Skeletal muscle transcriptional coactivator PGC-1α mediates mitochondrial, but not metabolic, changes during calorie restriction

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Calorie restriction (CR) is a dietary intervention that extends lifespan and healthspan in a variety of organisms. CR improves mitochondrial energy production, fuel oxidation, and reactive oxygen species (ROS) scavenging in skeletal muscle and other tissues, and these processes are thought to be critical to the benefits of CR. PGC-1α is a transcriptional coactivator that regulates mitochondrial function and is induced by CR. Consequently, many of the mitochondrial and metabolic benefits of CR are attributed to increased PGC-1α activity. To test this model, we examined the metabolic and mitochondrial response to CR in mice lacking skeletal muscle PGC-1α (MKO). Surprisingly, MKO mice demonstrated a normal improvement in glucose homeostasis in response to CR, indicating that skeletal muscle PGC-1α is dispensable for the whole-body benefits of CR. In contrast, gene expression profiling and electron microscopy (EM) demonstrated that PGC-1α is required for the full CR-induced increases in mitochondrial gene expression and mitochondrial density in skeletal muscle. These results demonstrate that PGC-1α is a major regulator of the mitochondrial response to CR in skeletal muscle, but surprisingly show that neither PGC-1α nor mitochondrial biogenesis in skeletal muscle are required for the whole-body metabolic benefits of CR.

Calorie restriction (CR) is the only environmental intervention known to extend lifespan and delay aging in species ranging from yeast to humans (1). Molecularly, CR provides many benefits, including sensitized insulin signaling, strengthened stress response pathways, and improved mitochondrial functions (2, 3). Whereas the precise mechanisms underlying the antiaging benefits of CR are unknown, the beneficial effects of CR on mitochondrial functions are well established, and several studies suggest that CR stimulates mitochondrial biogenesis. CR increases mitochondrial DNA content in several mouse tissues (4). Likewise, culturing HeLa cells with serum from CR rats induces expression of mitochondrial genes, and these changes are associated with an increased number of mitochondria per cell compared with cells cultured in serum from ad libitum fed rats (5). Certainly, CR induces expression of genes involved in mitochondrial energy metabolism in many tissues, including skeletal muscle, heart, and white adipose tissue (4, 6–8). The skeletal muscle response to CR is particularly interesting for two reasons. First, skeletal muscle is a major energy-consuming organ and must consequently undergo considerable metabolic alterations to adapt to a reduced-calorie diet. Second, age-associated declines in mitochondrial function are especially pronounced in postmitotic organs such as skeletal muscle (9). Not surprisingly, many groups have demonstrated that CR improves several parameters of mitochondrial function in skeletal muscle (2, 10, 11). Significantly, the skeletal muscle response to CR is conserved in humans: short-term CR results in clear evidence of mitochondrial biogenesis in human skeletal muscle (8). Given the pivotal role of mitochondria in ATP production, energy metabolism, reactive oxygen species (ROS) detoxification, and cellular signaling, it is important to understand the molecular mechanisms that mediate the effects of CR on these mitochondrial functions.

The transcriptional increase of genes involved in mitochondrial energy metabolism and ROS detoxification during CR is strongly correlated with elevated expression of the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (2, 12, 13). PGC-1α coactivates a wide variety of nuclear receptors and other transcription factors to coordinate context-specific programs of gene expression. In several studies, PGC-1α has been shown to induce mitochondrial biogenesis, increase mitochondrial respiration and fat oxidation, and regulate ROS detoxification and metabolic homeostasis (14, 15). For example, in mouse skeletal muscle, PGC-1α overexpression induces mitochondrial biogenesis (16), whereas deletion reduces expression of genes involved in mitochondrial metabolism and energy production (17, 18). The fact that CR and PGC-1α exert similar effects on mitochondrial gene transcription, coupled with the robust observation that CR induces PGC-1α expression in many tissues, has led to the widespread speculation that PGC-1α is the primary mediator of the effects of CR on mitochondrial functions (2, 5, 19).

In this study, we examined the direct requirement of skeletal muscle PGC-1α in the mitochondrial and metabolic adaptation to CR. Using mice with a skeletal muscle-specific deletion of PGC-1α and their control littermates (18, 20), we demonstrated that skeletal muscle PGC-1α is not required for the improvements in whole-body metabolic homeostasis conferred by CR. In contrast, the strong induction of mitochondrial gene expression during CR does require PGC-1α. Moreover, detailed analysis of oxidative (red) and glycolytic (white) fiber types revealed that induction of mitochondrial biogenesis is limited to oxidative muscle fibers and also requires PGC-1α. Together, these results provide a comprehensive view of the mitochondrial and metabolic changes that occur during CR and demonstrate that skeletal muscle PGC-1α is required for the full induction of mitochondrial gene expression and mitochondrial density triggered by CR.

