**NCoR1 Is a Conserved Physiological Modulator of Muscle Mass and Oxidative Function**

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**SUMMARY**

Transcriptional coregulators control the activity of many transcription factors and are thought to have wide-ranging effects on gene expression patterns. We show here that muscle-specific loss of nuclear receptor corepressor 1 (NCoR1) in mice leads to enhanced exercise endurance due to an increase of both muscle mass and of mitochondrial number and activity. The activation of selected transcription factors that control muscle function, such as MEF2, PPARβ/δ, and ERRs, underpins these phenotypic alterations. NCoR1 levels are decreased in conditions that require fat oxidation, resetting transcriptional programs to boost oxidative metabolism. Knockdown of gei-8, the sole C. elegans NCoR homolog, also robustly increased muscle mitochondria and respiration, suggesting conservation of NCoR1 function. Collectively, our data suggest that NCoR1 plays an adaptive role in muscle physiology and that interference with NCoR1 action could be used to improve muscle function.

**INTRODUCTION**

Transcription factors are key mediators in homeostatic circuits, as they process environmental signals into transcriptional changes (Desvergne et al., 2006; Francis et al., 2003). Transcriptional coregulators have recently emerged as equally important modulators of such adaptive transcriptional responses. The fact that the activity of coactivators and corepressors is tightly regulated through the spatial and temporal control of their expression and activity levels opens another avenue to adapt transcription to environmental cues (Feige and Auwerx, 2007; Rosenfeld et al., 2006; Smith and O’Malley, 2004; Spiegelman and Heinrich, 2004). Interestingly, many of these coregulators do not operate in isolation but are part of large multiprotein complexes that integrate complex signaling pathways. The convergence of an elaborate coregulator network on the peroxisome proliferator-activated receptor (PPAR) coactivator α (PGC)-1α illustrates this principle well, as its activity depends on several other coregulators, including the steroid receptor coactivators, NR-interacting protein 1 or RIP140, CREB-binding protein, p300, protein arginine methyltransferase 1, general control of amino acid synthesis 5, and SIRT1 (Fernandez-Marcos and Auwerx, 2011; Handschin and Spiegelman, 2006). The corepressor (NCoR1) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT or NCoR2) are also acting as cofactor scaffolding platforms. NCoR1 and SMRT hardwire corepressor pathways that incorporate several deacetylases (including class I [HDAC3], class II [HDAC4, 5, 7, and 9], and class III [SIRT1] HDACs), transducin beta-like 1 (TBL1) and TBLR1, two highly related F box/WD40-containing factors, and the G protein pathway suppressor 2 (reviewed in Perissi et al., 2010). Because germline NCoR1−/− and SMRT−/− mice are embryonically lethal (Jepsen et al., 2000, 2007), information on the role of these proteins in adult physiology is limited. Studies of mice with mutations in the NR interaction domains (RIDs) 1 and 2 of SMRT (SMRTmRID), which solely disrupts its interaction with NRs, indicated that lethality of SMRT−/− mice is caused by non-NR transcription factors (Nofsinger et al., 2008). Work in 3T3-L1 cells in which NCoR1 or SMRT expression was reduced by RNA interference demonstrated that they repress adipogenesis by inhibiting PPARγ (Yu et al., 2005). In line with this, adipogenesis was enhanced in mouse embryonic fibroblasts (MEFs) from SMRTmRID mice (Nofsinger et al., 2008). Interestingly, SIRT1 is also part of the NCoR1/SMRT complex and contributes to the inhibition of PPARγ (Picard et al., 2004).

Contrary to adipose tissue, the function of NCoR1/SMRT in skeletal muscle has not yet been established. We here report the generation and characterization of muscle-specific NCoR1−/− (NCoR1skm−/−) mice, which displayed a remarkable enhanced exercise capacity. This was the result of increased muscle mass and a muscle fiber type shift toward more oxidative fibers, coordinated by the induction of genes involved in mitochondrial biogenesis and function, ensuing from the activation...
of PPARδ, the estrogen-related receptors (ERRs), and myocyte-specific enhancer factor 2 (MEF2). Worms with a muscle-selective knockdown of \textit{gei-8}, the sole \textit{C. elegans} NCoR homolog, also had improved mitochondrial activity. These data combined with the specific reduction in the expression levels of NCoR1, but not SMRT, in situations of enhanced fat oxidation establish NCoR1 as a key physiological regulator of muscle mass and function.

