SIRT3 is crucial for maintaining skeletal muscle insulin action and protects against severe insulin resistance in high fat fed mice

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ABSTRACT

Protein hyperacetylation is associated with glucose intolerance and insulin resistance suggesting that the enzymes regulating the acetylome play a role in this pathological process. SIRT3, the primary mitochondrial deacetylase, has been linked to energy homeostasis. Thus, it is hypothesized that the dysregulation of the mitochondrial acetylation state, via genetic deletion of SIRT3, will amplify the deleterious effects of a high fat diet (HFD). Hyperinsulinemic-euglycemic clamp experiments show, for the first time, that mice lacking SIRT3 exhibit increased insulin resistance due to defects in skeletal muscle glucose uptake. Permeabilized muscle fibers from HF-fed SIRT3 KO mice showed that TCA cycle substrate-based respiration is decreased while fatty acid-based respiration is increased, reflecting a fuel switch from glucose to fatty acids. Consistent with reduced muscle glucose uptake, hexokinase II (HKII) binding to the mitochondria is decreased in muscle from HF-fed SIRT3 KO mice, suggesting decreased HKII activity. These results show that absence of SIRT3 in HF-fed causes profound impairments in insulin-stimulated muscle glucose uptake, creating an increased reliance on fatty acids. Insulin action was not impaired in the lean SIRT3 KO mice. This suggests that SIRT3 protects against dietary insulin resistance by facilitating glucose disposal and mitochondrial function.
INTRODUCTION

Lysine acetylation has recently emerged as an important post-translational modification. Consisting of the reversible transfer of the acetyl group from acetyl-CoA to a lysine residue, this highly regulated event modulates diverse cellular processes, including protein-protein interactions, protein subcellular localization, protein stability and enzymatic activity (1-3). Lysine acetylation is also increasingly linked to metabolism regulation and energy balance (4), and it has been shown that high fat feeding induces a shift in the acetylation balance, causing protein hyperacetylation in liver (5). This dysregulation of the acetylation balance could be due to diet-induced increases in acetyl-CoA levels, as observed in liver and muscle (6; 7). In a setting of over-nutrition, deacetylases such as Sirtuins should play a key protective role by counterbalancing increased protein acetylation.

Sirtuins are NAD$^+$-dependent deacetylases and as such are metabolic sensors (8). The Sirtuin family is comprised of seven members that differ by their subcellular distribution, substrate specificity and cellular functions (SIRT1-7) (9). Recent studies have highlighted the importance of mitochondrial Sirtuins in the regulation of metabolism (10). Indeed, numerous mitochondrial proteins involved in metabolism are regulated by acetylation (11). It now appears that lysine acetylation could be the most important regulatory protein modification event within the mitochondria. Recent evidence suggests that mitochondrial protein acetylation is solely regulated by deacetylases, with acetylation being the consequence of unregulated non-enzymatic reactions, driven by acetyl-CoA levels (12). SIRT3 in particular has been identified as the primary mitochondrial deacetylase (13), and has been linked to the regulation of lipid metabolism, reactive oxygen species levels, and energy production (14; 15).

SIRT3 has specifically been shown to direct mitochondrial respiration, and its targets include subunits of the respiratory chain complex (16; 17). Importantly, SIRT3 has been implicated in the development of metabolic disease in both humans and rodents (5). SIRT3
activity is increased by nutrient distress such as fasting, caloric restriction, and forced exercise, while SIRT3 activity is decreased in skeletal muscle of models of type 1 and type 2 diabetes (18). Given that SIRT3 is implicated in mitochondrial function, understanding how SIRT3 is involved in the regulation of metabolism is critical in understanding the link between mitochondrial function and metabolic disease. Mice lacking SIRT3 exhibit a decrease in oxygen consumption in isolated mitochondria from liver (16) and reduced glucose tolerance when placed on a HFD (5). However, the *in vivo* contribution of distinct peripheral tissues to this phenotype remains to be elucidated, as efforts to decipher the distinct roles of SIRT3 in tissue-specific models have remained inconclusive. Indeed, while various global SIRT3 knockout (KO) models consistently exhibit metabolic impairments (5; 16; 18), liver and muscle-specific KO models have no overt metabolic phenotype (19) as assessed by primary screening tools (20).

Here, we report, for the first time, the assessment of insulin sensitivity in chow and HF-fed SIRT3 KO mice using the hyperinsulinemic-euglycemic clamp. In this study, we show that HF-fed SIRT3 KO mice have exacerbated insulin resistance compared to their WT littermates, and that this phenotype is due to defects in skeletal muscle metabolism. Furthermore, we show that SIRT3-deficient muscle exhibits profound mitochondrial dysfunction, with decreased reliance on glycolytic substrates and increased reliance on fatty acid substrates, as assessed by high-resolution respirometry on permeabilized muscle fibers.
RESEARCH DESIGN AND METHODS

Mouse models

Mice lacking the SIRT3 protein (SIRT3 KO) and wild-type littermates (WT) on a C57BL/6J strain (16) were fed chow (13.5% calories from fat; 5001, LabDiet) or HFD (60% calories from fat; F3282, BioServ) for 12 weeks from 3 weeks of age. Mice were housed in a temperature/humidity-controlled environment (light cycle: 6am-6pm). Studies were performed on 15-week 5h-fasted male mice. Vanderbilt IACUC approved procedures. Body composition was determined by NMR.

Hyperinsulinemic-euglycemic clamp

One week before hyperinsulinemic-euglycemic clamps, carotid artery and jugular vein catheters were surgically placed for sampling and infusions. Mice were 5h-fasted then clamped as described (21; 22). [3-3H]glucose was primed and continuously infused ($t=-90-0\min$; $0.04\mu\text{Ci}/\text{min}$). The clamp was initiated at $t=0\min$ with a continuous insulin infusion ($4\mu\text{U}/\text{kg}/\text{min}$). Arterial glucose was monitored every 10min, to provide feedback for adjustment of the glucose infusion rate (GIR) (50% dextrose+[3-3H]glucose). [3-3H]glucose kinetics were determined at $-10\min$ and from 80-120min. $2^{[14]}\text{C}$deoxyglucose ($[^{14}\text{C}]2\text{-DG}, 13\mu\text{Ci}$) was administered at 120min to determine $R_g$ (tissue-specific glucose uptake). Plasma insulin was determined by ELISA. Plasma non-esterified fatty acids and tissue triglycerides were measured spectrophotometrically. See Supplemental Material for details.

