HSP60 reduction protects against diet-induced obesity by modulating energy metabolism in adipose tissue

Robert Hauffe¹23, Michaela Rath¹2, Mareike Schell²35, Katrin Ritter²4, Kai Kappert⁷, Stefanie Deubel⁶, Christiane Ott⁵, Markus Jähnert³5, Wenke Jonas³4, Annette Schürmann⁴5, André Kleinridders¹2³⁴

ABSTRACT

Objective: Insulin regulates mitochondrial function, thereby propagating an efficient metabolism. Conversely, diabetes and insulin resistance are linked to mitochondrial dysfunction with a decreased expression of the mitochondrial chaperone HSP60. The aim of this investigation was to determine the effect of a reduced HSP60 expression on the development of obesity and insulin resistance.

Methods: Control and heterozygous whole-body HSP60 knockout (Hsp60⁺⁻) mice were fed a high-fat diet (HFD, 60% calories from fat) for 16 weeks and subjected to extensive metabolic phenotyping. To understand the effect of HSP60 on white adipose tissue, microarray analysis of gonadal WAT was performed, ex vivo experiments were performed, and a lentiviral knockdown of HSP60 in 3T3-L1 cells was conducted to gain detailed insights into the effect of reduced HSP60 levels on adipocyte homeostasis.

Results: Male Hsp60⁺⁻ mice exhibited lower body weight with lower fat mass. These mice exhibited improved insulin sensitivity compared to control, as assessed by Matsuda index and HOMA-IR. Accordingly, insulin levels were significantly reduced in Hsp60⁺⁻ mice in a glucose tolerance test. However, Hsp60⁺⁻ mice exhibited an altered adipose tissue metabolism with elevated insulin-independent glucose uptake, adipocyte hyperplasia in the presence of mitochondrial dysfunction, altered autophagy, and local insulin resistance.

Conclusions: We discovered that the reduction of HSP60 in mice predominantly affects adipose tissue homeostasis, leading to beneficial alterations in body weight, body composition, and adipocyte morphology, albeit exhibiting local insulin resistance.

Keywords Mitochondria; Stress response; Obesity; Glucose homeostasis; Insulin resistance; Adipose tissue

1. INTRODUCTION

The current obesity pandemic is a consequence of an imbalance of nutrient intake and energy expenditure. The resulting positive energy balance leads to obesity and can cause insulin resistance, a feature of metabolic syndrome and type 2 diabetes (T2D). Mitochondria are essential for metabolism, as they convert nutrients and metabolites into energy in the form of ATP. Mitochondrial dysfunction and the concomitant release of reactive oxygen species (ROS) are linked to insulin resistance and obesity development [1]. To ensure proper mitochondrial function, mitochondria possess a protein quality control system consisting of mitochondrial chaperones and proteases. An integral part of this mitochondrial quality control system represents the mitochondrial chaperone heat shock protein 60 (HSP60). HSP60 interacts with its co-chaperone HSP10 and enables the proper folding of more than 300 mitochondrial matrix proteins and, thereby, mitochondrial function [2,3]. Important folding substrates of this complex include the antioxidative enzyme SOD2 or acyl-CoA dehydrogenases (e.g., medium-chain acyl-CoA dehydrogenase), suggesting that HSP60 influences obesity development by affecting oxidative stress and lipid utilization [4,5]. Indeed, reports show that patients deficient in HSP60 die early and exhibit marked alterations in fatty acid metabolism [6,7]. In rodents, HSP60 deficiency is embryonically lethal [8], and reduced expression of HSP60 is present in metabolic disorders ([9,10], Attie lab diabetes database http://diabetes.wisc.edu/).

HSP60 is also part of mitochondrial stress responses (MSR), such as the mitochondrial unfolded protein response (UPR(mt)), a mitochondria-to-nuclear signaling pathway to control mitochondrial proteostasis by regulating expression of mitochondrial proteins, chaperones, and proteases (e.g., SIRT3, HSP10, ClpP, or LONP1 [11]). Interestingly, insulin signaling regulates mitochondrial function via HSP60 in neurons
[10], showing the close interaction between UPR\textsuperscript{mt} and metabolic pathways. Though a dysregulation of the mitochondrial quality control has been associated with metabolic diseases [12,13], the deletion of different UPR\textsuperscript{mt} genes results in various, even opposing, metabolic outcomes. Thus, SIRT3 deficiency accelerates the development of the metabolic syndrome, whereas ClpP deletion protects against diet-induced obesity [14–16]. Controversial data about the influence of mitochondrial function on metabolism also exist, especially regarding adipose tissue biology. Here, both protection from diet-induced insulin resistance (TFAM deletion [17]) and induction of insulin resistance (PGC1-alpha deletion [18]) are described. Conversely, enhancing mitochondrial function by the overexpression of MitofNEET preserves insulin sensitivity under obese conditions [19].

These data reveal that increased mitochondrial activity in adipose tissue cannot be generally linked to improved metabolic outcomes. It even suggests that a mild decrease in adipose tissue mitochondrial function can be beneficial for metabolism. This finding is of interest, as proper mitochondrial function is a prerequisite of adipocyte differentiation, and impaired differentiation capacity impairs metabolism [20]. Accordingly, low steady-state oxidative stress, often a by-product of mitochondrial dysfunction, impairs adipocyte differentiation [21].

