Metabolic Characterization of a Sirt5 deficient mouse model

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Sirt5, localized in the mitochondria, is a member of sirtuin family of NAD\(^+\)-dependent deacetylases. Sirt5 was shown to deacetylate and activate carbamoyl phosphate synthase 1. Most recently, Sirt5 was reported to be the predominant protein desuccinylase and demalonylase in the mitochondria because the ablation of Sirt5 enhanced the global succinylation and malonylation of mitochondrial proteins, including many metabolic enzymes. In order to determine the physiological role of Sirt5 in metabolic homeostasis, we generated a germline Sirt5 deficient (Sirt5\(^{2/2}\)) mouse model and performed a thorough metabolic characterization of this mouse line. Although a global protein hypersuccinylation and elevated serum ammonia during fasting were observed in our Sirt5\(^{2/2}\) mouse model, Sirt5 deficiency did not lead to any overt metabolic abnormalities under either chow or high fat diet conditions. These observations suggest that Sirt5 is likely to be dispensable for the metabolic homeostasis under the basal conditions.

Sirt5 is one of the seven members of the sirtuin family of NAD\(^+\)-dependent deacetylases. Together with Sirt3 and Sirt4, Sirt5 localizes in the mitochondria. Activation of Sirt1, the founding member of the sirtuin family, has many beneficial effects on energy and metabolic homeostasis, improving health span. The role of mitochondrial sirtuins as metabolic sensors in different metabolic pathways is only beginning to be appreciated.

Sirt3 is thought to be the predominant mitochondrial protein deacetylase based on the fact that the absence of Sirt3 in mice, but not Sirt4 or Sirt5, enhances global acetylation of mitochondrial proteins. Sirt3 regulates the acetylation level and enzymatic activity of a vast set of metabolic enzymes such as long-chain acyl-CoA dehydrogenase, glutamate dehydrogenase (GDH), isocitrate dehydrogenase 2, ornithine transcarbamoylase. In line with the important molecular function of Sirt3, germline Sirt3\(^{2/2}\) mice are sensitized to high fat diet (HFD)-induced obesity, hyperlipidemia, insulin resistance, and steatohepatitis. Despite the fact that liver and skeletal muscle are the most important tissues determining whole body metabolism, specific deletion of Sirt3 in liver or skeletal muscle has almost no effect on the global metabolic homeostasis, pointing to the importance of other tissues in Sirt3 signaling.

Sirt4 lacks deacetylase activity but instead demonstrates an ADP-ribosyl transferase activity. The ability of Sirt4 to ADP-ribosylate and inactivate GDH indicates the involvement of Sirt4 in glutamine metabolism. Indeed, Sirt4 expression is suppressed by mammalian target of rapamycin complex 1 (mTORC1), thus activating GDH and promoting glutamine metabolism and cell proliferation in human cancer.

Sirt5 was shown to deacetylate and activate carbamoyl phosphate synthase 1 (CPS1), the rate-limiting enzyme catalyzing the first step of the urea cycle for ammonia disposal. The activation of CPS1 in Sirt5\(^{2/2}\) mice is impaired during fasting with a consequent accumulation of ammonia in the blood. Sirt5 is furthermore reported to exhibit potent desuccinylase and demalonylase activities. Sirt5 knockout mice show global protein hypersuccinylation and hypermalonylation including hypersuccinylated CPS1, indicating Sirt5 is the major protein desuccinylase and demalonylase in mitochondria. The ability of Sirt5 to remove acyl modifications, including acetylation, succinylation, and malonylation, on lysine residues establishes Sirt5 as a deacylase rather than as a deacetylase. Of note in this context, Sirt6 is recently discovered to efficiently remove long-chain fatty acyls, such as myristoyl groups, from lysine residues, further supporting the emerging recognition of sirtuins as deacylases.

Malonylation and succinylation were detected in multiple metabolic enzymes in mitochondria. A very recent succinylome study further revealed that Sirt5 desuccinylates a mast set of metabolic enzymes in mitochondria that are involved in amino acid degradation, the tricarboxylic acid (TCA) cycle, and fatty acid metabolism. All these
molecular evidences point toward possible important role of Sirt5 in metabolic homeostasis. Since the physiological role of Sirt5 in metabolic homeostasis at the level of whole body has not been reported, we generated a Sirt5 deficient mouse model and performed a thorough metabolic characterization of this mouse line using a standardized phenotyping protocol. Surprisingly, under the conditions that we studied, deletion of Sirt5 leads only to minor metabolic consequences. The absence of a phenotype under basal conditions hence warrants further study to define the biological role of Sirt5 in challenged conditions where Sirt5 function may be solicited.

