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E2F transcription factor-1 regulates oxidative metabolism

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Abstract

Cells respond to stress by coordinating proliferative and metabolic pathways. Starvation restricts cell proliferative (glycolytic) and activates energy productive (oxidative) pathways. Conversely, cell growth and proliferation require increased glycolytic and decreased oxidative metabolism¹. E2F transcription factors regulate both proliferative and metabolic genes^{2,3}. E2Fs have been implicated in the G1/S cell cycle transition, DNA repair, apoptosis, development, and differentiation²⁻⁴. In pancreatic β -cells, E2F1 gene regulation facilitated glucose-stimulated insulin secretion^{5,6}. Moreover, mice lacking E2F1 (*E2f1*^{-/-}) were resistant to diet-induced obesity⁴. Here, we show that E2F1 coordinates cellular responses by acting as a regulatory switch between cell proliferation and metabolism. In basal conditions, E2F1 repressed key genes that regulate energy homeostasis and mitochondrial functions in muscle and brown adipose. Consequently, *E2f1*^{-/-} mice had a marked oxidative phenotype. An association between E2F1 and pRb was required for repression of genes implicated in oxidative metabolism. This repression was alleviated in a constitutive active CDK4 (CDK4^{R24C}) mouse model or when adaptation to energy demand was required. Thus, E2F1 represents a metabolic switch from oxidative to glycolytic metabolism that responds to stressful conditions.

E2f1^{-/-} mice are resistant to diet-induced obesity; thus, E2F1 may participate in the control of energy homeostasis⁴. *E2f1*^{-/-} mice exhibited increased oxygen (O₂) consumption compared to *E2f1*^{+/+} mice (Fig. 1a) and lower respiratory exchange ratio (Fig. 1b), indicating enhanced fatty acid oxidation. This could be secondary to higher brown adipose tissue (BAT) regulation of heat production. Indeed, *E2f1*^{-/-} rectal temperatures were increased in response to cold and fasting conditions (Fig. 1c, supplementary Fig. S1a), and *E2f1*^{-/-} BAT exhibited a marked red color and decreased lipid droplets compared to controls (Supplementary Fig. S1b, c). *E2f1*^{-/-} BAT and gastrocnemius muscle (GN) had

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more mitochondria (Fig. 1d and f; Supplementary Fig. S1d-f) and higher oxygen consumption compared to control tissues (Fig. 1e, g) as well as mitochondrial enzyme activity (Supplementary Fig. S1g-i). *E2f1* inactivation resulted in increased thermogenesis and mitochondrial activity, suggesting increased oxidative metabolism. Typically, GN is predominantly glycolytic; thus, in response to succinate and ADP, O₂ consumption was not induced in GN of *E2f1*^{+/+} mice, but was increased in GN of *E2f1*^{-/-} mice (Fig. 1g). This suggested that the GN muscles of *E2f1*^{-/-} mice switched to oxidative metabolism. Moreover, *in vivo* electroporation of E2F1 DNA into the GN of *E2f1*^{-/-} mice caused a reduction of mitochondrial activity (Fig. 1g). E2F1 expression in electroporated muscles was demonstrated by immunofluorescence staining (Supplementary Fig. S1j). Furthermore, *E2f1*^{-/-} GN showed increased protein and mRNA expression of slow-twitch oxidative myofiber genes (MyHC I and IIa) and reduced expression of the fast-twitch glycolytic myofiber gene, MyHC IIb compared to *E2f1*^{+/+} mice (Fig. 1h, 1i and Supplementary Fig S2a-e).

MyHC I and IIa fibers are resistant to fatigue; therefore, we tested the endurance of weight-matched *E2f1*^{+/+} and *E2f1*^{-/-} mice with treadmill running to exhaustion. *E2f1*^{-/-} mice ran at least twice the distance (mean=1700m) of *E2f1*^{+/+} mice (mean=850m) before exhaustion (Fig. 1j and Supplementary Fig. S2f). Moreover, electroporation of E2F1 in *E2f1*^{-/-} GN reduced the running endurance (1400m; p<0.05) (Fig 1k; Supplementary Fig S2g). These results suggested that E2F1 negatively regulated oxidative metabolism, which decreased thermogenesis and physical endurance.

