Dietary restriction of mice on a high-fat diet induces substrate efficiency and improves metabolic health

Loes P M Duivenvoorde, Evert M van Schothorst, Annelies Bunschoten and Jaap Keijer
Department of Human and Animal Physiology, Wageningen University, Marijkeweg 40, 6709 GP Wageningen, PO Box 338, 6700 AH Wageningen, The Netherlands

(Correspondence should be addressed to E M van Schothorst; Email: evert.vanschothorst@wur.nl)

Abstract

High energy intake and, specifically, high dietary fat intake challenge the mammalian metabolism and correlate with many metabolic disorders such as obesity and diabetes. However, dietary restriction (DR) is known to prevent the development of metabolic disorders. The current western diets are highly enriched in fat, and it is as yet unclear whether DR on a certain high-fat (HF) diet elicits similar beneficial effects on health. In this research, we report that HF-DR improves metabolic health of mice compared with mice receiving the same diet on an ad libitum basis (HF-AL). Already after five weeks of restriction, the serum levels of cholesterol and leptin were significantly decreased in HF-DR mice, whereas their glucose sensitivity and serum adiponectin levels were increased. The body weight and measured serum parameters remained stable in the following 7 weeks of restriction, implying metabolic adaptation. To understand the molecular events associated with this adaptation, we analyzed gene expression in white adipose tissue (WAT) with whole genome microarrays. HF-DR strongly influenced gene expression in WAT; in total, 8643 genes were differentially expressed between both groups of mice, with a major role for genes involved in lipid metabolism and mitochondrial functioning. This was confirmed by quantitative real-time reverse transcription-PCR and substantiated by increase in mitochondrial density in WAT of HF-DR mice. These results provide new insights in the metabolic flexibility of dietary restricted animals and suggest the development of substrate efficiency.

Journal of Molecular Endocrinology (2011) 47, 81–97

Introduction

Obesity is a major health concern that affects millions of people worldwide (Kopelman 2000, Ogden et al. 2010). Although white adipose tissue (WAT) plays an important role in mammalian energy homeostasis by preventing lipotoxicity and providing a source of energy in times of need, excess amounts of WAT relates to reduction in the number of mitochondria and inflammation of the tissue (Semple et al. 2004, Dahlman et al. 2006), subsequently resulting in metabolic dysfunction (Trayhurn & Beattie 2001, Keijer & van Schothorst 2008). Obesity is associated with several serious complications including type 2 diabetes, coronary heart disease, and certain types of cancer and, therefore, significantly increases morbidity risks in those affected (Sobel et al. 2003). The current prevalence of obesity most likely resulted from the notable decrease in overall physical activity and increase in consumption of readily available energy-dense foods (Ledikwe et al. 2006). Fast foods, for example, as supplied by some typical outlets, contain an average energy density that is more than twice the energy density of a recommended healthy diet, and the energy density of food highly correlates with the fat content of a meal (Prentice & Jebb 2003).

Consistently, the WHO concluded that, in the period from 2000 to 2003, the average European diet consisted of 35–40% of energy available from fat; in France and Spain, this proportion was even higher (WHO/Europe, European HFA Database, July 2010).

Restriction on dietary intake (dietary restriction, DR) is known to robustly improve metabolic health (Lee et al. 1999, Rezzi et al. 2009). Long-term DR extends maximum lifespan and opposes the development of a broad array of age-associated biological and pathological changes in a diverse range of organisms (Weindruch et al. 1988). DR studies in mice and other species have led to detailed descriptions of changes in gene expression in different tissues (Lee et al. 1999, 2002, Higami et al. 2004, van Schothorst et al. 2006, Linford et al. 2007) and various mechanisms that are implicated in the beneficial health effects. Among the DR-related biomarkers, there is increase in mitochondrial density in adipocytes (Lambert et al. 2004) and increase in insulin sensitivity (Lara-Castro et al. 2008). Most studies examining the effects of DR, however, have been performed in standard rodent diets that are characterized by a low-fat content (7–9% energy from fat). Only few studies focused on the effect of DR using high-fat diets (HF-DR; Petro et al. 2004, Kalupahana et al. 2011, de Meijer et al. 2010), which is, in fact, more relevant to the current dietary status in most developed countries.
In this study, we therefore examined the effects of DR in the context of a semi-synthetic AIN93-based diet that contains 30% energy from fat and compare metabolic adaptation and metabolic health of mice that received the diet either on a restricted (30%) or ad libitum (HF-AL) basis. We show that, although an animal is fed a HF (western) diet, the moderate reduction in the total amount of calories improves metabolic health, as measured by a number of parameters, such as glucose tolerance and serum cholesterol, leptin, and adiponectin levels. Whole genome gene expression analysis of WAT revealed a strong upregulation of genes involved in mitochondrial function and lipid metabolism by HF-DR. Relative to results obtained with standard diets, in particular, lipid handling is affected, implying the development of substrate efficiency in HF-DR mice. Finally, we observed increase in mitochondrial density in WAT of HF-DR mice.

Materials and methods

Animals and dietary manipulations

We used 36 male C57BL/6J OlahHsd mice (Harlan Laboratories, Horst, The Netherlands) aged 10 weeks for this study. The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands. Mice were individually housed and maintained under environmentally controlled conditions (temperature 21 °C, 12 h light:12 h darkness cycle, 45% humidity) and had, during the first 3 weeks (the adaptation phase), access to food and water ad libitum. The food consisted of a pellitized diet (Research Diets Services B.V., Wijk bij Duurstede, The Netherlands) with a fat content of 30%, which resembles a western human diet (Table 1). The amount of lard and corn oil are also based on average human intake levels. The diet that was used for DR was in ratio supplemented with a vitamin and mineral premix to guarantee the same level of intake as in the non-restricted group to prevent deficiencies. As the mineral and vitamin premixes contain cornstarch, the HF-DR diet is corrected in the carbohydrate fraction.

At t=0 weeks, animals were stratified on body weight and divided into groups of 12 animals. The first treatment group (t=0) was directly culled after the adaptation phase, the HF-AL group remained under the same conditions, whereas the HF-DR group received a DR of 30%, which lasted for 12 weeks. The amount of restricted food depended on the amount individual mice consumed at the end of the adaptation phase and remained unchanged during the whole intervention phase. The bodyweight and food intake of individual mice were monitored on a weekly basis. The HF-DR group received a fixed amount of feed on a daily basis. All mice were culled at the end of the 12-week intervention.

<table>
<thead>
<tr>
<th>Component</th>
<th>HF-AL (g/kg feed)</th>
<th>HF-DR (g/kg feed)</th>
<th>AL intake HF-AL</th>
<th>DR intake HF-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid casein</td>
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<tr>
<td>L-cystine</td>
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<tr>
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<td>331.3</td>
<td>336.7</td>
<td>231.9</td>
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<tr>
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<td>110.4</td>
<td>112.25</td>
<td>77.3</td>
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<tr>
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<td>84.1</td>
<td>85.5</td>
<td>58.9</td>
</tr>
<tr>
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<td>50</td>
<td>38.3</td>
<td>50</td>
<td>26.8</td>
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<tr>
<td>Lard</td>
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<td>101.5</td>
<td>71.1</td>
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<tr>
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<td>30</td>
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<tr>
<td>Energy % from proteins</td>
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<td></td>
</tr>
<tr>
<td>Energy % from carbohydrates</td>
<td>50</td>
<td>50</td>
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</tr>
</tbody>
</table>

WAT and blood collection (at time of section)

The HF-AL and t=0 mice were fasted for 2 h before sectioning. To match the level of food intake before sectioning to the HF-AL group, HF-DR mice received the regular (restricted) amount of food on the habitual time in the morning and were sectioned 6 h afterward. At the time point of section, mice were anesthetized by inhalation of 5% isoflurane. Blood was sampled after eye extraction and collected in Mini collect serum tubes (Greiner Bio-one, Longwood, FL, USA) and centrifuged for 10 min at 3000 g and 4 °C to obtain serum. Serum samples were aliquoted and stored at −80 °C. After blood collection, mice were killed using cervical dislocation and epididymal WAT was dissected, weighted, and snap frozen in liquid nitrogen and stored at −80 °C.

