Mice Deficient in the Respiratory Chain Gene Cox6a2 Are Protected against High-Fat Diet-Induced Obesity and Insulin Resistance

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Abstract
Oxidative phosphorylation in mitochondria is responsible for 90% of ATP synthesis in most cells. This essential housekeeping phosphorylation is mediated by nuclear and mitochondrial genes encoding subunits of complex I to V of the respiratory chain. Although complex IV is the best studied of these complexes, the exact function of the striated muscle-specific subunit COX6A2 is still poorly understood. In this study, we show that Cox6a2-deficient mice are protected against high-fat diet-induced obesity, insulin resistance and glucose intolerance. This phenotype results from elevated energy expenditure and a skeletal muscle fiber type switch towards more oxidative fibers. At the molecular level we observe increased formation of reactive oxygen species, constitutive activation of AMP-activated protein kinase, and enhanced expression of uncoupling proteins. Our data indicate that COX6A2 is a regulator of respiratory uncoupling in muscle and we demonstrate that a novel and direct link exists between muscle respiratory chain activity and diet-induced obesity/insulin resistance.

Introduction
The worldwide prevalence of type 2 diabetes and obesity has reached epidemic proportions as the result of the interaction of a Westernized lifestyle with genetic determinants that are prevalent in human populations. Obesity often predisposes to the development of insulin resistance and type 2 diabetes, and therefore weight loss by promotion of a healthy lifestyle is often the initial therapeutic target of intervention for obese, type 2 diabetic patients. However, this strategy has only short-term success in 95% of the cases [1]. Thus, it has been proposed that long-term strategies for inducing weight loss should also aim at increasing the patients’ metabolic rates (energy expenditure) by decreasing their metabolic efficiency. This can be achieved in mice by induction of mitochondrial uncoupling proteins (UCPs), which dissipate energy as heat [2–5]. UCPs can be induced by different mechanisms among which reactive oxygen species (ROS) [6], or via activation of AMP-activated protein kinase (AMPK) [7,8], which is a master regulator of energy homeostasis.

The role of mitochondrial dysfunction in the etiology of insulin resistance, type 2 diabetes and obesity is still unclear. Early reports indicated that mitochondrial dysfunction was a causative factor [9–12] and was the result of the accumulation of metabolites such as ROS and long chain fatty acids [13–15]. However, over the past few years, several studies have suggested that changes in mitochondrial function may be compensatory or even protective, rather than a cause of these diseases [16–18]. Moreover, it seems that mitochondrial dysfunction in muscle and liver in mice protects against obesity and diabetes [19], whereas mice with a progressive muscle-specific respiratory chain deficiency have...
reduced blood glucose levels and increased peripheral glucose uptake [20].

COX6A protein is one of the thirteen subunits of the respiratory chain complex IV (cytochrome c oxidase). Its exact role in this complex is not yet known; possible functions may be assembly of the complex as well as regulation of the catalytic properties of the core subunits. Mammalian COX6A is represented by two different isoforms, COX6A-L (COX6A1, liver-type) and COX6A-H (COX6A2, heart-type), which are encoded in the mouse by the Cox6a1 and Cox6a2 genes, respectively. Two differences between these isoforms are their expression and regulation. The COX6A1 subunit is ubiquitously expressed, whereas expression of COX6A2 is restricted to striated muscles [21,22]. Unlike COX6A1, the COX6A2 subunit has the capability to bind ADP and ATP [23]. Binding of ADP results in an increase in catalytic activity which can be abolished by competition with a monoclonal antibody [24]. Moreover, at high intramitochondrial ATP/ADP ratios (i.e. at rest), reconstituted bovine heart complex IV has decreased proton pumping capacity, also referred to as intrinsic uncoupling or “slip” [25]. This mechanism has been suggested to play a role in thermogenesis in heart and skeletal muscle at rest [26] as well as in the protection against the formation of mitochondrial ROS [27,28]. Interestingly, in the stepwise assembly of the mammalian complex IV, the COX6A subunit is only added in the final step [29], suggesting that this subunit plays a major role in the expression and enzymatic activity of functional complex IV.

Until present, the functional consequence of a global deletion of the Cox6a2 gene in mice (Cox6a2−/−) has only been investigated for the heart: the phenotype was described as mild diastolic dysfunction at elevated workload, resulting in decreased stroke work [30]. Because Cox6a2 is also expressed in skeletal muscle and because of the specific regulatory properties of COX6A2 subunit in oxidative phosphorylation, we were interested in the effect of COX6A2 deficiency on skeletal muscle function as well as whole body energy metabolism and glucose homeostasis. Our data demonstrate a previously unrecognized link between presence of the Cox6a2 gene in mice and the susceptibility to develop high-fat diet-induced obesity, insulin resistance and glucose intolerance.

Results

COX6A2 deficiency results in a moderate decrease in complex IV enzymatic activity and increased production of ROS in skeletal muscles

In agreement with previous reports [31,32], COX6A1 protein expression was very low in three types of skeletal muscle: soleus (slow oxidative, mainly type I fibers), gastrocnemius (fast glycolytic, mainly type IIb fibers) and diaphragm (fast oxidative, mainly type IIA fibers) (Fig. 1A). On the contrary, COX6A2 protein was abundant not only in heart, but also in diaphragm and to a lesser extent in soleus and gastrocnemius muscle (Fig. 1A). In order to assess a direct functional consequence of Cox6a2 deletion in mice, we measured mitochondrial complex IV enzymatic activity, both in heart and skeletal muscle and observed a moderate but significant reduction in all of these tissues (Fig. 1B). In contrast, oxygen consumption rates of isolated diaphragm were not affected by the Cox6a2 deletion (Fig. 1C), indicating the existence of compensatory mechanisms. Since reduced complex IV activity is often associated with enhanced ROS production, and because intrinsic uncoupling of oxidative phosphorylation has been proposed to protect against the formation of ROS [28] we also measured steady state ROS levels in the diaphragm and hindlimbs of Cox6a2−/− and WT mice utilizing dihydroethidium, which preferentially indicates superoxide [33]. As is demonstrated in figures 1D and E, elevated ROS levels were detected both in diaphragm and in hindlimbs muscles of Cox6a2−/− mice as compared to WT mice with the largest difference being measured in the diaphragm. Together, our results indicate that COX6A2 deficiency causes moderate loss of complex IV activity and increased ROS production in skeletal muscle.