We then analyzed the expression of potential E2F1 target genes implicated in mitochondrial function. *E2f1*^{-/-} BAT and/or GN showed increased expression in the mitochondrial respiratory chain (*Atp5g1*, *Cox5a*, *Nduf1c*, *Sdha*, *Uqcrl*), TCA cycle (*Idh3a*), uncoupling respiration (*Ucp1*, 2), transcription regulation (*Ppargc1a*, *Esrr*, *Tfam*), and fatty acid oxidation (*Acadl*, *Pdk4*, *Cpt-1*) (Fig. 2a, b; Supplementary Table 1). Interestingly, expression of two classical E2F1 target genes involved in cellular proliferation, *Dhfr* or *Tk-1*, were unchanged (Fig. 2a, b). We observed increased mitochondrial DNA content and function (Supplementary Fig. S3a-b) in distinct *E2f1*^{-/-} muscles (diaphragm, tibialis) and in other tissues (liver), which was consistent with increased expression of genes involved in mitochondrial activity, as described above (Supplementary Fig. S3c). Mitochondrial DNA content, function and oxidative gene expression were also increased in *E2f1*^{-/-} mouse embryonic fibroblasts (MEFs; Supplementary Fig. S4a-d). Altogether, these results suggested that E2F1 modulated oxidative metabolism in different organs and cell types. Moreover, *E2f1* gene inactivation resulted in increased O₂ consumption in diaphragm and liver. Interestingly this effect was independent of PGC-1, since expression of this factor was not changed in these tissues in *E2f1*^{-/-} mice.

Computational analysis of the promoter region of oxidative genes revealed the presence of an E2F binding site in the regulatory sequences of *Cox5a*, *Cpt-1*, *Pdk4*, *Ppargc1a*, *Ucp1*, *Ucp2*, *Tfam*, *Esrra* and *Sdha* (data not shown). *In vivo* knockdown experiments of these genes by muscle electroporation of short hairpin RNAs in an *E2f1*-null background decreased oxygen consumption of mitochondria isolated from GN (Fig. 2c, d) or permeabilized MEFs (Supplementary Fig. S4e, f). Therefore, the partial rescue of the wild-type phenotype in *E2f1*^{-/-} cells by specific shRNAs suggested that each of these genes contributed to the effect of E2F1 on oxidative metabolism.

Under cold and fasting conditions, oxidative metabolism gene expression was increased in BAT and muscle (Fig. 3a, b). We investigated whether E2F1 and pRb complexes mediated the metabolic response under those conditions. Co-immunoprecipitation assays from muscle extracts indicated that E2F1 and pRb were associated in the same protein complex

(Supplementary Fig. S5a). Since this is known as a repressor complex we then hypothesized that cold or fasting conditions in BAT and muscle trigger pRb phosphorylation and release the pRb-E2F1 repressor complex. This was supported by our results showing enhanced pRb phosphorylation in BAT of mice placed at +4°C for 4 hr, as well as in muscle of 24 hr fasted mice (Fig. 3c-d; Supplementary Fig. S5b-c). Interestingly treatment of mice with isoproterenol, which is a 3-adrenergic receptor agonist that mimics fasting conditions also resulted in pRb phosphorylation (Fig. 3e); Supplementary Fig. S5d). Mitochondrial activity was increased in the presence of isoproterenol, which suggested that the cdk4-pRB-E2F1 pathway mediate the effects of isoproterenol. Consistent with this hypothesis was the observation that cdk4 inhibition results in the abrogation of the increased mitochondrial activity by isoproterenol (Fig. 3f). Moreover we found that E2F1 directly bound to the promoters of several genes implicated in oxidative metabolism (Fig. 3g-i; Supplementary Fig. S5e). Under basal temperature and nutrient availability, which elicit low oxidative activity, E2F1 was associated with pRB on the promoters of most of the studied genes in BAT and muscle (Fig. 3g-i). Consistent with pRb phosphorylation status, when oxidative metabolism was triggered by cold or fasting conditions, pRB was released from E2F1, which enabled transcription (Fig. 3a, b). Furthermore, upon return to basal conditions, acetylated histone H4, an epigenetic marker of transcription, was reduced, suggesting decreased transcription (Supplementary Fig. S5f, g).

To further prove our hypothesis of the pRb-dependent regulation of gene expression during the adaptive metabolic responses we analyzed mice expressing a mutant CDK4 that cannot bind the cell-cycle inhibitor p16Ink4a (CDK4^{R24C/R24C}). This mutation renders CDK4 hyperactive and deregulated, resulting in pRb constitutively phosphorylated and consequently dissociated from E2Fs. ⁷O₂ consumption (Fig. 4a) and running endurance (Fig. 4b) were increased in the CDK4^{R24C/R24C} background. Moreover, mRNA levels of oxidative genes were modulated during fasting in wild type mice, whereas their expressions were increased independently of feeding or fasting conditions (Fig. 4c). Interestingly, pRb was recruited on E2F1 target genes in the muscles of wild type mice in the fed state, whereas pRb was not found in these promoters in any condition tested in muscles of CDK4^{R24C/R24C} mice (Fig. 4d). The phenotype of the double mutant E2f1^{-/-} and R24C mice was not different of the E2f1^{-/-} mice further indicating that E2F1 mediates the observed effects in the cdk4R24C mice (data not shown).