RNA isolation, cDNA synthesis, and microarray hybridization

RNA isolation from WAT was performed as described (Van Schothorst et al. 2005). Briefly, WAT was homogenized in liquid nitrogen using a cooled mortar and pestle. Total RNA was isolated using TRIzol reagent, chloroform, and isooamyl alcohol (PCI; Invitrogen) followed by purification with RNeasy columns (Qiagen) using the instructions of the manufacturer. RNA concentration and purity were measured using the NanoDrop (IsoGen Life Science, Maarssen, The Netherlands). Approximately, 30 µg total RNA was isolated with...
A260/A280 ratios above 2 and A260/A230 ratios above 1.9 for all samples, indicating good RNA purity. RNA quality was additionally checked on the Experion automated electrophoresis system (Bio-Rad) using Experion StdSense chips (Bio-Rad).

For transcriptome analysis, the 4×44k Agilent whole-mouse genome microarrays (G4122F, Agilent Technologies, Inc., Santa Clara, CA, USA) were used. Preparation of the samples and the microarray hybridizations were carried out according to the manufacturer’s protocol with a few modifications as described previously (van Schothorst et al. 2007, van Helden et al. 2010). All materials and reagents are from Agilent Technologies, Palo Alto, CA, USA, unless stated.

In brief, cDNA was synthesized for each animal from 1 μg WAT RNA using the Agilent Low-RNA Input Fluorescent Linear Amplification Kit without addition of spikes. Thereafter, samples were split into two equal amounts to synthesize cyanine 3-CTP (Cy3) and cyanine 5-CTP (Cy5) labeled cRNA, using half the amounts per dye as indicated by the manufacturer. Labeled cRNA was purified using RNeasy columns (Qiagen). Yield and Cy-dye incorporation were examined for every sample using the Nanodrop. All samples met the criteria of a cRNA yield higher than 825 ng and a specific activity of at least 8·0 pmol Cy-dye per μg cRNA. Then, Cy3-labeled cRNA samples were pooled into an equimolar basis and used as a common reference pool. Individual 825-ng Cy5-labeled cRNA and 825-ng pooled Cy3-labeled cRNA were fragmented in 1× fragmentation and 1× blocking agent at 60°C for 30 min and thereafter mixed with GEx hybridization buffer HI-RPM. Individual Cy5-labeled cRNA and pooled Cy3-labeled cRNA were hybridized to the arrays in a 1:1 ratio at 65°C for 17 h in the Agilent Microarray Hybridization Chamber rotating at 10 r.p.m. Samples were randomly divided over the slides and positions on the slides. After hybridization, slides were washed according to the manufacturer’s wash protocol. Arrays were scanned with an Agilent Technologies Scanner G2505B with 10 and 100% laser power intensities.

Spots with an average Cy5 and Cy3 signal twice above background were selected and log transformed. The Cy5 signal was normalized against the Cy3 intensity as described before (Pellis et al. 2003). All arrays are deposited in GEO under the number GSE27213. Supervised principal component analysis and heat mapping were performed using GeneMaths XT. Volcano plots were made using GraphPad Prism version 5.03 (Graphpad Software, San Diego, CA, USA). Gene expression levels of the HF-DR versus the HF-AL groups were analyzed by Student’s t-tests using a multiple testing correction according to Benjamini–Hochberg (Hochberg & Benjamini 1990). Corrected P values <0.01 were considered as statistically significant. Fold change is expressed as the ratio of the HF-DR group over the HF-AL group. For downregulated genes, the fold change is converted to the corresponding negative value. Pathway analysis was performed using MetaCore (GeneGo, St Joseph, MI, USA), GO overrepresentation analysis, and literature mining.

Quantitative real-time reverse transcription-PCR

Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed on individual samples as described previously (de Boer et al. 2006, van Schothorst et al. 2009b) to validate the microarray data. RNA (1 μg) of all individual samples was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCRs were performed with iQ SYBR Green Supermix (Bio-Rad) using the MyIQ single-color real-time PCR detection system (Bio-Rad). Each reaction (25 μl) contained 12·5 μl iQ SYBR Green Supermix, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 8·5 μl RNase-free water, and 2 μl 100× diluted cDNA. The following cycles were performed: one initialization step of 3 min at 95°C, 40 amplification cycles (15 s at 95°C and 45 s at the optimal annealing temperature), and one final cycle of 1 min at 95°C and 1 min at 65°C. Finally, a melting curve was prepared (60 cycles of 10 s at 65°C with an increase of 0·5°C per 10 s). A standard curve using serial dilutions of pooled sample (cDNA from all samples), a negative control without cDNA template, and a negative control without reverse transcriptase (−RT) were taken along with every assay. Only standard curves with efficiency between 90 and 110% and a correlation coefficient >0·99 were accepted. Individual samples were measured in duplicate. Data were normalized against the geometrical mean of the reference genes calnexin (Canx), β-2 microglobulin (B2m), ribosomal protein S15 (Rps15), and endoplasmic reticulum chaperone SIL1 homolog (Sil1), which were chosen based on stable gene expression levels between the mice on the microarray and confirmed with the resulting qRT-PCR data (geNorm, Ghent University Hospital, Ghent, Belgium). Primers were designed using the NCBI Primer-Blast.

Normalization and statistical analysis of microarray data

Signal intensities for each spot were quantified using Feature Extraction version 9.5.3.1 (Agilent Technologies). Median density values and background values of each spot were extracted for both the individual samples (Cy5) and the pooled samples (Cy3). Quality control for every microarray was performed visually by using ‘quality control graphs’ from Feature extraction and M-A plots and box plots, which were made using limmaGUI in R (Wettenhall & Smyth 2004). Data were imported into GeneMaths XT 2.0 (Applied Maths, Sint-Martens-Latem, Belgium).
(NCBI Web site) and Beacon designer (Premier Biosoft International, Palo Alto, CA, USA). The primer sequences and PCR annealing temperatures for each gene are summarized in Table 2.

**Oral glucose tolerance test**

Oral glucose tolerance was tested in all mice of the HF-AL and HF-DR group at \( t = 5, 8, \) and 11 weeks of the dietary intervention. At \( t = 0 \) weeks, before the switch to the different dietary interventions, glucose tolerance was tested in three mice per group. On the days that the oral glucose tolerance tests (OGTTs) took place, HF-DR mice received their daily amount of food after the OGTT. Food of all mice was removed at the start of the light phase and 5 h afterward tail blood (\( \sim 50 \mu l \)) was collected and partly used to determine the glucose concentration with an automated blood glucose monitoring system (Freestyle, Abbott Diabetes Care). Remaining blood was transferred to Mini collect serum tubes (Greiner Bio-one) and centrifuged for 10 min at 3000 \( \times \) g and 4 \( ^\circ \)C to obtain serum; serum samples were stored at \( -80 \) \( ^\circ \)C. Glucose (2 g/kg BW) was given orally by gavage at the start of each experiment. Tail blood (3 \( \mu l \)) was collected 15, 30, 60, 90, and 120 min after glucose administration and used to determine the glucose concentration with the automated blood glucose monitoring system. The incremental area under the curve of the changes in blood glucose over time per mouse was calculated using GraphPad Prism.