Mice heterozygous for the Hsp71 gene, encoding HSP60, (Hsp60\textsuperscript{+/−} mice) can serve as a genetic model for diabetes-related mitochondrial dysfunction, as db/db mice exhibit an approximately 50% reduction in HSP60 ([9,22,23], Attie Lab diabetes database http://diabetes.wisc.edu). This model allows for an answer to the question of whether a mild generalized impairment in mitochondrial function due to decreased chaperone activity, as seen in diabetes, affects insulin action and obesity development. Thus, we investigated the metabolism of Hsp60\textsuperscript{+/−} mice in diet-induced obesity.

In the present study, we show that the reduction of HSP60 protects against diet-induced obesity in male mice, with a pronounced reduction in adipocyte size. The adipose tissue exhibits altered energy metabolism with decreased mitochondrial function, altered autophagy, and increased insulin-independent glucose uptake. In summary, the mild global impairment of mitochondrial function protects against diet-induced obesity by modulating adipose tissue homeostasis.

2. RESULTS

To understand the effect of global impaired mitochondrial protein homeostasis on obesity development, we fed control and Hsp60\textsuperscript{+/−} mice on a C57BL/6N background (B6N), displaying an average 50% reduction in HSP60 expression across all tissues (Suppl. Fig. 1A, B), a normal chow diet (NCD, 10% kcal from fat) and a high-fat diet (HFD, 60% of kcal from fat). Feeding control and Hsp60\textsuperscript{+/−} mice an NCD did not cause gross metabolic alterations. Here, heterozygous mice exhibited unaltered body weight, lean mass, fat mass, and unchanged insulin and glucose tolerance (Suppl. Fig. 1C-G). However, male Hsp60\textsuperscript{+/−} mice exhibited increased energy expenditure, indicating mild effects on energy metabolism (EE, Suppl. Fig. 1H).

Interestingly, HFD-fed Hsp60\textsuperscript{+/−} males exhibited reduced weight gain compared to the control, with a 16% reduction in body weight at 20 weeks of age (Figure 1A). The reduction in body weight was not due to altered food intake or fecal energy content as measurements of energy intake and clearance (Suppl. Fig. 2A, B) nor due to differences in body temperature between control and Hsp60\textsuperscript{+/−} mice (Suppl. Fig. 2C).

The observed decreased body weight may be a consequence of increased EE, as observed in Hsp60\textsuperscript{+/−} males fed an NCD. Indeed, EE relative to body weight was elevated in Hsp60\textsuperscript{+/−} mice, with a more pronounced effect during the dark phase (Figure 1B, C). Usually, skeletal muscle is the driving factor of EE. To this end, we determined body weight composition in control and Hsp60\textsuperscript{+/−} mice and related EE to lean mass. This analysis revealed unaltered lean mass but decreased fat mass (by 33%) in heterozygous compared to control mice (Figure 1D). However, regression adjustment for either body weight or lean mass using ANCOVA revealed no significant difference between the genotypes (Tables 1 and 2), thus questioning whether an elevated EE is indeed responsible for lower body weight in male Hsp60\textsuperscript{+/−} mice. There was no difference between animal groups at light or dark phases when relating lean mass to EE (Figure 1E), confirming the previous finding. The positive correlation usually seen between lean mass and EE was absent in Hsp60\textsuperscript{+/−} mice compared to control mice (Ctrl: R\textsuperscript{2} = 0.482, P = 0.0177, Hsp60\textsuperscript{+/−}: R\textsuperscript{2} = 0.165, P = 0.1901; Figure 1F), hinting at potential physiological alterations of adipose tissue.

This metabolic phenotype was sex-specific, as female HFD-fed Hsp60\textsuperscript{+/−} mice exhibited increased body weight compared to the control, with a significant increase in lean mass and unaltered fat mass assessed by NMR (Suppl. Fig. 3A, B). Heterozygous females displayed a specific increase in heart mass and an overall 10% increase in combined muscle mass (Suppl. Fig. 3C, D). Female Hsp60\textsuperscript{+/−} mice showed a decreased EE relative to lean mass, but adjusting for either body weight or lean mass using an ANCOVA showed no significant genotype effect (Suppl. Fig. 3E, Supplementary Tables 1 and 2).

However, the increase in body weight could be linked to decreased body temperature in female Hsp60\textsuperscript{+/−} mice (Suppl. Fig. 3F). As determined by NMR in male Hsp60\textsuperscript{+/−} mice, a decrease in fat mass is often linked to improved glucose tolerance and insulin action. Male Hsp60\textsuperscript{+/−} mice showed unaltered insulin sensitivity and glucose tolerance compared to controls, assessed by insulin and glucose tolerance tests (Figure 2A, B). However, when we measured insulin levels during a glucose tolerance test, Hsp60\textsuperscript{+/−} mice secreted (and thus needed) less insulin to maintain comparable blood glucose levels throughout the test (Figure 2C). Determining insulin resistance and sensitivity by calculating the HOMA-IR (Homeostatic Model of Insulin Resistance) and the Matsuda insulin sensitivity index revealed significantly lower insulin resistance (HOMA-IR, Ctrl = 7.51 vs. Hsp60\textsuperscript{+/−} = 2.52, P = 0.0091) and higher insulin sensitivity scores (Matsuda Index, Ctrl = 2.02 vs. Hsp60\textsuperscript{+/−} = 4.18, P = 0.0462) in heterozygous than control mice (Figure 2D, E). The decreased insulin release was not due to decreased insulin content in islets, as Hsp60\textsuperscript{+/−} mice showed similar total pancreatic insulin content to control mice (Figure 2F).