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Results

Improvements in Metabolic Homeostasis During CR Do Not Require Skeletal Muscle PGC-1α. To examine the specific requirement for PGC-1α in the response to CR, we took advantage of a previously described mouse model with skeletal muscle-specific deletion of PGC-1α (18, 20). Several groups have shown that CR induces expression of both mitochondrial genes and PGC-1α in skeletal muscle (2, 8, 21). Moreover, skeletal muscle is a major site of glucose disposal, and skeletal muscle mitochondrial dysfunction is linked to deterioration of glucose homeostasis (22, 23). We reasoned that putting mice with skeletal muscle-specific PGC-1α deletion (MKO) and their control littermates (flanked by LoxP, FLOX) on a CR diet would enable us to test the role of PGC-1α in the skeletal muscle response to CR, while circumventing potential confounding effects of PGC-1α deletion in multiple tissues. Before initiating CR, both FLOX and MKO mice had similar metabolic profiles: the groups had equivalent body weights, blood glucose levels, and daily food intake (Fig. S1 *A–C*), allowing matching comparison of the responses to CR. We divided the genotypes into two groups and put one group on a control diet and the second group on a CR diet for 3 mo. As expected, CR reduced body weight and fed blood glucose levels in control animals (Fig. 1 *A* and *B*). These effects were identical in mice lacking skeletal muscle PGC-1α expression (Fig. 1 *A* and *B*). CR is associated with a robust improvement in glucose homeostasis, which can be assessed using a glucose tolerance test (24). Again, both MKO and FLOX CR mice demonstrated striking improvement in glucose tolerance compared with control diet counterparts (Fig. 1 *C* and *D*). Consistent with the enhanced insulin sensitivity characteristic of CR, we found that both FLOX and MKO mice had approximately a 50% reduction in serum insulin levels (Fig. S1*D*). These results demonstrate the unexpected finding that the improvements in glucose homeostasis conferred by CR are independent of skeletal muscle PGC-1α.

To further probe the role of skeletal muscle PGC-1α in whole body energy homeostasis during CR, we performed indirect calorimetry experiments measuring the metabolism of the mice for 2 consecutive days. FLOX CR mice showed a trend toward increased whole-body oxygen consumption compared with their control diet counterparts (Fig. 1*E*), consistent with previous reports of increased oxygen consumption and energy expenditure per unit weight during CR (25–27). This marker of increased metabolism did not require skeletal muscle PGC-1α: CR significantly increased oxygen consumption in MKO mice (Fig. 1*E*). Furthermore, CR significantly lowered the respiratory exchange ratio (RER) in FLOX mice, indicating increased reliance on fat oxidation to support energy expenditure during CR, and again this response was preserved in MKO mice (Fig. 1*F*). The increases in oxygen consumption and lipid oxidation were particularly pronounced during the light phase, when CR mice were more active (Fig. 1*G* and Fig. S1 *E–G*). Together, these results show that CR induces profound metabolic shifts, including improved glucose tolerance, increased oxygen consumption, and enhanced fat oxidation, and surprisingly, that skeletal muscle PGC-1α is not required for any of these whole-body metabolic changes.

To identify further the metabolic signatures of FLOX and MKO mice accompanying CR, we used liquid chromatography/mass spectrometry (LC/MS) to measure 256 metabolites from the serum of FLOX and MKO mice on either a control or CR diet. In general, FLOX and MKO mice on the control diet exhibited very similar profiles (Dataset S1). However, consistent with published results demonstrating that MKO mice have decreased expression of genes involved in mitochondrial substrate oxidation (17, 18), serum lactate was higher in MKO mice, suggestive of either reduced pyruvate oxidation or increased glycolytic rate in MKO mice (Fig. 2*A*). Transcriptional changes in skeletal muscle correlated with increased serum lactate: glycolytic genes were increased and tricarboxylic acid cycle (TCA) genes were reduced in the skeletal muscle of MKO mice (Fig. S1*H*). Strikingly, CR rescued this defect and restored serum lactate to control levels (Fig. 2*A*). CR also slightly reduced serum levels of alanine and