**Serum measurements**

Serum samples of the starting point (\( t = 0 \) min) of each of the OGTTs were used to measure serum leptin, insulin, and adiponectin in fasted animals with the mouse serum adipokine Lincoplex Kit (Linco Research, Nuclilab, Ede, The Netherlands). Preparation of the samples and binding to the antibody-coupled beads were carried out according to the manufacturer's protocol with a few modifications as described previously (van Schothorst et al. 2009a). In short, sera were diluted 5 times in HPE buffer (Sanquin, Amsterdam, The Netherlands), debris was removed by spinning the sample using SpinX columns (Corning, Schiphol-Rijk, The Netherlands), and possible inhibition of the immunological Ig fraction in serum was circumvented by an incubation at gentle shaking for 1 h at room temperature in 96-well Reacti-BindTM protein L-coated plates (Pierce, Rockford, IL, USA) before adipokine assaying. The assays were measured using the Lumexin X100 system (Bio-Rad) with Starstation software (Applied Cytometry Systems, Dinnington, Sheffield, UK). Sample concentrations were calculated using a standard curve of the metabolite of interest (supplied by the kit). All individual samples were analyzed in duplicate and averaged.

Serum total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglycerides were measured using liquicolor enzymatic colorimetric tests (Human, Wiesbaden, Germany). Measured serum triglyceride concentrations were corrected for free serum glycerol concentrations, using a glycerol colorimetric method (Instruchemie, Delfzijl, The Netherlands). Serum samples (2 \( \mu l \)) were measured in duplicate and averaged. Sample concentrations were calculated using a standard curve of the metabolite of interest (supplied by the kit). Serum low-density lipoprotein (LDL)-cholesterol concentrations were calculated using the modified (for rodents) Friedewald formula (LDL-cholesterol = total cholesterol − HDL-cholesterol − (triglycerides × 0.16)) (Friedewald et al. 1972).

**Mitochondrial mass measurement**

To estimate the mitochondrial density in WAT of HF-AL, HF-DR, and \( t = 0 \) mice, we determined the
ratio of mitochondrial DNA to nuclear DNA with qRT-PCR according to Lagouge et al. (2006). Total DNA was isolated from 25 mg WAT using the QIAamp DNA mini kit (Qiagen). Quality and quantity of DNA in each sample was analyzed with the Nanodrop and each sample was diluted to the same concentration. qRT-PCR was performed with mitochondrial DNA and genomic DNA-specific primers (Lagouge et al. 2006). A standard curve for both genes was generated using serial dilutions of a pooled sample (DNA from all samples). Only standard curves with an efficiency between 90 and 110% and a correlation coefficient >0.99 were accepted. Samples were measured in duplicate. The individual relative mitochondrial density was calculated as the $C_t$ value of the mitochondrial gene compared with the $C_t$ value of the nucleic gene in the same sample.

**Statistical analysis of serum samples, WAT weight, and OGTT**

Data are expressed as mean ± S.E.M.; statistical analyses were performed using GraphPad Prism. All individual measurements within the different treatment groups were checked for normality using the D’Agostino-Pearson normality test. Test results that were not normally distributed were log transformed. Measurements at single time points between two groups were analyzed by Student’s t-tests. Measurements at single time points among three groups were analyzed by one-way ANOVA and Bonferroni post hoc analysis. $P < 0.05$ was considered as statistically significant.

**Results**

**HF-DR decreased body weight and WAT weight**

Body weight of HF-DR animals declines after the start of the intervention and stabilizes after ~5 weeks, whereas HF-AL-fed mice gained weight throughout the study (Fig. 1A). At the time point of section, 12 weeks after the adaptation phase, HF-DR mice showed a significant decrease in absolute and relative epididymal WAT weight compared with HF-AL mice (Fig. 1B and C).

**HF-DR mice performed better on the OGTT and had decreased fasting glucose and insulin levels**

In a fasted state, HF-AL and HF-DR mice significantly differed in serum levels of markers that predict the onset of insulin resistance; at 5, 8, and 11 weeks after the start of the intervention, OGTTs indicated that the insulin sensitivity of the HF-DR mice was considerably higher than that of HF-AL mice (Fig. 2A and B). Secondly, the HF-DR group had significantly lower serum levels of glucose and insulin (Fig. 2C and D).

It should be noted that insulin levels of most of the HF-DR animals (~9 per measurement) were below the detection limit of the serum adipokine kit; these values were set to half the value of the detection limit (0.13 nM).

**Serum leptin, adiponectin, and cholesterol levels were significantly altered in HF-DR mice**

Leptin concentrations were significantly lowered in HF-DR animals (Fig. 3A), whereas serum adiponectin levels were significantly elevated after 5, 8, and 11 weeks of DR (Fig. 3C). At the end of the dietary intervention, total cholesterol was significantly lowered in HF-DR mice (Fig. 3B). Triglycerides and HDL cholesterol levels remained unchanged between both groups (data not shown). Serum LDL cholesterol was decreased in HF-DR mice at time point of section, which caused a significant increase in the HDL/LDL-cholesterol ratio in these mice (Fig. 3D).

**Gene expression in epididymal WAT was drastically altered between HF-DR and HF-AL mice**

Gene expression in epididymal WAT was measured after 12 weeks of HF-DR or HF-AL diet. Of the more than 43 000 transcripts that were studied, 24 146 transcripts were found to be expressed in WAT. After filtering the transcripts that encode for the same gene and are similarly regulated, 16 995 genes appeared to be significantly different between HF-DR and HF-AL mice. Of the more similar regulated, 16 995 genes appeared to be significantly different between HF-DR and HF-AL mice.
The majority of the genes in this group is known to be under transcriptional control of Srebf2 (denoted with asterisks in Table 3). Indeed, all genes involved in cholesterol synthesis (Cyp51, Fdps, Hmgcr, Insig1, Lss, Mvd, Sfle, and Tm7sf2) and fatty acid synthesis and elongation (Acaca, Acl, Elovl3, Elovl6, and Pecr) were significantly upregulated as was Me1 that plays a central role in adipose metabolism, linking gluconeogenesis and fatty acid metabolism by producing NADPH. This confirms earlier findings of long- and short-term DR, although on a low-fat diet (Higami et al. 2004, van Schothorst et al. 2006), but is in contrast with short-term HF-DR of only 12 days (van Schothorst et al. 2006). Interestingly, Sfle and Hmgcr are rate limiting enzymes in cholesterol synthesis, while Elovl6 controls the first, rate limiting condensation step of fatty acid elongation (Leonard et al. 2004). The transcription of Ppara and Pparg, two other key regulators of lipid metabolism, were not altered by the restriction regime (also see Table 4). HF-DR upregulated the expression of two genes encoding lipolysis-related proteins (Aspg, Lpca13) and it upregulated the expression of two StAR-related lipid transfer proteins (Stard4, Stard5) that play a role in the transfer of cholesterol to different cellular compartments, such as the endoplasmic reticulum and the Golgi apparatus (Rodriguez-Agudo et al. 2008) and Rdh11 and Sorl1 that play a role in the reduction of short-chain (fatty) aldehydes and the uptake of lipoproteins and proteases respectively.