These results demonstrated first, that CDK4 is a major regulator of E2F1 activity in the context of metabolic control through phosphorylation of pRb; and second, that E2F1 mediated the adaptive oxidative metabolic responses to cold and fasting.

The effects of E2F1 were cell autonomous, because E2f1^{-/-} primary myoblasts also showed increased mitochondrial activity (Supplementary Fig. S6a) and increased oxidative metabolic gene expression (Supplementary Fig. S6b). Interestingly, E2f1^{-/-} and E2f1^{+/+} myoblasts showed similar efficiency for differentiating into myotubes (Supplementary Fig. S6c). This suggested that E2F1 was not required for myoblast proliferation or differentiation *in vitro*. Importantly, mitochondrial activity in E2f1^{-/-} differentiated myofibers (Supplementary Fig. S6d) was reminiscent of that observed *in vivo*. Primary E2f1^{-/-} myoblast differentiation gave rise to oxydative myofibers (MyHC I) with a concomitant decrease in glycolytic fibers (MyHC IIb) (Supplementary Fig. S6e); in contrast, E2f1^{+/+} myotubes comprised increased expression of MyHC IIb compared to MyHC I, as demonstrated by western blot analysis (Supplementary Fig. S6f, g). Accordingly, the transcriptional program of differentiated E2f1^{-/-} myotubes was directed towards oxidative metabolism (Supplementary Fig. S6h), and their glycolytic gene expression was decreased compared to E2f1^{+/+} myotubes (Supplementary Fig. S6i). When compared to myotubes mRNA levels of only 4 out of 18 glycolytic genes were decreased in the GN muscles of

E2f1^{-/-} mice (Supplementary Fig. S6j). These results support our data in adult muscles, although it should be interpreted with caution since these cells have features of stem cells. A decrease in serum lactate levels was observed (Supplementary Fig. S6k), suggesting a role of E2F1 in the control of the glycolytic pathway in mice.

E2Fs function as activator or repressor of transcription⁸. E2F1 proteins have demonstrated paradoxical functions in the control of cell proliferation and apoptosis⁹, which might be dictated by promoter sequence and/or promoter context¹⁰. Since *Dhfr* and *Tk* mRNA levels were not changed in muscle of *E2f1*^{-/-} mice, nor upon fasting/refeeding conditions (Fig. 5a), we investigated the molecular mechanisms underlying the differential regulation of proliferative *versus* metabolic E2F1 target genes. E2F1 recruitment was not significantly increased on proliferative promoters compared to IgG, as evidenced by ChIP assays (Fig. 5b). Computational analysis of *Dhfr* and *Tk* promoters revealed the presence of CpG islands in genomic regions known to interact with E2F1. Most importantly, we demonstrated that these DNA sequences were methylated in wild-type muscle. This epigenetic modification blocks E2F1-mediated gene transactivation of these proliferative genes (Fig. 5c). Conversely, the *Pparg1a* promoter was not methylated in muscle, as evidenced by the absence of PCR amplification of this promoter region (Fig. 5c). Taken together, these data demonstrated that the differential regulation of proliferative *versus* metabolic genes in muscle is operating, at least in part through CpG islands methylation of E2F1 responsive elements.

The physiological implications of our findings are not limited to the control of oxidative metabolism, but also concern the relationship between cell growth, proliferation, and function. The E2F1-pRB repressor complex translates signals that sense the metabolic needs of the cell into a transcriptional response. Usually, when biosynthetic processes are required, such as when cell division is increased, cells switch to glycolytic metabolism, and decrease oxidative phosphorylation. We showed that E2F1 orchestrates a complex control of oxidative and glycolytic metabolisms, necessary for cell proliferation and adaptations to energy demands notably during fasting, and cold (Supplementary Fig. 5d). *E2f1*^{-/-} mice had increased mitochondrial activity under normal conditions in glycolytic muscle and BAT. Genetic inactivation of *E2f1* released the inhibition of oxidative pathway genes implicated in mitochondrial function and biogenesis required to provide energy to the cell to overcome the lack of nutrients, and to generate heat to fight cold temperature¹¹.