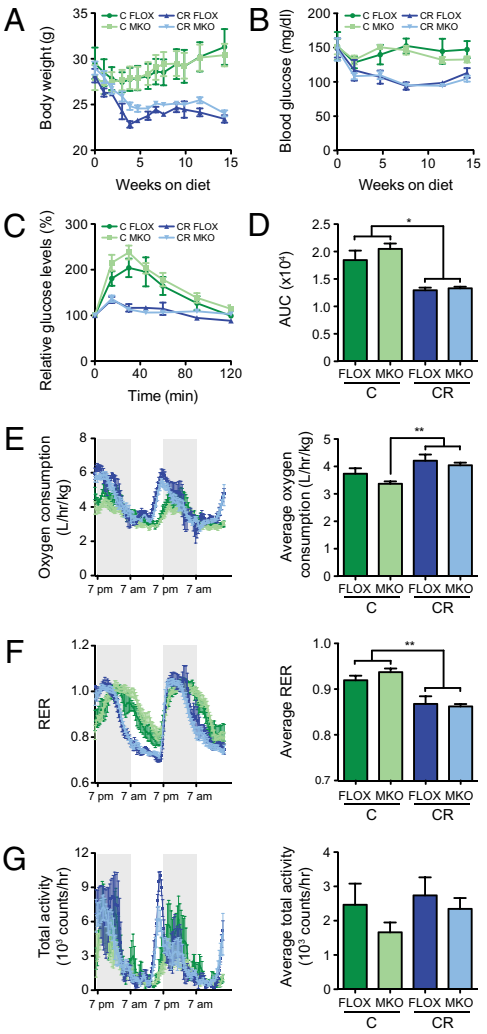

Fig. 1. FLOX and MKO mice have equivalent whole-body metabolic responses to CR. (*A*) Body weights of FLOX and MKO mice on control (C) and calorie restricted (CR) diets. (*B*) Basal blood glucose levels of control and CR FLOX and MKO mice. (*C*) Glucose tolerance tests were performed on fasted FLOX and MKO mice and glucose was monitored at the indicated intervals. Glucose levels relative to baseline (0 min, 100%) are shown. Glucose tolerance was measured as the area under the curve in *D*. (*E–G*) Metabolic cage analyses showed that FLOX and MKO mice have similar metabolic responses to CR. (*E*) Oxygen consumption was measured by indirect calorimetry. (*F*) Respiratory exchange ratio (RER) was significantly decreased by CR in both FLOX and MKO mice. (*G*) Both FLOX and MKO CR mice had a burst of activity before feeding time (5:00 PM), but no changes in total activity with CR. All experiments were performed on two independent cohorts of mice. Both cohorts showed equivalent results. All results shown are from a single cohort, n = 4–8 per group. All bars, SEM. Significance was assessed by two-way ANOVA followed by Bonferroni posttest. *$P < 0.05$, **$P < 0.01$.

pyruvate, metabolites that can equilibrate with lactate (Fig. S1 *I* and *J*). Although few metabolites were significantly ($P < 0.05$) altered between FLOX and MKO mice, we noted that CR could significantly reverse these changes in 11/18 cases (14/18 with $P < 0.07$) (Dataset S1). As most of the metabolites altered in MKO mice were lipid species, these data hint that CR can rescue subtle defects in lipid or glucose metabolism in MKO mice.

In contrast to the mild metabolomic differences caused by skeletal muscle PGC-1α deletion, CR altered levels of many metabolites, particularly amino acids and lipid species, in both FLOX and MKO mice (Dataset S1). Consistent with our measurements of blood glucose, LC/MS analysis of serum

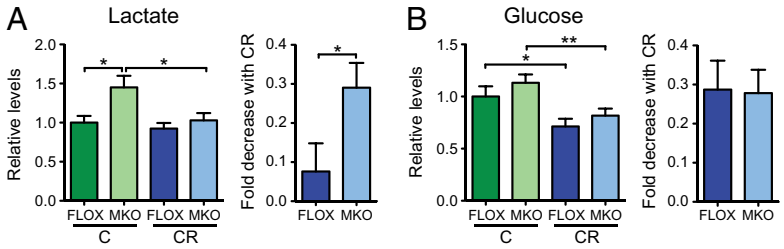

Fig. 2. Metabolite profiling underscores the similar metabolic responses of FLOX and MKO mice to CR. (*A*) Lactate and (*B*) glucose were measured by LC/MS in serum from FLOX and MKO mice on control or CR diets. Fold decrease with CR represents the fold change with CR relative to control mice of the same genotype. All experiments were performed on two independent cohorts of mice. Both cohorts showed equivalent results. For metabolomics, data from both cohorts were pooled, $n = 9$–16 per group. All bars, SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

revealed a significant reduction in glucose in both FLOX and MKO mice on CR (Fig. 2*B*). Furthermore, creatine, recently identified as a metabolite elevated with mitochondrial dysfunction (28), was lowered by CR, particularly in MKO mice (Fig. S1*K*). Analysis of serum triglycerides (TGs) revealed that CR reduced many individual TG species (Dataset S1) and total TG levels (Fig. S1*L*), consistent with elevated lipid oxidation and reduced fat stores during CR, as other groups have shown (29, 30). Altogether, even though CR triggered large shifts in serum metabolomic profiles, both FLOX and MKO mice showed broadly similar responses to CR. These data provide detailed molecular evidence that the mice have very similar metabolic responses to CR and suggest that CR can even normalize subtle metabolic defects in MKO mice.

PGC-1α Coordinates the Expression of Mitochondrial Genes During CR. As PGC-1α is a transcriptional coactivator, we next tested the requirement for PGC-1α in the transcriptional response to CR. We performed genome-wide expression profiling on RNA isolated from skeletal muscle of FLOX and MKO mice on control and CR diets followed by pairwise gene set enrichment analysis (GSEA) to identify the biological pathways affected by diet or genotype. Strikingly, cellular metabolic pathways dominated the list of gene sets significantly induced by CR in FLOX mice (Table S1) but not in MKO mice (Table S2). Although a few metabolic pathways were enriched in CR MKO mice relative to control MKO mice, many of the pathways enriched by CR related to DNA replication and RNA processing (Table S2). Thus, unbiased genome-wide analysis indicated that shifts in expression of genes involved in cellular metabolism are the primary transcriptional changes associated with CR in skeletal muscle and that this transcriptional response requires expression of PGC-1α.

We next looked specifically at how CR and PGC-1α influenced transcription of genes encoding mitochondrial proteins. As expected, given the role of PGC-1α in regulating the expression of mitochondrial genes (17, 18), we found that a set of 402 mitochondrial genes (31) was down-regulated in skeletal muscle of MKO mice relative to FLOX mice (Fig. 3*A*). CR robustly increased expression of these same genes in the muscle of FLOX mice (Fig. 3*A*), corroborating earlier studies that found increased expression of a wide variety of mitochondrial genes during CR (6–8). Importantly, these genes remained down-regulated when comparing FLOX and MKO mice on a CR diet (Fig. 3*A*).