Microarray analysis on highly significantly regulated genes revealed a major role for genes involved in lipid metabolism and mitochondrial functioning

**Lipid metabolism**

A large proportion of the significantly regulated genes (\(P<10^{-11}\)) play a role in lipid metabolism. All genes in this group exhibit a strong upregulation compared with the expression in HF-AL mice, except for BC005764 that serves a function in triglycerides formation and was downregulated. Among the upregulated genes in this group, sterol regulatory element-binding protein 2 (Srebf2) functions as a key transcriptional regulator of genes involved in fatty acid and cholesterol synthesis. The majority of the genes in this group is known to be HF-AL mice (\(P<0.01\) Benjamini–Hochberg FDR adjusted). Initially, we focused on the 96 genes that have a \(P\) value \(<10^{-11}\) (Table 3). The majority of these 96 genes is involved in lipid metabolism and mitochondrial function. A substantial part is involved in other metabolic processes (particularly in carbohydrate metabolism) and in the cellular response to stress (Fig. 5). In total, 24 genes have an unknown function. The 96 genes that were differentially expressed between HF-DR and HF-AL mice were not significantly modulated by HF feeding on itself, as analyzed between HF-AL and \(t=0\) mice (Fig. 4B and C).

**Figure 2** HF-DR improved glucose tolerance and decreased fasted glucose and insulin levels. (A) Oral glucose tolerance was tested at the start of the intervention and after 5, 8, and 11 weeks and improved during the course of the experiment. (B) 5, 8, and 11 weeks after the start of the restriction, HF-DR mice had increased glucose sensitivity, as measured by the incremental area under the curve. (C and D) At 5, 8, and 11 weeks, HF-DR mice exhibit a decrease in serum glucose and insulin levels. Data are mean ± S.E.M. of 12 mice (results of the tests in week 0 are based on three mice per group). *\(P<0.05\); ***\(P<0.001\).
transcription of this group fulfills a role in mitochondrial activity. It is downregulated by HF-DR. The majority of genes in sodium/hydrogen exchanger, of which transcription biogenesis, except for seem to promote mitochondrial functioning and significantly regulated by HF-DR (with \( P \leq 10^{-11} \)). The same holds true for Dnm1l, Mtfp1, March5, Mfn1, Opa1, Oma1, and Stoml2, the genes that play an important role in mitochondrial fission and fusion (Yoon et al. 2010). Mtor, a transcription factor that is involved in the suppression of autophagy and cell death (Jung et al. 2010), is upregulated in HF-DR animals. Ulk2 promotes autophagy and cell death in the absence of Mtor and is consistently downregulated. The expression of Park2 that is involved in the induction of mitophagy and the promotion of cell survival (Narendra et al. 2008) is, mitochondrial functioning

All eight mitochondrial genes that were highly significantly regulated by HF-DR (with \( P \leq 10^{-11} \)) seem to promote mitochondrial functioning and biogenesis, except for C80638, a mitochondrial sodium/hydrogen exchanger, of which transcription is downregulated by HF-DR. The majority of genes in this group fulfills a role in mitochondrial activity. Transcription of Fxn, for example, a gene that promotes mitochondrial function and oxidative phosphorylation, was increased by HF-DR. The same holds true for L2hgdh and Slc25a1 that both play a promoting role in the mitochondrial citric acid cycle. Transcription of mitochondrial localized Ifit27 protein that is expected to inhibit adipocyte differentiation and mitochondrial biogenesis and function (Li et al. 2009) was downregulated.

Since the genes mentioned above are not known as the ‘classical’ regulators of mitochondrial functioning, we further analyzed the complete list of regulated genes \( (P < 0.01) \) for genes that are better established as key regulators in mitochondrial biogenesis, dynamics, and autophagy (Table 4). Ppargc1a, Ppargc1b, Esrra, Nrf1, and Tfam, which are known to be of major importance for mitochondrial biogenesis (Scarpulla 2008), were indeed all upregulated in HF-DR animals. The same holds true for Dnm1l, Mtfp1, March5, Mfn1, Opa1, Oma1, and Stoml2, the genes that play an important role in mitochondrial fission and fusion (Yoon et al. 2010). Mtor, a transcription factor that is involved in the suppression of autophagy and cell death (Jung et al. 2010), is upregulated in HF-DR animals. Ulk2 promotes autophagy and cell death in the absence of Mtor and is consistently downregulated. The expression of Park2 that is involved in the induction of mitophagy and the promotion of cell survival (Narendra et al. 2008) is,
Table 3 Categorization and expression profiles of strongly regulated genes (P < 10^-11) between HF-AL and HF-DR mice. Genes are ordered based on function and within functional categories based on alphabetic order. Genes indicated with an asterisk are transcriptionally regulated by Srebfl2.

<table>
<thead>
<tr>
<th>Gene symbols</th>
<th>Protein name</th>
<th>FC</th>
<th>P value</th>
<th>Function</th>
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<tr>
<td>Lipid metabolism</td>
<td>Cyp51*</td>
<td>Cytochrome P450, family 51</td>
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<td>Fatty acids pyrophosphate synthetase</td>
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<td>Lipid metabolism</td>
<td>BC005764</td>
<td>Lipid phosphate phosphatase-related protein type 3</td>
<td>-2-8</td>
<td>2.50 × 10^-13</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Elovi3</td>
<td>Elongation of very long-chain fatty acids protein 3</td>
<td>28-6</td>
<td>0</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Elovi6*</td>
<td>Elongation of very long-chain fatty acids protein 6</td>
<td>47-2</td>
<td>0</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Pecr</td>
<td>Peroxisomal trans-2-enoyl-CoA reductase</td>
<td>2-8</td>
<td>3.50 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Acaca*</td>
<td>Acetyl-coenzyme A carboxylase 2</td>
<td>5-7</td>
<td>2.30 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Acly</td>
<td>ATP-citrate synthase</td>
<td>12-3</td>
<td>3.90 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Aspg</td>
<td>60 kDa lysophospholipase</td>
<td>12-8</td>
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<td>Lipid metabolism</td>
<td>Lpcat3</td>
<td>Lysophospholipid acyltransferase 5</td>
<td>2-7</td>
<td>2.10 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Rdh11*</td>
<td>Retinol dehydrogenase 11</td>
<td>9-6</td>
<td>6.10 × 10^-13</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Srebfl2</td>
<td>Sterol regulatory element-binding protein 2</td>
<td>2-0</td>
<td>7.90 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Stard4*</td>
<td>StAR-related lipid transfer domain containing 4</td>
<td>2-1</td>
<td>3.80 × 10^-13</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Stard5</td>
<td>StAR-related lipid transfer protein 5</td>
<td>3-3</td>
<td>2.80 × 10^-12</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Sort1*</td>
<td>Sortilin-related receptor</td>
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<td>9.10 × 10^-13</td>
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<tr>
<td>Mitochondrial function</td>
<td>1700020C11 Rik</td>
<td>Mitochondrial 18 kDa protein</td>
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<td>3.56 × 10^-12</td>
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<td>Mitochondrial function</td>
<td>C80638</td>
<td>Mitochondrial sodium/hydrogen exchanger</td>
<td>-9-0</td>
<td>0</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Fxn</td>
<td>Frataxin, mitochondrial</td>
<td>2-3</td>
<td>6.58 × 10^-12</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Ifi27</td>
<td>Interferon, x-inducible protein 27 like 2A</td>
<td>-11-1</td>
<td>6.15 × 10^-12</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>L2greh</td>
<td>L-2-hydroxylglutarate dehydrogenase</td>
<td>2-0</td>
<td>2.77 × 10^-12</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Ppil</td>
<td>Peptidyl-prolyl cis-trans isomerase, mitochondrial</td>
<td>3-2</td>
<td>6.42 × 10^-12</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Slc25a1</td>
<td>Solute carrier family 25 (mitochondrial citrate transporter), member 1</td>
<td>8-2</td>
<td>7.59 × 10^-12</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Timm9</td>
<td>Translocase of inner mitochondrial membrane 9 homolog transcript variant 1</td>
<td>3-1</td>
<td>5.83 × 10^-13</td>
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<tr>
<td>Other metabolic genes</td>
<td>Acp1</td>
<td>Acid phosphatase 1</td>
<td>-5-6</td>
<td>0</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Adh1</td>
<td>Alcohol dehydrogenase 1</td>
<td>2-4</td>
<td>3.30 × 10^-12</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Me1</td>
<td>NADP-dependent malic enzyme</td>
<td>9-0</td>
<td>0</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Pdk1</td>
<td>Pyruvate dehydrogenase kinase isozyme 1</td>
<td>5-0</td>
<td>1.12 × 10^-13</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Pglk1</td>
<td>Phosphoglycerate kinase 1</td>
<td>2-8</td>
<td>9.36 × 10^-13</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Pgp</td>
<td>Phosphoglycolate phosphatase</td>
<td>2-1</td>
<td>2.51 × 10^-13</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Ppitr3b</td>
<td>Protein phosphatase 1 regulatory subunit 3B</td>
<td>17-8</td>
<td>0</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Prei4</td>
<td>Putative glycerophosphodiester phosphodiesterase</td>
<td>2-7</td>
<td>6.70 × 10^-13</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Psph</td>
<td>Phosphoserine phosphatase</td>
<td>5-0</td>
<td>7.31 × 10^-13</td>
</tr>
<tr>
<td>Other metabolic genes</td>
<td>Rorc</td>
<td>Nuclear receptor ROR-γ</td>
<td>5-7</td>
<td>8.49 × 10^-13</td>
</tr>
</tbody>
</table>