Skeletal muscle adapts to different physiopathological conditions by changing its physical, contractile, and metabolic properties to accommodate alterations in functional demands¹². Thus, slow (type I) fibers, rich in mitochondria, typically work under oxidative conditions; fast (type II) fibers, with fewer mitochondria, typically work under anaerobic conditions¹³. Regulation of fiber-type determination in skeletal muscle is still not well understood, especially at the transcriptional level, although some studies demonstrated the key participation in the switch to oxidative muscles fibers of the nuclear receptor PPAR¹⁴, the transcription factor NFAT¹⁵ or the transcriptional coregulators PGC-1 and RIP-140^{16,17}. We showed that E2F1 is also one of such transcriptional modulators of the metabolic oxidative response. Compared to other factors, E2F1 is unique because it can coordinate both a metabolic and proliferative response. In this study we show that E2F1 silenced oxidative metabolic gene expression and increased glycolytic gene expression. This is consistent with the observed correlation of increased E2F1 activity with abnormal mitochondrial respiration and function in highly proliferative cancer cells¹⁸. Recent studies have shown that, in dividing cells, E2Fs function as transcriptional activators, but in differentiating cells, E2F-pRb complexes repress transcription¹⁹. Here, we showed that the E2F1 formed a complex with pRb to modulate oxidative gene expression. This is consistent

with recent results that suggested adipose-tissue specific *pRB*^{-/-} mice had a strong metabolic phenotype, markedly similar to *E2f1*^{-/-} mice²⁰.

Muscle adaptation to nutrient availability, cold conditions or exercise requires signaling pathways that will ultimately activate E2F1 transcriptional activity. In line with this hypothesis, we observed increased phosphorylation of pRb during these processes. However, the pathways regulating the CDK4-E2F1-pRb complexes in muscles were still unknown. We showed here that catecholamines, which secretion is increased during fasting/cold conditions, play a role in activating CyclinD/CDK4 complexes.

In summary our results underscored an important role for E2F1 in the control of mitochondrial oxidative metabolism. E2F1 and pRb complexes repressed oxidative metabolism.

Methods

Materials and oligonucleotides

Chemicals, unless stated otherwise, were purchased from Sigma (St Louis, MO, USA). PD0332991 was purchased from Euromedex (France). Anti-E2F1 (C-20 and KH-95), and anti-pRb (C-15) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-acetylated H4 (Lys12) and anti-S780phospho-pRb were from Cell Signaling. Anti-MyHCl (BA-F8), IIa (SC-71) and IIb (BF-F3) were purchased from the Developmental Studies Hybridoma Antibody Core at the University of Iowa. Oligonucleotide sequences appear in Supplementary table 1.

Animal experiments

E2f1^{+/+} and *E2f1*^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *CDK4*^{R24C/R24C} were described previously⁷. Mice were maintained according to European Union guidelines for use of laboratory animals. *In vivo* experiments were performed in compliance with the French guidelines for studies on experimental animal (Animal House agreement no. B-34-172-27, Authorization for Animal Experimentation no. 34.324). Energy expenditure was measured with an Oxymax apparatus (Columbus Instruments, Columbus, OH, USA). Cold test was performed on animals individually housed at 4°C for 4hr as described²⁰. Endurance was tested in 2-hr fasted mice with a treadmill equipped with an electrical shock grid (Bioseb, Chaville, France)²¹. Briefly, animals were acclimatized to the test using a habituation protocol one day before the test consisting of a run at 27cm/s for 10 min. The endurance test was performed on 2 hr fasted weight-matched *E2f1*^{+/+} and *E2f1*^{-/-} mice at the beginning speed of 18 cm/s for 10 min. The speed was progressively increased (around 4cm/s each 10 min) to reach at the end of the running test the maximal speed of 38 cm/s. The distance run and the number of shocks were recorded all along the test and a mouse was considered exhausted and removed from the experiment when it received 180 shocks. For intra-muscular electrotransfer, mice were randomized and anaesthetized by intra-peritoneal injection of a ketamine (30 mg/Kg) and xylazine (10 mg/Kg) solution. The paws were shaved, and 70 µg of pCMV expressing E2F1 or empty plasmid were injected intra-muscularly (i.m.) in 70 µl into the gastrocnemius muscle of *E2f1*^{+/+} and *E2f1*^{-/-} mice. For *in vivo* silencing experiments, 50µg of pRNATU6 (control), pRNATU6-sh*Ucp2*, -sh*Ppargc1a*, -sh*Esrra*, -sh*Pdk4* were injected i.m. into the gastrocnemius muscle. Following i.m. injections, echographic gel was applied and transcutaneous electric pulses were applied using two custom-made stainless steel plate electrodes placed on either side of the hind limb as previously described²². Experiments were carried out 4 days after electroporation, except if stated otherwise. For fasting/refeeding experiments, mice were fasted 24hr or fasted 20hr et refed 4h before harvesting

tissues. For cold experiments, mice were maintained at +23°C or placed at +4°C for 4hr and fasted during the time of the experiment. For isoproterenol treatment, mice were fasted 4hr and treated for 30 min by intraperitoneal (i.p.) injection of isoproterenol at a dose of 10 mg/Kg body weight.