High-resolution respirometry on permeabilized muscle fibers

Fibers from gastrocnemius of 5h-fasted mice were prepared in BIOPS (23). Respiration was measured in Buffer Z at 37°C (Oxygraph O2k) with 2mM malate+10mM glutamate or 2mM
malate+75µM palmitoyl-carnitine (24). With 2mM ADP (MG+ADP; MPC+ADP), coupled respiration is maximally stimulated. Citrate synthase activity was measured spectrophotometrically (25).

Immunoprecipitations and Western blots

Whole tissue lysates were obtained by homogenization in Lysis Buffer. Mitochondrial fractions were obtained by homogenization in Buffer M. The mitochondrial pellet was washed twice before resuspension in Lysis Buffer. For immunoprecipitations, lysates were incubated 1h with anti-IRS1 or anti-VDAC, then overnight with A/G-PLUS-Agarose. Beads were washed four times in PBS then resuspended in Loading Buffer. Samples were applied to 4-12% SDS-PAGE. Quantifications were performed using the Odyssey system.

Immunostaining

CD31 staining was assessed by immunohistochemistry in paraffin-embedded gastrocnemius sections. Gastrocnemius cryosections were stained with antibodies anti-GLUT4 and anti-CAV3 followed by visualization with fluorescent secondary antibodies. Images were acquired at 40X magnification (Zeiss LSM 510 confocal microscope). Image analysis was performed using ImageJ (see Supplemental Material for details).

Statistics

Data are expressed as mean±SE. Student t-test or two-way ANOVA followed by Tukey post hoc tests were performed. p<0.05 was considered significant. Additional method details can be found in Online Supplemental Material.
RESULTS

Mice lacking SIRT3 exhibit increased skeletal muscle mitochondrial protein acetylation

SIRT3 protein in vastus lateralis was measured in muscle from WT and SIRT3 KO mice fed chow or HFD for 12 weeks. As expected, SIRT3 protein is absent in SIRT3 KO muscle (Figure 1A). HF-feeding does not affect SIRT3 protein expression in WT skeletal muscle. We were interested in determining how SIRT3 deletion or a HFD would impact protein acetylation in the mitochondrial and cytosolic compartments. SIRT3 deletion increased acetylation exclusively in skeletal muscle mitochondria (Figure 1B and 1C). HF-feeding alone induced an important increase in mitochondrial protein acetylation in muscle from both WT and SIRT3 KO mice, but did not affect protein acetylation in the cytosol. Interestingly, the gene and diet effects were additive, as SIRT3 KO mice fed a HFD exhibited the highest mitochondrial protein acetylation.

High fat-fed SIRT3 KO mice have increased skeletal muscle insulin resistance

To study the role of SIRT3 in the pathogenesis of insulin resistance, we fed WT and SIRT3 KO mice a chow or HFD for 12 weeks. We observed no difference in weight gain within their respective diets (Table 1). The WT and SIRT3 KO chow-fed mice were not different with respect to basal fasting blood glucose, plasma insulin, plasma NEFAs and liver triglycerides (Table 1). HF-feeding raised fasting blood glucose comparably in both genotypes. Basal plasma insulin was higher in HF-fed SIRT3 KO mice compared to their WT littermates, which is indicative of exaggerated insulin resistance (Table 1). We observed no difference in plasma NEFA and liver triglycerides between HF-fed WT and SIRT3 KO mice.

To assess whole body insulin sensitivity, we performed hyperinsulinemic-euglycemic clamps. During the clamp, blood glucose was clamped at 130 mg/dL in all groups (Figures 2A and 2D), and insulin was raised to comparable levels between groups (Table 1). Circulating
NEFAs were suppressed in all groups, suggesting equivalent insulin-induced suppression of lipolysis (Table 1). The GIR necessary to maintain euglycemia in the chow-fed animals was similar between genotypes (Figure 2B). However, on a HFD the GIR was significantly lower in the SIRT3 KO mice, indicating increased insulin resistance (Figure 2E). EndoRa was completely suppressed in both chow groups during the clamp (Figure 2C and 2G). On a HFD, SIRT3 KO mice have a slightly lower basal EndoRa and a lower clamp EndoRa (Figure 2F), likely due to the slightly higher insulin levels in these mice (Table 1). However, the insulin-induced suppression of EndoRa and %EndoRa Suppression were similar between genotypes (Figure 2F and 2G). In addition, the P-Akt/Akt ratio in WT and SIRT3 KO livers were not different (Figure 2H).

Basal and clamp Rd, and consequently the insulin-stimulated increase in Rd, were not different between genotypes on a chow diet (Figure 3A). In addition, Rg, a measure of tissue-specific glucose uptake, was not different between chow-fed WT and SIRT3 KO mice in gastrocnemius, vastus lateralis, soleus, diaphragm and heart (Figure 3B). These data are consistent with the similar GIR observed in lean WT and SIRT3 KO mice, suggesting SIRT3 is not a critical determinant of insulin action in chow-fed mice. However, when fed a HFD, both basal and clamp Rd were significantly decreased in SIRT3 KO mice (Figure 3C). In addition, the insulin-stimulated increase in Rd was significantly lower in SIRT3 KO mice, indicating that the ability of insulin to stimulate peripheral glucose uptake is greatly diminished in HF-fed SIRT3 KO mice (Figure 3C). Gastrocnemius, vastus lateralis, soleus and diaphragm Rg were also dramatically reduced in HF-fed SIRT3 KO mice (Figure 3D). These data are consistent with the reduced GIR, as they reflect higher insulin resistance in SIRT3 KO mice. Moreover, these results clearly indicate that skeletal muscle insulin resistance to muscle glucose uptake is responsible for the observed whole body phenotype.
Mice lacking SIRT3 exhibit normal skeletal muscle insulin signaling and GLUT4 translocation

To investigate whether skeletal muscle insulin resistance in HF-fed SIRT3 KO mice was associated with decreased insulin signaling, we performed Western blots for key proteins of the insulin signaling pathway. The P-Akt/Akt and P-AS160/AS160 ratios were increased by insulin during the clamp in muscle from both WT and SIRT3 KO HF-fed mice, with no difference between genotypes (Figure 4A). We also investigated phosphorylation of GSK3β as a surrogate marker for Akt activity, and did not observe differences in P-GSK3β/GSK3β (Figure S1). By immunoprecipitating IRS1 in insulin-stimulated muscle homogenates, we showed that p85 binding to IRS1, while decreased by HF-feeding, was not different between genotypes (Figure 4B). Furthermore, the ratio of phospho-IRS1 to IRS1 was not different between groups (Figure 4B). Western blotting for GLUT4 and GLUT1 in insulin-stimulated muscle lysates revealed GLUT4 and GLUT1 levels were not different between groups (Figure 4C). In addition, GLUT4 intensity at the muscle plasma membrane, as detected by immunofluorescence, was not different, suggesting no defects in the insulin-induced GLUT4 translocation event (Figure 4D). Taken together, these results suggest that the insulin signaling pathway was not impaired in SIRT3 KO muscle and does not explain the insulin resistance observed in mice lacking SIRT3.