(continued)
Other metabolic genes

Next to the genes involved in lipid metabolism and mitochondrial function, we found a number of genes that are involved in other metabolic processes. Most of

However, increased in these animals; the same holds true for the expression of Rab7 and Bnip3l that are involved in the induction of autophagy (Jager et al. 2004, Zhang & Ney 2009).

Table 3 Continued

<table>
<thead>
<tr>
<th>Gene symbols</th>
<th>Protein name</th>
<th>FC</th>
<th>P value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc2a5</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 5</td>
<td>35-0</td>
<td>5.69 × 10⁻¹²</td>
<td>Glucose uptake</td>
</tr>
<tr>
<td><strong>Cellular response to stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blcap</td>
<td>Bladder cancer-related protein</td>
<td>-4-5</td>
<td>9.77 × 10⁻¹²</td>
<td>Regulation of cell proliferation and apoptosis (by similarity)</td>
</tr>
<tr>
<td>C8g</td>
<td>Complement component 8, γ-subunit</td>
<td>2-5</td>
<td>3.26 × 10⁻¹²</td>
<td>Complement pathway (by similarity)</td>
</tr>
<tr>
<td>Egln3</td>
<td>EGL nine homolog 3</td>
<td>7-4</td>
<td>2.36 × 10⁻¹²</td>
<td>Cellular response to stress (by similarity)</td>
</tr>
<tr>
<td>Fastkd3</td>
<td>FAST kinase domains 3</td>
<td>2-2</td>
<td>6.59 × 10⁻¹²</td>
<td>Regulation of apoptosis (by similarity)</td>
</tr>
<tr>
<td>Gclm</td>
<td>Glutamate–cysteine ligase regulatory subunit</td>
<td>2-2</td>
<td>8.49 × 10⁻¹³</td>
<td>Glutathione metabolism; response to oxidative stress (by similarity)</td>
</tr>
<tr>
<td>Klf11</td>
<td>Krueppel-like factor 11</td>
<td>-1-8</td>
<td>4.69 × 10⁻¹³</td>
<td>Transcriptional regulation of apoptosis (by similarity)</td>
</tr>
<tr>
<td>Tmem49</td>
<td>Transmembrane protein 49</td>
<td>2-1</td>
<td>9.10 × 10⁻¹²</td>
<td>Cellular response to stress (by similarity)</td>
</tr>
<tr>
<td>Traf4</td>
<td>Tnf receptor associated factor 4</td>
<td>6</td>
<td>1.68 × 10⁻¹²</td>
<td>Proapoptotic cellular signaling</td>
</tr>
<tr>
<td><strong>Other functions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abcc3</td>
<td>ATP-binding cassette, sub-family C</td>
<td>-3-0</td>
<td>4.35 × 10⁻¹³</td>
<td>Transmembrane organic anion transport</td>
</tr>
<tr>
<td>Ank</td>
<td>Progressive ankylosis</td>
<td>4-3</td>
<td>5.29 × 10⁻¹²</td>
<td>Transmembrane inorganic pyrophosphate transport</td>
</tr>
<tr>
<td>Atp1a3</td>
<td>Na⁺/K⁺-transporting ATPase subunit α-3</td>
<td>16-9</td>
<td>0</td>
<td>Transmembrane ion transport</td>
</tr>
<tr>
<td>Anxa6</td>
<td>Annexin A6</td>
<td>-2-6</td>
<td>1.12 × 10⁻¹³</td>
<td>Transmembrane ion release</td>
</tr>
<tr>
<td>Kcnj11</td>
<td>K⁺ voltage-gated channel, Isk-related family, member 1-like</td>
<td>-4-6</td>
<td>3.52 × 10⁻¹²</td>
<td>Transmembrane ion release</td>
</tr>
<tr>
<td>Kctd15</td>
<td>K⁺ channel tetramerization domain containing 15</td>
<td>-2-4</td>
<td>3.13 × 10⁻¹²</td>
<td>Transmembrane ion release</td>
</tr>
<tr>
<td>Bmpr</td>
<td>BMP-binding endothelial regulator</td>
<td>-3-3</td>
<td>1.95 × 10⁻¹²</td>
<td>Cell differentiation</td>
</tr>
<tr>
<td>Col1a1</td>
<td>Collagen α-1(I) chain</td>
<td>-3-9</td>
<td>8.00 × 10⁻¹³</td>
<td>Cell development</td>
</tr>
<tr>
<td>Cry1</td>
<td>Cryochrome 1 (photolyase-like)</td>
<td>3-6</td>
<td>0</td>
<td>Regulation of the circadian rhythm</td>
</tr>
<tr>
<td>Dixc1</td>
<td>DIX domain containing 1</td>
<td>3-1</td>
<td>1.94 × 10⁻¹²</td>
<td>Multicellular organisinal development</td>
</tr>
<tr>
<td>Dock11</td>
<td>Dedicator of cytokinesis 11</td>
<td>-4-6</td>
<td>5.69 × 10⁻¹²</td>
<td>Activation of G proteins</td>
</tr>
<tr>
<td>Ift122</td>
<td>Intraflagellar transport 122 homolog</td>
<td>-2-7</td>
<td>5.14 × 10⁻¹²</td>
<td>Cilia formation</td>
</tr>
<tr>
<td>Nnat</td>
<td>Neuronatin, transcript variant 1</td>
<td>-4-0</td>
<td>0</td>
<td>Regulation of cell proliferation (in neurons and pancreas)</td>
</tr>
<tr>
<td>Pcsk4</td>
<td>Proprotein convertase subtilisin/kexin type 4</td>
<td>5-8</td>
<td>1.99 × 10⁻¹³</td>
<td>Conversion of secretory precursor proteins</td>
</tr>
<tr>
<td>Recq4</td>
<td>RecQ protein-like 4</td>
<td>9-7</td>
<td>4.87 × 10⁻¹³</td>
<td>DNA recombination</td>
</tr>
<tr>
<td>S100b</td>
<td>S100 protein, β-polypeptide</td>
<td>-4-0</td>
<td>1.17 × 10⁻¹²</td>
<td>Regulation of proinflammatory cytokines (in neurons)</td>
</tr>
<tr>
<td>Sar1b</td>
<td>SAR1 gene homolog B</td>
<td>2-0</td>
<td>1.43 × 10⁻¹²</td>
<td>Protein transport from ER to Golgi</td>
</tr>
<tr>
<td>Syngr1</td>
<td>Synaptogyrin 1, transcript variant 1b</td>
<td>3-0</td>
<td>3.30 × 10⁻¹²</td>
<td>Protein targeting for synaptic like microvesicles</td>
</tr>
<tr>
<td>Tmem41b</td>
<td>Transmembrane protein 41B</td>
<td>2-4</td>
<td>9.71 × 10⁻¹³</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>Tmem79</td>
<td>Transmembrane protein 79</td>
<td>4-5</td>
<td>0</td>
<td>Transmembrane protein (Regulation of) transcription</td>
</tr>
<tr>
<td>Polr3g</td>
<td>Polynucleotide (RNA) III (DNA directed) polypeptide G</td>
<td>4-1</td>
<td>0</td>
<td>Regulation of transcription</td>
</tr>
<tr>
<td>1500003O22Rik</td>
<td>Ribosomal RNA processing 8, methyltransferase, homolog</td>
<td>1-9</td>
<td>2.74 × 10⁻¹²</td>
<td>RNA splicing</td>
</tr>
<tr>
<td>Zbtb20</td>
<td>Zinc finger and BTB domain containing 20</td>
<td>-2-0</td>
<td>3.56 × 10⁻¹²</td>
<td></td>
</tr>
<tr>
<td>6330548G22Rik</td>
<td>Small nuclear ribonucleoprotein 35</td>
<td>1-7</td>
<td>2.21 × 10⁻¹²</td>
<td></td>
</tr>
</tbody>
</table>
the genes in this category are involved in carbohydrate metabolism. \( Pdk1 \), for example, inhibits glycolysis and was strongly upregulated by the restriction regime. Similarly, \( Ppp1r3b \) promoting glycogen synthesis and \( Slc2a5 \) promoting glucose uptake are both increased in HF-DR animals.