Again, this phenotype was sex-specific, as female Hsp60\textsuperscript{+/−} mice showed unaltered insulin sensitivity compared to control mice (Suppl. Fig. 4A-E).

To assess organ-specific insulin sensitivity in male mice, we performed vena cava insulin injections in control and Hsp60\textsuperscript{+/−} mice and evaluated insulin signaling via western blot analyses. The insulin-induced serine 473 phosphorylation of AKT in the liver, hypothalamus, and quadriceps was unaltered (Suppl. Fig. 5A-C). In line with this effect, metabolic biomarkers, such as adiponectin, glucagon, triglyceride, and non-esterified fatty acid levels in plasma, showed no differences between Hsp60\textsuperscript{+/−} and control mice (Suppl. Fig. 5D-G).

As total fat mass was decreased in male Hsp60\textsuperscript{+/−} mice, we analyzed different adipose depots in more detail. Though subcutaneous and gonadal white adipose tissue (scWAT and gWAT) and brown adipose tissue (BAT) revealed only minor changes in weight, the combination resulted in a significant, global 25% decrease in fat mass per mouse (Figure 3A), concurrent with our NMR data (Figure 1D).
Accordingly, leptin levels were decreased by 45 ± 20% (Figure 3B), indicating enhanced leptin sensitivity.

To further delineate the effect of reduced HSP60 on adipose tissue development, we analyzed BAT, scWAT, and gWAT in detail. However, BAT did not show major differences in neither morphology, expression of brown adipocyte markers, except for a minor increase in P2RX5, nor in insulin sensitivity (Suppl. Fig. 6). Though not significantly different in weight, subcutaneous and gonadal adipocytes of Hsp60+/− mice were

Table 1  — Energy expenditure from male mice fed HFD, adjusted for body weight using ANCOVA.

| Variables   | Estimate | StdError | P value |
|-------------|----------|----------|---------|
| Intercept   | 0.2556   | 0.0847   | 0.0068  |
| Body weight | 0.0105   | 0.0035   | 0.0065  |
| Genotype    | 0.0036   | 0.0127   | 0.7770  |

Table 2  — Energy expenditure from male mice fed HFD, adjusted for lean mass using ANCOVA.

| Variables   | Estimate | StdError | P value |
|-------------|----------|----------|---------|
| Intercept   | 0.4810   | 0.0551   | 2.964E-08 |
| Lean        | 0.0008   | 0.0015   | 0.5807  |
| Genotype    | −0.0003  | 0.0173   | 0.9871  |

Figure 1: Male Hsp60+/− mice are protected from DIO and insulin resistance. A: Body weight development of male Ctrl and Hsp60+/− mice fed an HFD for 16 weeks (N = 12). B, C: Energy expenditure relative to body weight of male Ctrl and Hsp60+/− mice as a function of time (B) or divided into light and dark phase (C) after 14 weeks of HFD (N = 12). D: Body composition of male Ctrl and Hsp60+/− mice measured via NMR after 14 weeks of HFD (N = 12). E: Energy expenditure relative to lean mass of male Ctrl and Hsp60+/− mice as a function of time (N = 12). F: Correlation of energy expenditure and lean mass of male Ctrl and Hsp60+/− from (F). *P < 0.05, and **P < 0.01, after two-tailed Student’s t-test. #P < 0.01 after two-way ANOVA. All data are presented as mean ± SEM.

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smaller and more numerous than control mice, with an increased frequency of small compared to large adipocytes, (Figure 3C-E), a phenotype preserved in the female sex (Suppl. Fig. 4F). Gene expression analysis of these depots showed an unchanged expression of proinflammatory markers TNFα or F4/80 and adipocyte differentiation markers (Figure 3F,G). HSP60 can also be released from adipocytes and cause inflammation [25]. Unexpectedly, plasma HSP60 protein levels were not markedly different between groups either, despite the expected reduction in the tissue expression of 50% of Hsp60+/− mice (Figure 3H, Suppl. Fig. 1A, B).