Cox6a2−/− mice are lean and protected against diet-induced obesity

Accumulation of ROS in skeletal muscle is often associated with mitochondrial dysfunction, insulin resistance and obesity. Therefore, we investigated the metabolic phenotype of Cox6a2−/− mice and compared animals that were fed a regular diet to those fed a high-fat diet (HFD). As shown in figure 2A, WT mice gained extra weight when fed a HFD as compared to a regular diet. However, Cox6a2−/− mice had superimposable weight gain curves on regular diet or HFD (Fig. 2A). Absolute food intake was reduced in HFD-fed Cox6a2−/− mice (Fig. 2B, left panel) but when corrected for body weight, food intake was significantly increased in Cox6a2−/− mice (Fig. 2B, middle panel). The difference in weight gain between WT and Cox6a2−/− mice can be attributed to decreased feed efficiency in the Cox6a2−/− strain (Fig. 2B, right panel). The increment in whole body weight after a HFD was primarily caused by increased mass of adipose tissue (both in gonadal and subcutaneous fat pads) in control animals (Fig. 2C). We also observed slightly increased heart weights in Cox6a2−/− mice (Fig. 2C) in which conforms the previous observation of cardiac hypertrophy caused by diabetic dysfunction [30].

The reduced mass of the gonadal fat pad in Cox6a2−/− mice fed a HFD was associated with decreased adipocyte size and increased cellular density 12 weeks after the start of HFD feeding (Fig. 2D, E). A similar trend was observed in adipocytes from subcutaneous fat (Fig. 2E). Therefore, at the whole organism level, deficiency of COX6A2 protects against HFD-induced fat mass accumulation.

Increased glucose tolerance and insulin sensitivity in Cox6a2−/− mice fed a HFD

Development of diet-induced obesity in WT mice was associated with increased plasma levels of insulin, leptin, cholesterol and triglycerides as compared to Cox6a2−/− mice (Table S1). Despite similar random fed blood glucose levels, the difference in plasma insulin levels between WT and Cox6a2−/− mice suggested that a difference in insulin sensitivity existed between the two strains. To further investigate this idea, we first performed intraperitoneal glucose tolerance tests in mice that were either on a regular diet or a HFD for 9 or 22 weeks. Although Cox6a2−/− mice fed a regular diet had significantly lower fasting glucose levels, no difference in glucose tolerance between Cox6a2−/− and WT mice was observed (Fig. 3A, left panel). However, while WT mice fed a HFD developed progressive glucose intolerance with a marked degree of hyperglycemia two hours after glucose injection (Fig. 3A), no difference in glucose tolerance was observed in Cox6a2−/− mice after 22 weeks of HFD versus regular diet (Fig. 3A). To assess whether the preservation of glucose tolerance in Cox6a2−/− mice after HFD was a result of increased insulin sensitivity, we performed hyperinsuliniemic, euglycemic clamps on mice fed a HFD for 15 weeks. During hyperinsulinemia, plasma insulin levels were comparable between WT and Cox6a2−/− mice (Fig. 3B). Within 5 min after start of the i.v. administration of insulin, Cox6a2−/− mice exhibited a significant drop in blood glucose levels, required a significantly higher glucose infusion rate...
Energy expenditure and adaptive thermogenesis are increased in Cox6a2−/− mice

In order to determine whether the lean phenotype of the Cox6a2−/− mice was due to an increased basal metabolic rate and energy expenditure, indirect calorimetric measurements were performed on weight-matched male mice fed a regular diet. The body composition of these mice was assessed using MRI, and revealed a small but statistically significant decrease in percent lean body mass in the Cox6a2−/− mice as compared to WT mice (Fig. S1A), whereas there was no difference in % fat mass in mice fed a regular diet (Fig. S1B). Daily food intake was similar between Cox6a2−/− and WT mice, but the daily intake of water was slightly lower in Cox6a2−/− mice (Fig. S1C). The indirect calorimetric measurements showed that, compared to WT mice, Cox6a2−/− mice consumed more oxygen and generated more heat during the day (Fig. 4A, B), which confirms an increased metabolic rate in Cox6a2−/− mice compared to WT mice. During the night, there was a non-significant increase in oxygen consumption and heat generation in Cox6a2−/− mice, despite a 25% decrease in spontaneous activity compared to WT mice (Fig. 4C). The calorimetric studies also revealed no difference in the respiratory exchange ratio (RER) between WT and Cox6a2−/− mice (Fig. 4D), suggesting that the ratio of carbohydrate over fat oxidation is unaltered in Cox6a2−/− mice. These data also indicated that the increased energy expenditure and thermogenesis in Cox6a2−/−

Characterisation of the Cox6a2 Knockout Mouse

Figure 1. COX6A1 and COX6A2 expression in skeletal muscles and heart of WT mice. Disruption of the gene leads to reduced complex IV activity and enhanced ROS generation. (A) Representative images of western blots for COX6A1 and COX6A2 in different skeletal muscles. COX6A1 expression was only observed in the heart, whereas COX6A2 protein expression is highest in the heart and diaphragm and lowest in the gastrocnemius muscle, suggesting that COX6A2 expression correlates with muscle oxidative capacity. Sol: soleus muscle; Gast: gastrocnemius muscle; Diaph: diaphragm. (B) Complex IV activity measurements in skeletal muscles and heart of WT and Cox6a2−/− mice (n = 3). (C) Oxygen consumption rate in diaphragm of WT vs Cox6a2−/− mice (n = 3). Inhibition of complex IV by NaN₃ was used to validate the method. (D) Measurement of steady state ROS levels by DHE staining. Representative images of DHE stained sections of the diaphragm (upper) and hindlimbs (lower) of WT and Cox6a2−/− mice. (E) Quantification of the number of positive myocytes stained with DHE (n = 6). *p<0.05, **p<0.01. Data represent mean±SEM. doi:10.1371/journal.pone.0056719.g001
Figure 2. *Cox6a2<sup>−/−</sup>* mice are protected against high-fat diet-induced obesity. (A) Mice were fed a regular diet or a HFD starting from 6 weeks of age (n = 5 for WT, n = 14–16 for *Cox6a2<sup>−/−</sup>* mice). Body weight was monitored weekly. The right panel shows representative images of WT and *Cox6a2<sup>−/−</sup>* mice after 12 weeks of HFD feeding. (B) Absolute food intake, relative food intake and feed efficiency of mice fed a HFD (n = 5). (C) Average weight of gastrocnemius muscle, heart, liver, subcutaneous WAT and gonadal WAT dissected from mice that were fed a HFD for 12 weeks.
mice were the main reasons for the lean phenotype of these mice, rather than differences in fuel selection for mitochondrial oxidation.