**RESULTS**

\textbf{NCoR1\textsuperscript{skm/−} Mice Have Increased Muscle Mass}

Given the embryonic lethality of germline \textit{NCoR1\textsuperscript{+/−}} mice (Jepsen et al., 2000) (Table S1 available online), we generated a floxed \textit{NCoR1} mouse line in which exon 11 of the \textit{NCoR1} gene (Hörlein et al., 1995) was flanked with LoxP sites, priming it for subsequent deletion using the Cre-LoxP system. These mice, bearing floxed \textit{NCoR1} L2 alleles, were then bred with a skeletal muscle (skm)-specific Cre driver (human α-skeletal actin promoter) (Miniau et al., 1999) to yield \textit{NCoR1\textsuperscript{skm/−}} and \textit{NCoR1\textsuperscript{skm/+}} mice (Figure S1). As expected, \textit{NCoR1} mRNA expression was significantly decreased in soleus, gastrocnemius, and quadriceps and modestly reduced in the heart muscle of \textit{NCoR1\textsuperscript{skm/−}} mice, but not altered in other tissues (Figure 1A). No compensatory induction of the related corepressor SMRT/NCoR2 (Chen and Evans, 1995) was observed (Figure 1A). We also tried to determine \textit{NCoR1} protein levels in muscle but failed to detect the endogenous protein with the currently available NCoR1 antibodies.

\textit{NCoR1\textsuperscript{skm/−}} mice were indistinguishable from \textit{NCoR1\textsuperscript{skm/+}} mice upon visual inspection, and no gross organ anomalies were revealed upon autopsy. The relative mass of the soleus muscle was higher, whereas the mass of the gastrocnemius showed a trend toward an increase, which did not reach statistical significance (Figures S2A and S2B). The soleus was also more intensely red, and there were larger sections with reddish
color in the gastrocnemius in NCoR1<sup>skm</sup>/−/− mice (Figure S2C). Body weight evolution and food intake of male NCoR1<sup>skm</sup>/−/− and NCoR1<sup>skm</sup>/−/+ mice after weaning were comparable both on chow diet (CD) and on high-fat diet (HFD) (Figure S2A and data not shown). On CD, carbohydrate and lipid profiles were similar except for LDL cholesterol, which was reduced in NCoR1<sup>skm</sup>/−/− mice (Figure 1B). In addition to the lower LDL cholesterol on CD, total and HDL cholesterol levels were also reduced in NCoR1<sup>skm</sup>/−/− mice on HFD (Figure 1B). Furthermore, glucose edged down (p = 0.074) in the wake of similar insulin levels on HFD. The slightly reduced area under the curve in intraperitoneal insulin tolerance test (IPITT; Figure S2D) and the delayed recovery from hypoglycemia during intraperitoneal insulin tolerance test (IPITT; Figure S2E) in mutant mice on HFD may suggest a discrete improvement in insulin sensitivity but without a clear impact on glucose tolerance.

Enhanced Exercise Performance in NCoR1<sup>skm</sup>/−/− Mice

We next evaluated energy expenditure by indirect calorimetry and actiometry in CD- and HFD-fed mice (Figures 1C and S2F). Total locomotor activity was significantly higher in NCoR1<sup>skm</sup>/−/− mice. Consistent with this, O<sub>2</sub> consumption (VO<sub>2</sub>) was increased under both CD and HFD. Interestingly, the NCoR1<sup>skm</sup>/−/− mice displayed a marked decrease in the respiratory exchange ratio (RER) on a HFD (Figure 1C), indicating an enhanced use of fat as a main energy source. NCoR1<sup>skm</sup>/−/− mice were also more cold tolerant, as they maintained their body temperature better when exposed to 4°C (Figure 1D).

Exercise performance was strikingly improved in NCoR1<sup>skm</sup>/−/− mice (Figures 1E, 1F, and S3A–S3D). In endurance exercises, NCoR1<sup>skm</sup>/−/− mice ran for a significantly longer time and distance before exhaustion (Figures 1E, S3A, and S3B). The increase of the VO<sub>2</sub> values (ΔVO<sub>2</sub>) during exercise and the maximal ability to utilize oxygen during exercise (VO<sub>2max</sub>) which critically determines the endurance performance of skeletal muscle, was slightly higher in NCoR1<sup>skm</sup>/−/− mice on both CD (Figure S3D and data not shown) and HFD (Figure 1F). Despite the moderate reduction in NCoR1 mRNA levels in cardiac muscle of NCoR1<sup>skm</sup>/−/− mice, heart rate, blood pressure, cardiac morphology, and function were not changed (Figures S3E–S3G and Table S2).