Next, we investigated adipose tissue insulin sensitivity after vena cava insulin injections in control and Hsp60+/− mice. In contrast to other tissues (Suppl. Fig. 5A-C and 6D), the gWAT of Hsp60+/− mice exhibited insulin resistance compared to control mice, evidenced by a 73 ± 23% reduction in insulin-induced AKT S473 phosphorylation (Figure 4A), whereas insulin action in scWAT was indistinguishable.
between both groups (Figure 4B). Therefore, we focused on gWAT homeostasis. As HSP60 controls mitochondrial function and insulin resistance is linked to mitochondrial dysfunction, we next assessed mitochondrial respiration. Explants from the gWAT of male mice fed an HFD for 16 weeks were analyzed in the Seahorse Flux Analyzer, and clear signs of deteriorated mitochondrial function were revealed. Basal and maximal respiration were significantly reduced in heterozygous mice by 68% and 21% respectively (Figure 4C,D). Following the decreased mitochondrial respiration in the gonadal adipose tissue from Hsp60+/- mice, the gWAT showed 73% lower ATP content,
Another critical metabolism effector up-regulated during mitochondrial MSR in gWAT (Suppl. Fig. 7B). Thus, we isolated the stromal vascular fraction (SVF) from the gWAT of male mice fed an HFD and performed an adipocyte differentiation protocol. After 10 days of differentiation, Hsp60+/− mice displayed a 130% increase in 3-Nitrotyrosine cations in the form of carbonylated proteins. Indeed, Hsp60+/− mice exhibited a 93% increase in 3-Nitrotyrosine level, another biomarker of oxidative stress, specifically in gWAT (Suppl. Fig. 7F). Furthermore, the antioxidative enzyme glutathione peroxidase 1 (GPx1) expression was significantly increased in the gWAT of Hsp60+/− mice compared to control mice, revealing a potential compensatory mechanism (Figure 4G). Insulin signaling and mitochondrial function are essential for proper adipocyte differentiation. Therefore, we analyzed insulin-dependent glucose uptake to investigate the physiological consequences of decreased HSP60 expression on carbohydrate metabolism further. Here, gWAT explants from HFD-fed and insulin-resistant control mice showed a trend toward increased glucose uptake after insulin stimulation (P = 0.08). In contrast, explants from Hsp60+/− mice exhibiting enhanced global insulin sensitivity but impaired insulin response in gWAT (Figure 4A) were unresponsive to measured by ATP turnover by the firefly luciferase in gWAT lysates (Figure 4E). Interestingly, the reduction of HSP60 did not lead to differences in mitochondrial DNA content, suggesting a specific defect in mitochondrial function rather than mitochondrial number (Suppl. Fig. 7A). However, the reduction of HSP60 did not induce the MSR in gWAT (Suppl. Fig. 7B).
Examples of Hsp60 insights into the underlying mechanisms of this phenotype, RNA morphology and functionality, with an apparent dissociation of the heterozygosity for HSP60 had a considerable effect on gWAT lipidated LC3-II at the autophagosomal membranes [29]. gWAT samples from the gWAT of control and Hsp60+/− mice fed an HFD for 16 weeks were submitted to microarray transcriptomic analysis. In total, 926 differentially expressed genes were found. Of these, 220 were down-, and 706 were up-regulated in Hsp60+/− mice (Figure 5A). The down-regulated genes showed no enrichment in specific pathways or gene ontology terms (data not shown). However, in the subset of up-regulated genes, the Ingenuity Pathway Analysis and the gene ontology analysis showed an enrichment of genes involved in the cellular process of autophagy (Figure 5B,C, Supplementary Table 3). Autophagy represents a process by which damaged organelles can be degraded and their components recycled. In addition, it is a vital process in nutrient-depleted conditions or decreased energy production due to organelle dysfunction, allowing for survival by recycling intracellular components [28].

Cell components targeted for autophagy are sequestered in a membrane vesicle, the autophagosome, and directed to the lysosomes to form autolysosomes. The induction of autophagy involves the recruitment of microtubule-associated protein 1A/1B light chain 3B (LC3) and its subsequent conversion from the cytosolic form LC3-I to lipidated LC3-II at the autophagosomal membranes [29]. gWAT samples of Hsp60+/− mice showed a significant increase in LC3-I and LC3-II levels by 105 ± 37% and 251 ± 49%, respectively, in the gWAT (Figure 6A), indicating overall enhanced autophagosomal presence and activity, in line with our microarray results. Activation of mammalian target of rapamycin (mTOR), an important regulator of cellular growth, is regulated by nutrient availability and inhibits autophagy. Insulin phosphorylates mTOR at S2448, leading to its activation [30]. Interestingly, S2448 phosphorylated mTOR was reduced by 55% in gWAT samples of Hsp60+/− mice, indicating decreased energy availability and an altered regulation of autophagy and insulin resistance (Figure 6B).

However, the sole presence of the transcripts or proteins involved in autophagy is insufficient to draw conclusions about its activation. To investigate this aspect, 3T3-L1 fibroblasts were transfected with either a non-target (NT) siRNA or an siRNA directed against Hsp60 mRNA (Hsp60 KD). This approach resulted in a 51 ± 3% decrease of Hsp60 mRNA expression of Hsp60 KD compared to control cells (Figure 6C). Hsp60 KD cells displayed no differences in cell viability, as determined by an MTT assay, but reduced uptake of glucose from the insulin/IGF-containing medium, confirming our hypothesis of a different mechanism of energy supply (Suppl. Fig. 8A, B). Thus, we assessed lysosomal activity using a Neutral Red assay. This assay revealed slightly increased lysosomal activity in Hsp60 KD cells than control cells, as evidenced by enhanced Neutral Red incorporation into active lysosomes (Figure 6D). Finally, to gain further insight into the autophagic processes in vivo, we investigated this pathway in gWAT samples of Ctrl and Hsp60+/− HFD-fed mice. Protein lysates were prepared under non-denaturing conditions, and the lysosomal and proteasomal activities were measured by substrate turnover. Here, the gWAT of Hsp60+/− mice showed unaltered proteasomal activity (Suppl. Fig. 8C, D), but exhibited an almost two-fold increase in lysosomal activity, an indication of increased lysosomal/autophagic activity and a potentially altered energy supply (Figure 6E). The combined evidence from our in vitro and ex vivo analysis indicate that Hsp60+/− mice are protected against diet-induced obesity by an unexpectedly increased basal glucose uptake in adipose tissue and enhanced energy turnover due to insufficient mitochondrial energy production in the presence of local gWAT insulin resistance.

