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Double *Myod* and *Igf2* inactivation promotes brown adipose tissue development by increasing *Prdm16* expression

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ABSTRACT Brown fat or brown adipose tissue (BAT), found in newborn mammals as small depots localized in the interscapular region, plays a prominent role in regulating thermogenesis perinatally. The physiological importance of functional BAT has been recently reasserted in human adults. Because myoblasts and adipoblasts emerge from a common mesodermal precursor, we investigated developmental determination and the reciprocal relationship between muscle and adipocyte commitment. Here we show that a mutant mouse defective for both *Igf2* and *Myod* genes exhibits massive BAT hypertrophy compared with wild-type and single-mutant newborns. The increased adipocyte proliferation in BAT of double-mutant newborns was associated with overexpression of the brown fat-specific marker *Ucp1*. More strikingly, expression of the master key gene *Prdm16* involved in the switch between myogenic and brown adipogenic lineages was drastically enhanced. We further demonstrate that concomitant *Myod* and *Igf2* inactivation accelerates differentiation of a brown preadipocyte cell line and induces lipid accumulation and increased *Ucp1* and *Prdm16* expression. This *in vitro* approach brings additional support for the implication of both *Myod* and *Igf2* in BAT development. These results provide the first *in vivo* evidence that a myogenic regulator together with a growth factor act simultaneously but through independent pathways to repress *Prdm16*, which opens potential therapeutic perspectives for human metabolic disorders.—Borensztein, M., Viengchareun, S., Montarras, D., Journot, L., Binart, N., Lombès, M., Dandolo, L. Double *Myod* and *Igf2* inactivation promotes brown adipose tissue development by increasing *Prdm16* expression. *FASEB J.* 26, 4584–4591 (2012). www.fasebj.org

Key Words: adipogenesis • transcription factors • UCP1 • differentiation

ADIPOSE TISSUES CONTAIN two distinct types of fat, white and brown. White fat cells are associated with chemical storage of energy, whereas brown fat cells dissipate chemical energy in the form of heat. Adipose tissue is of mesodermal origin, as are muscle, bone, and cartilage. Its differentiation is controlled by multiple factors, such as peroxisome proliferator-activated receptor (PPAR) γ 2, ZFP423, and CCAAT/enhancer-binding protein (C/EBP), that are adipose-specific genes for both white and brown fat (1, 2).

Brown fat or brown adipose tissue (BAT) is found in newborn mice as small depots in the interscapular region. Its development starts around embryonic day 15.5 (E15.5) continues until the end of gestation in rodents. It plays a prominent role in regulating thermogenesis in mammals (3, 4). In humans, brown fat is present at birth, but recent evidence has shown that BAT is also present in adults and may play a role in protecting against obesity (5–8).

It was long hypothesized that both white adipose tissue (WAT) and BAT had a common embryonic origin. More recently, it was suggested that in fact white and brown fat cells had distinct origins. Cell fate experiments using the *Engrailed-1* (*En-1*) gene showed that cells expressing this gene in the dermomyotome of the early embryo (E9.5) gave rise not only to some epaxial muscles but also to BAT in the interscapular

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Abbreviations: BAT, brown adipose tissue; C/EBP, CCAAT/enhancer-binding protein; DM, double mutant; E, embryonic day; IRS1, insulin receptor substrate-1; MRF, myogenic regulatory factor; PPAR, peroxisome proliferator-activated receptor; SM, single mutant; UCP1, uncoupling protein-1; WAT, white adipose tissue; WT, wild-type

dorsal cervical region (9, 10). It was then discovered, on differentiation of white and brown preadipocytes from primary cultures, that brown fat cells could express myogenic regulatory factors (MRFs), such as MYOD, MYF5, and MYOGENIN (7, 11). Finally, the PRDM16 transcription factor was found to be an essential player in brown adipocyte determination (12). On inactivation of *Prdm16*, brown adipocytes produce *in vitro* long fibers expressing myogenic genes, suggesting that this gene could act in a cell autonomous manner to induce a switch between skeletal myoblasts and brown fat cells. In addition, another cell fate experiment has shown that *Myf5*-expressing cells give rise to both skeletal muscle cells and brown adipocytes in the BAT (13). Taken together, these recent data suggest that muscle and adipocytes share a common embryonic origin. In *Myf5*-positive progenitors, PRDM16 represses the expression of myogenic genes and induces the differentiation of BAT (14).