We also assessed the effect of COX6A2 deficiency on adaptive thermogenesis by exposing mice to cold (4°C) for three hours and monitoring body temperature. Body temperature remained relatively constant in Cox6a2−/− mice fed either a regular diet (Fig. 4E) or a HFD (Fig. 4F), indicating enhanced non-shivering thermogenesis. Moreover, we also found that, in contrast to WT mice, Cox6a2−/− mice fed a HFD had increased core body temperature as compared to mice fed a regular diet (Fig. 4G). This may represent an additional mechanism to explain the resistance to diet-induced obesity we observed in Cox6a2−/− mice.

To rule out a compensatory thermoregulatory mechanism via the pituitary-thyroid axis, we also assessed thyrotropin (TSH) bioactivity (a measure for TSH content) and serum T4 levels. These assays revealed that both hormones were not significantly altered in Cox6a2−/− mice (Fig. S1D), suggesting that the pituitary-thyroid axis was not responsible for the increased thermogenesis observed in Cox6a2−/− mice exposed to cold. Therefore we wondered whether the COX6A2 subunit might be also expressed in thermogenic brown adipose tissue (BAT) or subcutaneous white adipose tissue (SC WAT), which has recently been shown to contain brown-like cells, called “beige fat” [34]. Thus, we analyzed public microarray data of BAT (GSE7623, [35]) and SC WAT (E-MEXP-1636, [36]). In both data sets, we found very heterogeneous Cox6a2 signals (Fig. S2) which may be

Figure 3. Increased glucose tolerance and insulin sensitivity in Cox6a2−/− mice is associated with constitutive activation of AMPK.

(A) After a 16–18 h fast, mice were injected with 2.5 mg/g BW glucose and blood glucose levels were monitored for 2 h (n = 7–9 for RD, n = 4–11 for 9 weeks of HFD, n = 3–4 for 22 weeks of HFD). Note that WT animals become progressively glucose intolerant when receiving HFD, whereas Cox6a2−/− mice are completely protected against HFD-induced glucose intolerance. (B) Hyperinsulinemic, euglycemic clamps were performed on mice (21 weeks old) fed a HFD for 15 weeks (n = 6–8). Plasma insulin levels before (10 min: B1; 0 min: B2) and after (70 min: H1; 80 min: H2; 90 min: H3) insulin infusion, (C) The glucose infusion rate (GIR) was monitored for 90 min after administration of a hyperinsulinemic solution via the tail vein, (D) Blood glucose levels before insulin infusion (basal) and at the end of the clamp (hyperinsulinaemia), (E) Whole body glucose disposal (left) and hepatic glucose production (right) were measured during the basal period and under hyperinsulinemic conditions, (F) Western blot analysis of insulin-stimulated phosphorylation of Akt in soleus muscle of regular diet fed mice. No difference was observed between fasted (18 h) WT and Cox6a2−/− mice (n = 3), (G) Western blot analysis of AMPK phosphorylation in response to fasting. Mice (n = 3) on a regular diet were either fed ad libitum or fasted overnight (18 h) before dissection of soleus muscles. *p<0.05, **p<0.01, ***p<0.001. In all panels, data represent mean±SEM.

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reflective of either sample contamination, or expression of the gene in only a subpopulation of the cells, as would be expected in SC WAT. In the case of the latter, a high degree of correlation in the expression between Cox6a2 and the thermogenic protein UCP1 was to be expected. However, whereas expression of UCP1 was also very heterogeneous among the different SC WAT samples, there was no correlation with the expression of Cox6a2 (Fig. S2B). Instead, both in BAT and SC WAT we found very strong correlation between Cox6a2 and skeletal muscle markers (Fig. S2), indicating that when Cox6a2 expression is found in these tissues, it is most likely the result of contaminating skeletal muscle tissue in the samples.

Together, these data indicate that increased thermogenesis in Cox6a2−/− mice, which may be partly responsible for their protection against diet-induced obesity, results from loss of the gene in skeletal muscles, rather than more established thermogenic tissues such as BAT. These results also indicate that the COX6A2 subunit is an important regulator for whole-body metabolic rate.

**Cox6a2−/− mice lose body weight faster upon short-term starvation**

The protection from diet-induced obesity, the inefficient energy metabolism and the increase in energy expenditure predicted that Cox6a2−/− mice would tolerate starvation less well than control mice. We tested this prediction by a starvation experiment with free access to water for 36 h at room temperature. The observation was that both male and female Cox6a2−/− mice lost significantly more weight compared to WT animals (Fig. S3A, B). In fact, two out of ten Cox6a2−/− male mice had to be refed after 24 h, because they were lethargic. Between 24 h and 36 h of starvation...
failing, the calculated average weight loss in the remaining eight \textit{Cox6a2}^{−/−} male mice (slope = −0.32±0.03% weight loss per h) was 2-fold greater as compared to the male controls (slope = −0.28±0.02%; p<0.001) (Fig. S3A). This period corresponds to the "scoping phase" of body weight loss, in which primarily fat and ketone bodies are used as an energy source [37]. We also evaluated the effect of fasting on \textit{Cox6a2} expression in different muscles and found that during fasting, \textit{Cox6a2} expression, both at the mRNA (Fig. S3C) as well as the protein level (Fig. S3D), was increased in the soleus muscle but not the gastrocnemius or the diaphragm of WT mice. This suggests that the feeding-dependent regulation of \textit{Cox6a2} expression is restricted to type I myofibers.