3. DISCUSSION

Mitochondrial function and insulin sensitivity are crucial signaling nodes to engage a healthy metabolism. In general, the healthy expansion of adipocytes is characterized by elevated proliferation, known as adipocyte hyperplasia. The unhealthy expansion of adipose tissue is characterized by adipocyte hypertrophy, the enlargement of adipocytes. Hypertrophic adipocytes often exhibit mitochondrial dysfunction and secrete TNF-α, causing insulin resistance [31,32]. The healthy expansion of adipose tissue depends on proper insulin action and mitochondria. Therefore, it was unexpected that a reduction of HSP60 in our study led to decreased fat mass and adipocyte hyperplasia in adipose tissue, albeit with the presence of mitochondrial dysfunction and insulin resistance in gWAT, though male Hsp60+/− mice were protected against diet-induced obesity. The question of why insulin resistance was only present in adipose tissue remains to be answered. Gonadal adipose tissue is insulin resistant, yet Hsp60+/− mice exhibit unaltered glucose tolerance, showing that local insulin resistance does not need to impact overall metabolic health. One explanation for this unexpected phenotype includes the elevated insulin-independent glucose uptake under basal conditions, which is comparable to insulin-induced glucose uptake rates and may allow normoglycemia (Figure 2A—C and 4H).

An important consequence of adipose tissue insulin action is the inhibition of lipolysis and induction of lipogenesis. Here, our transcriptomic data shows that genes for beta oxidation were significantly increased or showed a strong tendency, whereas the expression of the lipogenic transcription factor Srebf2 was decreased (Acadm P = 0.07, Acadm P = 0.1, Acadl P = 0.013, AcadP = 0.054, AcadB P = 0.07, Acad11 P = 0.054, Srebt2 P = 0.03, Supplementary Table 4), also suggesting an impairment of lipogenesis. Accordingly, lipid accumulation into adipocytes was decreased (Figure 4G), further confirming that the reduction of HSP60 causes gonadal adipose tissue insulin resistance. Why this insulin resistant phenotype is specific for gonadal but not subcutaneous adipose tissue is still unclear but might relate to the differences in the occurrence of oxidative stress, which was absent in the scWAT of Hsp60+/− mice (Figure 4F, Suppl. Fig. 7E and F) [33,34]. Thus, oxidative stress is a prerequisite for insulin resistance induced by decreased HSP60 levels, explaining the unaltered insulin sensitivity phenotype in scWAT [9]. This observation might also clarify the difference between the insulin-sensitive phenotype in Hsp60+/− mice fed an NCD on C57BL/6N (Suppl. Fig. 1F, G) compared to C57BL/6J background [9], as only C57BL/6J mice exhibited increased oxidative stress [35]. In addition, C57BL/6J displayed insulin resistance and alterations in their metabolic response to diet-induced obesity compared to C57BL/6N mice, further highlighting the importance of the genetic background when assessing metabolism [36—38].
The decreased mitochondrial function and reduced ATP production contradict our observation of potentially altered energy expenditure driven by adipose tissue. A compensation for decreased mitochondrial function can be facilitated by elevated autophagy [39], which is not as energy efficient as mitochondrial energy production [28] but might partially account for alterations in body weight development. Accordingly, gWAT from Hsp60+/−/C0mice exhibited decreased ATP levels, suggesting partial compensation through autophagy (Figure 4E). It remains unclear why Hsp60+/−/mice exhibited decreased body weight (Figure 1A and Tables 1 and 2). We have examined other parameters of energy balance but did not find any difference in food intake, fecal energy content, or body temperature. As such, it seems likely that the difference in final body weight stems from accumulating minor differences of the parameters mentioned above, which cannot be captured by our methodology. Another possibility includes alterations in housing during the measurement of energy expenditure. Mice were single-housed for the measurement of energy expenditure, which alters social interaction. It has been shown that alterations in housing can influence metabolism [24,40]. Of note, ClpP−/−/mice showed a similar decreased body weight and energy expenditure but no difference in EE after ANCOVA adjustment [41]. It remains unclear why an alteration of the mitochondrial unfolded protein response (ClpP and HSP60 are members of this response) leads to reduced body weight. Further research is required to address this vital aspect.

In obese human conditions, increased autophagy in adipose tissue correlates with the degree of obesity and adipocyte hypertrophy [42]. Therefore, increased autophagy due to mitochondrial dysfunction during obesity development could represent a protective mechanism to

Figure 5: Transcriptomic analysis of the gWAT from HFD-fed male mice indicates increased autophagy. A: Expression pattern of 926 differentially expressed genes (DEGs) after analysis of the microarray transcriptomic data. Figure was generated with the online tool ClustVis (https://biit.cs.ut.ee/clustvis/). B: Result of the ingenuity pathway analysis of up-regulated genes generated with Ingenuity Pathway Analysis (IPA) from QIAGEN. C: Genes belonging to the macroautophagy process, enriched in the subset of up-regulated genes, generated with the Ontology enrichment analysis and visualisation tool Gorilla (http://cbl-gorilla.cs.technion.ac.il/).
Figure 6: The gWAT of HFD-fed male Hsp60+-/ mice shows signs of increased autophagy and lysosomal activity. 