**Pathway analysis**

In addition to analysis of strongly significantly regulated genes, we performed a pathway analysis on the complete set of regulated genes (Table 5). This analysis confirmed that the primary changes in gene expression are related to lipid metabolism and mitochondrial functioning. Of the top ten named pathways, eight pathways (oxidative phosphorylation, cholesterol biosynthesis, citric acid cycle (ubiquinone metabolism), regulation of lipid metabolism, mitochondrial fatty acid unsaturated and long chain \( \beta \)-oxidation, and regulation of fatty acid metabolism) are involved in these functions.

**Microarray results were confirmed with qRT-PCR**

qRT-PCR was used to validate the microarray results for seven genes with an important role in lipid metabolism. This resulted in a significant upregulation of all seven genes with fold changes ranging between 4.0 and 513.3 (microarray data ranged between 2.0 and 48.0 for these genes) (Fig. 6).

**HF-DR mice have higher levels of mitochondrial mass in WAT**

Mitochondrial and nuclear DNA content in WAT of HF-AL, HF-DR, and \( t=0 \) mice was compared with qRT-PCR in order to estimate mitochondrial density. HF-DR mice had a significantly higher mitochondrial DNA content compared with both HF-AL and \( t=0 \) mice (Fig. 7). HF feeding (HF-AL versus \( t=0 \)) reduced mitochondrial density, although non-significantly.

**Discussion**

**DR of a HF diet improves metabolic health and promotes a metabolic adaptation toward lipid metabolism**

We demonstrate that DR of a HF diet restores glucose sensitivity, increases the mitochondrial density in WAT, decreases the serum levels of cholesterol and leptin, and increases serum adiponectin levels. The bodyweight and serum parameters of HF-DR mice remained stable during the last 7 weeks of restriction, implying that within 5 weeks, HF-DR mice have adapted to the restriction regime and exhibit a steady energy balance. The beneficial effects on health are comparable to previous reports that demonstrate that DR on a standard mouse diet improves health, by enhancing insulin sensitivity (Dhahbi et al. 2001); decreasing serum cholesterol (Choi et al. 1988), glucose, and leptin levels (Reimer et al. 2010); and increasing serum adiponectin (Combs et al. 2003). To our knowledge, this is the first study that exclusively reports improvements in markers of the metabolic syndrome by DR with a HF diet. Two previous studies, in fact, reported unfavorable effects in mice that were pair-fed on a HF diet to match the caloric intake of mice on a low-fat diet. A certain iso-caloric treatment was found to result in a decrease in insulin sensitivity in the restricted HF group (Petro et al. 2004), whereas a second study reported that HF pair-fed mice develop insulin resistance, hepatic steatosis, and obesity (de Meijer et al. 2010). The diets used in both studies, however, contained almost twice as much energy available from fat compared with the diet we used in our study (58 and 60% energy from fat, compared with 30%), which might have evoked a different coping strategy. A third study on HF-DR in mice, consistently,
reported decreased fasting insulin levels; although it also reported a decrease in serum adiponectin levels and only minor effects of HF restriction on inflammations in WAT (Kalupahana et al. 2011). An important difference with our study is that the authors used a run-in period on the HF diet that is much longer than the actual restriction period. In this case, 2 months of DR might simply be insufficient to reverse the negative effects of 6 months HF feeding ad libitum and to stabilize energy balance as was seen in our study. The occurrence of beneficial effects of DR with a HF diet seems, consequently, to depend on both the duration of restriction and the fat content of the diet.

To additionally examine the molecular events associated with the observed adaptation to HF-DR, we analyzed whole genome expression in WAT, the organ...
that was notably affected by HF-DR (Figs 1B, C and 3A, C) and is known to play an important role in the development of metabolic complications in obesity (Trayhurn & Beattie 2001). Among the drastic shifts in gene expression in WAT, the changes in genes involved in lipid metabolism and mitochondrial function were most pronounced (Table 3 and Fig. 5). In our study, the majority of lipid metabolism-related genes plays a role in cholesterol synthesis and fatty acid synthesis and elongation and was upregulated by the restriction regime. For this analysis, we initially focused on the functional interpretation of genes with the strongest P values. To further validate this approach, we also conducted a pathway analysis on the complete set of regulated genes, which revealed significant regulation of several pathways involved in both lipid metabolism and mitochondrial function (Table 5), which further supports the functional interpretation of our initial approach. To our surprise, pathway analysis also pointed toward an upregulation of mitochondrial fatty acid oxidation, a result that seems contradictory to the observed increase in fatty acid synthesis and elongation. Earlier studies to the effects of DR on gene expression in WAT, however, also reported changes in genes and pathways involved in both storage and usage of energy; although in these studies, a standard rodent diet was used for DR and compared to our results, the effects on lipid metabolism are less pronounced (Higami et al. 2004, Park & Prolla 2005). We propose that the HF-DR mice have developed an enhanced substrate efficiency for the abundant fat component in the diet and that upregulation of both fat storage and release pathways relates to the beneficial effects on metabolic health we observed in this study.