\textit{Cox6a2}^{−/−} mice display elevated expression of uncoupling proteins in muscles, heart and adipose tissue

Uncoupling proteins serve a critical role in the regulation of cell metabolism and overall energy expenditure and are often induced in animal models that are protected against obesity. We therefore measured the \textit{Usp} transcript levels not only in skeletal muscle, but also in heart, and adipose tissue (WAT and BAT). A significant relative increase in UCP2 expression was measured in muscle and fat of \textit{Cox6a2}^{−/−} mice, but there were tissue-specific differences (Fig. 5A) with the highest increase being measured in the diaphragm. Furthermore, UCP1 mRNA expression in the diaphragm, soleus muscle and heart of \textit{Cox6a2}^{−/−} mice was upregulated by 4- to 9-fold (Fig. 5B). In WAT of mice that were fed a HFD we found that both UCP1 and UCP2 mRNA expression was further increased 3-to 5-fold in \textit{Cox6a2}^{−/−} mice as compared to mice fed a regular diet (Fig. S4A). Interestingly, a similar increase in UCP2 expression in response to HFD was seen in WAT of obesity-resistant C57BL/KsJ and A/J mice, whereas in the obesity-prone C57BL/6J mouse strain no difference in UCP2 expression could be seen in HFD fed mice [38]. Also in gastrocnemius muscle of HFD fed \textit{Cox6a2}^{−/−} mice, expression of UCP2 was further elevated compared to \textit{Cox6a2}^{−/−} mice on a regular diet (Fig. S4B).

Fiber type switch in skeletal muscle of \textit{Cox6a2}^{−/−} mice

The changes in \textit{Usp} gene expression and ROS levels in muscle of \textit{Cox6a2}^{−/−} mice led to the question whether more global differences of the gene expression exist. Since most of the changes in mRNA expression were small, a gene set enrichment analysis (GSEA) was undertaken to identify statistically significant, coordinate changes in gene expression of \textit{a priori} defined sets of genes. In both the gastrocnemius muscle and diaphragm, expression of genes associated with mitochondrial function as well as oxidoreductase activity was elevated in \textit{Cox6a2}^{−/−} mice as compared to WT mice (Table S2). Significant enrichment was found for instance for antioxidative enzymes (Table S3) as well as the subunits of the respiratory chain (Fig. 6A,B) which were, with the obvious exception of \textit{Cox6a2}, almost all slightly (10–70%) upregulated (Fig. 6B). Similarly to the induction of ROS production (Fig. 1E), the effects at the level of gene expression were more outspoken in the diaphragm compared to the gastrocnemius muscle.

The upregulation at the level of the respiratory chain coincided with enhanced expression of markers for oxidative (type I and IIA) myofibers, both in gastrocnemius muscle and in diaphragm of \textit{Cox6a2}^{−/−} mice (Fig. 6A, right panel). In contrast, markers for glycolytic (type IIB) myofibers were downregulated (Fig. 6A, right panel). These data indicate that in \textit{Cox6a2}^{−/−} mice, skeletal muscle fibers have switched to a slower, more oxidative phenotype. This change was confirmed at the histochemical level by performing a metachromatic ATPase assay, by which the fiber type composition within a skeletal muscle group can be determined. As is demonstrated in figures 6C and 6D, we observed an increase in the number of oxidative type IIA fibers within the gastrocnemius muscle of \textit{Cox6a2}^{−/−} mice. Moreover, the total number of myofibers per mm² was significantly increased in \textit{Cox6a2}^{−/−} gastrocnemius muscle, and the cross-sectional areas of all the fiber types in \textit{Cox6a2}^{−/−} mice were significantly reduced (Fig. 6E). Interestingly, we found that \textit{Fndc5}, the gene that codes for the recently discovered antidiabetic hormone irisin [39] was also significantly increased in \textit{Cox6a2}^{−/−} diaphragm (data not shown). This is probably also a result of the fiber type switch since we found in the publicly available data from a microgenomic analysis of individual fast and slow myofibers [40] that \textit{Fndc5} is preferentially expressed in slow fibers.

Together with a change in gene expression and fiber type, we also observed a change in mitochondrial size. Perinuclear as well as internymfibrillar mitochondria were larger in the diaphragm of \textit{Cox6a2}^{−/−} mice (Fig. 6F, G, H), although we did not observe a difference in size of the small internymfibrillar mitochondria located near the Z-discs. The alignment of myofilbrils as well as the dense packaging of cristae within the mitochondria were not affected in \textit{Cox6a2}^{−/−} mice. The increase in mitochondrial size we observed in the diaphragm was associated with increased expression of the peroxisome proliferator-activated receptor γ coactivator, PGC-1α (Fig. 6I), a key regulator of mitochondrial

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Figure 5. Increased UCP2 (A) and UCP1 (B) expression in metabolically active tissues of \textit{Cox6a2}^{−/−} mice. Quantitative RT-PCRs were performed on cDNA from gastrocnemius muscle, diaphragm, soleus muscle, heart, white and brown adipose tissue (n = 3–5). Gene expression in WT mice was set at 1.0 for each individual tissue. UD = Undetectable. Please note that UCP1 mRNA expression in BAT is about 100-fold that of other tissues. *p<0.05, **p<0.01, ***p<0.001. In all panels, data represent mean ± SEM.

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biogenesis [41] and of skeletal muscle fiber type determination [42]. Also mitofusin-1 (Mfn1) and optic atrophy 1 (Opa1), both of which regulate mitochondrial fusion and therefore mitochondrial size [43], were increased in the diaphragm of Cox6a2−/− mice compared to WT mice (Fig. 6J).