Ultra structural evaluation

GN muscle and BAT were immersed in a solution of 3.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) overnight at 4°C, subsequently rinsed in phosphate buffer and post-fixed in a 1% osmic plus 0.8% potassium ferrocyanide for 2 hr in the dark at room temperature. After two rinses in a phosphate buffer, tissues were dehydrated in a graded series of ethanol solutions (30-100%) and then embedded in EmBed 812 DER 736. Thin sections (85 nm; Leica-Reichert Ultracut E) were collected at different levels of each block. These sections were counterstained with uranyl acetate and lead citrate and observed using a Hitachi 7100 transmission electron microscope in the Centre de Ressources en Imagerie Cellulaire de Montpellier (France).

Oxygen consumption

Measurements of oxygen consumption were performed in liver, muscle and BAT isolated mitochondria or myoblasts, myotubes and MEFs permeabilized with 100 µg/ml of digitonin. Briefly, tissues were homogenized into isolation buffer (250mM mannitol, 10mM EDTA, 45mM Tris-HCl, 5mM Tris-Base, pH 7.4) using a Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 2000 rpm for 10 min. Mitochondria were isolated from supernatant by spinning twice at 10000 rpm for 10 min. The mitochondrial pellet was resuspended in isolation buffer and kept on ice. Mitochondrial protein was measured by Bradford method (BioRad). The rate of mitochondrial oxygen consumption was measured at 37°C in an incubation chamber with a Clark-type O₂ electrode as described in ²¹ (Strathkelvin Instruments, Glasgow, UK) filled with 1 ml of respiration medium (15mM KCl, 30mM KH₂PO₄, 25mM Tris Base, 45mM sucrose, 12mM mannitol, 5mM MgCl₂, EDTA 7mM, 20mM Glucose, 0.2% BSA). Cells were collected by trypsinisation and washed twice in respiration medium (0.25M sucrose, 20mM Hepes pH7.1, 10mM MgCl₂ for MEFs; PBS, 35mM Hepes, 5.55mM glucose, 1% BSA for myoblasts and myotubes). Cells pellets were kept on ice. Prior to the addition of the cells in the chamber, the medium was supplemented with 2mM KH₂PO₄, 5mM malate, 100µg/ml digitonin. Concentrated cells were added to 1ml respiration medium. All measurements were performed using mitochondria (3 mg/ml) or MEFs (3 millions cells), myoblasts (4 millions cells) and myotubes (1 million cells) incubated with succinate (5mM) as substrate, in the presence or in the absence of 1 mM ADP. The incubation medium was constantly stirred with a magnetic stirrer. Respiration values were normalized to mitochondrial protein content for tissues or cell number for MEFs, myoblasts and myotubes.

mtDNA quantification, RNA extraction, RT-PCR and quantitative RT-PCR (Q-PCR)

For DNA extraction, tissues were digested in a lysis buffer containing proteinase K and 10% Chelex 100 resin (BioRad, Hercules, CA, USA). Q-PCR was performed on DNA diluted 200 times using mitochondrial (ND2) and nuclear (28S) specific primers. For gene expression analysis, total RNA extraction from *E2f1*^{+/+} and *E2f1*^{-/-} tissues and primary MEFs, myoblasts and myotubes and reverse transcription were performed as described ^{6,23}. Q-PCR was carried out using a LightCycler 480 and the DNA double strand specific Power SYBR Green master mix for detection (Applied Biosystems, Foster City, CA, USA). Q-PCR was performed using gene-specific oligonucleotides and results were then normalized to 18S levels (Supplementary Table 1).

Histology, immunofluorescence, biochemical analysis and MitoTracker staining

Hematoxylin staining, immunohistochemistry (phospho-pRb, dilution 1/50) and immunofluorescence (MyHC, 2 μ g/section; E2F1, dilution 1/50) analysis were performed on 5 μ m formalin-fixed paraffin-embedded tissue sections and succinate dehydrogenase activity on 10 μ m frozen sections as described^{24,25}. Citrate synthase activity was determined on gastrocnemius homogenates as previously described^{21,26}. Serum lactate levels were measured at the Service Phenotypage de l'IFR 150 (Toulouse, France) on 16h fasted mice. For mitotracker staining primary MEFs were cultured on coverslips overnight and then incubated for 30 min with 100 nM MitoTracker redCMX-ROS (Molecular Probes, Carlsbad, CA, USA). Cells were subsequently rinsed with PBS and fixed with 3.7% formaldehyde, for 15 min. After washing, cells were mounted with fluorescent medium (Dako, Glostrup, Denmark) and observed under a fluorescent microscope (Leica Microsystems SAS, Rueil Malmaison, France).