Decreased muscle vascularization is sufficient to induce insulin resistance in mice (26). We therefore investigated whether this factors into the muscle insulin resistance observed in HF-fed SIRT3 KO mice. We found that staining for endothelial cell marker CD31 in gastrocnemius was not different between genotypes, regardless of diet (Figure S2).

Mitochondrial respiration is decreased in skeletal muscle of SIRT3 KO mice
We hypothesized that loss of SIRT3 would impair mitochondrial respiration, as SIRT3 tightly regulates respiration in isolated mitochondria (16; 18). We performed high-resolution respirometry on permeabilized fibers prepared from red gastrocnemius obtained from chow and HF-fed WT and SIRT3 KO mice. We observed that respiration was decreased in fibers from chow-fed SIRT3 KO mice in presence of malate/glutamate, both in basal (MG) and ADP-stimulated (MG+ADP) states (Figure 5A). This indicates decreased respiration through Complex I in SIRT3 KO muscle. We observed no difference in respiration between genotypes in the presence of fatty-acid substrates (Figure 5C).

As in chow-fed mice, respiration was decreased in MG and MG+ADP conditions in HFD SIRT3 KO fibers (Figure 5B). Interestingly, when supplied with MPC, oxygen consumption was significantly higher in both basal and ADP-stimulated conditions in SIRT3 KO fibers (Figure 5D). Citrate synthase activity and OXPHOS proteins were not different between groups, indicating similar mitochondrial abundance in these muscles (Figure 5E and S3)(27). Therefore the differences in respiration observed between WT and SIRT3 KO mice cannot be attributed to differences in mitochondrial content. In addition, HF-fed SIRT3 KO mice have decreased muscle triglyceride content compared to their WT littermates (Figure 5F). Taken together, these results show that SIRT3 KO muscle fibers have defective Complex I-dependent respiration, and when subjected to a HFD, have increased reliance on fatty acids to provide substrates for the respiratory chain.

**Hexokinase II association with mitochondria is decreased in skeletal muscle of SIRT3 KO mice**

To further examine the defects in muscle glucose uptake in SIRT3 KO mice, we investigated hexokinase II (HKII) cellular compartmentation. HKII binds to the outer mitochondrial membrane and this directs its activity, with active HKII being bound to voltage-
dependent anion channel (VDAC), an outer-mitochondrial transmembrane protein (28). Immunoblots for HKII performed on muscle mitochondrial and whole tissue fractions showed that mitochondria-bound HKII was significantly decreased in HF-fed SIRT3 KO muscle, as compared to WT controls (Figure 6A). As no difference in total HKII was observed between groups, the ratio of mitochondria-bound to total HKII, indicative of overall HKII activity, was significantly reduced in muscle of HF-fed SIRT3 KO mice (Figure 6A). Consistent with this finding, we observed decreased HK activity in gastrocnemius and vastus lateralis in HF-fed SIRT3 KO mice compared to their WT littermates (Figure 6B). As a control, HK activity was not affected in the brain. In addition, the ability of insulin to stimulate glycogen synthesis during the clamp was severely blunted in muscle of HF-fed SIRT3 KO mice (Figure 6C). In accordance with this result, the net glycogen synthetic rate was reduced in these tissues (Figure 6D).

HKII docking to the mitochondria is dependent on binding of VDAC to the adenine nucleotide transporter (ANT), located in the inner mitochondrial membrane. We therefore hypothesized that VDAC binding to ANT would be decreased, causing dissociation of the HKII-VDAC-ANT complex in muscle from SIRT3 KO mice. VDAC was immunoprecipitated from mitochondrial protein fractions to assess the association of HKII, VDAC, and ANT. In accordance with our previous results, HKII binding to VDAC was decreased in SIRT3 KO mice (Figure 7A and B). We also showed that the VDAC-ANT association was decreased in skeletal muscle lacking SIRT3 (Figure 7A and C). Taken together, these results show that HF-fed SIRT3 KO mice exhibit decreased HKII binding to the mitochondria associated with decreased VDAC-ANT complex formation.
DISCUSSION

High fat feeding in mice is a well-described model of over-nutrition, and has been shown to induce liver mitochondrial protein hyperacetylation (5). Nutrient overload is associated with increased levels of acetyl-CoA, substrate for protein acetylation, in liver (7) and muscle (6). This is consistent with the idea that mitochondrial protein acetylation is driven by acetyl-CoA abundance (12). In the present study, we challenged WT and SIRT3 KO mice with nutrient overload to amplify protein acetylation. Our results show that SIRT3 is a crucial regulator of glucose fluxes under conditions of over-nutrition, as whole body insulin sensitivity and insulin-stimulated muscle glucose uptake are markedly impaired. We further report the novel finding of the disassociation of the mPTP complex and decrease in mitochondrial HKII binding. Importantly, HKII activity is a key site of control of muscle glucose uptake (29-31). In addition, SIRT3 knockout resulted in impaired mitochondrial carbohydrate-based substrate oxidation.

Consistent with the role of SIRT3 as the major mitochondrial deacetylase, we find that SIRT3 deletion increases skeletal muscle protein acetylation specifically in the mitochondrial compartment. HFD increases acetyl-CoA in skeletal muscle, expanding the precursor pool for acetylation (6). This resulted in greater acetylation of mitochondrial proteins. This imbalance is relatively small in the presence of SIRT3 but is increased synergistically in the absence of SIRT3. This is in line with the hypothesis that SIRT3 is a master regulator of the mitochondrial acetylome. Its presence effectively counteracts hyper-acetylation. Accordingly its absence combined with nutrient overload causes a marked increase in mitochondrial protein acetylation with functional consequences.