Figure 6. Fiber type switch and increased mitochondrial size in muscles of Cox6a2−/− mice. (A) Heat maps of top subsets of genes of a Gene Set Enrichment Analysis of gene expression in diaphragm of WT versus Cox6a2−/− mice on a regular diet (n = 3). Genes were ranked according to their signal-to-noise ratio. Left: Electron transport chain genes. Right: Striated muscle contraction genes. Red color indicates high expression, green color indicates low expression. KO: Cox6a2−/−, (B) Relative changes in expression of genes of the electron transport chain in diaphragm and gastrocnemius muscle of Cox6a2−/− mice compared to WT mice. Horizontal bars indicate mean difference in expression. For complex IV, Cox6a2 was not taken into account for calculation of the mean difference in expression. Cl, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V (ATP synthase); CytC, cytochrome c. (C) Fiber typing of gastrocnemius muscle by a metachromatic ATPase assay. ATPase activity stains type I fibers dark blue, type IIA fibers light blue, and type IIB fibers very light blue. Representative images of fiber typing in the gastrocnemius muscle from WT and Cox6a2−/− mice are shown. Scale bar = 100 µm. I, IIA, and IIB refer to type I, type IIA, and type IIB fibers, (D) Quantification of muscle fiber types in gastrocnemius muscle of WT and Cox6a2−/− mice (n = 20–30). Note the increase in the total number of fibers in gastrocnemius muscle from Cox6a2−/− mice, (E) Cross-sectional area of different fiber types in gastrocnemius of WT and Cox6a2−/− mice (n = 30–60), (F–G) Transmission electron micrographs of WT and Cox6a2−/− diaphragm. Perinuclear (F) and intermyofibrillar mitochondria (G) are shown. N: nucleus; M: mitochondrion; IM: intermyofibrillar mitochondria; Z: small intermyofibrillar mitochondria located near Z-discs. (H) Quantification of the mitochondrial size of the diaphragm of WT and Cox6a2−/− mice (n = 3) was measured using qRT-PCR, (J) mRNA expression signals for Mfn1 and Opa1 as measured by microarrays. doi:10.1371/journal.pone.0056719.g006

Isolated skeletal muscles of Cox6a2−/− mice are more resistance to fatigue

The switch in muscle fiber composition towards a more oxidative profile in Cox6a2−/− mice was also reflected by the mechanical properties of these muscles. The specific force production (force/cross-sectional area) of soleus and extensor digitorum longus (EDL) muscles (Fig. 7A), as well as the grip strength (Fig. 7B) were similar between WT and Cox6a2−/− mice. However, in a fatiguing protocol, isolated soleus muscle of Cox6a2−/− mice was more resistant to fatigue and recovered much faster as compared to WT muscle (Fig. 7D), which is consistent with an increase in oxidative muscle fibers. However, this was not reflected at the level of the whole animal since we observed that in an endurance experiment Cox6a2−/− mice had...
decreased exercise capacity as compared to WT mice when running uphill. When mice were forced to run downhill there was no significant difference in performance, although there was again a tendency towards reduced performance in Cox6a2<sup>−/−</sup> mice (Fig. 7C). We ascribe this discrepancy between the in vitro and in vivo muscle performance to the cardiac phenotype of these mice (i.e. diastolic dysfunction), which is only observed under high workloads [30]. Finally, despite the loss of COX6A2 expression, ATP levels within the skeletal muscles of Cox6a2<sup>−/−</sup> and WT mice were similar (Fig. 7E), which is consistent with previous observations in the heart [30]. Thus, Cox6a2<sup>−/−</sup> mice have developed compensatory mechanisms that allow production of sufficient amounts of ATP to perform mechanical work.

**Discussion**

In this study, we explored the metabolic phenotype of Cox6a2<sup>−/−</sup> mice and we found a previously unrecognized direct functional link between complex IV of the respiratory chain, and whole body energy metabolism. We propose on basis of these observations that skeletal muscle COX6A2 acts as a key regulator of whole body energy homeostasis, thermogenesis and response to the fasted state. Although COX6A2 protein expression is known to be abundant in heart and skeletal muscle, a previous analysis of Cox6a2-deficient mice was restricted to the heart. These mice were reported to be viable but developed mild diastolic dysfunction during high work load [30]. One could speculate that this cardiac phenotype would compromise a metabolic characterization of the mice because it might affect oxygen and nutrient delivery to various tissues. However, Cox6a2<sup>−/−</sup> mice do not have systolic dysfunction and therefore their circulation is not impaired. As a result, Cox6a2<sup>−/−</sup> mice have developed compensatory mechanisms that allow production of sufficient amounts of ATP to perform mechanical work.

![Figure 7](image-url)
mice do not develop tissue hypoxia due to hypoperfusion, a consequence often seen in both humans and animals with systolic heart failure. As Cox6a2 is both expressed in the heart and in skeletal muscles, our current study focused on the metabolic profile as well as the muscle phenotype of the Cox6a2−/− mice. The present study in mice links Cox6a2-deficiency to protection to the metabolic changes of obesity, insulin resistance and glucose intolerance that are observed in control mice after a HFD. Cox6a2−/− mice are characterized by an elevated metabolic rate which results in increased energy expenditure, thermogenesis and susceptibility to fasting-induced weight loss. We interpret our observations as being caused by loss of COX6A2 expression in skeletal muscle, which is, like BAT, an important tissue for non-shivering thermogenesis [44]. The increase in energy expenditure is observed despite a decrease in spontaneous activity. Although decreased spontaneous activity may be suggestive of additional underlying pathologies, we observed no other obvious behavioral differences between WT and Cox6a2−/− mice. In fact, there are several other mouse models with genetic or pharmacological protection against obesity due to increased energy expenditure which also show decreased physical activity [45–50]. Thus, one possible explanation is that mice with a higher basal metabolic rate move less to compensate the energy balance.

