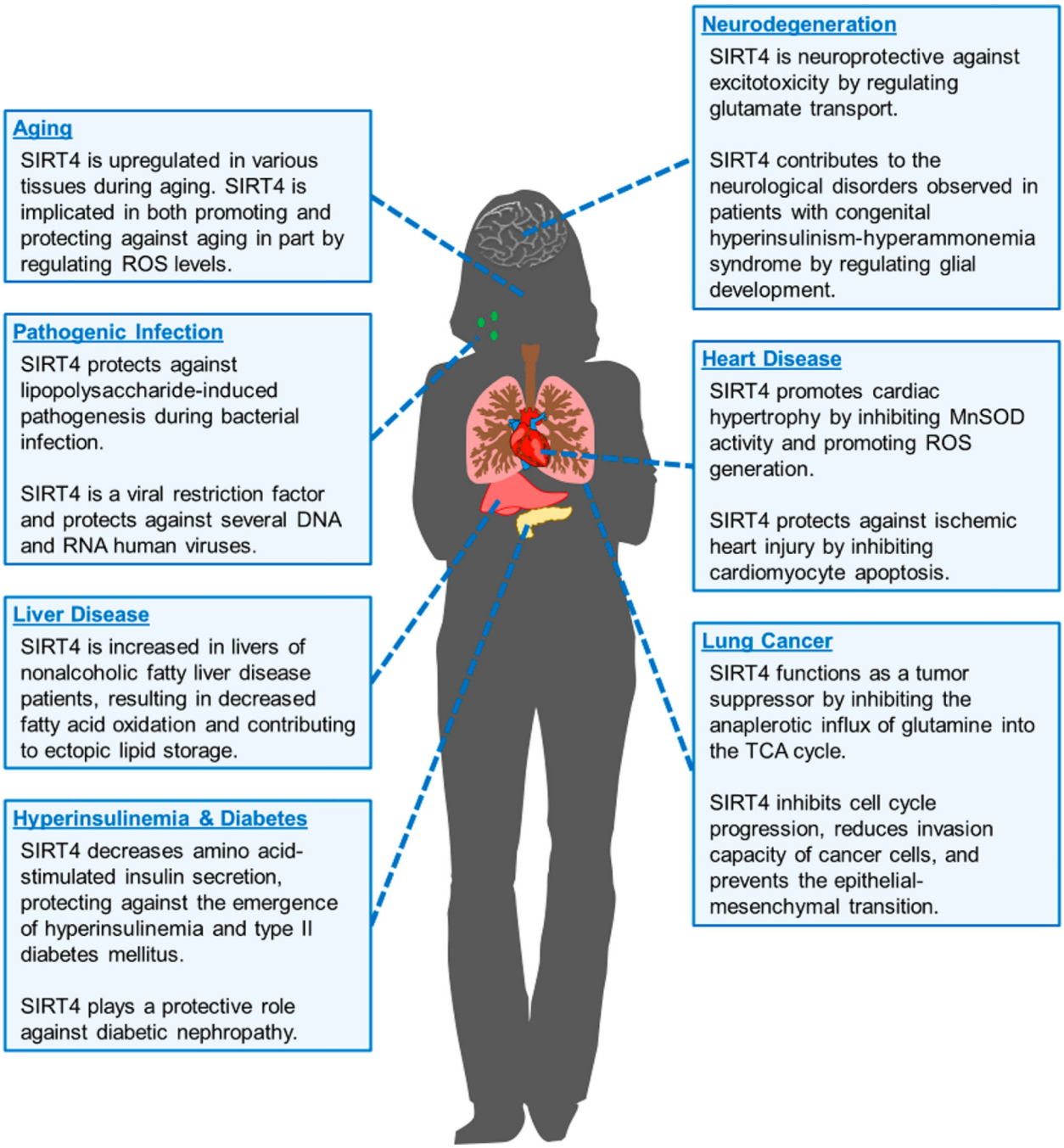


**Figure 3.**

The link between SIRT4 and human disease. SIRT4 has been implicated in the development and progression of hyperinsulinemia and diabetes, liver disease, cancer, neurodegeneration, heart disease, aging, and pathogenic infections. Depending on the tissue type and disease, SIRT4 can be either detrimental or ameliorative to human health.

**Table 1.**

SIRT4 Enzymatic Activities and Substrates

| Enzymatic activity | Substrates | Literature |
|---|---|---|
| ADP-ribosyltransferase | Glutamate dehydrogenase (GDH) | Haigis et al., 2006[19] |
| Deacetylase | Malonyl-CoA decarboxylase (MCD) | Laurent et al., 2013[22] |
| | mtHsp60 | Rauh et al., 2013[21] |
| | Stress-70 | |
| | NAD(P) transhydrogenase (NNT) | |
| | Mitochondrial trifunctional protein $\alpha$-subunit (MTP$\alpha$) | Guo et al., 2016[23] |
| Lipoamidase | Pyruvate dehydrogenase complex (PDH) | Mathias et al., 2014[25] |
| Deacylase | Methylcrotonyl-CoA carboxylase (MCCC) | Anderson et al., 2017[28] |