For the first time, insulin sensitivity was assessed in conscious SIRT3 KO mice on chow and HF-fed mice. Consistent with previous studies (5; 13), we showed that lean SIRT3 KO mice have no overt metabolic dysfunction, despite protein hyperacetylation present in the muscle mitochondria. In contrast, HF-feeding dramatically increased insulin resistance in SIRT3 KO.
Thus, while SIRT3 may not be critical to muscle insulin action in lean mice, it becomes crucial in the face of nutrient overload, where it is needed to offset the increased mitochondrial protein acetylation. Interestingly, clamp studies revealed that this insulin resistant phenotype was due solely to effects on skeletal muscle. Indeed, the glucose rate of disappearance Rd was strongly reduced in HF-fed SIRT3 KO mice at basal and during the clamp, and the insulin-induced increase in Rd was also markedly decreased. In accordance with this result, muscle glucose uptake was strongly impaired in skeletal muscle. Indeed, the insulin-induced and % suppression of EndoRa is equal between genotypes, suggesting that insulin action on the liver is similar between genotypes. In addition, liver triglycerides were similar between genotypes. Others have concluded that the liver is a driver of the metabolic phenotype in SIRT3-deficient mice (5; 15). The techniques used in those studies, such as glucose tolerance tests, do not delineate whether the mouse is more insulin sensitive, nor do they identify the tissues responsible for phenotypic differences. More importantly, those studies used 24h-fasted mice, as opposed to our 5h-fasted mice. These differences in fast duration are critical in mice, particularly as liver fatty acid and glucose metabolism are extremely different in those two states (32; 33). By combining the insulin clamp technique with isotopic methods, we show that SIRT3 deletion in HF-fed mice strongly impairs skeletal muscle metabolism and that this induces the severe insulin resistant phenotype observed in our mice.

Fernandez-Marcos et al. concluded that muscle-specific SIRT3 KO mice were not affected by HF-feeding, as assessed by glucose and insulin tolerance tests (19). The techniques as employed were not designed to assess insulin sensitivity, but rather glucose tolerance and the hypoglycemic-responsiveness of the hypothalamic-pituitary-adrenal axis (34; 35). Furthermore, effects on muscle were not assessed in that study. One cannot rule out that SIRT3 deficiency in multiple tissues may be necessary to exacerbate insulin resistance in HF-fed mice. We have shown that a reduction in capillary density is sufficient to cause muscle insulin resistance (26).
We therefore hypothesized that absence of SIRT3 in endothelial cells could affect capillary density and aggravate the muscle insulin resistance in our model. However, our results show that the endothelial cell marker CD31 is not different in WT and SIRT3 KO mice.

Studies have shown that SIRT3 deletion impairs ATP generation (15; 17), and this could be due to decreased mitochondrial respiration. Indeed, SIRT3 activity has been shown to positively correlate with oxygen consumption in isolated muscle mitochondria (16) and cell lines (18; 36). Assessment of muscle respiratory capacity by high-resolution respirometry on permeabilized fibers, as performed here, enables analysis of mitochondrial respiration in situ, with intact inter-mitochondrial morphology, cytoskeleton and myofilaments (23; 37). We showed that respiration is markedly decreased in the presence of Complex I substrates in SIRT3 KO fibers. This result is consistent with studies showing that Complex I is a direct SIRT3 target, and that SIRT3 deletion decreases Complex I activity (16). Moreover, we showed that SIRT3 KO muscle undergoes a fuel switch, whereby the mitochondria rely more on fatty acids as substrates for oxidative phosphorylation. While this trend is observed in chow-fed mice, it is dramatic in HF-fed mice. Interestingly, fatty acids feed substrates to Complexes II and III, suggesting that mitochondria of SIRT3 KO muscle may compensate for Complex I deficiency by increasing electron flux through Complexes II and III. Given that these mice have markedly reduced muscle glucose uptake, we hypothesize that muscle mitochondria in SIRT3 KO mice increase their reliance on fatty acid substrates to compensate for reduced glycolytic substrates. In support of this hypothesis, we show that muscle triglycerides are reduced in HF-fed SIRT3 KO mice. However, despite this compensatory mechanism, the overall oxygen consumption remains reduced in these tissues. This likely contributes to the decreased ATP seen in the skeletal muscle of this model (17).

Finally, regulatory events that couple mitochondrial function to muscle glucose uptake were investigated. Hexokinase activity is a key determinant of muscle glucose uptake (38).
Hexokinase binding to the mitochondria forms the most direct link from mitochondria function to muscle glucose uptake. The mPTP is upstream of the respiration chain and links oxidative phosphorylation to glucose uptake by forming a complex with HKII on the outer mitochondrial membrane. HKII is a crucial step in determining glucose uptake, as it is responsible for creating the downhill concentration gradient that favors glucose transport into the cell. It irreversibly commits glucose to glucose-utilizing pathways such as glycolysis in skeletal muscle (39). HKII activity is modulated by its binding to VDAC on the outer mitochondrial membrane (39). VDAC itself binds ANT in the inner mitochondrial membrane to form the mPTP, a pore that allows exchange of ADP and ATP across the mitochondrial membrane (28; 40). The binding of HKII to mPTP increases HKII activity and gives it “privileged access” to its substrate ATP (41; 42), while allowing rechanneling of its product ADP directly back into the mitochondria (39). Therefore, the highly efficient HKII-mPTP complex promotes high rates of glycolysis. In HF-fed SIRT3 KO muscle, we found that not only was HKII dissociated from the mPTP and its activity reduced, but VDAC and ANT were disassociated, suggesting a closed mPTP and reduced rates of glycolysis. In line with this hypothesis, we found reduced insulin-stimulated glycogen content and glycogen synthetic rate in HF-fed SIRT3 KO muscle.

The manner in which SIRT3 modulates HKII binding to VDAC is currently unknown, and there are many different mechanisms by which this could occur. We know acetylation status is changing within the mitochondria, and we believe acetylation events are modulating the formation of the mPTP. However we do not know if HKII binding is a consequence of a disrupted mPTP, or if pore dissociation is subsequent to HKII dissociation. Given that little is known about the regulation of HKII binding to the mPTP in muscle, we cannot state whether it is a direct or indirect event, or whether it is mediated from within the mitochondria or from the cytosol. The latter is indeed a possibility since both HKII and VDAC have post-translational modifications that have been described to affect HKII binding (42-44). The pathways and
proteins involved in these events could include SIRT3 targets and signaling events that are, as of yet, unidentified. Further studies are necessary to identify specific SIRT3 targets that mediate HKII binding to VDAC.