A plausible mechanism is that the observed changes in metabolic rate are due to an increase in expression of uncoupling proteins in skeletal muscles and brown as well as white fat. It is already well known from the use of 2,4-dinitrophenol and β-adrenoreceptor agonists that a pharmacological increase of mitochondrial uncoupling leads to weight reduction, but these agents can have very serious side effects. Protective effects against obesity have also been attributed to the uncoupling proteins by means of genetic induction in mice [2–5,51]. UCPI is mainly expressed in brown fat where it generates heat by uncoupling the mitochondrial proton gradient from ATP production. However, a moderate induction of UCPI expression in white fat (10% of brown fat levels) [52] or skeletal muscle (1% of brown fat levels) [5] can be sufficient to protect against obesity, even despite a 35% decline in brown fat mass [5]. Recently, it was shown that a subpopulation of subcutaneous white fat cells (beige cells) have endogenous UCPI-dependent thermogenic properties upon stimulation [53]. The exact physiological roles of UCPII and UCPIII, which are more ubiquitously expressed, is still under debate [54]. Both UCPII and UCPIII knockout mice respond normally to cold exposure and are not obese, at least under room temperature [55–57]. However, it has been suggested that UCPII as well as UCPIII may well be significantly thermogenic when fully activated by endogenous or exogenous effectors such as superoxide [58]. It is also known that ROS can induce the expression [6] as well as the activity of UCPIs [59]. Therefore, our observation that the increase in expression of UCPIs is associated with elevated skeletal muscle ROS production is significant. Interestingly, expression of UCPIs is not only induced in skeletal muscle and the heart of Cox6a2−/− mice, but also in brown and white adipose tissue. This suggests the existence of a crosstalk between skeletal muscle and adipose tissue, as was recently shown with the identification of a new hormone, irisin, which stimulates browning and UCPII expression in WAT [39]. The Fndc5 gene, which codes for irisin, seems to be a marker of slow myofibers and is induced in the diaphragm of Cox6a2−/− mice. Whether this increased Fndc5 expression also results in increased irisin levels in Cox6a2−/− mice remains to be established.

Although the COX6A2 subunit has been proposed to be involved in thermogenesis at rest [26], the Cox6a2−/− mice actually have increased thermogenesis. We therefore hypothesize that the intrinsic uncoupling activity of COX6A2 is compensated by extrinsic uncoupling via UCPs, which increase thermogenesis in a more constitutive manner. Thus, increased uncoupling via UCPs, is most likely the primary mechanism underlying the protection against obesity in Cox6a2−/− mice. This further corroborates the idea that increasing energy expenditure via uncoupling of ATP synthesis may be an effective method for reducing body weight.

The enhanced ROS levels in muscles of Cox6a2−/− mice, support the hypothesis that proton pump slipping protects against ROS formation in the mitochondria [28]. It was an unexpected observation that skeletal muscles which generate more ROS display increased insulin sensitivity, especially in the setting of data suggesting that elevated ROS levels are associated with mitochondrial dysfunction and insulin resistance [13–15]. However, over the past few years, several studies have shown that mild mitochondrial dysfunction both in skeletal muscles, liver and adipose tissues, might even protect against the development of glucose intolerance, insulin resistance and obesity [19,20,60] and it has been suggested that mitochondrial dysfunction is a consequence rather than a cause of these conditions [16–18]. Also, emerging data supports an important role of ROS as second messengers in the regulation of cell signaling in skeletal muscle. For instance, it has been shown that moderate physical exercise results in increased muscle ROS generation followed by induction of endogenous antioxidant defense mechanisms [61]. This can be prevented by antioxidant intake which negated the health-promoting effects of physical exercise on insulin resistance in humans [62], forming the basis for the concept of mitohormesis [63]. This novel concept suggests that increased ROS production in the mitochondria results in an adaptive antioxidative response which eventually leads to a long-term reduction of oxidative stress. In support of these findings, Tigges and colleagues revealed that ROS can actually enhance insulin sensitivity and that mice with elevated ROS levels in various tissues can be protected against HFD-induced insulin resistance [64]. Another recent study showed that aging and cellular senescence were accelerated in Tap73 knockout mice. This phenotype resulted from increased ROS production due to a reduction of Cox4I1 expression and subsequent impaired complex IV activity. Importantly, these mice gained less weight and were more insulin sensitive compared to control mice when fed a HFD, although oxygen consumption was decreased [65].

In view of these data, it is interesting to note that it was recently shown that ROS can activate AMPK [66], which is constitutively activated in Cox6a2−/− mice (Fig. 3G). AMPK is a key regulator of energy homeostasis, responding to an elevated [AMP]/[ATP] ratio for instance due to increased mitochondrial uncoupling via UCPI [6,67], and initiating a signal cascade that accelerates catabolic pathways while inhibiting anabolic pathways. The AMPK metabolic sensor also activates [7] and induces gene expression [66] of Pgc-1α, which can subsequently lead to increased mitochondrial biogenesis as well as induction of PGC-1α target genes such as Ucp2, Ucp3 and Slc2a4, which codes for the facilitated glucose transporter GLUT4 [7]. Although we have not been able to identify the exact mechanism of activation of UCPIs, AMPK and PGC-1α, we believe that signaling via ROS may play a central role. Indeed, it has been shown that uncoupling proteins [6], AMPK [66] as well as PGC-1α [60] can be directly activated by ROS. Together, our data provide further evidence that increased ROS levels in skeletal muscle are not sufficient to induce insulin resistance and that mild mitochondrial dysfunction may even protect against metabolic disorders.
Another aspect of the phenotype of \textit{Cox6a2}\textsuperscript{−/−} mice which may be attributed to the increased expression of PGC-1\textalpha, was the switch in skeletal muscle fibers towards a more oxidative profile in \textit{Cox6a2}\textsuperscript{−/−} mice, resulting in more fatigue-resistant skeletal muscles with larger mitochondria. These findings contrast with those observed in mice with a skeletal muscle-specific knockout of \textit{Cox10} which develop a severe, progressive myopathy, with decreased muscle performance resulting in premature death at 3 to 4 months of age [69]. Unlike the \textit{Cox6a2}\textsuperscript{−/−} mice, however, \textit{Cox10}\textsuperscript{−/−} mice also displayed a severe reduction in skeletal muscle complex IV activity (below 5\% of control) leading to significantly decreased ATP levels (35\% of control). The discrepancy between both phenotypes may potentially be explained by cell-free reconstitution experiments: the COX10 subunit is a chaperone needed for the early steps to form the complex; in contrast, a late assembly intermediate forms without COX6A protein, which binds as the last subunit completing the formation of the holoenzyme [29]. These cell-free experiments and the data in the present study suggest that the COX6A subunit plays a role in the regulation of the activity and the expression of the fully functional complex, as the cell would be able to rapidly increase the amount of functional complex IV by synthesizing COX6A alone, rather than all 13 subunits. In agreement with this idea, incomplete complex IV in COX6A knockdown cells retains residual electron transfer potential [29]. Thus, the phenotypic abnormalities in \textit{Cox6a2}\textsuperscript{−/−} mice are more subtle than a general respiratory chain defect and appear more regulatory in nature.