Results
Generation and validation of a Sirt5 deficient mouse model. To investigate the impact of Sirt5 on whole body metabolism, we generated a mouse line in which the exon 4 of the Sirt5 locus was flanked with LoxP sites using a standard gene targeting strategy. The resulting Sirt5flxed mice were bred with CMV-Cre transgenic mice to generate germline Sirt5 mice. mRNA analysis demonstrated that the Sirt5 gene is successfully inactivated in different tissues in the Sirt5 mice (Fig. 1b). Protein analysis further supported a complete disruption of Sirt5 expression in the knockout animals (Fig. 1c). To generate experimental cohorts, Sirt5 mice were bred to generate Sirt5, Sirt5, and Sirt5 littermates after 24-hour of fasting. In agreement with published data showing the involvement of Sirt5 in the control of the urea cycle, there was a marked increase in blood ammonia level in Sirt5 mice during fasting (Fig. 1f).

Metabolic characterization of chow fed Sirt5 deficient mice. We then subjected chow fed Sirt5 male mice to a standardized phenotyping protocol (Fig. 2a). Although the curve of weight gain

![Figure 1](https://www.nature.com/scientificreports)

**Figure 1 | Generation and validation of a Sirt5 deficient mouse model.** (a) Targeting strategy for disruption of the mouse Sirt5 gene. LoxP sites were inserted to flank exon 4 of the Sirt5 gene. The resulting Sirt5 mice were bred with CMV-Cre transgenic mice to generate Sirt5 mice. (b) Sirt5 mRNA levels in selected tissues of Sirt5 and Sirt5 mice. Three pairs of 8-week-old wild-wt(wt) and knockout male mice were used for the validation. (c) Sirt5 protein levels in liver and lung of Sirt5 and Sirt5 mice. Two pairs of 8-week-old wt and knockout male mice were used for protein analysis. (d) The birth and sex ratio of Sirt5, Sirt5, and Sirt5 mice. 328 mice were genotyped and analyzed. (e) Global protein succinylation in liver and gastrocnemius muscle. 3 pairs of 12-week-old wt and knockout male mice were fasted for 24 hours and sacrificed for protein analysis. (f) Serum ammonia levels. 12-week-old Sirt5 and Sirt5 male mice were fasted for 24 hours and sacrificed. Blood was collected by cardiac puncture. Serum was immediately frozen in liquid nitrogen and used for measurement of ammonia levels. N = 4. * P < 0.05.
of the Sirt5−/− mice was continuously lower than that of Sirt5+/+ littermates, the difference was not significant (Fig. 2b). The body composition was similar between Sirt5+/+ and Sirt5−/− littermates (Fig. 2c). Furthermore, during indirect calorimetry using the comprehensive lab animal monitoring system (CLAMS), food intake, body heat production, spontaneous locomotor activity, oxygen consumption, respiratory exchange ratio (RER) were comparable between Sirt5+/+ and Sirt5−/− mice (Fig. 2d). The mice were then subjected to different physiological challenges. The cold test revealed a similar tolerance of Sirt5+/+ and Sirt5−/− mice to the cold (Fig. 2e). In an endurance run, the distance run to exhaustion of Sirt5−/− mice was also similar to that of wt controls (Fig. 2f). Sirt5−/− mice demonstrated a slight trend towards improved glucose tolerance compared to wt littermates during an ipGTT test, although only the 2 hr time point reached statistical significance (Fig. 2g). In line with the results of the ipGTT, Sirt5−/− animals are more sensitive to insulin than Sirt5+/+ mice, an effect that was particularly noted during the later phases of the ipITT test (Fig. 2h).

We also measured the levels of major metabolites in the plasma of Sirt5−/− and Sirt5−/− mice. The aspartate transaminase (AST), alanine transaminase (ALT), triglycerides, non-essential fatty acids (NEFA), cholesterol, high-density lipoprotein (HDL)-cholesterol, and low density lipoprotein (LDL)-cholesterol were comparable in the plasma of chow-fed Sirt5+/+ and Sirt5−/− mice (Fig. 3a). At the molecular levels, we examined the liver and skeletal muscle for the expression of a vast set of metabolic genes involved in transcriptional regulation of metabolism (Foxo1, Ppars, Pgc1s, Sirt1), mitochondrial function (Atp5g1, Cs, Cycs), fatty acid oxidation (Acox1, Lcad, Mcad, Cpt1a), lipogenesis (Fasn, SREBP1c), and glucose metabolism (PEPCK, G6Pase) (Fig. 3b and 3c). The deletion of Sirt5 had, however, no significant impact on the expression of genes involved in the major metabolic pathways.

Metabolic characterization of Sirt5 deficient mice fed a HFD. Since no striking metabolic phenotypes were observed in Sirt5−/− mice fed chow diet, we challenged male Sirt5+/+ and Sirt5−/−...
littermates with HFD for 10 weeks and then reevaluated their metabolic profiles using a similar phenotyping protocol as described in Fig. 2a (Fig. 4a). No significant difference in weight gain was observed between \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice (Fig. 4b).