The reduced activity and binding of HKII to VDAC is a critical component in our model, as it is established that glucose phosphorylation by HKII is a key step in muscle glucose uptake in insulin-stimulated conditions (29; 30; 45). We have shown that a 50% reduction in HKII in mice is sufficient to impair muscle glucose uptake (29). We therefore postulate that decreased HKII binding to the mitochondria contributes to the SIRT3 KO mouse muscle phenotype. In addition to decreased HKII activity in HF-fed SIRT3 KO muscle, we show a dissociated mPTP, as evidenced by decreased VDAC-ANT binding, likely leading to reduced trafficking of nucleotides in and out of the mitochondria. Taken together, these events may lead to decreased glycolytic rates and glucose-based respiration, and a compensatory increase in fatty acid-based respiration. Our work is consistent with previous studies showing reduced glycolytic rates and increased fatty acid utilization in SIRT3-deficient muscle (36). It has long been suggested that SIRT3 is involved in reprogramming metabolism during calorie restriction, allowing respiration to continue during times of low-nutrient availability (46). We show here that SIRT3 is absolutely critical in times of over-nutrition, when high acetyl-CoA levels lead to mitochondrial protein hyperacetylation. SIRT3, as the major mitochondrial deacetylase, becomes crucial for removal of these nonspecific acetylations, and ultimately maintains efficient respiration, glycolysis, and ATP generation in the muscle.

Our findings demonstrate for the first time an insulin resistant permissive phenotype in the SIRT3 KO mice, due specifically to skeletal muscle insulin resistance. Moreover, we show that SIRT3 promotes, from within the mitochondria, muscle glucose uptake and subsequently insulin sensitivity. SIRT3 is therefore a major mitochondrial protein that could, when activated, effectively protect against severe insulin resistance by promoting muscle glucose uptake and
mitochondrial respiration. SIRT3 is in fact associated with metabolic disease in humans, as a single nucleotide polymorphism recently identified in the human Sirt3 gene causing decreased SIRT3 activity is associated with metabolic syndrome (5). Therefore, novel therapeutic approaches targeting SIRT3 activity may be key in providing new opportunities to treat insulin resistance and type 2 diabetes.
Acknowledgments

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No potential conflicts of interest relevant to this article were reported.

L.L. researched data and wrote the article. A.S.W. researched data, contributed to discussion and reviewed article. I.M.W., K.K.Y., D.P.B., M.G. and F.D.J. researched data. D.R.G. supplied material, contributed to discussion and reviewed article. D.H.W. oversaw the project, supplied material, contributed to discussion and reviewed the article. D.H.W is the guarantor of the work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Table 1: Characteristics of the SIRT3 knockout mice. Data are expressed as mean ± SE. ** p< 0.01 WT vs. SIRT3 KO

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FIGURE LEGENDS

Figure 1: Skeletal muscle mitochondrial protein acetylation is increased in chow and HF-fed SIRT3 KO mice.
A. Protein homogenates extracted from vastus lateralis were applied to a 4-12% SDS-PAGE. Western blotting was performed using the anti-SIRT3 and β-actin antibodies. Quantifications below the blot were obtained by normalizing SIRT3 band intensity to β-actin band intensity for each lane (n=4).
B and C: Mitochondrial and cytosolic proteins were extracted from vastus lateralis isolated from 5h fasted WT or SIRT3 KO mice fed a chow of HFD for 12 weeks. Mitochondrial (B) and cytosolic (C) protein lysates were subjected to a 4-12% SDS-PAGE and Western blotting was performed using an anti-acetyl-lysine antibody. Integrated intensities of acetyl-lysine bands from mitochondrial (B) or cytosolic (C) proteins were obtained by the Odyssey software and normalized to VDAC or GAPDH respectively (n=5). (*p<0.05; **p<0.01; ***p<0.001)

Figure 2: HF-fed SIRT3 KO mice exhibit increased insulin resistance during the hyperinsulinemic-euglycemic clamp.
A and D: Blood glucose was monitored throughout the clamp at 10min intervals by sampling from the arterial catheter. Blood glucose was maintained at euglycemia (130 ~ 140 mg/dL) in both chow (A) and HF-fed (D) mice.
B and E: Rate of glucose infused in the venous catheter in order to maintain euglycemia in chow (B) or HF-fed (E) mice.
C and F: Endogenous glucose production (EndoRa), a marker of hepatic glucose production, in chow (C) or HF-fed (F) mice, determined by administration of [3-3H]glucose. The insulin-induced suppression of EndoRa is the difference between basal and clamp EndoRa.
G: Insulin-induced suppression of EndoRa expressed in % of Basal.
H: Liver protein homogenates obtained from 5h-fasted (Basal) or clamped (Insulin) mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for Akt and P-Akt (Ser473). Integrated intensities were obtained by the Odyssey software. (*p<0.05; **p<0.01; ***p<0.001)

Figure 3: HF-fed SIRT3 KO mice have increased skeletal muscle insulin resistance.
A and C: Glucose disappearance rate (Rd) in chow (A) or HF-fed (C) WT and SIRT3 KO mice. The insulin-induced increase in Rd is the difference between clamp and basal Rd and represents the ability of insulin to stimulate peripheral tissue glucose uptake.
B and D: Glucose uptake (Rg) in gastrocnemius, vastus lateralis, soleus, diaphragm and cardiac muscles determined by administration of nonmetabolizable glucose 2[14C]deoxyglucose in chow (B) or HF-fed (D) WT and SIRT3 KO mice. (*p<0.05; **p<0.01; ***p<0.001)

Figure 4: Insulin signaling and GLUT4 translocation are not affected in skeletal muscle of SIRT3 KO mice
A: Gastrocnemius protein homogenates obtained from 5h-fasted (Basal) or clamped (Insulin) HF-fed mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for Akt, P-Akt (Ser473), AS160 and P-AS160 (Thr588). Integrated intensities were obtained by the Odyssey software.
B: IP from gastrocnemius extracts obtained from clamped (Insulin) mice were performed with total IRS-1 antibody; then, immunoblots were completed for IRS1, P-IRS1 (Ser612) and the p85 subunit of PI3K (n=6). Integrated intensities were obtained by the ImageJ software.
C: Western blotting was performed for GLUT1 and GLUT4 on gastrocnemius protein homogenates obtained from 5h-fasted (Basal) or clamped (Insulin) mice (n=4). Integrated intensities were obtained by the Odyssey software and normalized to GAPDH intensities.