A: Western Blot and densitometric analysis of LC3 I/II in the gWAT of male Ctrl and Hsp60+-/ mice after 16 weeks of HFD. 

B: Western Blot and densitometric analysis of phosphorylated and total mTOR protein levels in the gWAT of male Ctrl and Hsp60+-/ mice after 16 weeks of HFD. 

C: mRNA expression of HSP60 in 3T3-L1 fibroblasts infected with siRNA targeting either non-target (NT) or Hsp60 (Hsp60 KD). 

D: Neutral Red absorption in NT or Hsp60 KD 3T3-L1 cells incubated with neutral red for 2 h. 

E: Lysosomal activity measured via fluorogenic substrate turnover in lysates of gWAT from male Ctrl and Hsp60+-/ mice after 16 weeks of HFD (N = 12). **P < 0.01, ***P < 0.001, and ****P < 0.0001 after two-tailed Student's t-test. All data are presented as mean ± SEM.
reduce the metabolic burden. This mechanism is specific to mitochondrial dysfunction-induced autophagy, as HFD-fed mice with hyperactive autophagy due to a Beclin-1 mutation had normal adiposity and even exhibited enhanced insulin sensitivity in adipose tissue [43]. As mentioned earlier, a siRNA-mediated knockdown of GLUT8 in hepatocytes was recently shown to reduce basal glucose uptake and impair sucralse-induced autophagy [27]. This observation could help explain the discrepancy between local adipose tissue insulin resistance and unaltered blood glucose levels, which is compensated by basal, insulin-independent glucose uptake.

The decrease in HSP60 levels did not induce MSR activation (Suppl. Fig. 7B), indicating that the inability to cleave unfolded or misfolded proteins primarily causes induction of MSR in adipose tissue as evidenced by CipP deficiency [15]. Indeed, in C. elegans, it has been shown that the accumulation of mitochondrial peptide fragments is a major activator of this response [44,45]. It is important to note that a reduction of HSP60 sex-specifically affects diet-induced obesity. Overall, both animals showed an improved insulin sensitivity, mice were kept on HFD for 16 weeks and then anaesthetized. Then, 5 U of insulin was injected directly into the vena cava. Organs were harvested after the time points quoted in parentheses: Liver (2 min), quadriceps (5 min), gonadal and subcutaneous white adipose tissue (5 min), and brain and brown adipose tissue (10 min). Mice were individually caged for 48 h with free access to food and water for fecal energy content and body temperature measurements. Body temperature was measured, and feces were collected twice daily. Fecal energy content was measured in a caloric bomb.

4.2. Genotyping

PCR Typing for heterozygosity was performed using specific primers for the deleted part of the Hsp60 gene: Hsp60 P1 (5′-TAAGACGAGATTGCCTGGTAG-3′), Hsp60 P2 (5′-CTGAGTGTTGGGATTATG-CAG-3′), Hsp60 P3 (5′-GCCAGTCCTCGATTGAC-3′).

4.3. Isolation of stroma vascular fraction

The gonadal adipose tissue of C57BL/6N Ctrl and Hsp60+/− mice fed an HFD for 16 weeks was collected, digested with Collagenase II, filtered through a 50 μm mesh, and the resulting SVF cells plated at a density of 2.5 ×104 cells/cm2. Differentiation was induced as described below.

4.4. Cell culture

White 3T3-L1 preadipocyte cells were cultured Gibco DMEM Gluta-MAX™ (Thermo Fisher Scientific Inc., USA) culture medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Thermo Fisher Scientific Inc.), and 1% pyruvate (Thermo Fisher Scientific Inc.) at 37 °C and 5% CO₂. 3T3-L1 differentiation and Oil-Red-O staining were performed as previously described [47].

4.5. Ex vivo 14C-deoxy-D-glucose uptake

Glucose uptake was measured using 14C-labeled deoxy-D-glucose (14C-DOG). Tissue explants from mice fed an HFD for 16 weeks were taken and weighed and incubated in Krebs-Ringer-Hepes buffer (KRH) with 1% BSA for 45 min with either 250 nM insulin or 10 μM Cytochalasin B before the addition of 2 mM 14C-DG for 10 min. The tissue was then lysed and incubated with Ultima Gold™ scintillation liquid (Perkin Elmer Inc.). Radioactivity in samples was measured with the Beckman Coulter LS6500 Liquid Scintillation Counter, and the resulting values were related to explant weight.

4.6. Ex vivo seahorse assay

Tissue explants from mice fed an HFD for 16 weeks were taken, weighed, and incubated in Agilent Seahorse media. Mitochondrial respiration was monitored using the Seahorse XF Mito Stress Test Kit and the Seahorse XF24 Extracellular Flux Analyzer, measuring the oxygen consumption rate (OCR) of tissue explants. Final compound concentrations were 2 μM oligomycin, 1 μM FCCP, and 1 μM/2 μM rotenone/antimycin A.