Next, we measured the enrichment of the mitochondrial gene set using GSEA. Mitochondrial genes were significantly enriched in FLOX mice compared with MKO mice on a control diet (enrichment score = 0.65, $P = 0.002$) (Fig. 3*B*). The same mitochondrial gene set was significantly enriched comparing FLOX control and CR mice (enrichment score = –0.43, $P = 0.002$) (Fig. 3*C*). Conversely, the mitochondrial gene set was not significantly enriched when comparing MKO control mice to MKO CR mice (enrichment score = –0.31, $P = 0.142$) (Fig. 3*C*). Thus, GSEA demonstrated that the transcriptional response to CR requires PGC-1α for the enrichment of metabolic and mitochondrial pathways.

To confirm that PGC-1α is required for the up-regulation of the mitochondrial pathway in skeletal muscle during CR, we quantified the average expression level of the 402 mitochondrial genes in FLOX and MKO control and CR mice. In line with our previous results, expression of the mitochondrial gene set was significantly ($P < 0.0001$) reduced by PGC-1α deletion and CR significantly ($P < 0.0001$) increased the average expression of

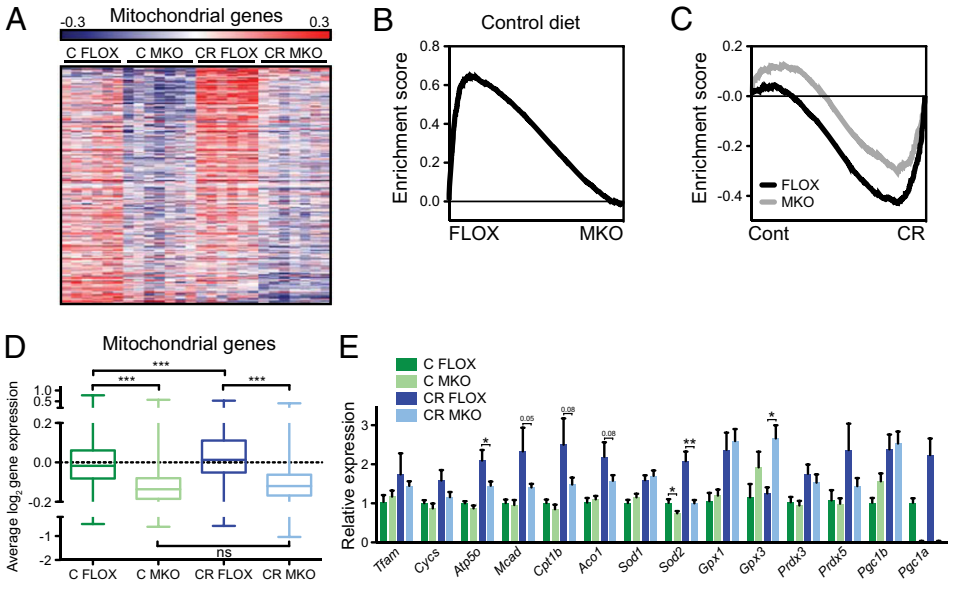

Fig. 3. CR induces a mitochondrial gene expression program in skeletal muscle through PGC-1α. Gene expression profiling was performed on RNA extracted from the tibialis anterior and extensor digitorum longus (TA/EDL) muscles of control (C) and calorie restricted (CR) mice. (*A*) Heat map showing relative levels of the 402 genes in the mitochondrial pathway. Scale is based on changes in $\log_2$ expression relative to the median. (*B*) GSEA analysis revealed that the mitochondrial pathway is highly enriched in skeletal muscle of FLOX relative to MKO animals on a control diet. (*C*) The mitochondrial pathway is highly enriched in skeletal muscle of FLOX CR animals relative to FLOX control animals, and PGC-1α deletion blunts the enrichment of the mitochondrial pathway in CR animals. See *SI Materials and Methods* for details on GSEA enrichment plots. (*D*) Average $\log_2$ expression of the 402 mitochondrial genes is graphed as a box and whisker plot, with whiskers showing minimum to maximum values. Significance was assessed by two-way ANOVA followed by Bonferroni posttest. NS, not significant. $^{***}P < 0.001$, $n = 6$–7. (*E*) Gene expression was analyzed using qRT-PCR of RNA from TA/EDL muscles. $^{*}P < 0.05$, $^{**}P < 0.005$. Bars, SEM. $n = 4$–8.

mitochondrial genes in FLOX mice (Fig. 3*D*). Importantly, there was no increase in the average mitochondrial gene expression with CR in MKO mice (Fig. 3*D*), indicating that PGC-1α is required for the coordinated increase in the expression of mitochondrial genes in skeletal muscle during CR.