PRDM16 plays its regulatory function by binding to the nuclear factors proliferator-activated receptor γ coactivator (PGC)-1 α and PGC-1 β , which then activate genes such as *Ppar γ* and *C/ebp*. BAT is characterized by the expression of the late differentiation marker uncoupling protein-1 (UCP1), a protein responsible for the uncoupling of the respiratory chain in mitochondria of brown adipocytes (1, 15).

In our study, we produced a double mutant (DM) lacking both the *Igf2* and *Myod* genes. These mutant mice die at birth of respiratory failure, due to atrophy of their diaphragm (unpublished results). On dissection of the DM E18.5 embryos, we surprisingly discovered an increase in the brown fat mass in the interscapular region. We investigated this phenotype further and compared DMs with single *Igf2* and *Myod* mutants and with wild-type (WT) embryos. Our results show that both *Myod* and *Igf2* genes are required for proper control of BAT development in newborn mice.

To further validate the *in vivo* results, we performed knockdown experiments of *Myod* and *Igf2* transcripts using a siRNA strategy in a brown preadipose cell line that was induced to differentiate. Double si-*Igf2*/si-*Myod* cells showed accelerated differentiation compared with single siRNA-treated cells and control cells. Collectively, our findings reveal an essential role for both the *Myod* and *Igf2* genes *via Prdm16* expression in the control of brown adipocyte proliferation and differentiation.

MATERIALS AND METHODS

Mouse strains

All experimental procedures are in agreement with guidelines of the animal ethics committee of the Ministère de l'Agriculture (France). *Myod*^{-/-} mice were bred on a C57BL/6/CBA outbred background and maintained as heterozygous females and males. The *Igf2*^{-/-} strain was on a 129/Sv background. Matings between *Myod*^{+/-} females and *Myod*^{+/-} *Igf2*^{+/-} males were plugged to collect E18.5 embryos (day of plug E0.5). Offspring genotypes were determined by PCR analysis of tail DNA (Supplemental Table S1).

Embryo collection

E18.5 embryos were fixed in 4% paraformaldehyde and embedded in paraffin; 5- μ m sections were stained with hematoxylin and eosin.

BAT collection

E18.5 embryos were weighed before dissection of the interscapular mass in the dorsal cervical region. BAT was weighed and snap-frozen in liquid nitrogen.

Cell culture and brown adipocyte differentiation

T37i cells were cultured as described previously (16). Differentiation into mature brown adipocytes was achieved by treatment with 2 nM triiodothyronine (Sigma-Aldrich, St. Louis MO, USA) and 20 nM insulin (Sigma-Aldrich) for 5 d.

Gene expression analysis

Total RNA from tissues and cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μ g) treated with DNase I (Invitrogen) was reverse-transcribed using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY, USA). Samples were diluted 10-fold, and 1/20 of the RT reaction was used for quantitative PCR (qPCR) using Fast SYBR Green Master Mix (Life Technologies, Foster City, CA, USA) or using the TaqMan assay for *Myod* in T37i (probe 4331182; Life Technologies) with 300 nM concentrations of specific primers (Supplemental Table S1) and performed on a StepOnePlus Real-Time PCR System (Life Technologies). Amplicons of each gene were subcloned into pGEM-T-Easy plasmid (Promega, Madison, WI, USA) and used as standards with serial dilutions spanning 6 orders of magnitude. Data are presented as means \pm se. The level of gene expression was normalized to the geometric mean of the expression level of *Tbp*, *Gapdh*, and 18S housekeeping genes with geNorm 3.4 software (17).