Consistently, body composition (Fig. 4c), food intake, spontaneous locomotor activity, oxygen consumption, and RER (Fig. 4d) were similar between the two genotypes. In addition, both the systolic blood pressure and heart rate were indistinguishable between \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice (Fig. 4e). Also on HFD, the tolerance of \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice to cold was comparable (Fig. 4f). Although during an endurance run, the distance run to exhaustion of \textit{Sirt5}^{−/−} mice tended to be less than that of control mice, the difference was not significant (Fig. 4g). Consistent to what was observed under chow diet, \textit{Sirt5}^{−/−} and \textit{Sirt5}^{+/+} littermates on HFD showed only a slight difference in glucose tolerance during the later phases of an ipGTT (Fig. 4h). Both genotypes showed equal insulin sensitivity during the ipITT test under HFD, although the reverse AUC indicated a difference between \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice (Fig. 4i).

Upon sacrifice, we weighed the metabolic organs and determined the levels of plasma metabolites in HFD-fed \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice. The weights and gross morphology of \textit{Sirt5}^{−/−} brown adipose tissue (BAT), heart, and liver were similar to those of wt mice (Fig. 5a). The epididymal white adipose tissue (eWAT) of \textit{Sirt5}^{−/−} mice weighed less than \textit{Sirt5}^{+/+} littermates. The levels of AST, ALT (Fig. 5b), triglycerides, and NEFA (Fig. 5c) were comparable in the plasma of mice with the two genotypes. Plasma cholesterol was higher in \textit{Sirt5}^{−/−} mice, probably accounted for by the increased level of HDL-cholesterol (Fig. 5d).

At the molecular level, we again examined the livers of HFD fed mice for the expression of metabolic genes. Similar to the results in chow fed mice, the deletion of the \textit{Sirt5} gene had no striking impact on the expression of genes involved in the major metabolic pathways (Fig. 5e, f). Besides reduced expression levels of Acox1, SREBP1c and SREBP2, most of the mRNAs were unchanged in the livers of HFD fed \textit{Sirt5}^{−/−} mice.

### Discussion

We report here the generation and metabolic characterization of whole body \textit{Sirt5} knockout mice. The germline deletion of \textit{Sirt5} caused mice to be born at an abnormal Mendelian ratio, with the number of live-born \textit{Sirt5}^{−/−} offspring being reduced by 40%. This partial loss of \textit{Sirt5}^{−/−} offspring pointed towards a potential important role of \textit{Sirt5} in embryogenesis or early development.

The surviving \textit{Sirt5}^{−/−} mice were characterized by a global protein hypersucyclination in both liver and skeletal muscle. The \textit{Sirt5}^{−/−} mice showed elevated levels of blood ammonia during fasting, but otherwise were metabolically similar to their wt littermates under basal conditions. The general metabolic and physiological parameters of the two genotypes (weight gain, body composition, spontaneous activity, oxygen consumption, response to cold exposure, and endurance) were comparable between \textit{Sirt5}^{+/+} and \textit{Sirt5}^{−/−} mice. \textit{Sirt5}^{−/−} mice tended to have a slightly improved glucose tolerance.
tolerance and a trend towards insulin sensitization, although the changes were rather minimal. Under HFD, the absence of Sirt5 did not protect or sensitize mice to the development of HFD-induced obesity, hypertension, and insulin resistance. Of note, the obese Sirt5^−/− mice may tolerate glucose slightly better than Sirt5^+/+ mice and showed increased serum cholesterol levels. At the molecular level, Sirt5 deficiency did not lead to any overt expression change of the genes involved in the major metabolic pathways, except for the reduced expression of Acox1, SREBP1c, and SREBP2 under HFD.

The fact that multiple and high-level of acylation (acetylation, succinylation, and malonylation) occurs on various mitochondrial proteins favors the possibility of mitochondrial sirtuins being involved in the sensing and removal of inadvertent acyl modifications16,17,19,24,25. This detoxifying and regulatory mechanism may become particularly important under extreme stress conditions, as was demonstrated for Sirt3. Initially Sirt3^−/− mice were reported to be metabolically normal under basal conditions6. Similarly, skeletal muscle and liver-specific Sirt3 deficiency did not induce any phenotypic abnormalities under standard chow and HFD conditions10. However, when exposed to extreme stress, such as seen after fasting followed by cold exposure, 48 hours of fasting6, unfortunately, we are unable to test such stringent stress conditions because of ethical concerns in Switzerland.