4.7. Analytical procedures

Blood glucose was measured with a Glucometer — Contour XT (Bayer). Plasma Insulin and Adiponectin were measured with ELISAs from Alpco (Alpco Salem, BioCatGmbH). Plasma HSP60 was measured with an ELISA from Elabscience (Elabscience Inc.). Leptin was measured using an ELISA from R & D Systems (R & D Systems/Bio-Techne GmbH) and Plasma Glucagon levels with an ELISA from Yanaihara (Yanaihara Institute Inc.). FGF-21 was measured using an ELISA from Biovendor (BioVendor R&D). All ELISAs were performed according to
the manufacturer’s guidelines. Triacylglycerols were measured with Triglyceride Reagent ABX, and NEFAs were measured with the NEFA-HR assay according to manufacturer’s guidelines (FUJIFILM Wako Chemicals Europe GmbH). The glucose in the supernatants of cultured 3T3-L1 cells was determined by the hexokinase method (Roche Diagnostics GmbH).

4.8. Immunohistochemistry
Fresh tissue samples were submerged in 4% paraformaldehyde for 24 h at 4 °C, washed in PBS, and embedded in paraffin. Slices were fixed on glass slides and stained with hematoxylin and eosin. Calculations of adipocyte size were performed with ImageJ Fiji version 1.53c.

4.9. RNA isolation from tissue
Total RNA was extracted from 5 to 20 mg tissue using the RNaseasy Mini Kit (Qiagen) following the manufacturer’s manual.

4.10. Analysis of gene expression by quantitative real-time PCR
RNA was reverse transcribed using oligo(dT)15 primers, random primers, Thermo Scientific dNTP-Set, and M-MLV reverse transcriptase (Promega Corporation). Real-time quantitative PCR was performed with 10 ng cDNA using GoTaq qPCR master mix (Promega Corporation) and gene-specific primers (Supplementary Table 5). Fluorescence was analyzed in the Viia-7-Real-Time-PCR System (Applied Biosystems, Thermo Fisher Scientific Inc.). Gene expression was calculated applying the ΔΔCt method using Tbp (TATA binding protein) as a reference gene for mRNA analysis or Chdh1 for mitochondrial DNA content analysis.

4.11. Western blot analysis
Western Blots were performed on 10–15 μg total protein lysates loaded on 10% SDS-PAGE gels and transferred to PVDF membranes (GE HealthCare Life Science) for 3 h at 90 V. After the transfer, membranes were blocked in Starting Block (StartingBlockTM T20 (TBS) Blocking Buffer Thermo Scientific #37543) for 1 h. Following blocking, membranes were probed with the primary antibody (Supplementary Table 6) overnight at 4 °C. Membranes were then incubated with peroxidase-conjugated secondary antibodies (anti-rabbit DiaNovo #711-065-152, 1:10000, anti-mouse DiaNovo #715-065-150, 1:10000) at room temperature for 1h. Specific bands were detected with chemiluminescence assay (WesternBright ECL Biozym 541005X) using the ChemiDoc Touch Imaging System (BioRad). Ponceau staining or Ponceau staining or β-Actin were used as loading controls. To remove the phospho-epitope of a protein and run the total antibody, membranes were incubated in stripping buffer (Restore™ PLUS Western Blot Stripping Buffer Thermo Scientific #46430) for 20 min at room temperature, re-blocked for 30 min and re-probed. Band intensities were quantified via densitometric analysis using Image Lab 5.2.1 software and ImageJ Fiji version 1.53c.

4.12. Protein carbonylation
Carbonylated proteins were detected as previously described [48]. Briefly, after western blot transfer membranes were incubated for derivatization with 2 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl and probed afterward with an anti-DNP primary antibody (Merck KGaA).

4.13. Infection with lentiviral particles
3T3-L1 cells were infected using lentiviral transduction particles with pLKO.1 plasmid containing shRNA, targeting either the Hsp60 gene (HSPD1 MISSION shRNA Lentiviral Transduction Particles, SHCLNV-NM_010477; Merck KG) or control transduction particles targeting no known mammalian genes (MISSION pLKO.1-puro Non-Mammalian shRNA Control Transduction Particles, SHC9002V; Merck KG) according to manufacturer’s instructions. 12 μg/ml hexamethylenetetramine bromide (polybrene) was added for incubation overnight. For the antibiotic selection of infected cells, 5 μg/ml of puromycin was added to the media. Selection continued for at least one week.

4.14. Neutral red assay
The compound Neutral Red (3-Amino-7-dimethylamino-2-methylenephene hydrochloride) incorporates into active lysosomes. Cells were incubated with 40 μg/ml Neutral Red for 2 h at 37 °C. After cell lysis with 1% acetic acid in 50% ethanol for 5 min, absorption was measured at 540 nm in a microplate reader.

4.15. MTT assay
The tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was reduced by NAD(P)H dependent cellular oxidoreductases to Formazan. In a 96-well plate, 20 μl of a 5 mg/ml MTT solution was applied and incubated for 3 h at 37 °C, then washed with PBS. Cells were lysed with DMSO, and Formazan absorption was measured at 560 nm.

4.16. Luciferase assay
Homogenized tissue was lysed in a lysis buffer (Promega Dual Luciferase Assay Kit) and briefly centrifuged to assess ATP content in target tissues. The supernatant was mixed with a reaction mix containing Firefly Luciferase and Luciferin (Thermo Scientific). The measured luminescence is a direct result of the presence of free ATP, which fuels the conversion of Luciferin to Oxyluciferin by the Luciferase. Luminescence was determined in the Promega GlowMAX.