NCoR1<sup>skm</sup>/−/− Muscle Demonstrates Increased Oxidative Capacity

The enhanced exercise capacity, associated with the increase in overall muscle mass and change in muscle appearance, led us to examine muscle morphology. Upon staining muscles with hematoxylin/eosin or toluidine blue, not only was the diameter of single muscle fibers larger, but also the connective tissue between muscle bundles was less abundant in NCoR1<sup>skm</sup>/−/− mice (Figures 2A, 2B, and S4A). The increased number of intensely stained fibers upon succinate dehydrogenase (SDH) and cytochrome oxidase (COX) (Figures 2C and 2D) staining further testified to increased mitochondrial activity in the NCoR1<sup>skm</sup>/−/− gastrocnemius. Two mitochondrial DNA markers, cyclooxygenase 2 (Cox2) and 16S ribosomal RNA, normalized by genomic DNA markers (uncoupling protein 2 [Ucp2] and hexokinase 2 [Hk2]) were both significantly higher in NCoR1<sup>skm</sup>/−/− muscle, indicative of increased mitochondrial content (Figure 3B). This observation was also underscored by electron microscopy, which revealed more abundant and larger mitochondria with normal structure (Figure 3A). Immunohistochemical analysis of the myosin heavy-chain (MyHC) isoforms (Schiaffino et al., 1989) demonstrated a decreased number of the more glycolytic MyHC2b fibers, with a concomitant increase in the number of more oxidative MyHC2x and 2a fibers in the NCoR1<sup>skm</sup>/−/− gastrocnemius (Figure 3C). This observation was consolidated by analysis of MyHC isoform mRNAs, which indicated an increased expression of the mRNAs of MyHC2x and 2a (more oxidative fibers) compared to that of MyHC2b (more glycolytic) in both NCoR1<sup>skm</sup>/−/− gastrocnemius and quadriceps (Figure 3D). In quadriceps, but not gastrocnemius, the expression of MyHC1 mRNA was also increased. Finally, staining of platelet-endothelial cell adhesion molecule (PECAM)-1, an endothelial cell marker of angiogenesis and tissue vascularization that contributes to enhanced myocellular aerobic capacity, also increased in NCoR1<sup>skm</sup>/−/− muscle (Figure S5C).

The Control of Muscle Mitochondria by NCoR1 Is Conserved in C. elegans

To investigate whether the effects of NCoR1 deficiency are evolutionary conserved, we took advantage of the power of C. elegans genetics. A protein blast search indicated that GEX-interacting protein family member 8 (gei-8) is the only putative NCoR1 homolog in the C. elegans genome. Further analysis showed that the total amino acid sequence of gei-8 is 43% homologous to mouse NCoR1 and contained conserved SANT (switching-defective protein 3 [Swi3], adaptor 2 [Ada2], nuclear receptor corepressor [N-CoR], transcription factor [TF] IIIB) domains (34% identical/77% similar for SANT1; 20% identical/57% similar for SANT2) (Figure 3E). Other important functional domains (repressor domain [RD] and nuclear receptor interaction domain [ID]) were also conserved (Figure 3E). Upon the robust gei-8 knockdown in worms expressing a mitochondrial GFP reporter driven by the muscle-specific myo-3 promoter, a striking enlargement of the mitochondria was observed in body wall muscle (Figure 3F). This result is not due to an indirect effect on transcriptional activity through the myo-3 promoter because no increase in GFP expression is observed with another strain carrying the<sup>myo-3::</sup>GFP reporter (Figure S4B). We also measured O<sub>2</sub> consumption in NR350 transgenic worms fed with gei-8 dsRNA. NR350 worms lack rde-1, an essential component of the RNAi machinery encoding a member of the PIWI/STING/Argonaute family, in all tissues except the body wall muscle in which the wild-type rde-1 gene has been rescued using the hli-1 promoter (Durieux et al., 2011). Consistent with the effects observed in the mouse, also the muscle-specific knockdown of gei-8 enhanced O<sub>2</sub> consumption in these NR350 worms (Figure 3G), suggesting that the function of gei-8 to control mitochondrial metabolism is conserved through evolution.

NCoR1 Negatively Correlates with Key Mitochondrial and Myogenic Genes

After establishing these striking mitochondrial effects of NCoR1 in mice and worms, we exploited a complementary systems
A genetics approach to evaluate NCoR1s molecular coexpression partners in the mouse (Argmann et al., 2005; Houtkooper et al., 2010). Expression of NCoR1 in two panels of genetically heterogeneous mice, made by intercrossing C57BL/6 with C3H/HeJ (the B × H F2 cross) or C57BL/6 with DBA/2J (the B × D genetic reference population) mice, varied ± 1.5-fold between cases in both lung and muscle (Figure 4A). A large number of transcripts covaried significantly with NCoR1 in the different mice lines belonging either to the B × H cross or B × D strains. Most distinctively, only a fraction of these covariates were negative, which was against the dogma expected for a corepressor such as NCoR1s (Tables S4 and S5). In skeletal muscle from the B × H cross (n = 124 females), strong covariates of NCoR1 include Mef2d, myoglobin (Mb), muscle creatine kinase (Mck), and glucose transporter type 4 (Glut4) (van Nas et al., 2010). A similar analysis of lung tissue from the B × D cross (n = 51 strains) includes genes such as cytochrome c oxidase subunit IV (CoxIV), Pdk4, uncoupling (Ucp2 and Ucp3), fatty acid uptake, and metabolism (Cd36 and Lcad), were robustly induced in NCoR1skm/C0/C0 muscle. In addition, mRNA levels of Vegfb and its receptor Flt1, which regulates trans-endothelial fatty acid transport (Haegberg et al., 2010), were also induced. Interestingly, the expression of hypoxia-inducible factor (Hif) 1α and of its targets, glucose transporter 1 (Glut1), fibroblast growth factor (Fgf), and Fgf-receptor 2 (Fgfr2), were unchanged (Figure S5A), whereas all three Vegfa isoforms, i.e., Vegfa-121, -165, and -189, were induced in NCoR1skm/C0/C0 quadriceps, gastrocnemius, and covariates, with several of them being consistent with increased mass and mitochondrial biogenesis observed in NCoR1skm/C0/C0 muscle.