D: Confocal imaging of GLUT4 and plasma membrane marker Caveolin-3 (CAV3) were performed on clamped (insulin) gastrocnemius cryosections (n=5-8). (*p<0.05; **p<0.01; ***p<0.001)

**Figure 5: Oxygen flux is reduced in permeabilized fibers from gastrocnemius of SIRT3 KO mice fed a chow or HFD.**

A and B: High-resolution respirometry is performed on permeabilized red gastrocnemius fiber bundles isolated from 5h fasted WT or SIRT3 KO mice fed a chow (A) or HFD (B). Oxygen flux is first measured in presence of malate (2mM) and glutamate (10mM) (MG). Saturating ADP (2mM) is then added to the chamber (MG + ADP). Oxygen flux is normalized to fiber dry weight (n=8-10).

C and D: High-resolution respirometry is performed on permeabilized red gastrocnemius fiber bundles isolated from 5h fasted WT or SIRT3 KO mice fed a chow (C) or HFD (D), in presence of malate (2mM) and palmitoyl-carnitine (75μM) (MPC), followed by addition of saturating ADP (2mM) (MPC + ADP). Oxygen flux is normalized to fiber dry weight (n=8-10).

E: Citrate synthase activity was measured in red gastrocnemius from 5h fasted WT or SIRT3 KO mice fed a chow or HFD (n=8).

F: Triglyceride content was determined in vastus lateralis from 5h fasted WT or SIRT3 KO chow or HF-fed mice (n=6-9). (*p<0.05; **p<0.01; ***p<0.001)

**Figure 6: SIRT3 deletion decreases HKII binding to the mitochondria**

A: Mitochondrial or whole muscle proteins were extracted from vastus lateralis isolated from 5h fasted WT or SIRT3 KO mice, and lysates were subjected to a 4-12% SDS-PAGE (n=4-6). Western blotting was performed using anti-HKII, anti-VDAC, anti-GAPDH or anti-β-actin antibodies. HKII band intensities from the mitochondrial fraction were quantified using the Odyssey software and normalized to VDAC. HKII band intensities from the whole muscle fraction were quantified using the Odyssey software and normalized to β-actin. The ratio of mitochondria-bound HKII to total HKII intensities was then calculated.

B: HK activity was determined by dividing the amount of 2[^14]C]deoxyglucose-6-phosphate by the amount of 2[^14]C]deoxyglucose found in gastrocnemius, vastus lateralis, and brain in 5h fasted WT or SIRT3 KO HF-fed mice at the end of the insulin clamp (n=6-9).

C: Glycogen content was determined in vastus lateralis obtained from 5h fasted WT or SIRT3 KO mice fed a HFD (n=6-9).

D: The Glycogen Synthetic Rate was determined in vastus lateralis in 5h fasted WT or SIRT3 KO HF-fed mice by measuring the rate of incorporation of [3-^3H]glucose into glycogen during the insulin clamp (n=6-9). (*p<0.05; **p<0.01; ***p<0.001)

**Figure 7: SIRT3 deletion affects mitochondrial Permeability Transition Pore (mPTP) formation in skeletal muscle of HF-fed mice.**

A: Mitochondrial proteins were extracted from vastus lateralis isolated from 5h fasted WT or SIRT3 KO mice, and IP for VDAC was performed on 400μg of proteins. Western blotting was performed on immunoprecipitated proteins using anti-HKII, anti-ANT and anti-VDAC antibodies. Quantifications of bands for HKII and ANT obtained by Odyssey are presented in panels B and C respectively (n=4-6). (*p<0.05; **p<0.01; ***p<0.001)
**Figure A**

Bar graph showing integrated intensity normalized to VDAC (arbitrary units) for SIRT3 and β-actin in WT and SIRT3 KO mice on Chow and High Fat Diet. Values are presented as mean ± SEM. The asterisks indicate statistical significance: *p<0.05, **p<0.01, ***p<0.001.

**Figure B**

Western blot images showing Acetyl-lysine in Mitochondria. Acetyl-lysine bands are quantified in integrated intensity normalized to VDAC (arbitrary units). 

**Figure C**

Western blot images showing Acetyl-lysine in Cytosol. Acetyl-lysine bands are quantified in integrated intensity normalized to GAPDH (arbitrary units).
A. Arterial Glucose

B. Glucose infusion rate

C. Hepatic glucose production

D. Plasma glucose (mg/dL)

E. Glucose infusion rate (mg/kg/min)

F. Insulin-induced suppression of EndoRa (mg/kg/min)

G. % Suppression of EndoRa

H. Liver P-Akt / Akt

Insulin-induced suppression of EndoRa was significantly decreased in SIRT3 KO compared to WT, as indicated by the p=0.05 comparison.
**Glucose rate of disappearance**

- **A:**
  - WT Chow
  - SIRT3 KO Chow
  - Basal vs. Clamp
  - Insulin-induced increase in Rd (mg/kg/min)

- **B:**
  - WT Chow
  - SIRT3 KO Chow
  - Tissue-specific glucose uptake

**Diabetes**

- **C:**
  - WT High Fat
  - SIRT3 KO High Fat
  - Basal vs. Clamp
  - Insulin-induced increase in Rd (mg/kg/min)

- **D:**
  - WT High Fat
  - SIRT3 KO High Fat
  - Tissue-specific glucose uptake

*Significance levels indicated with asterisks (*) and double asterisks (**) indicate statistical differences between groups.
A

B

C

D

Integrated intensity (arbitrary units)

Integrated intensity (arbitrary units)

Integrated intensity (arbitrary units)

Integrated intensity (arbitrary units)

GLUT1 / GAPDH

GLUT4 / GAPDH

GLUT4 Intensity at the Plasma Membrane
**Malate/glutamate-based respiration**

- A: Oxygen flux (pmol/s/mg dry weight)
  - WT Chow vs. SIRT3 KO Chow
  - MG vs. MG + ADP

- B: Oxygen flux (pmol/s/mg dry weight)
  - WT High Fat vs. SIRT3 KO High Fat
  - MG vs. MG + ADP

**Malate/acytelyl carnitine-based respiration**

- C: Oxygen flux (pmol/s/mg dry weight)
  - WT Chow vs. SIRT3 KO Chow
  - MPC vs. MPC + ADP

**Citrate Synthase Activity**

- E: Citrate Synthase Activity (U/g protein)
  - WT vs. SIRT3 KO
  - Chow vs. High Fat

**Malate/palmitoyl carnitine-based respiration**

- D: Oxygen flux (pmol/s/mg dry weight)
  - WT High Fat vs. SIRT3 KO High Fat
  - MPC vs. MPC + ADP

**Muscle Triglycerides**

- F: Muscle Triglycerides (mg/g muscle)
  - WT vs. SIRT3 KO
  - Chow vs. High Fat
  - p=0.1
A) Mitochondria-bound HKII and Total HKII levels were measured in WT and SIRT3 KO mice fed Chow or High Fat diets. Significant differences were observed (*p<0.05).