Although PGC-1α is critical for the CR-mediated induction of the mitochondrial transcriptional program, some individual mitochondrial genes were up-regulated by CR even in the absence of PGC-1α. For example, of the 402 mitochondrial genes, 102 are significantly ($P < 0.05$) increased during CR in FLOX mice, whereas 71 are significantly increased in MKO mice. Although the responses of FLOX and MKO mice are significantly different ($P = 0.0099$ by Fisher's exact test), reflecting the blunting of the mitochondrial pathway in MKO mice shown in Fig. 3*D*, it is clear PGC-1α is not required for increased expression of every mitochondrial gene during CR. To illustrate this point, we measured the expression of several genes involved in mitochondrial energy metabolism or the general antioxidant response by qRT-PCR (Fig. 3*E*). In some cases, CR could not increase gene expression in the absence of PGC-1α (e.g., *Sod2*) (Fig. 3*E* and Fig. S2*A*). In other cases, PGC-1α deletion merely blunted the response to CR (e.g., *Mcad* and *Atp5o*) or had no effect on CR-mediated gene induction (e.g., *Gpx1* and *Prdx3*). Mitochondrial genes showed a similar pattern of varied dependence on PGC-1α in gastrocnemius muscle (Fig. S2*B*). This variable dependence on PGC-1α was also seen in the protein levels of representative subunits of oxidative phosphorylation complexes II–V (Fig. S2*G*). As there is no single nuclear transcription factor responsible for the expression of all genes encoding mitochondrial proteins, coordinated changes in mitochondrial gene expression require integration of multiple transcriptional pathways, a feat achieved largely by PGC-1α. Consequently, whereas some mitochondrial genes can be induced even in the absence of PGC-1α, up-regulation of the mitochondrial transcriptional program is largely blocked in the skeletal muscle of MKO animals.

PGC-1α Is Required for CR-Mediated Increases in Mitochondrial Density in Oxidative Skeletal Muscle Fibers. Our gene expression data suggested that skeletal muscle PGC-1α is important for the coordinated induction of many mitochondrial genes during CR. We next queried whether PGC-1α was required for changes in mitochondrial morphology and density during CR. To systematically examine the effect of CR on skeletal muscle mitochondrial number and morphology, we used EM to image both the center and periphery of red and white muscle fibers. In contrast to white, glycolytic muscle fibers, red, oxidative fibers have a high mitochondrial content and high PGC-1α expression (16). Consequently, we reasoned that white and red skeletal muscle fibers might respond differently to CR. We were particularly interested in looking at how oxidative and glycolytic fibers may have different requirements for PGC-1α, and distinctions in their adaptive response to CR have not been systematically investigated.

Surprisingly, we found that CR actually decreased total mitochondrial number in white muscle (Fig. 4*A* and Fig. S3). There are several interpretations of this result. Glycolytic white fibers may conserve precious resources by reducing de novo mitochondrial biogenesis or catabolizing existing mitochondria for fuel. Alternatively, white fibers may increase the efficiency of their mitochondria to maintain output with fewer resources. Additionally, we noted a reproducible trend of decreased mitochondrial number in the white muscle of control MKO animals relative to FLOX mice (Fig. 4*A*), supporting the role of PGC-1α as an important player in the regulation of endogenous mitochondrial biogenesis. Neither mitochondrial length nor width was significantly altered by CR in white muscle fibers, although mitochondria appeared to be smaller in the center of white fibers (Fig. S4 *A* and *B*). As with mitochondrial number, mitochondria from white fibers of MKO mice tended to be smaller than mitochondria from FLOX mice, and CR abrogated this difference (Fig. S4 *A* and *B*). Thus, CR decreases mitochondrial content in white muscle fibers in a PGC-1α-independent manner.

In contrast to our observations in white fibers, mitochondria in red muscle fibers had a strikingly different response to CR. We found no evidence of changed mitochondrial number in red muscle fibers of either FLOX or MKO mice on CR (Fig. 4 *B* and *C* and Fig. S5). Whereas CR had little effect on mitochondrial size in white fibers, both mitochondrial length and width were increased in red fibers of FLOX mice on CR (Fig. 4 *D* and *E*). To determine whether the increase in average mitochondrial length and width were indicative of a true increase in mitochondrial density, we measured the total area covered by mitochondria in two sets of images from two independent cohorts of mice. CR induced a 30% increase in mitochondrial density and deletion of PGC-1α completely blocked the effects of CR (Fig. 4*F*). Interestingly, PGC-1α overexpression in mouse skeletal muscle enhances mitochondrial gene expression and drives increased mitochondrial density as measured by EM (32), similar to the effects of CR we observed in red muscle fibers of FLOX mice. Altogether, the EM data illustrate a previously unappreciated complexity of the mitochondrial response to CR. White muscle fibers respond to dietary restriction by reducing mitochondrial number and size. In contrast, red muscle fibers increase mitochondrial density. Together, our results illustrate that skeletal muscle PGC-1α is required for induction of both the mitochondrial transcriptional response to CR and the concurrent enhancement of mitochondrial density in oxidative muscle fibers.

To probe the functional consequences of increased mitochondrial gene expression and density, we next measured three critical mitochondrial activities: complex I and IV of the electron transport chain and citrate synthase, the rate limiting enzyme of the TCA cycle. CR tended to increase all three activities, and these increases were blocked by PGC-1α deletion (Fig. S6 *A*–*C*). In contrast, CR did not increase the activity of the glycolytic enzyme lactate dehydrogenase in any genotype (Fig. S6*D*). In sum, these data demonstrate that PGC-1α coordinates increases in mitochondrial gene expression, mitochondrial density, and oxidative capacity in response to CR.

Discussion

Studies of calorie restriction commonly assume that CR induces mitochondrial biogenesis through PGC-1α. Here, we directly test this theory by performing CR in animals lacking skeletal muscle PGC-1α. We demonstrate that PGC-1α is required for the induction of the mitochondrial transcriptional program and the increase in mitochondrial density observed during CR. Surprisingly, however, we find that skeletal muscle PGC-1α is dispensable for the improvements in whole-body metabolic homeostasis that occur with CR. Together, these results argue that neither skeletal muscle PGC-1α nor muscle mitochondrial biogenesis are necessary for the short-term metabolic benefits of CR.