This initial set of NCoR1 covariates (Figure 4B) was then included together with other potential candidates for qRT-PCR analysis in mixed fiber muscle, including the gastrocnemius and quadriceps (Figures 4C and S5A and data not shown). Whereas the mRNAs of most relevant NRs were unchanged, mRNA levels of PGC-1α and β (Ppargc1a and 1b) increased. Several genes involved in mitochondrial function, including those encoding for proteins involved in TCA cycle and oxidative phosphorylation (Cs, cytochrome c oxidase subunit IV [CoxIV], Pdk4), uncoupling (Ucp2 and Ucp3), fatty acid uptake, and metabolism (Cd36 and Lcad), were robustly induced in NCoR1skm/C0/C0 muscle. In addition, mRNA levels of Vegfb and its receptor Flt1, which regulates trans-endothelial fatty acid transport (Haegberg et al., 2010), were also induced. Interestingly, the expression of hypoxia-inducible factor (Hif) 1α and of its targets, glucose transporter 1 (Glut1), fibroblast growth factor (Fgf), and Fgf-receptor 2 (Fgfr2), were unchanged (Figure S5A), whereas all three Vegfa isoforms, i.e., Vegfa-121, -165, and -189, were induced in NCoR1skm/C0/C0 quadriceps, gastrocnemius,
and soleus (Figures 4C and 5B). Together with this increase in Vegfa, both Angpt2 and Pdgfb mRNA levels were induced (Figure 4C), suggesting that myocellular aerobic capacity is facilitated by an HIF1α-independent angiogenic pathway in NCoR1 skm+/−/− mice (Arany et al., 2008).

Enhanced PPARβ/δ and/or ERR Function in NCoR1 skm+/−/− Muscle

Several genes whose expression is changed in the absence of NCoR1 are PPARβ/δ and/or ERR targets (Figure 4). Because the expression of PPARβ/δ and/or ERR was unchanged in NCoR1 skm+/−/− mice (Figure S5A), a direct effect of NCoR1 on the expression of these targets through the activation of these NRs was expected. As cases in point to demonstrate the recruitment of NCoR1 to these genes, we selected the mouse Ucp3 and Pdk4 promoters, which contain three PPAR responsive elements (PPREs) (Figure 5A) and extended NR half-sites (NR1/2) known to bind members of the ERR subfamily (Zhang et al., 2006), respectively. We first used NIH 3T3 cells in which an epitope-tagged version of NCoR1 (NCoR1-FLAG) was expressed. The two PPREs adjacent to the Ucp3 transcription start site recruited NCoR1 more efficiently, compared to two control sequences in the Gapdh and Ucp3 promoter that lack PPREs (Figure 5B, left). Likewise, NCoR1 bound avidly to the mouse Pdk4 promoter NR1/2 site in transfected NIH 3T3 cells (Figure 5F, left). Although there is a two nucleotide difference in NR1/2 site of the human Pdk4 promoter (Figure 5E), NCoR1 and ERRα were also recruited to this site in human HEK293 cells (Figure 5H).
We then used a highly specific NCoR1 antibody, which we recently generated (G.D.B. and R.M.E., unpublished data), for ChiP experiments in C2C12 myotubes. Confirming our data in NIH 3T3 cells that express NCoR1-FLAG, endogenous NCoR1 occupied the same Ucp3 PPREs (Figure 5B, right). The recruitment of NCoR1 to the Ucp3 promoter was robustly inhibited by the addition of the selective PPARβ/δ ligand GW501516. Likewise, endogenous NCoR1 was readily detected on the NR1/2 in the Pdk4 promoter in C2C12 myotubes (Figure 5F, right).

Subsequently, we explored whether NCoR1 gene deletion in NCoR1<sup>−/−</sup> MEFs by means of adenoviral Cre recombination, or NCoR1 gene knockdown in C2C12 myotubes infected by an NCoR1 shRNA adenovirus, modulates histone H4 acetylation on the Ucp3 and Pdk4 promoters. Consistent with NCoR1 binding to these promoters in NIH 3T3 cells and C2C12 myotubes (Figures 5B and 5F), NCoR1 deletion or silencing induced H4 acetylation of both target promoters in MEFs and C2C12 cells, indicating chromatin opening (Figures 5C and 5G).

To further consolidate these observations, we analyzed whether NCoR1 interacts directly with PPARβ/δ or ERRα, using nuclear extracts of HEK293 cells, transfected with tagged versions of NCoR1, PPARβ/δ, or ERRα. Although a specific association between NCoR1 and PPARβ/δ was evident in these co-IP experiments (Figure 5D, lane 8), we failed to detect a similar interaction between ERRα and NCoR1 (data not shown).