In summary, we report here the generation and phenotypic characterization of a germline Sirt5^−/− mouse line. Strikingly, no overt phenotype was observed in Sirt5^−/− mice fed chow or HFD, indicating that Sirt5 may be dispensable for basal homeostasis, under these conditions. Further studies are, however, warranted to explore a role of Sirt5 in particular challenged and stressed conditions. Given the emerging role of protein succinylation in immune response28 and the genetic link between a single-nucleotide polymorphism (SNP) in Sirt5 and brain aging29, such studies should also focus on the role of Sirt5 in immune response and neurodegeneration, or carcinogenesis.

Methods

Generation and maintenance of the Sirt5^−/− mice. Sirt5^floxed mice were generated using standard gene targeting procedures22, using 129SV embryonic stem (ES) cells. These animals were crossed with CMV-Cre transgenic mice31 to generate germline Sirt5^−/− mice, which were subsequently backcrossed for 5 generations with C57BL/6J.
mice. All mice were maintained in a temperature-controlled (23 °C) facility with a 12 hr light/dark cycle and were given free access to food and water. Regular chow diet (2018) and high-fat diet (TD.06414) were obtained from Harlan. Clinical phenotyping of the Sirt5−/− mice. All animal work in the manuscript was performed according to the validated standard operating procedures (SOPs) as defined and validated by the Eumorphia program (see: http://empress.har.mrc.ac.uk/). Animal experiments were approved by the veterinary ethics committee of the canton of Vaud - Switzerland (Permit ID 2163). We subjected our mice to non-invasive monitoring of body fat and lean mass by EchoMRI. Indirect calorimetry to monitor food consumption, body temperature, O2 consumption, CO2 production, and animal activity was measured using CLAMS system (Columbus Instruments). The blood pressure and heart rate was measured by a computerized tail-cuff system (Visitech Systems) in conscious animals.

For the cold test, the body temperature was recorded using a thermometer (Bioseb) with a rectal probe (Physitemp Instruments). A variable speed belt treadmill enclosed in a plexiglass chamber with a stimulus device consisting of a shock grid attached to the rear of the belt (Panlab) was used for the endurance test. Exhaustion was assumed when mice received more than 50 shocks in a 2.5 min interval30. ipGTT and ipITT the rear of the belt (Panlab) was used for the endurance test. Exhaustion was assumed when mice received more than 50 shocks in a 2.5 min interval30. ipGTT and ipITT were performed under the Cornell Institutional Animal Care and Use Committee (IACUC) protocol #2011-0098. Food was depleted for 24 hours during the fasting, but animals could access water freely.

Quantitative RT-PCR. qRT-PCR was performed as described31. Briefly, RNA was extracted from tissues using TRIzol reagent (Invitrogen). cDNA was generated using QuantiTect Rev (Qiagen). The real-time PCR measurement of individual cDNAs was performed using SYBR green dye in the LightCycler System (Roche Diagnostics). A Student’s t-test. Data are expressed as mean ± SEM. Statistical significance is displayed as * (p < 0.05) or ** (p < 0.01).

Western blot analysis. Tissue samples were lysed in lysis buffer (50 mM Tris pH 7.9, 150 mM NaCl, 1 mM EDTA, 1% NP40, 10 mM sodium butyrate, 5 mM nicotinamide, protease inhibitor tablet). Liver and muscle samples were used to extract intact mitochondria2, which were lysed in lysis buffer and subjected to western blot. Protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Anti-Sirt5 was purchased from Abcam. Anti-Tubulin was purchased from Santa Cruz. Anti-succinyllysine antibody was purchased from PTM Biolabs.

Statistical analyses. Statistical analyses were performed with non-parametric Student’s t-test. Data are expressed as mean ± SEM. Statistical significance is displayed as * (p < 0.05) or ** (p < 0.01).

Figure 5 | Metabolic analysis of HFD-treated Sirt5−/− mice after sacrifice. The 33-week-old wt and knockout male mice after 25-week of HFD treatment were fasted for 6 hours and sacrificed. N = 8. (a). The weight of BAT, heart, liver, and eWAT. All the data were normalized to body weight. (b). Plasma AST and ALT. (c). Triglycerides and NEFA in plasma. (d). Cholesterol, LDL-cholesterol, and HDL-cholesterol in plasma. (e) and (f). The expression profile of a selected set of metabolic and inflammation genes in the liver. * P< 0.05, ** P < 0.01.

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**Author contributions**

J.Y. directed and performed most experiments. S.S. measured the serum ammonia level, succinylation in the muscle, and coordinated the collaboration at Cornell. L.N. participated in the metabolic phenotyping of the mouse models. NM measured serum metabolites and analyzed gene expression in the chow-fed mice. B.H. measured the succinylation in the liver. R.W. and H.L. directed the collaboration work at Cornell. K.S. and J.A. directed all the work. J.Y., K.S. and J.A. wrote the manuscript. All authors reviewed the manuscript.

**Additional information**

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