Protein extracts, co-immunoprecipitation and immunoblot analysis

Protein extracts and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrotransfer and immunoblotting were performed as described²⁷. Co-immunoprecipitation experiments were described elsewhere⁵. Antibody dilutions were 1/50000 for MHC, 1/5000 for tubulin, 1/5000 for actin, 1/1000 for pRb and 1/500 for E2F1.

Cell culture and shRNA experiments

Primary *E2f1*^{+/+} and *E2f1*^{-/-} MEFs were obtained from embryos at embryonic day 13.5 by standard methods. Primary *pRb*^{-/-} MEFs were described elsewhere²⁸. Monolayer cell cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM glucose and 10% foetal calf serum (FCS) (Invitrogen). *In vitro* shRNA experiments were performed by retroviral infection. Phoenix cells were transfected with either pSUPERretro (Oligoengine), pSUPERretro-*shTfam* (5'-GTGTGAAACGATCCGGAGA-3'), pSUPERretro-*shCpt-1* (5'-GAAGTTCATCCGATTCAAG-3'), pSUPERretro-*shUcp2* (5'-GCCTACAAGACCATTGCAC-3'), pSUPER-*shPpargc1a* (5'-CAGCCGAGGACACGAGGAA-3'), pSUPER-*shEsrra* (5'-GCTGTACGCCATGCCTGAC-3'), pSUPER-*shPdk4* (5'-GCCACATTGGAAGTATCGA-3') using JetPEI (Invitrogen). After 48 h of transfection, the medium containing retroviruses was collected, filtered, treated by polybrene (1 μ g ml⁻¹) and transferred to MEF target cells. 48hr after transfection, cells were harvested and processed for oxygen consumption. For *in vivo* experiments, shRNA oligonucleotides were cloned in pRNAT-U6 vector, and electroporated in GN muscle of *E2f1*^{+/+} and *E2f1*^{-/-} mice. Down regulation was verified by Q-PCR analysis, and 4 out of 6 shRNA were validated.

Primary muscle cell cultures were derived from gastrocnemius and tibialis anterior muscles of three week old mice, as described²⁹. Briefly, muscles were partly digested with four sequential 10-min incubations in DMEM/HamF12 medium containing 0.14% pronase (Gibco). The supernatants from the second, third and fourth digestions were pooled and filtered through a 100- μ m cell strainer. Cells were centrifuged, washed twice, counted and plated at low density (100 cells cm⁻²) on 12-well plates coated with gelatin (Type A from pig skin; Sigma). Myoblasts were grown in complete medium (DMEM/Ham F12, 2% Ultrosor G, 20% fetal calf serum, penicillin, streptomycin and L-glutamine) on gelatine-coated plates. After 1 week, wells containing myoblasts without contaminating fibroblasts were trypsinized, pooled and expanded. Experiments were performed on cells kept in culture for not more than 1 month (6-15 passages). Complete medium was changed every 2 days, and cultures were trypsinized before subconfluence to avoid differentiation. To differentiate

muscle cells, myoblasts were plated on Matrigel-coated dishes at 30,000 cells cm⁻² in DMEM/Ham F12 containing 2% horse serum.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously^{5,30} on mice subjected to 4hr at RT vs 4°C (for BAT) or 24hr fasted vs 20hr fasted and then 4hr refed. Briefly, proteins from BAT and muscle tissues were formaldehyde cross-linked to DNA. After homogenization, lysis and DNA sonication, proteins were then immunoprecipitated using purified IgGs, anti-E2F1, anti-pRb or anti-acetylated H4 antibodies. After washing, DNA-protein-complexes were subsequently eluted and cross-linking was reversed by heating the samples at 65°C for 16 hr. DNA was then purified using Qiagen PCR purification kit (Qiagen, Courtaboeuf, France) and Q-PCR was performed using promoter-specific primers (Supplementary Table 1). As a negative control, tissues from *E2f1*^{-/-} mice were used and Q-PCR analysis demonstrated no binding of E2F1 on its target genes.