**MEF2 Is Hyperacetylated and Activated in the Absence of NCoR1**

The increased muscle mass observed in NCoR1<sup><sup>−/−</sup></sup> mice indicated that the absence of NCoR1 not only induced oxidative metabolism, but also stimulated myogenesis. In line with this,
mRNA levels of two markers of myogenesis, *Mb* and *Mck*, were increased in *NCoR1*^skm−/−/^ quadriceps (Figure 6A). Among several myogenic regulatory factors, only the expression of two *Mef2* family members, i.e., *Mef2c* and *Mef2d*, negatively correlated with *NCoR1* expression in our systems genetics analysis (Figures 4 and S6A). The selective induction of *Mef2c* and *Mef2d* mRNA was furthermore confirmed by qRT-PCR of *NCoR1*^skm−/−/^ gastrocnemius and quadriceps, whereas no changes were found in *MyoD*, *Myf5*, and *myogenin* mRNA (Figures 6A and S6E and data not shown).

The activity of MEF2 family members is not only controlled by their expression levels, but is also modulated by their acetylation status. MEF2 is acetylated and activated by p300, whereas it is deacetylated by HDAC3 and HDAC4, which are part of the NCoR1 corepressor complex (Ma et al., 2005; Nebbioso et al., 2009). Because the expression of the *Mef2d* isoform is most...
prominently correlated with NCoR1 expression, we investigated MEF2D acetylation in gastrocnemius and found that its acetylation levels were enhanced in NCoR1 skm/C0/C0 mice (Figures 6A and 6B).

We then compared the acetylation of MEF2D in floxed NCoR1 L2/L2 MEFs, infected with an adenovirus expressing either GFP as control or Cre-recombinase to reduce NCoR1 protein expression (Figure 6C, left). Whereas MEF2D protein levels were stable in NCoR1/C0/C0 MEFs, perhaps due to the more acute nature of the deletion, MEF2D was robustly hyperacetylated when NCoR1 levels were attenuated (Figure 6C, right). Likewise, a slight but consistent MEF2D hyperacetylation was observed in C2C12 myotubes infected with Ad-shNCoR1 to knock down NCoR1 expression (Figures 6D and S6B–S6D), further underscoring the importance of MEF2D deacetylation by the NCoR1 complex.

Given the induction of MEF2D expression and its hyperacetylation and consistent with our systems genetics analysis (Figure 4B), mRNA levels of the MEF2 targets, Mb and Mck, were robustly induced in NCoR1 skm/C0/C0 gastrocnemius (Figure 6A). Silencing of NCoR1 in C2C12 myotubes also resulted in a similar induction of several MEF2 target genes, including Mb, Mck, Glut4, c-Jun, Nur77, PGC-1α, and PGC-1β (Figures 6E and S6B–S6D). In line with these data, endogenous NCoR1 was readily detected on MEF2-binding sites on these target promoters in C2C12 myotubes, as illustrated for the Mb

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**Figure 6. Enhanced MEF2 Activity in NCoR1 skm/C0/C0 Muscle**

(A) Gene expression of myogenesis-related genes was measured by qRT-PCR in NCoR1 skm+/+ and skm/C0/C0 quadriceps. n = 10.

(B–D) Acetylation levels of MEF2D were determined by western blot after immunoprecipitation with an Ac-Lys Ab from gastrocnemius (B), from NCoR1 L2/L2 MEFs infected with Ad-GFP or Ad-Cre recombinase (C, right), and from C2C12 myotubes infected with Ad-shLacZ or Ad-shNCoR1 (D). MEF2D expression in total protein extracts was shown in the bottom panels. The expression of NCoR1 and actin in NCoR1 L2/L2 MEFs was also shown (C, left).

(E) MEF2 target mRNAs determined by qRT-PCR in C2C12 myotubes infected with either Ad-shLacZ or Ad-shNCoR1. n = 6.

(F) NCoR1 recruitment to the MEF2 site of the mouseMb promoter determined by ChIP in C2C12 myotubes.

(G) Binding of either global acetylated histone 4 (H4) or H4 acetylated on K16 (H4K16) to the MEF2 site of the Mb gene was evaluated by ChIP from C2C12 myotubes infected as in (E).

Data are expressed as mean ± SEM. See also Figure S6 and Table S3.
promoter (Figure 6F). The induction of these MEF2 targets by NCoR1 knockdown was furthermore accompanied by H4K16 and global H4 hyperacetylation on their promoters (e.g., Mb, Glut4, and Mck) (Figures 6G and S6).

NCoR1 Levels Are Regulated in Response to Physiological Stimuli

We next investigated whether NCoR1 function could be altered by different physiological stimuli in vitro and in vivo. One hour after stimulation of 293T cells with 1 μM insulin, higher amounts of endogenous NCoR1 (Figure 7A) or transfected FLAG-NCoR1 (Figure S7A) were detected in nuclei, as evidenced by immunofluorescence and subcellular fractionation (Figures 7A–7C).