Our data indicate that the blunted mitochondrial response to CR in MKO mice is the direct result of the specific deletion of PGC-1α in skeletal muscle rather than a consequence of systemic differences between FLOX and MKO mice on a CR diet. First, MKO and FLOX mice show equivalent whole-body responses to CR (Figs. 1 and 2). Second, CR uniformly increased expression of representative mitochondrial genes (*Tfam*, *Atp5o*, *Cycs*, *Mcad*, and *Sod2*) in liver and heart in both FLOX and MKO animals (Fig. S2 *C*–*F*), indicating that the MKO mice do not have a general defect in their transcriptional response to CR. Thus, the failure of MKO mice to up-regulate mitochondrial gene expression in muscle is because skeletal muscle PGC-1α is required for coordination of a mitochondrial transcriptional program during CR.

As the largest site of glucose disposal, skeletal muscle is a critical organ in the maintenance of glucose homeostasis (23). Skeletal muscle mitochondrial dysfunction is increasingly implicated in the etiology of insulin resistance (22, 23, 33). Despite the well-established role for skeletal muscle PGC-1α in the regulation of mitochondrial oxidative metabolism, CR can improve glucose homeostasis even in the absence of skeletal muscle PGC-1α expression (Fig. 1). There are several possible interpretations of this result. First, PGC-1α-independent changes in mitochondrial

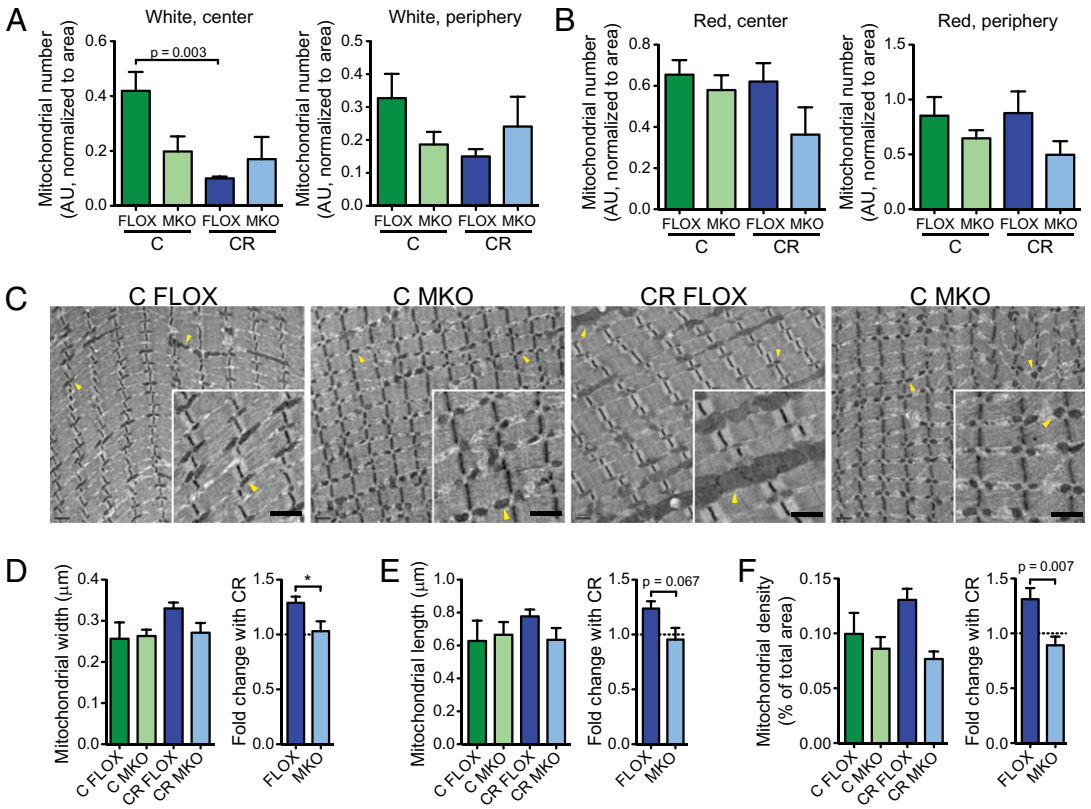

Fig. 4. PGC-1α is required for the increase in mitochondrial density in red muscle fibers during CR. (*A–F*) Electron microscopy (EM) was used to analyze red and white muscle fibers from the TA/EDL muscles of FLOX and MKO mice on control or CR diets. Images were taken from both the center of the myofiber and the periphery, and these regions were analyzed separately. Three separate fibers were imaged from each mouse, and three to four mice per group were analyzed. Mitochondrial number per unit area was quantified in white fibers (*A*) and red fibers (*B*). (*C*) Representative images of the center of red muscle fibers of control and CR FLOX and MKO animals. In each image, two representative mitochondria are indicated with an arrowhead. Increased magnification is shown on the *Right*, including one representative mitochondrion indicated by an arrowhead. (Scale bar, 1 μm.) (*D* and *E*) There is a trend of increased mitochondrial width (*D*) and length (*E*) with CR in the center of red muscle fibers of FLOX, but not MKO animals. (*F*) Mitochondrial density was calculated as the percentage of the total area covered by mitochondria using ImageJ software. Images from three separate fibers from each mouse and a total of six to eight mice per group were analyzed. All bars, SEM.