4.17. Transcriptome analysis of gWAT
To determine changes in the gene expression patterns in the gWAT between Ctrl and Hsp60+/− mice fed an HFD for 16 weeks, a transcriptomic analysis using a microarray approach was employed. RNA was isolated as described above, and RNA quality control was performed with the Agilent 2100 Bioanalyzer. The microarray was performed by OakLabs GmbH on an Agilent 8 × 60K chip. Visualization was performed with Pathway Analysis (IPA) software from Qiagen, the online tools ClustVis, and the Gene Ontology enrichment analysis and visualizAtion tool GOrilla.

4.18. Lysosome activity (cysteine cathepsin activity)
Adipose tissue samples were homogenized with a ball mill in 500 μl of 1 mM DTT/PBS and shaken in a thermomixer for 1 h at 4 °C. Afterward, samples were sonicated on ice for 2 min at 50% amplitude and centrifuged at 14,000 rpm for 20 min at room temperature. Fat tissue lysates (0.25 μg/well) were incubated with a lysosome activity incubation buffer (containing 24 mM Cystein·HCl, 150 mM Na-Acetate, 3 mM EDTA Dihydrate, pH 4.0) for 10 min at room temperature. To measure cysteine cathepsins, OmniCathepsin fluorogenic substrate Z-FR-AMC, Z-Phe-Arg-AMC (Enzo #BML-p-139) was used, and the final substrate concentration was 166 μM/Well. AMC liberation was measured in a black 96-well plate and monitored every 180 s for 90 min at 37 °C using a fluorescence microplate reader (excitation: 380 nm, emission: 480 nm). Proteolytic cathepsin activity was calculated using free 7-amino-4-methylcoumarin (AMC) as fluorogenic calibration standard. The protein concentration of fat lysates was determined using a Bradford protein assay, and proteolytic activity was
verified using a protease inhibitor cocktail (Sigma—Aldrich, P8340, diluted according to manufacturer’s instructions).

4.19. Proteasome activity (chymotrypsin-like activity)
Fat tissue samples were homogenized with a ball mill in 150 μl lysis buffer (containing 250 mM sucrose, 25 mM HEPES, 1 mM EDTA, 10 mM magnesium chloride, and freshly added 1.7 mM DTT, pH 7.8), followed by passing lysates through a 27-gauge needle attached to a 1 ml syringe 20 times, freeze—thaw cycles 3 times, and centrifugation at 13,400 rpm, for 10 min at 4 °C. Supernatants were used for the determination of protein, using a Bradford assay, and proteasome activity. For the activity assay, samples were adjusted to 1 mg/ml protein (final protein concentration was 10 μg/well) and incubated with a proteasome activity incubation buffer (containing 225 mM Tris buffer (pH 7.8), 7.5 mM magnesium acetate, 45 mM potassium chloride, 7.5 mM magnesium chloride, and freshly added 1 mM DTT). ATP was depleted, and 15 mM 2-deoxyglucose and 0.1 mg/ml hexokinase were added to the incubation buffer to measure 20S proteasome activity. Additional ATP was added to the incubation buffer to determine 26S proteasome activity, with a final ATP concentration of 2 mM. Samples were shaken in the respective incubation buffers for 2 min at 100 rpm, followed by a 10 min incubation at room temperature. Chymotrypsin-like proteasome activity was measured using fluorogenic peptide suc-Leu-Leu-Val-Tyr-7-AMC (Enzo, #BML-P802-0005, final concentration 166 μM/well). 7-amino-4-methylcoumarin (AMC) liberation was determined in a black 96-well plate and monitored every 180 s for 90 min at 37 °C using a fluorescence microplate plate reader (excitation: 360 nm, emission: 460 nm). Proteolytic activity was calculated using free AMC as fluorogenic calibration standard and verified using proteasome inhibitor lactacystin (Enzo, BML-PI104-1000).

4.20. Statistical analysis
All data are presented as mean ± SEM. Groups were compared using an unpaired two-tailed Student’s t-test. A two-way ANOVA was performed to detect interactions (e.g., between sex and treatment) and Sidak post hoc analysis was performed when appropriate. Correlates in mice were conducted using a linear regression model. The EE ANCOVA analysis performed for this work was provided by the NIDDK Mouse Metabolic Phenotyping Centers (MMPC, www.mmpc.org) using their Energy Expenditure Analysis page (http://www.mmpc.org/shared/regression.aspx) and supported by grants DK076169 and DK115255. All in vitro experiments were performed in independent triplicates. The statistical analyses and generation of graphs were performed using GraphPad Prism 7 Software (GraphPad Software Inc.). The graphical Abstract was generated in Inkscape version 1.01.

DECLARATION OF APPROVAL
All animal procedures were conducted according to German laws for the protection of animals and in compliance with protocols approved by local government authorities (State Agency of Environment, Health and Consumer Protection, State of Brandenburg, Germany).

DATA AND RESOURCE AVAILABILITY
All data generated or analyzed during this study are included in the published article (and its online supplementary files). No applicable resources were generated during the current study.

AUTHOR CONTRIBUTIONS
R.H. designed the study, researched data, and wrote the manuscript. M.R., M.S., K.R., K.K., S.D., C.O., M.J., W.J., and A.S. researched data and helped design experiments. A.K. designed the study, supervised all work, and wrote the manuscript. A.K. is the guarantor of this 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.

MATERIAL AND CORRESPONDENCE
Material request and correspondence should be addressed to Dr. André Kleinridders.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA
Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101276.

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