At the transcriptional level, NCoR1 mRNA changed in response to different concentrations of glucose in the culture media (Figures 7D and 7E). Growing MEFs in low glucose decreased NCoR1 mRNA (Figure 7D) and protein (Figure 7E) levels, concomitant with the induction of its target genes (Pdk4, Vgfb, Mef2d, etc.). Similar results, i.e., decreased mRNA levels of NCoR1 associated with increased expression of its targets (Pdk4, Ucp2, Ucp3, Vgfb, Mb, Mck, and Glut4) were also obtained in glucose-deprived C2C12 myotubes (Figure S7B). Interestingly, the reduction of glucose decreased mRNA levels of NCoR1, but not those of SMRT (Figure 7F). The tight dose-dependent correlation between NCoR1 expression and glucose levels in the culture medium (Figures 7E and 7F) suggested the possibility that NCoR1 could block the oxidation of lipid substrates when glucose was available. We therefore evaluated NCoR1 mRNA and protein in MEFs cultured in different fatty acid concentrations (Figure 7H and 7I). The addition of oleic acid (OA) to the medium to force fatty acid oxidation also decreased NCoR1 levels, independently of the glucose concentration (Figure 7H). These effects seemed again specific to NCoR1, as only a small difference in SMRT mRNA levels was observed with OA in the absence of glucose (data not shown). Together, these data indicate that settings that favor fatty acid oxidation (i.e., low glucose, low insulin, and high fatty acid) are all associated with a reduction of NCoR1.

We then tested whether different conditions that enhance fatty acid oxidation also modulate NCoR1 mRNA levels in the muscle in vivo. This was indeed the case, as muscle NCoR1, but not SMRT, mRNA levels decreased after exercise (3 hr after a resistance run), high-fat feeding (20 weeks of high fat feeding), fasting (after a 16 hr fast), and aging (6-month-old versus 2-year-old mice) (Figure 7J and data not shown). Interestingly, the reduction in NCoR1 mRNA levels matches well with the potentiation of lipid oxidation after exercise (Kiens and Richter, 1998; Pilegaard et al., 2000), high-fat feeding (Watanabe et al., 2006), and fasting (Storlien et al., 2004) and in aged mice (Houtkooper et al., 2011). Also in epididymal white adipose tissue, HFD feeding reduced specifically NCoR1, and not SMRT, mRNA (Figure S7C). Unlike for the muscle, where we were unable to detect NCoR1 with the available antibodies, NCoR1 protein levels almost disappeared from epididymal fat upon HFD (Figure S7D). Altogether, these data led us to suggest that NCoR1 is a negative transcriptional regulator of fatty acid oxidation and that a reduction of NCoR1 enables the muscle (and adipose tissue) to deal with lipid substrates more efficiently.

DISCUSSION

The increased muscle mass, associated with a strikingly improved exercise capacity, is the most prominent phenotypic outcome of the muscle-specific NCoR1 gene deletion. The enhanced exercise capacity is associated with a reprogramming of glycolytic to more oxidative muscle fibers and a corresponding stimulation of oxidative mitochondrial metabolism, indicative of an improved intrinsic quality of the muscles. The increased muscle quantity and oxidative profile in NCoR1<sup>skm</sup>-/– mice also contribute to the slight improvement in metabolic parameters after high-fat feeding and to the cold resistance subsequent to shivering thermogenesis (Cantó and Auwerx, 2009).

Combined, these properties suggest that NCoR1 acts as a master modulator of mitochondrial metabolism in the muscle, a hypothesis bolstered by the fact that the inhibition of the single worm NCoR homolog, gei-8, also robustly boosts muscle oxidative mitochondrial metabolism in C. elegans. The evolutionary conservation of the structure and function of this corepressor makes it tempting to speculate that NCoR may have evolved to facilitate metabolic adaptation of the mitochondria to energy availability, as has been described for other cofactors as SIRT1 (Cantó and Auwerx, 2009).

As to how the absence of NCoR1 in the muscle achieves these remarkable effects, it is important to recall that NCoR1 docks histone deacetylases, such as HDAC3 (Alenghat et al., 2008) and SIRT1 (Picard et al., 2004). The use of mice with a point mutation in the NCoR1 deacetylase activation domain, incapacitating its interaction with HDAC3, indicated that this interaction is a nodal point in epigenetic regulation (Alenghat et al., 2008; Feng et al., 2011). The physiological alterations in the NCoR1<sup>skm</sup>-/– muscle together with the study of the expression correlates of NCoR1 suggested that, rather than the generalized transcriptional activation expected upon ablation of a corepressor platform protein, only a small set of transcriptional pathways was selectively affected.