B) HK Activity in various tissues (Gastrocnemius, Vastus, Brain) was assessed between WT and SIRT3 KO mice fed Chow or High Fat diets. Significance was noted for P-2DG/2-DG levels (*p<0.05).

C) Glycogen content and Glycogen Synthetic Rate were measured in WT and SIRT3 KO mice fed High Fat diet. Insulin clamp significantly increased Glycogen content and Synthetic Rate (*p<0.05).

D) Glycogen Synthetic Rate was measured in WT and SIRT3 KO mice fed High Fat diet. Significant differences were observed (*p<0.05).
A) Western blot images showing the expression levels of HKII, ANT, and VDAC in WT and SIRT3 KO mice under Chow and High Fat conditions.

B) Integrated intensity normalized to VDAC (arbitrary units) for HKII.

C) Integrated intensity normalized to VDAC (arbitrary units) for ANT.
Supplemental Data

Antibodies
Antibodies against Acetylated-lysine, SIRT3, Hexokinase II, phospho-Akt (Ser473), Akt, phospho-IRS1 (Ser612), IRS1, phospho-AS160 (Thr588), GSK3β, phospho-GSK3β (Ser9) were purchased from Cell Signaling. The antibodies against p85 subunit of phosphoinositide 3-kinase and AS160 were from Millipore. Anti-GLUT4 and total Rodent OXPHOS antibody were from Abcam. Anti-IRS1 and anti-VDAC antibodies for immunoprecipitation were purchased from Santa Cruz. Equal loading was assessed using β-actin (Cell Signaling), GAPDH or VDAC (abcam). The anti-GLUT4 antibody used for immunofluorescence was a gift from Dr. Jeffrey E. Pessin (Albert Einstein College of Medicine). Anti-Cav3 was from Santa Cruz.

Buffer compositions
BIOPS: CaK₂EGTA (2.77 mM), K₂EGTA (7.23 mM), Na₂ATP (5.77 mM), MgCl₂*6H₂O (6.56 mM), Na₂Phosphocreatine (15 mM), Imidazole (20 mM), Taurine (20mM), K-MES (50 mM), pH 7.1
Buffer Z: KCl (30 mM), K-MES (105 mM), K2HPO4 (10 mM), MgCl₂*6H₂O (5 mM), EGTA (1 mM), K₂EGTA (7.23 mM), Imidazole (20 mM), Taurine (20mM), Dithiothreitol (0.5 mM), fatty acid free BSA (0.5g/L), pH 7.1
Lysis Buffer: 25mM Tris-HCl pH7.4, 10mM EDTA, 10% Glycerol, 1% Triton-X100, HALT protease and phosphatase inhibitor cocktail (Pierce) and deacetylase inhibitors Nicotinamide (10mM) and Trichostatin A (1µM).
Buffer M: 100mM KCl, 40mM Tris HCl, 10mM Tris base, 5mM MgCl2, 1mM EDTA, 1mM ATP, pH = 7.5 with HALT protease and phosphatase inhibitor cocktail (Pierce) and deacetylase inhibitors Nicotinamide (10mM) and Trichostatin A (1µM).
Loading Buffer: 6% SDS, 350mM Tris pH 6.8, 45% Glycerol, 5% β-mercaptoethanol, bromophenol blue.

Hyperinsulinemic-euglycemic clamp
One week before hyperinsulinemic-euglycemic clamps, catheters were surgically placed in a carotid artery and jugular vein for sampling and infusions respectively. Erythrocytes were replaced to prevent a decline in hematocrit with repeated blood sampling. [3-3H]glucose was primed and continuously infused between t=-90-0min (0.04µCi/min). The clamp was initiated at t=0min with a continuous insulin infusion (4mU/kg/min) maintained for 155min. Arterial glucose was monitored every 10min, to provide feedback to adjust the glucose infusion rate (GIR). [3-3H]glucose (0.06µCi/µL) was added to the glucose infusate to clamp both arterial glucose and glucose specific activity. [3-3H]glucose kinetics were determined at −10min and at 10min intervals between 80 and 120min as insulin action is in a steady state. A 13µCi bolus of 2[14C]deoxyglucose ([14C]2-DG) was administered at 120min and used to determine the glucose metabolic index (Rg), a measure of tissue-specific glucose uptake. Blood was collected at 2, 5, 15, 25 and 35min after injection to measure the disappearance of [14C]2-DG from the
plasma. At t=155min, mice were anesthetized with Pentobarbital and tissues were freeze-clamped for subsequent analyses. Whole-body glucose appearance (Ra) and endogenous glucose production (EndoRa), a measure of hepatic glucose production, were calculated as described (1; 2). Radioactivity of [3-3H]glucose, [14C]2DG and [14C]2DG-6-phosphate were determined by liquid scintillation counting (3). Glucose appearance (Ra) and disappearance (Rd) rates were determined using non-steady state equations (4). Endogenous glucose production (EndoRa) was determined by subtracting the GIR from total Ra. The glucose metabolic index (Rg) was calculated as previously described (5). The ratio of 2[14C]deoxyglucose-6-phosphate to 2[14C]deoxyglucose within tissues was calculated as an index of hexokinase activity. Glycogen was determined using the method of Chan and Exton (6). A full description of the surgery, clamp method and isotope calculations are publicly accessible on the Vanderbilt Mouse Metabolic Phenotyping Center website (https://labnodes.vanderbilt.edu/resource/view/id/10764/community_id/1418).