The fiber type switch in \textit{Cox6a2}\textsuperscript{−/−} mice also partly explains the increased insulin sensitivity and glucose tolerance of the mice. Indeed, oxidative fibers express more of the glucose transporter GLUT4, which is primarily responsible for glucose uptake in skeletal muscles. Moreover, several studies have shown that increased numbers of oxidative fibers (type I and/or type IIA fibers) result in enhanced insulin sensitivity and glucose tolerance [67,70–73]. Another compensatory mechanism, probably related to the fiber type switch, is the coordinate upregulation of virtually all of the nuclear encoded subunits of the respiratory chain in muscles of \textit{Cox6a2}\textsuperscript{−/−} mice (Fig. 7A and B). This correlates well with the modest increases of complex I, III and V protein that were observed in the heart of \textit{Cox6a2}\textsuperscript{−/−} mice [30]. A similar induction of genes involved in oxidative phosphorylation, associated with increased mitochondrial mass, was seen in mice treated with the PGC-1\textalpha activator resveratrol, which protects mice from developing obesity and insulin resistance [49]. Takanoyama et al. [49] showed that mice lacking the PGC-1\textalpha activator PGC-1\textalpha also displayed a reductive metabolic phenotype that results from loss of COX6A2 protein (complex IV is completely absent). In agreement with this idea, decreased complex IV activity (below 5\% of control) leading to significantly decreased ATP levels (35\% of control) was observed in the heart of \textit{Cox6a2}\textsuperscript{−/−} mice. More recently, it was shown that loss of COX6A2 protein (complex IV is completely absent) significantly alters overall mitochondrial metabolism in mice. However, full body \textit{Cox6a2}\textsuperscript{−/−} mice display mild cardiac dysfunction and are very sensitive to food deprivation. While the latter may have been the evolutionary driving force to express the protein in wild-type animals, it would compromise a therapy aimed at complete inhibition of COX6A2 activity.

In conclusion, our data show that COX6A2 an important and previously unrecognized role in thermogenesis and whole-body energy metabolism. Therefore, we believe that COX6A2 may be a potential new target for therapy against obesity and/or insulin resistance. However, full body \textit{Cox6a2}\textsuperscript{−/−} mice display mild cardiac dysfunction and are very sensitive to food deprivation.

## Experimental Procedures

### Animals and diet

Mice deficient in the \textit{Cox6a2} gene were generated as previously described [30]. Age- and sex-matched C57BL/6J mice (Harlan Laboratories) were used as controls. Mice were fed a regular rodent diet (R/M-H, Sniff, Soest, Germany). For high-fat diet studies, mice were given a diet containing 45\% fat (D12451, Research Diets Inc., New Brunswick, USA) starting from 6 weeks of age, unless otherwise stated. Body weight was measured weekly. Animals were kept on a 12 hr light/12 hr dark cycle at 20°C according to the guidelines approved by the KU Leuven animal welfare committee and the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. The ethics committee specifically approved this study with project number p085/2007. For body temperature measurements, rectal temperature was measured at room temperature (time 0) using a DT-610B thermocouple thermometer (ShenZhen Everbest Machinery Industry CO.,Ltd, Shiyan, China). Mice were subsequently deprived of food, caged individually, and transferred to a cold room (4°C). Rectal temperature was then measured every hour for three hours. For the fasting experiment, mice were deprived of food, and caged individually early into the light cycle (8 am). Body weight was then followed for 36 hours.

### Adipocyte size

The mean adipocyte size of subcutaneous and gonadal WAT was determined by computer-assisted image analysis of adipose tissue sections stained with hematoxylin and eosin as described previously [75].

### Glucose tolerance tests

Following an overnight fast (16 h), mice were injected with glucose (2.5 mg/g BW) in the intraperitoneal cavity. Blood samples were drawn from the tail vein at the indicated time.
points and glucose concentrations were measured using a Gluocard Memory PC (Arkray, Inc., Kyoto, Japan) glucometer.

Hyperinsulinemic, euglycemic clamps

Clamps were performed as described earlier [76], with modifications. Briefly, after an overnight fast mice were sedated using a mixture of xetranquil, dormicium and fentanyl and a canula was placed in the tail vein. A basal infusion containing 1-14C-glucose was subsequently injected for 1 hour at a speed of 50 µl/h. Blood samples were taken after 50 and 60 min for glucose and insulin measurement and tracer dilution. Next, a 30 µl bolus of 100 mU/ml of insulin (Actrapid, Novo Nordisk) was injected i.v., followed by infusion of an hyperinsulinemic solution containing 1-14C-glucose at a speed of 50 µl/h. To maintain blood glucose levels, a variable infusion of 12.5% glucose was started and the rate was adjusted to blood glucose levels measured at 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 and 90 min. At 70, 80 and 90 min after the start of the insulin infusion, blood samples were taken for glucose and insulin measurement and tracer dilution. Next, a 30 µl bolus of 100 mU/ml of insulin (Actrapid, Novo Nordisk) was injected i.v., followed by infusion of an hyperinsulinemic solution containing 1-14C-glucose at a speed of 50 µl/h. Blood samples were taken after 50 and 60 min for glucose and insulin measurement and tracer dilution. Next, a 30 µl bolus of 100 mU/ml of insulin (Actrapid, Novo Nordisk) was injected i.v., followed by infusion of an hyperinsulinemic solution containing 1-14C-glucose at a speed of 50 µl/h. Blood samples were taken after 50 and 60 min for glucose and insulin measurement and tracer dilution.