Several transcription factors control muscle differentiation and development, including MyoD, myogenin, Myf5, Myf6, and MEF2 (Black and Olson, 1998; McKinsey et al., 2001, 2002; Potthoff and Olson, 2007). MEF2 especially caught our attention, as only its expression negatively correlated with NCoR1 (Figures 4B and S6) and was induced in NCoR1<sup>skm</sup>-/– muscle (Figure 6A). MEF2 is key for muscle development and also participates in muscle stress response and remodeling in adulthood, such as occurs during muscle fiber type switch (Potthoff and Olson, 2007; Zhang et al., 2002). MEF2 activity is not only controlled at the level of its expression, but also by a wide range of intracellular signaling pathways and interacting coregulator molecules (reviewed by Potthoff and Olson, 2007). The histone acetyltransferases, p300/CBP, bind, acetylate, and activate MEF2 (Ma et al., 2005; McKinsey et al., 2001), whereas class I (HDAC3 [Grégoire et al., 2007]), class II (HDAC4, 5, 7, and 9 [Haberland et al., 2007; McKinsey et al., 2001]), and class III HDACs (SIRT1 [Zhao et al., 2005]) all are reported to interact with MEF2 and prevent the activation of its target genes (McKinsey et al., 2001). The absence of NCoR1 would hence favor the acetylation and activation of MEF2. The hyperacetylation of MEF2D and histone 4 in NCoR1-deficient MEFs, C2C12 myotubes, and muscles, which
translates into the induction of MEF2 targets and the gain of muscle mass in NCoR1^skm^−/− mice, is fully in line with this idea (Figure 6).

The increased expression of genes related to fatty acid catabolism and mitochondrial respiration underlies the oxidative mitochondrial changes observed in the NCoR1^skm^−/− mice. In the muscle, the expression of these gene sets is tightly controlled by NRs belonging to the PPAR and ERR families (Alaynick, 2008). PPARβ/δ, the predominant PPAR isoform in oxidative fibers, regulates oxidative capacity and enhances slow fiber-type function, resulting in improved exercise capacity and metabolic protection (Luquet et al., 2003; Tanaka et al., 2003; Wang et al., 2004). Among the ERRs, mainly ERRα and ERRγ seem to be involved in the coordination of muscle energy homeostasis

Figure 7. Localization and Expression of NCoR1 in Physiological Conditions

(A–C) Localization of NCoR1 protein was determined either by immunofluorescence and quantified (A and B) or by western blot (C). 293T cells grown without (−) or with (+) 1 μM insulin for 1 hr were stained with DAPI or anti-NCoR1 (A). Quantification of nuclear NCoR1 is shown in (B). Nuclear and cytosolic fractions were separated from FLAG-NCoR1-transfected 293T cells after a 1 hr stimulation without (−) or with 1 μM insulin (+), and protein levels were determined by western blotting (C).

(D) mRNA levels of NCoRα and its target genes determined by qRT-PCR in MEFs cultured for 24 hr in 5 or 25 mM glucose. n = 6.

(E) NCoR1 protein was determined by western blotting from MEFS cultured for 24 hr in 5 or 25 mM glucose.

(F) NCoR1 and SMRT mRNA were determined in MEFs cultured for 48 hr in 0, 5, and 25 mM glucose.

(G) NCoR1 protein determined by western blotting from MEFS cultured as indicated in (G).

(H) NCoR1 mRNA measured by qRT-PCR in muscles of resting mice or 3 hr after an endurance run (14 weeks old; n = 5), in mice that were fed for 20 weeks either HFD or CD (28 weeks old; n = 8), in mice that were fasted or fed for 16 hr (14 weeks old; n = 10), and in 6-month-old or 2-year-old mice (n = 10).

(I) Model schematizing how different levels of NCoR1 control transcription of muscle genes by controlling the activity of transcription factors (TFs), i.e., PPARβ/δ, ERR, and MEF2.

Data are expressed as mean ± SEM. See also Figure S7 and Table S3.
(reviewed in Giguère, 2008; Villena and Kralli, 2008). In line with this, a genome-wide location analysis of ERRα and ERRγ identified binding sites in genes of a large number of mitochondrial proteins (Dufour et al., 2007), and studies in mouse models show that they coordinate many aspects of muscle oxidative metabolism, including endurance capacity (Giguère, 2008; Narkar et al., 2011; Villena and Kralli, 2008). Several features of the NCoR1<sup>flx</sup> mice are suggestive of the activation of PPARγ and ERR. The demonstration that NCoR1 is recruited to the PPREs in the Ucp3 and the NR1/2 in Pdk4 promoters and that histone 4 is hyperacetylated on these promoters when NCoR1 is absent suggests that coactivators now activate the transcription of these genes in a fashion unopposed by the NCoR1 corepressor platform (Figure 7K). Given that PGC-1α is a key coactivator of PPARγ and ERR transcriptional programs (reviewed in Handschin and Spiegelman, 2006), it is no surprise that several phenotypic features, ranging from similarities in gene expression patterns over the induction of mitochondrial oxidative metabolism and exercise capacity to HIF1α-independent angiogenesis, are shared between mice that lack NCoR1 or that overexpress PGC-1α (Arany et al., 2008; Lin et al., 2002).