**Immunoprecipitations**

For IRS1 immunoprecipitation (IP), 500µg of gastrocnemius protein lysate was incubated 1h at 4°C with 3µg IRS1 antibody (Santa Cruz Biotechnology). Then, 20µL protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated overnight at 4°C. The mixture was centrifuged at 1,000g and the supernatant removed. The beads were washed four times with cold PBS. Beads were resuspended in 30µL SDS PAGE loading buffer and heated at 80°C for 5min before immunoblot was performed on 15µL. For VDAC IP, 400µg of the mitochondrial protein fraction from vastus lateralis was incubated 1h at 4°C with anti-VDAC antibody (Santa Cruz Biotechnology). 30µL protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated overnight at 4°C. The mixture was centrifuged at 1,000g and the supernatant removed. The beads were washed three times with cold PBS + 0.05% lauryl maltoside. For elution of proteins, beads were resuspended in 40µL 1% SDS for 10min, centrifuged at 1,000g and supernatant was mixed with 10µL SDS PAGE loading buffer. Immunoblot was performed on 25µL.

**Immunostaining**

**Immunohistochemistry**

CD31 staining was assessed by immunohistochemistry in paraffin-embedded tissue sections. Sections (5µm) were incubated with anti-CD31 primary antibody (BD Biosciences) for 60min. Slides were lightly counterstained with Mayer hematoxylin. The EnVision+HRP/DAB System (DakoCytomation) was used to produce localized, visible staining. Images were captured at 20x magnification using a Q-Imaging Micropublisher camera mounted on an Olympus upright microscope. Quantification of capillary density was performed using stereological techniques. Specifically, a test point grid was applied to each micrograph using ImageJ software. To quantify capillary density, the numbers of points falling within muscle tissue and on CD31+ structures were compared (7).

**Immunofluorescent staining**

Following hyperinsulinemic-euglycemic clamps, gastrocnemii were excised, mounted in optimal cutting temperature medium (Sakura), and flash frozen in liquid-nitrogen cooled
isopentane. 5μm sections were cut using a cryostat, mounted on charged slides, and stored at -80°C. On the day of immunostaining, slides were thawed for 30min at room temperature followed by a 5 minute fixation in 4% paraformaldehyde in Tris-buffered saline (TBS). After a series of TBS washes, non-specific secondary antibody binding was blocked by incubating slides in 5% normal goat serum (NGS; Jackson ImmunoResearch) for 1 hour at room temperature. To prevent mouse antibodies from binding endogenous immunoglobulins, sections were incubated in TBS containing ‘mouse on mouse’ blocking reagent (Vector Laboratories). Then, sections were incubated overnight at 4°C in rabbit anti-Glut4 (1:100; gift from Dr. Jeffrey E. Pessin, Albert Einstein College of Medecine) and mouse anti-Cav3 (1:100; Santa Cruz) in 5%NGS/TBS. The next day sections were washed in TBS followed by incubation in an AlexaFluor 555 conjugated goat anti-rabbit (1:100 dilution; Life Technologies) and an AlexaFluor 647 conjugated goat anti-mouse secondary antibody (1:100 dilution; Life Technologies) in 5%NGS/TBS. Finally, sections were rinsed, coverslipped with Vectashield mounting medium (Vector Laboratories), and stored at 4°C in the dark. Control sections stained in the same manner as described above but lacking primary antibodies were used to demonstrate specificity of the secondary antibodies. Similarly, sections stained with only one primary antibody were used to ensure that secondary antibodies only recognized the correct primary antibody.

Image Acquisition
Images were acquired using an inverted Zeiss LSM 510 confocal microscope equipped with two helium-neon (HeNe) lasers (543nm and 633nm) and a 40X 1.3NA oil immersion Plan Neofluar objective (Zeiss). Alexa Fluor 555 was excited with the 543nm laser line of the HeNe laser and emitted light was collected using a 560-615nm bandpass emission filter. Alexa Fluor 647 was excited with the 633 nm laser line of the HeNe laser and emitted light was collected using a longpass 650nm filter. Sampling was performed at a resolution fulfilling the Nyquist criteria and with the pinhole set to give an optical section of 2μm. The two fluorophores were excited sequentially to prevent bleed-through and frame averaged 4 times. Detector settings were kept constant throughout imaging to allow for quantitative comparisons. 2 images were collected per section from 4 serial sections per mouse.

Image Analysis
All image analysis was performed using ImageJ (NIH). Initially, images were background corrected using the rolling ball radius background subtraction method. Then, automatically thresholded CAV3 immunostaining was used to create a mask demarcating the myofiber plasma membrane. This mask was applied to the GLUT4 (AlexaFluor 555) channel and the mean GLUT4 fluorescence intensity within this mask was measured. All image acquisition and analysis was performed by an investigator blind to diet and genotype.
References:
Supplemental Figure legends

Supplemental Figure S1:
Gastrocnemius protein homogenates obtained from 5h-fasted (Basal) or clamped (Insulin) HF-fed mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for GSK3β, P-GSK3β (Ser9) and GAPDH. Integrated intensities were obtained by the Odyssey software and normalized to GAPDH.

Supplemental Figure S2:
Paraffin-embedded gastrocnemius were stained for CD31. Representative images are presented. Number of CD31 positive structures were normalized to tissue area (n=8).

Supplemental Figure S3:
Gastrocnemius protein homogenates obtained from 5h-fasted mice were applied to a 4-12% SDS-PAGE (n=4). Western blotting was performed for total OXPHOS. Integrated intensities were obtained by the Odyssey software and normalized to GAPDH.
Supplemental Figure S1

- **Chow**
  - WT
  - SIRT3 KO

- **High Fat Diet**
  - WT
  - SIRT3 KO

**P-GSK3β**

**GSK3β**

**GAPDH**

**Integrated intensity (arbitrary units)**

**P-GSK3β/GAPDH and GSK3β/GAPDH**

**Ratio Integrated intensity (arbitrary units)**

- **WT Chow**
- **SIRT3 KO Chow**
- **WT HF**
- **SIRT3 KO HF**

**P-GSK3β / GSK3β**

- **Basal**
- **Insulin**

- **P-GSK3b**
- **GSK3b**
Supplemental Figure S2

Diabetes

CD31 structures

Chow

WT

SIRT3 KO

HF

Supplemental Figure S2

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Supplemental Figure S3: Integrated intensity of OXPHOS/GAPDH bands for Complexes I-V.

- WT Chow
- KO Chow
- WT HF
- KO HF

Integrated intensity (arbitrary units) vs. Complexes I-V.