Whole body glucose disposal (µmol/min·kg) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of 1-14C-glucose (dpm/µmol). The ratio was corrected for body weight. The hyperinsulinemic HGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Indirect calorimetry

Utilizing the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, Ohio), in vivo metabolic rates were measured in weight-, age-, and sex-matched WT and Cox6a2−/− mice fed a regular diet. The metabolic studies were undertaken as previously described [77].

Quantitative RT-PCR

Quantitative RT-PCR was performed as described [78]. Gene expression was normalized to RNA Pol II or beta actin expression and the expression level for each gene in WT mice fed a regular diet was set at 1.0. Primer and probe sequences are summarized in Table S4.

Assessment of Reactive Oxygen Species (ROS) levels

Utilizing the fluorescent probe dihydroethidium (DHE; Molecular Probes/Invitrogen, Carlsbad, California) in situ assessment of ROS levels within the diaphragm and hindlimbs of WT and Cox6a2−/− mice was performed. The diaphragm and hindlimbs of 2 to 4 months old male mice were harvested and cryopreserved. Multiple frozen sections (6 µm in thickness, n = 6 in each group) from each muscle group were cut onto glass slides and then incubated with 2 µM DHE in a light-restricted, humidified chamber at 37°C for 30 min. A control set of slides was incubated with a PBS-based vehicle without DHE under similar conditions. Upon completion of the incubation period, vectashield (Vector Laboratories; Burlingame, California) and coverslips were placed over the tissue sections. Assessment of ROS levels was performed utilizing a photomicroscope equipped with fluorescence (Leica DM-2000; Wetzlar, Germany). Quantification of the ROS levels on a tissue section was undertaken by measuring the number of DHE positive nuclei per unit area.

Metachromatic fiber typing of skeletal myofibers

The gastrocnemius muscles from WT and Cox6a2−/− mice were harvested and cryoembedded in gum tragacanth without fixation and stored at −80°C. Subsequently, fiber typing was performed on cryosections (8 µm thickness) utilizing a metachromatic dye-ATPase assay as previously described [79].

Myofiber cross-sectional area

Measurements of cross-sectional areas of skeletal muscle fibers were made on images of transverse cryosectioned muscle stained with metachromatic ATPase and were quantified using NIH software Imagej 1.37V. The software was used to draw boundaries and calculate areas for any individual cell within a snapshot. The appropriate square micron scale was set up prior to measuring the area. From each snapshot, multiple cells were measured for each of the three fiber types, and multiple snapshots were used per mouse.

Statistical analysis

All data are shown as means±SEM. Differences between experimental groups were determined by the unpaired (paired when appropriate), two-tailed Student’s t-test. Whenever the criteria for applying the t-test were not met, we used the Mann-Whitney rank-sum test. A p-value <0.05 was considered to indicate statistical significance.

Other methods used in this manuscript are available as supporting information (M&M S1).

Supporting Information

Figure S1 Total body composition, food intake and thyroid and pituitary hormones of WT versus Cox6a2−/− mice fed a regular diet. (A–B) % lean mass (A), and % fat mass (B) were assessed by NMR, (C) Food and water consumption measured in 12–16 weeks old mice (n = 4–5). Measurements were performed over a 5-day period, (D) Thyrotropin (TSH) bioactivity (left panel) was measured by a standard bioassay (n = 5). Thyroxine (T4) levels (right panel) were assayed by RIA (n = 5). *p<0.05. In all panels, data represent mean±SEM. (TIF)

Figure S2 Cox6a2 is not expressed in thermogenic adipose tissues. (A–B) Left panels: Microarray hybridization signals from public data in BAT (A: GSE7623) and a comparison performed over a 3-day period, (D) Thyrotropin (TSH) bioactivity (left panel) was measured by a standard bioassay (n = 5). Thyroxine (T4) levels (right panel) were assayed by RIA (n = 5). *p<0.05. In all panels, data represent mean±SEM. (TIF)

Figure S3 Accelerated body weight loss of fasted Cox6a2−/− mice. (A–B) Male (A) (n = 10) and female (B) (n = 3–7) mice were deprived of food at 7 am. Throughout the experiment, they had unlimited access to water. Body weight was measured for 36 h. Percentage body weight loss per hour of fasting (slope) is shown on the right. Note that two out of ten male Cox6a2−/− mice were eliminated from the experiment after 24 h, (C) Cox6a2 mRNA expression in soleus muscle, gastrocnemius muscle (gastroc) and diaphragm of overnight fasted (16 h) WT mice. Gene expression in fed mice was set at 1.0 for each individual tissue, (D) Cox6a2 protein abundance in soleus muscle, gastrocnemius muscle (gastroc) and diaphragm of overnight fasted (16 h) WT mice. Cox6a2 protein expression in WT mice was set at 1.0 for each individual tissue. *p<0.05, **p<0.01, ***p<0.001. In all panels, data represent mean ± SEM. (TIF)
Figure S4  Ucp1 and Ucp2 mRNA expression in WAT and gastrocnemius muscle of mice fed a HFD. (A–B) Quantitative RT-PCR was performed on cDNA from white adipose tissue (WAT), (C) and gastrocnemius muscle (gastroc), (D) n = 5. Gene expression in wild-type mice on a regular diet was set at 1.0 for each individual gene (dashed line). Note that Ucp1 mRNA was not detectable in gastrocnemius muscle of mice fed a HFD.

Table S1  Plasma parameters in fed WT and Cox6a2−/− mice. (PDF)

Table S2  Gene Set Enrichment Analysis. (PDF)

Table S3  Expression of antioxidant enzymes in Cox6a2−/− vs WT mice. (PDF)

Table S4  Primers and probes used for quantitative PCR. (PDF)

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M&M SI  Supportive Experimental procedures. (DOCX)

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Author Contributions
Conceived and designed the experiments: RQ, SS, KD MG PV SL DL JMS PJV JAR JMS LS HRL PJ V PC PMAP FCS. Performed the experiments: RQ, SS, KD MG PV SL DL JMS PJV AS IOC MV VC LV LT GD. Analyzed the data: RQ, SS, KD MG PV SL DL JMS PJV JAR JMS LS HRL PJ VC PMAP FCS. Contributed reagents/materials/analysis tools: PPAM. Wrote the paper: RQ, FCS.
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