DNA methylation analysis

Methylation status of *Dhfr* and *Tk* promoters were analyzed *in silico* using <http://www.urogene.org/methprimer/> and *in vivo* on isolated genomic DNA using the MethylCollector kit (Active Motif, Rixensart, Belgium) following manufacturer's instructions. Briefly, DNA was extracted from GN muscle using DNeasy Blood and Tissue Kit (Qiagen). 4 µg of DNA was digested 2 hours at 37°C by *MseI* and incubated with purified methyl-CpG binding domain 2b (MBD2b) proteins. DNA-protein complexes were captured by using magnetic beads and DNA was recovered after washing and elution. Methylation status of the promoter was subsequently analyzed by PCR using promoter-specific primers.

Statistical analysis

Data are presented as means SEM. Statistical analyses were performed with unpaired Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Energy expenditure, adaptative thermogenesis, mitochondrial function, and physical activity

(a) Energy expenditure (VO₂). n=4 animals/group.

(b) Respiratory exchange ratio (V_{CO2}/V_{O2}) during total, light and dark phase of the experiment. n=5 animals/group.

(c) Rectal temperatures under room temperature, fed (control), cold, or fasted conditions. n=4 animals/group.

(d) Mitochondrial DNA (mtDNA) measured relative to nuclear DNA in BAT tissues of *E2f1*^{+/+} and *E2f1*^{-/-} mice. n=5 animals/group.

(e) Mitochondrial oxygen consumption without (-) and with (+) succinate. n=4 animals/group.

(f) Mitochondrial DNA (mtDNA) content in *E2f1*^{+/+} and *E2f1*^{-/-} muscles. n=5 animals/group.

(g) GN mitochondrial O₂ consumption measured after electrotransfer of pCMV or pCMV-E2F1, without (-) and with (+) ADP and succinate. n=4 animals/group.

(h) Relative gene expression of gastrocnemius myosin heavy chain (MyHC) type I, IIa, IIX, and IIb. Results were normalized to the expression of mouse 18S RNA. n=7 animals/group.

(i) Immunofluorescence analysis of serial gastrocnemius sections showing expression of MyHC I, MyHC IIa and MyHC IIb (red) in fibers. Nuclei are stained with Hoechst reagent. Percentage of positive stained fibers is indicated. Scale bars, 100 μm.

(j) *E2f1*^{+/+} and *E2f1*^{-/-} mice were tested for physical endurance. Individual animal performances are shown. n= 11 *E2f1*^{+/+} animals; n= 14 *E2f1*^{-/-} animals.

(k) The effect of E2F1 rescue in *E2f1*^{-/-} mice was evaluated with an endurance test. Individual performances are represented for *E2f1* wild type electroporated with empty vector (*E2f1*^{+/+} pCMV), knock-out (*E2f1*^{-/-} pCMV) and knock-out-rescued animals (*E2f1*^{-/-} pCMV-E2F1). n= 4 animals for *E2f1*^{+/+} pCMV group; n= 3 animals for *E2f1*^{-/-} pCMV group; n= 3 animals for *E2f1*^{-/-} pCMV-E2F1 group.

Values represent means ± SEM. *p<0.05. **p<0.01.

Figure 2. Increased *E2f1*^{-/-} oxidative metabolic gene expression

(a, b) Relative expression of relevant mitochondrial genes in (a) brown adipose tissue (BAT) and (b) gastrocnemius (GN). Results were normalized to mouse 18S RNA expression. n=7 animals/group.

(c) Relative expression levels of oxidative genes in *E2f1*^{-/-} GN electroporated with an empty vector (pRNAT-control) or a plasmid expressing shRNA targeting *Ucp2*, *Pparg1a*, *Esrra* or *Pdk4*. mRNA levels are represented relative to 18S mRNA. n=3 animals.

(d) 72h post-electroporation, O₂ consumption was measured in isolated mitochondria from GN using a Clark electrode. O₂ was measured in the absence and presence of succinate and ADP. n=3 animals.

Values represent means $\pm$ SEM. *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Cold and fasting modulate gene expression through the pRb-E2F1 complex

(a,b) Q-PCR quantification of the expression of relevant genes involved in oxidative metabolism in (a) BAT and (b) gastrocnemius of $E2f1^{+/+}$ and $E2f1^{-/-}$ mice in cold/room temperature and fasted/refed conditions, respectively (See Supplementary Table 1 for sequences). Results were normalized to the expression of mouse 18S RNA and are expressed as means $\pm$ SEM of three independent experiments.