metabolism may underlie enhanced glucose metabolism. For example, PGC-1β may function redundantly with PGC-1α to induce the targets that are critical for CR-mediated metabolic benefits. If this is true, however, then the critical events in muscle mitochondrial reprogramming must be independent of mitochondrial biogenesis per se, because MKO mice had improved glucose tolerance in the absence of increased mitochondrial density. Although PGC-1β was expressed at similar levels in FLOX and MKO mice on CR (Fig. 3*E*), it will be interesting for future studies to examine the requirement for skeletal muscle PGC-1β in the response to CR. Alternatively, improved glucose homeostasis during CR could be the consequence of changes in glucose metabolism in organs other than skeletal muscle. Ultimately, however, signaling must occur within skeletal muscle itself, because this is the most important site of glucose disposal. Our data indicate that this final signal to skeletal muscle must occur independently of PGC-1α and of increases in mitochondrial density.

Similarly, our metabolic analyses demonstrated that CR increases whole-body fatty acid oxidation independently of skeletal muscle PGC-1α. CR increased oxygen consumption and reduced the RER and serum triglycerides in both FLOX and MKO mice on a CR diet (Fig. 1 *E* and *F* and Fig. S1*L*). Indeed, the MKO mice appeared to have a subtle oxidative defect on a control diet: oxygen consumption trended lower, RER trended higher, and serum lactate was elevated in MKO mice. These differences were erased by CR. Together, these observations suggest that other pathways, independent of PGC-1α in skeletal muscle, activate fatty acid oxidation in mice during CR and can actually rescue any inherent defect exhibited by MKO mice on a control diet.

Our study illustrates the complex effect of CR on skeletal muscle mitochondrial morphology and density. Our results reveal the striking specificity of the response to CR. Individual muscle fibers within the same tissue can have drastically different mitochondrial responses to CR. Glycolytic, mitochondria-poor white muscle fibers surprisingly decrease their total mitochondrial content during short-term CR. In contrast, mitochondria-rich, high PGC-1α-expressing red muscle fibers exhibit a PGC-1α–dependent increase in mitochondrial density during CR. In aggregate, the effects on red fibers dominate over those in white fibers, because there are far more mitochondria in red fibers. Together, these results underscore the importance of careful fiber-type selection in CR studies.

Intriguingly, the effects of CR on mitochondrial density are due predominantly to an increase in mitochondrial size rather than mitochondrial number. Our findings are in line with a previous study, which found that mitochondria isolated from liver of CR mice were larger than mitochondria isolated from control mice (34). Little is known about the regulation of mitochondrial size in skeletal muscle or what impact this parameter has on mitochondrial function. Modulation of mitochondrial fusion and/or fission is likely to play a role, although again little is known of how CR affects these processes.

In sum, we demonstrate that skeletal muscle PGC-1α is required for the mitochondrial, but not the metabolic, response to CR. Similarly, the transcription factor NF-E2 related factor 2

(NRF2) was shown to regulate the antioxidant response to CR, with important consequences for the anticarcinogenesis effects of the diet, but not for the increases in insulin sensitivity and lifespan with CR (35). These studies illustrate the complexity of the response to CR and the importance of unraveling the genetic pathways underlying the many benefits of the diet. Intriguingly, the NAD-dependent deacetylatse sirtuin 1 (SIRT1) is induced in several tissues in response to CR, and SIRT1 can deacetylate and activate both PGC-1α and PGC-1β (36, 37). It will be interesting for future studies to examine the role of sirtuins, acetyltransferases, and other energy-sensing pathways in the response to CR. Dissecting the molecular pathways underpinning the metabolic benefits of CR will be critical to our understanding of how CR can improve lifespan and healthspan in mammals.

Materials and Methods

Animal Studies. Animal studies were performed according to protocols approved by the institutional animal care and use committee, the standing committee on animals at Harvard. PGC-1α FLOX and MKO mice (mixed C57/BL6 and 129 background) were described previously (18, 20). Littermates were used for all experiments and all mice were housed individually throughout the duration of the study. Calorie restriction studies were performed according to a modified version of a published protocol (38). Mice were fed AIN-93M diet (BioServe) ad libitum for at least 10 d until baseline food intake was determined. Mice were randomly divided into control (C) or calorie restricted (CR) groups. Control mice were fed 90% and CR mice were fed 80% of their ad libitum food intake. After 1 wk, CR mice were switched to AIN-93M 40% CR diet and fed 60% of their ad libitum intake until sacrifice ~11 wk later. Diet compositions (percentage by weight for control, CR, respectively): protein: 12.4, 20.8; fat: 4, 6.7; fiber: 3.8, 5.5; ash: 3.6, 5.8; and carbohydrate: 71.3, 57. Mice were fed 2× their daily food allotment every other day at 5:00 PM. CR mice consumed most of their allotted food on the first day. Body weight and blood glucose were measured weekly and biweekly, respectively, at 9:00 AM the morning after feeding. To assess glucose tolerance, mice were fasted for 16 h before i.p. injection of 2 g/kg glucose and glucose levels were measured from blood obtained from the tail vein at the indicated intervals. Serum insulin was analyzed by the Vanderbilt Mouse Metabolic Phenotyping Center.