NCoR1 action is tightly regulated by various physiological challenges, and this is achieved through at least two different mechanisms. First, nuclear levels of NCoR1 are regulated. Our results show how insulin, which stimulates glucose oxidation at the expense of fatty acid oxidation, increases NCoR1 levels in the nucleus, enabling it to subsequently repress lipid oxidation genes. Interestingly, this effect of insulin on nuclear NCoR1 accumulation is consistent with the positive effect of mTORC1 on nuclear NCoR1 accumulation, recently reported in hepatocytes (Sengupta et al., 2010). The second mechanism involves the modulation of NCoR1 expression. Exposing cells to media with low glucose and/or high fatty acid levels reduces specifically NCoR1<sup>α</sup> mRNA and protein levels, ultimately derepressing genes that control oxidative lipid metabolism. Likewise, endurance exercise, fasting, high-fat feeding, and aging—conditions paired with increased fat oxidation—also are characterized by attenuated muscle NCoR1 mRNA expression. Although we are unable to detect NCoR1 protein in the muscle with the currently available antibodies, our data in adipose tissue unequivocally show that the specific reduction in NCoR1<sup>α</sup> mRNA observed in that tissue after HFD is matched with a spectacular drop in NCoR1 protein. If the reduced NCoR1<sup>α</sup> mRNA levels in muscle also translate to corresponding changes in NCoR1 protein, they will prime the muscle for mitochondrial oxidation. These selective effects of NCoR1 to repress muscle fatty acid oxidation hence suggest that changes in NCoR1 levels adapt transcriptional outcomes to physiological energy needs (Figure 7K).

In conclusion, we demonstrated here that NCoR is an evolutionary conserved negative regulator of both muscle mass and mitochondrial oxidative metabolism in nematode and mammals. In the mouse, NCoR1 achieves these effects through controlling a rather selected set of functional pathways, which are governed by MEF2, PPARγ, and the ERRs. The NCoR1<sup>flx</sup> muscle phenotype furthermore mirrors many features of the stimulation of PGC-1α, a coactivator, whose action is less constrained by the absence of the NCoR1 corepressor scaffold. Our work also provides evidence that NCoR1 expression is regulated in a dynamic fashion and, as such, could play a role similar to PGC-1α in transcriptional adaptation to physiological challenges. Moreover, pharmacological inhibition of NCoR1 and/or its interaction with deacetylases may be a viable approach to improve muscle mass and oxidative metabolism. The fact that the inhibition of HDACs increases muscle cell size supports this concept (Iezzi et al., 2004). It is also tempting to speculate that the beneficial effects of the inhibition of mTORC1 and insulin signaling on health and life span may, in part, rely on the attenuation of NCoR1 activity and the subsequent induction of oxidative metabolism in the muscle (reviewed in Houtkooper et al., 2010).

**EXPERIMENTAL PROCEDURES**

**Animal Studies**

NCoR1<sup>flx</sup> (<sup>+/−</sup>), NCoR1<sup>flx/flx</sup>, and <sup>+/−</sup> mice were generated at the Mouse Clinical Institute (Strasbourg, France) and phenotyped (Champy et al., 2004, 2008) according to standard procedures that are described in detail in the Extended Experimental Procedures. C. elegans strains, RNAi feeding experiments, and GFP expression analysis are described in the Extended Experimental Procedures. C. elegans O<sub>2</sub> consumption was measured in 200 2-day-old worms using a Seahorse XF24, as described in the Extended Experimental Procedures.

**Histological and EM Analyses**

Staining of muscles with hematoxylin and eosin, immunohistochemical and EM analysis, and analysis of enzymatic activity of SDH and COX were carried out as described (Lagouge et al., 2008).

**mRNA Analysis and Identification of NCoR1-Correlated Genes**

The mRNA expression levels were measured in cells and tissues using qRT-PCR (Lagouge et al., 2006) The GeneNetwork program (http://www.gene-network.org) was used to generate a broad range of NCoR1-correlated genes that may contribute to the phenotype of NCoR1<sup>flx/flx</sup> mice. Skeletal muscle mRNA expression was analyzed using 124 females from a classic F2 intercross between C57BL/6J and C3H/HeJ (UCLA BHHBF2 Muscle; van Nas et al., 2010). GEO GSE12795). NCoR1 (10024414685, 3′ UTR) was compared across all transcripts to find muscle covariates. Lung mRNA in a recombinant inbred intercross between C57BL/6J and DBA/2J was analyzed across 51 strains (H2I BXD Lung M430v2 [Apr08] RMA; Alberts et al., 2011). Four NCoR1 probe sets from this microarray were analyzed (1423200_at, 3′UTR; 1435914_at, 3′UTR; 1423202_a_at, exonic and 3′UTR; 1423201_at, exonic). For all probe sets, the top correlates were calculated (Tables S4 and S5). Strong or interesting correlates were selected for validation by qRT-PCR.

**Cell Culture, Adenoviral Infections, ChIP, co-IP, and Western Blot Experiments**

Experimental details are provided in the Extended Experimental Procedures.

**Statistical Analyses**

Statistical analyses were performed with a Student’s t test for independent samples. Data are expressed as mean ± SEM, and p values smaller than 0.05 were considered as statistically significant. *p < 0.05; **p < 0.01; ***p < 0.001.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and five tables and can be found with this article online at doi:10.1016/j.cell.2011.10.017.
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