(c, d, e) Immunohistochemistry analysis of pRb phosphorylation on serine 780 (S^{780} p-pRb). (c) BAT was obtained from mice placed at +23° or +4°C (d) GN was obtained from mice fasted for 24hr or fasted for 20hr and refed for 4hr. (e) Mice were fasted for 4hr, injected i.p. with 0.9% NaCl or 0.9% NaCl+isoproterenol and GN were harvested 30 min after injection. Tissues were subsequently processed for immunohistochemistry as described in the methods section. Scale bars, 100 μ m.

(f) Mitochondrial activity, measured as oxygen consumption in $E2f1^{+/+}$ embryonic cells in the presence or absence of isoproterenol and the cdk4 inhibitor PD0332991 as indicated. Results were normalized to protein levels and are expressed as means $\pm$ SEM of four independent experiments per conditions.

(g,h,i) Chromatin immunoprecipitation demonstrates binding of E2F1 and pRb to oxidative metabolic gene promoters in (g) BAT obtained at room temperature (23°) or after 4-h cold exposure (4°C) or in (h-i) muscle in 24hr fasting (F) and 4hr refed (R) conditions. Values represent means $\pm$ SEM of six independent experiments. Immunoprecipitates (IP) were

analyzed by Q-PCR (g, i) or classical PCR (h) with specific primers for the E2F-RE identified in these promoters (See Supplementary Table 1 for sequences). Values represent means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (*) $p < 0.05$ *E2f1*^{+/+} fast (24 hours) vs refed (4 hours); (£) $p < 0.05$ *E2f1*^{+/+} fast vs *E2f1*^{-/-} fast; and (#) $p < 0.05$ *E2f1*^{+/+} refed vs *E2f1*^{-/-} refed.

Figure 4. Increased O₂ consumption, running time and expression of oxidative genes in *CDK4^{R24C/R24C}* mice

(a) Mitochondrial oxygen consumption without (–) and with (+) succinate. *n*=6 animals.

(b) *WT* and *CDK4^{R24C/R24C}* mice were tested for physical endurance. Average running time to exhaustion is shown. *n*= 6 animals/group.

(c) Q-PCR quantification of the expression of relevant genes involved in oxidative metabolism in gastrocnemius of *WT* versus *CDK4^{R24C/R24C}* mice in fasted /refed conditions. Results were normalized to the expression of mouse 18S RNA and are expressed as means ± SEM of three independent experiments. *n*= 3 animals/group.

(d) Chromatin immunoprecipitation demonstrates binding of E2F1 and pRB to oxidative metabolic gene promoters in muscle in 24hr fasting and 5hr refeed conditions. *n*=3 animals. Values represent means ± SEM **p*<0.05; ***p*<0.01; (*)*p*<0.05 *E2f1^{+/+}* fast (24 hours) vs refeed (4 hours); (£)*p*<0.05 *E2f1^{+/+}* fast vs *CDK4^{R24C/R24C}* fast; and (#) *p*<0.05 *E2f1^{+/+}* refeed vs *CDK4^{R24C/R24C}* refeed.

Figure 5. DNA methylation of proliferative target genes modulates E2F1 transcriptional activity in muscle

(a) Q-PCR quantification of the expression of *Dhfr* and *Tk-1* in WT mice in fasted /refed conditions (See Supplementary Table 1 for sequences). Results were normalized to the expression of mouse 18S RNA and are expressed as means $\pm$ SEM. n= 6 animals/group.

(b) Chromatin immunoprecipitation demonstrates binding of E2F1 relative to IgG to proliferative gene promoters in muscle in 24hr fasting or 20hr fasting/4hr refeed conditions. n=3 animals.

(c) Methylation status of *Dhfr*, *Tk-1*, and *Ppargc1a* promoters on CpG island in fasted muscles. PCR analysis of methylated DNA-MBD2b complexes in genomic DNA from muscle. A sample corresponding to genomic DNA before incubation with MBD2b was included in the PCR (Input). As a positive control (+), a PCR reaction using A431 methylated DNA and *Dhfr*, *Tk-1* and *Ppargc1a* promoter specific primers was performed. A negative control (–) was performed using unmethylated Hela genomic DNA as a template and *Dhfr*, *Tk-1* and *Ppargc1a* promoter specific primers.

(d) In basal (fed/room temperature) conditions, E2F1/pRb complexes can repress the oxidative gene program (Oxphos) by switching off those genes. External stimuli activate E2F1 transcriptional activity through the release of pRb, which induces the cell to switch to oxidative metabolism. This molecular mechanism enables the transcription of an oxidative metabolic gene program, allowing the cell to adapt to energy demands and triggers physiological processes such as thermogenesis in BAT or enhanced physical activity in muscle. On the other hand, classical proliferative E2F1 target genes are not subjected to E2F1 regulation due to epigenetic mechanisms.