As both groups of mice were kept under the same rearing conditions and on diets with equal vitamin and mineral intake and identical macronutrient composition, most plausible alternative explanations for the differences in gene expression between HF-DR and

**Table 5** Top ten pathways that are significantly regulated between HF-AL and HF-DR mice. Pathway analysis was performed on the complete set of regulated genes (P<0.01). The top ten pathways (based on P value) consist of several pathways involved in lipid metabolism and mitochondrial function. The table additionally shows the total number of genes that is involved in the pathway and the number of these genes that was either upregulated or downregulated in our dataset.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>P value</th>
<th>Total number of genes involved in pathway</th>
<th>Number of upregulated genes in dataset</th>
<th>Number of downregulated genes in dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidative phosphorylation</td>
<td>1·80×10⁻¹¹</td>
<td>114</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>2. Cholesterol biosynthesis</td>
<td>1·16×10⁻⁶</td>
<td>22</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>3. Citric acid cycle</td>
<td>2·49×10⁻⁶</td>
<td>20</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>4. Regulation of lipid metabolism via LXR, NF-Y, and SREBF</td>
<td>7·75×10⁻⁶</td>
<td>35</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>5. Ubiquinone metabolism</td>
<td>1·03×10⁻⁵</td>
<td>52</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>6. BAD phosphorylation</td>
<td>1·08×10⁻⁵</td>
<td>88</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td>7. Mitochondrial long-chain fatty acid β-oxidation</td>
<td>1·14×10⁻⁵</td>
<td>18</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>8. EDG3 signaling</td>
<td>3·11×10⁻⁵</td>
<td>68</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>9. Insulin regulation of fatty acid metabolism</td>
<td>5·12×10⁻⁵</td>
<td>55</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>10. Mitochondrial unsaturated fatty acid β-oxidation</td>
<td>5·13×10⁻⁵</td>
<td>16</td>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 6** Confirmation of microarray results by real-time qRT-PCR. The expression of (A) Acaca, (B) Acly, (C) Fasn, (D) Sqle, (E) Sreb1f, (F) Elovl3, and (G) Elovl6 in HF-DR and HF-AL mice was analyzed by qRT-PCR (left) using stable reference genes Ccna, B2m, Silt, and Rps15. Data obtained with microarray are shown for comparison (right). Data represent the mean ± S.E.M. of 12 mice, in which the mean expression of HF-AL mice is set to 1.0. *P<0.05; ***P<0.001.

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HF-AL can be excluded. It should, nonetheless, be noted that, in order to match the levels of food intake, animals of both groups were treated differently. HF-DR animals received their restricted amount of food at the start of the light phase and were sectioned 6 h afterward, whereas HF-AL animals were sectioned 3 h after start of the light phase, which might have influenced expression patterns of genes that display a diurnal pattern, such as Bmal, Cry1, Elavl3, and Elavl6. Indeed, we found a significant upregulation of the circadian clock regulator Bmal in HF-DR mice (data not shown). However, we found the expression of Cry1 to be increased, while it is expected to be stable within the time frame just mentioned (Ando et al. 2005). Accordingly, the expression level of Elavl3 is increased instead of decreased, as would be expected if the difference was due to the circadian rhythm of this gene (Anzulovich et al. 2006). Finally, the increase in expression of Elavl6 is well beyond the increase in expression that is normally seen during this time frame in the light phase (a twofold increase that is normally seen compared with a 47.2-fold increase observed in this study) (Cretenet et al. 2010). It seems, therefore, unlikely that the differences observed in the expression of metabolic genes, which is the focus of this study, are primarily due to the differences in feeding and sectioning time.

**Srebf1 and Srebf2 are expected to play a major role in the adaptation to a HF diet in HF-DR animals**

Srebf1 and -2 play an important role in the regulation of lipid metabolism and both were highly expressed in HF-DR mice. Although Srebf1 did not belong to the list of genes with the strongest P values (Table 3), the same list contains various genes that are under transcriptional control of both Srebf isoforms, such as Elavl6, Acly, and Acaca (Matsuzaka et al. 2002). SREBP s (the human annotation of Srebf s) are known as crucial cellular molecules that mediate adaptive metabolic responses to changing dietary exposures (Strable & Ntambi 2010). Nuclear translocation of SREBP s is regulated by intracellular fatty acid and sterol levels. When cellular sterols are abundant, SREBP s are inactive in the endoplasmic reticulum membrane. On sterol depletion, SREBP s are cleaved by regulated proteolysis to release the mature transcription factor domain, which translocates to the nucleus. SREBP s then bind promoter sterol regulatory elements (SREs) to activate genes involved in the biosynthesis and uptake of cholesterol and fatty acids (Brown & Goldstein 1997). The expression of Ppara and Pparg, two other key regulators of lipid metabolism, is not significantly altered between HF-DR and HF-AL, implying the significance of both Srebf s in upregulating lipid metabolism in our study.

Consistent to the upregulation of both Srebf s, cholesterol synthesis appeared to be significantly changed in HF-DR mice (Fig. 5); in fact, 20 of the 22 genes that are involved in this metabolic process were upregulated by our treatment. At first sight, it might appear strange for HF-DR mice to increase the rate of cholesterol and fatty acid synthesis. The HF-AL mice consumed 3·94 kcal from fat per day; this was 2·76 kcal for HF-DR mice, whereas a chow-fed mouse eats about 1·08 kcal from fat per day (Champy et al. 2008). Secondly, mice on a standard chow diet receive no cholesterol, whereas HF-DR mice do consume cholesterol, although to a lesser extent compared with HF-AL mice. It can, therefore, be assumed that both HF-DR and HF-AL mice received enough energy from fat. Similar results on Srebf s functioning and upregulation of energy metabolism have, however, earlier been observed in dietary restricted animals. At first, HF diets are known to induce expression of Srebf1 in the liver (Lin et al. 2005), and studies on both mice and humans showed that the expression of SREBP s is downregulated in obese versus lean subjects (Nadler et al. 2000, Soukas et al. 2000, Oberkofler et al. 2002). These authors subsequently hypothesize that the down-regulation of Srebf s correlates with the dedifferentiation of adipocytes in obesity, while enhanced differentiation of adipocytes is rather known as a characteristic of DR (Higami et al. 2004). Secondly, it was shown that re-feeding after fasting stimulates the expression of Srebf1 (Liang et al. 2002), which implies that the dietary restricted animals in our study are still (6 h after feeding) in a postprandial state. A result that is consistent to a study by Bruss et al. (2010) is that DR elevates fatty acid synthesis in mice until 4–6 h after food provision compared to normal fed mice as measured by their respiratory exchange ratio. Our study confirms this finding and implicates an important role for Srebf1 and Srebf2 in this metabolic adaptation.
Finally, upregulation of metabolic rate has earlier been observed in ‘normal’ low-fat DR studies in mice (Higami et al. 2004), as well as in dietary restricted nematodes (Houthoofd et al. 2002) and flies (Hulbert et al. 2004). The main difference in these studies is, however, that our results primarily point in the direction of lipid metabolism, implying that mice are capable of adjusting their metabolism specifically to their diet. As a result, HF-DR mice seem to be better equipped to deal with the excess amount of fat in their diet compared to the HF-AL mice. Several aspects of the microarray results fit within this theory. The upregulation of, for example, mitochondrial fatty acid β-oxidation (Fig. 5), in which acyl-CoA molecules are oxidized to generate acetyl-CoA that can be subsequently used in the citric acid cycle, enables HF-DR animals to synthesize ATP during scarcity of pyruvate. Pdk1 that was also upregulated by HF-DR complements this process by protecting carbohydrate stores in muscle (Peters et al. 2001) and, in this way, stimulates cells to shift to fat utilization and spare carbohydrate stores. Finally, the upregulation of Starld4 and Starld5 could make the metabolism of cholesterol more efficient by regulating the transfer of cholesterol to different cellular compartments. Apart from that, by binding, transporting, and positioning dietary cholesterol, STARD4 protein increases cholesteryl ester formation and is, therefore, postulated to play a role in the determination of serum total cholesterol and LDL-cholesterol (Riegelhaupt et al. 2010), which were found to be significantly decreased in serum of HF-DR mice (Fig. 3).