Metabolic Analyses. Mouse metabolic analyses were performed using the TSE LabMaster (TSE Systems). Mice were acclimated to the chambers for 2 d and then gas exchanges and locomotor activity were measured every 27 min for 48 h. Mice were fed every day while in the cages.

Statistical Analysis. Significance was assessed using an unpaired Student's *t* test, unless otherwise noted. All experiments were performed on two independent cohorts of mice. Both cohorts showed equivalent results. All results shown are from a single cohort, unless otherwise noted.

Additional methods are provided in *SI Materials and Methods*.

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1. Fontana L, Partridge L, Longo VD (2010) Extending healthy life span—from yeast to humans. *Science* 328:321–326.
2. Anderson RM, Weindruch R (2010) Metabolic reprogramming, caloric restriction and aging. *Trends Endocrinol Metab* 21:134–141.
3. Ristow M, Schmeisser S (2011) Extending life span by increasing oxidative stress. *Free Radic Biol Med* 51:327–336.
4. Nisoli E, et al. (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* 310:314–317.
5. López-Lluch G, et al. (2006) Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc Natl Acad Sci USA* 103:1768–1773.
6. Park SK, Prolla TA (2005) Gene expression profiling studies of aging in cardiac and skeletal muscles. *Cardiovasc Res* 66:205–212.
7. Baur JA, et al. (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 444:337–342.
8. Civitarese AE, et al.; CALERIE Pennington Team (2007) Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Med* 4:e76.
9. Wallace DC (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. *Annu Rev Genet* 39:359–407.
10. Hunt ND, et al. (2006) Bioenergetics of aging and calorie restriction. *Ageing Res Rev* 5:125–143.
11. Merry BJ (2004) Oxidative stress and mitochondrial function with aging—the effects of calorie restriction. *Aging Cell* 3:7–12.
12. Finley LW, Haigis MC (2009) The coordination of nuclear and mitochondrial communication during aging and calorie restriction. *Ageing Res Rev* 8:173–188.
13. López-Lluch G, Irusta PM, Navas P, de Cabo R (2008) Mitochondrial biogenesis and healthy aging. *Exp Gerontol* 43:813–819.
14. Scarpulla RC (2011) Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* 1813:1269–1278.
15. Handschin C, Spiegelman BM (2006) Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr Rev* 27:728–735.
16. Lin J, et al. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797–801.
17. Handschin C, et al. (2007) Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. *J Biol Chem* 282:30014–30021.
18. Handschin C, et al. (2007) Abnormal glucose homeostasis in skeletal muscle-specific PGC-1alpha knockout mice reveals skeletal muscle-pancreatic beta cell crosstalk. *J Clin Invest* 117:3463–3474.
19. Guarente L (2008) Mitochondria—a nexus for aging, calorie restriction, and sirtuins? *Cell* 132:171–176.
20. Chinsomboon J, et al. (2009) The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle. *Proc Natl Acad Sci USA* 106:21401–21406.
21. Cerqueira FM, Laurindo FR, Kowaltowski AJ (2011) Mild mitochondrial uncoupling and calorie restriction increase fasting eNOS, akt and mitochondrial biogenesis. *PLoS ONE* 6:e18433.
22. Anderson EJ, et al. (2009) Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest* 119:573–581.
23. Lowell BB, Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384–387.
24. Hempenstall S, Picchio L, Mitchell SE, Speakman JR, Selman C (2010) The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice. *Mech Ageing Dev* 131:111–118.
25. Selman C, et al. (2005) Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mech Ageing Dev* 126:783–793.
26. Masoro EJ (2005) Overview of caloric restriction and ageing. *Mech Ageing Dev* 126:913–922.
27. Boily G, et al. (2008) SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS ONE* 3:e1759.
28. Shaham O, et al. (2010) A plasma signature of human mitochondrial disease revealed through metabolic profiling of spent media from cultured muscle cells. *Proc Natl Acad Sci USA* 107:1571–1575.
29. Liepa GU, Masoro EJ, Bertrand HA, Yu BP (1980) Food restriction as a modulator of age-related changes in serum lipids. *Am J Physiol* 238:E253–E257.
30. Fontana L, Meyer TE, Klein S, Holloszy JO (2004) Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proc Natl Acad Sci USA* 101:6659–6663.
31. Mootha VK, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273.
32. Choi CS, et al. (2008) Paradoxical effects of increased expression of PGC-1alpha on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proc Natl Acad Sci USA* 105:19926–19931.
33. Morino K, Petersen KF, Shulman GI (2006) Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes* 55 (Suppl 2):S9–S15.
34. Weindruch RH, Cheung MK, Verity MA, Walford RL (1980) Modification of mitochondrial respiration by aging and dietary restriction. *Mech Ageing Dev* 12:375–392.
35. Pearson KJ, et al. (2008) Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction. *Proc Natl Acad Sci USA* 105:2325–2330.
36. Rodgers JT, et al. (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 434:113–118.
37. Kelly TJ, Lerin C, Haas W, Gygi SP, Puigserver P (2009) GCN5-mediated transcriptional control of the metabolic coactivator PGC-1beta through lysine acetylation. *J Biol Chem* 284:19945–19952.
38. Pugh TD, Klopp RG, Weindruch R (1999) Controlling caloric consumption: Protocols for rodents and rhesus monkeys. *Neurobiol Aging* 20:157–165.