HF-DR significantly enhanced mitochondrial function and density in WAT

The mitochondrion serves a critical function in the maintenance of cellular energy stores, thermogenesis, and apoptosis. Proper control of mitochondrial function is, therefore, of major importance for cell survival and the maintenance of metabolic health. In our list of strongly regulated genes, we identified several genes that are involved in mitochondrial function (Table 3). The expression patterns of these genes pointed toward an improved mitochondrial function. To further confirm this finding, we analyzed our complete set of regulated genes for genes that are better established to play a key role in the regulation of mitochondrial function (Table 4). In the first place, we aimed at genes involved in the process of mitochondrial biogenesis. Mitochondrial biogenesis is mainly determined by genes that promote the formation of elements of the respiratory chain and the maintenance of mitochondrial DNA. Pparge1a, Pparge1b, and Esra are known as the key transcriptional regulators within this process and were all significantly upregulated in HF-DR animals. Nrf1, Gabpa, and Tfam that work as the direct effectors of Pparge1a and stimulated mitochondrial biogenesis (Scarpulla 2008) were consistently upregulated in these animals. In addition, we found a significant increase in the expression of CoxIV and CoxVb that are under transcriptional control of Nrf1 and Gabpa and serve a direct function in the formation of the cytochrome oxidase subunits (Kelly & Scarpulla 2004).

Secondly, we looked at genes involved in mitochondrial dynamics, which can be characterized by the degree of fission and fusion of mitochondria. Fission and fusion refers to the formation and breakdown of tubules between mitochondria, which plays an important role in the regulation of mitochondrial morphology. Dysregulation of genes involved in fission and fusion relates to the development of various disorders, such as diabetes (Yoon et al. 2010). In mammals, fission is primarily regulated by Dnm1l and Fis1, both of which are upregulated by HF-DR, although the regulation of Fis1 is only marginally significant. Fusion of mitochondrial tubules is mediated by Mfn1 and Opa1, both of which are significantly upregulated in HF-DR mice. Opa1 is specifically involved in the fusion of the mitochondrial inner membrane and was found to be cleaved and activated by Oma1, which is also significantly upregulated in our dataset. Finally, we also found a significant upregulation in the expression of Stomi2 and Dhadh that play a role in stress-induced fusion and the transcriptional regulation of fission respectively. Under normal conditions, fission and fusion proteins are expressed in response to the mitochondrial activity of the cell. Artificial overexpression of one of either fission or fusion proteins disrupts the balance and disturbs normal cell function (Yoon et al. 2010). Fis1 overexpression in liver cells, for example, causes mitochondrial fragmentation and impaired glucose-stimulated insulin secretion, whereas overexpression of Mfn1 causes similar perturbations of the metabolism–secretion coupling of glucose (Park et al. 2008). Regarding the improved health status of HF-DR mice, we hypothesize that the upregulation of both fission and fusion proteins relates to the observed improvement of mitochondrial function.

Fission and fusion proteins are, additionally, involved in the regulation of apoptosis by the elimination of damaged mitochondria by autophagy. Selective autophagy of mitochondria, or mitophagy, has extensively been described in yeast, where it is known to play an important role in the determination of cell survival or cell death after oxidative stress (Kanki & Klionsky 2010). In short, damaged, aged, or excess mitochondria are a risk factor for the cell, and proper elimination of such organelles is important to maintain optimal cellular homeostasis. To also investigate the end stage of mitochondrial function, we analyzed a number of genes involved in mitochondrial autophagy. In mammals,
mitophagy is regulated by Bnip3, Bnip3L, and Park2 (Narendra et al. 2008, Zhang & Ney 2009), of which the latter two were significantly upregulated in HF-DR mice. Finally, we analyzed the expression of Mtor, a serine/threonine protein kinase transcription factor that responds to nutrient starvation, oxidative stress, and availability of growth factors. In fact, nutrient starvation and oxidative stress inhibit Mtor transcription, which stimulates autophagy and apoptosis (Jung et al. 2010). Mtor appeared to be significantly highly expressed in HF-DR animals, which implies a decreased prevalence of oxidative damage and apoptosis. The downstream effectors of Mtor, Ulk1 and Ulk2, that initiate apoptosis in the absence of Mtor were either not regulated or significantly downregulated.

It should be noted that DR is often related to downregulation of Mtor (Kenyon 2010). In this case, increase in autophagy is seen as a strategy to survive low nutrient concentrations by recycling their own cell content. In our study, however, the restriction of the HF diet still provides the animals with an amount of calories that is similar to the amount of calories that mice consume on a standard low-fat rodent diet: the HF-DR mice received 9.2 kcal/day, whereas an ‘average’ male C57BL6/J mouse eats around 10.8 kcal/day (Champy et al. 2008). It can, therefore, be hypothesized that the HF-DR mice received sufficient nutrients and therefore did not need to switch to salvage pathways such as autophagy.

To summarize, microarray analysis provided us with a convincing amount of evidence for the upregulation of mitochondrial function in HF-DR mice. The increase in the ratio of mitochondrial DNA in WAT of HF-DR mice, which can be seen as an estimation of the mitochondrial density (Lagouge et al. 2006), further confirms our findings regarding the upregulation of mitochondrial genes. Although the promotion of mitochondrial biogenesis induced by caloric restriction was recently re-evaluated in an extensive restriction study in rats, in which no changes were found in the amount of mitochondrial proteins in restricted animals (Hancock et al. 2010), improvements of mitochondrial function in WAT were earlier observed in other restricted animals and is thought to be related to the beneficial effects of restriction (Higami et al. 2004, Linford et al. 2007), in which our study is the first to report effects on mitochondrial function in mice restricted on a HF diet.

Conclusions
The results of our study prove that DR can reverse the adverse effects of an initial unhealthy HF diet. Our treatment had a large influence on gene expression in WAT. An in-depth study on genes with the strongest P-values revealed a major role for genes involved in lipid metabolism and mitochondrial functioning. It is hypothesized that the restriction procedure has forced the metabolism of mice toward the utilization of especially lipids in order to effectively make use of the diet, spare carbohydrates, and to create a stable energy balance. The effective use of fat seems to protect HF-DR mice from the increase in serum cholesterol and leptin levels that were found in HF-AL mice, as well as from the decrease in mitochondrial density in WAT, and decrease in glucose sensitivity and serum adiponectin levels.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
The research leading to these results has received funding from the European Union’s Seventh Framework Programme FP7 2007-2013 under grant agreement no. 244995 (BIOCLAIMS Project).

Acknowledgements
We thank all members of the Department of Human and Animal Physiology and RIKILT Food Bioactives group for their helpful contributions, especially Melissa Bekkenkamp and Marianne van der Mark for their help with the OGTT and qRT-PCR, and everyone from the animal facility who was involved in this study.

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