Gene expression knockdown by siRNA

T37i cells were seeded at a density of 0.6×10^5 cells in 12-well plates. On the following day, cells were incubated for 3 h in Opti-MEM serum-free medium (Life Technologies) before transfection with 180 pmol of siRNA using Lipofectamine RNAiMAX (Life Technologies). Stealth Select RNAi sequences for *Myod*, *Igf2*, and scramble siRNA (medium GC concentration) are presented in Supplemental Table S1. Cells were cultured for 2 d before induction of adipocyte differentiation in the presence of insulin and triiodothyronine. Total RNA was extracted at d 1 and d 4. Oil Red O staining was performed at d 5.

Oil Red O staining

Cells were fixed for 1 h in 10% buffered formalin and stained for 1 h with Oil Red O solution [0.6% (w/v) in isopropanol-water (60:40)]. After extensive washing to remove unbound dye, cells were photographed and solubilized in 10% SDS, and optical density was measured at 520 nm.

Statistical analysis

Statistical significance of the different experiments was determined using a Kruskal-Wallis test followed by *post hoc* paired

comparisons or a Mann-Whitney test, using Prism 5.0 software (GraphPad, San Diego, CA, USA). Results were considered statistically significant at values of $P < 0.05$ compared with WT or scramble.

RESULTS

BAT is increased in $Myod^{-/-}$ $Igf2^{+/-}$ DM embryos

We developed DM mice lacking both *Myod* and *Igf2* genes to analyze their phenotype and their main characteristics. Because *Igf2* is imprinted, a paternal heterozygous mouse ($Igf2^{mat+/pat-}$) does not express *Igf2*. Thus, mating between a heterozygous *Myod* female ($Myod^{+/-}$) and a heterozygous male ($Myod^{+/-}Igf2^{+/-}$) will produce the 4 genotypes of interest in the same litter: WT, $Igf2^{+/-}$ mutant, $Myod^{-/-}$ mutant, and $Myod^{-/-}Igf2^{+/-}$ DM.

On dissection of the embryos, we discovered a strong hypertrophy of the BAT mass in the interscapular region in DM compared with WT or single mutant (SM) newborns. Histological examinations of E18.5 hematoxylin and eosin-stained sections confirmed the BAT enlargement in DM embryos (Fig. 1A). However, a slight increase of BAT in $Igf2^{+/-}$ embryos was also

noted compared with that in WT and $Myod^{-/-}$ embryos. The BAT weight of E18.5 embryos of each genotype was compared with their body weight, because the $Igf2^{+/-}$ and DM embryos are 40% smaller than the WT or $Myod^{-/-}$ mutants, as reported previously (18).

In WT embryos, whereas interscapular BAT represented 0.9% of the total body mass (Fig. 1A, bottom panel), BAT weight of $Igf2^{+/-}$ mutants displayed a significant difference compared with BAT weight of WT and $Myod^{-/-}$ embryos (+25%). More interestingly, the DM BAT showed a striking increase in weight of 64% compared with the BAT weight of their WT or $Myod^{-/-}$ littermates. The DM interscapular BAT therefore represented >1.5% of the total body mass (Fig. 1A, bottom panel). Absence of IGF2 protein therefore induces a weak but significant BAT mass enlargement, yet this was clearly enhanced by the simultaneous deletion of the *Myod* gene.

To examine whether a compensation for these two genes occurs in BAT, we measured *Igf2* and *Myod* expression by RT-qPCR in E18.5 WT, $Igf2^{+/-}$, $Myod^{-/-}$, and DM BAT (Fig. 1B). No obvious difference was observed, either for *Igf2* expression in $Myod^{-/-}$ embryos or for *Myod* expression in $Igf2^{+/-}$ embryos. These

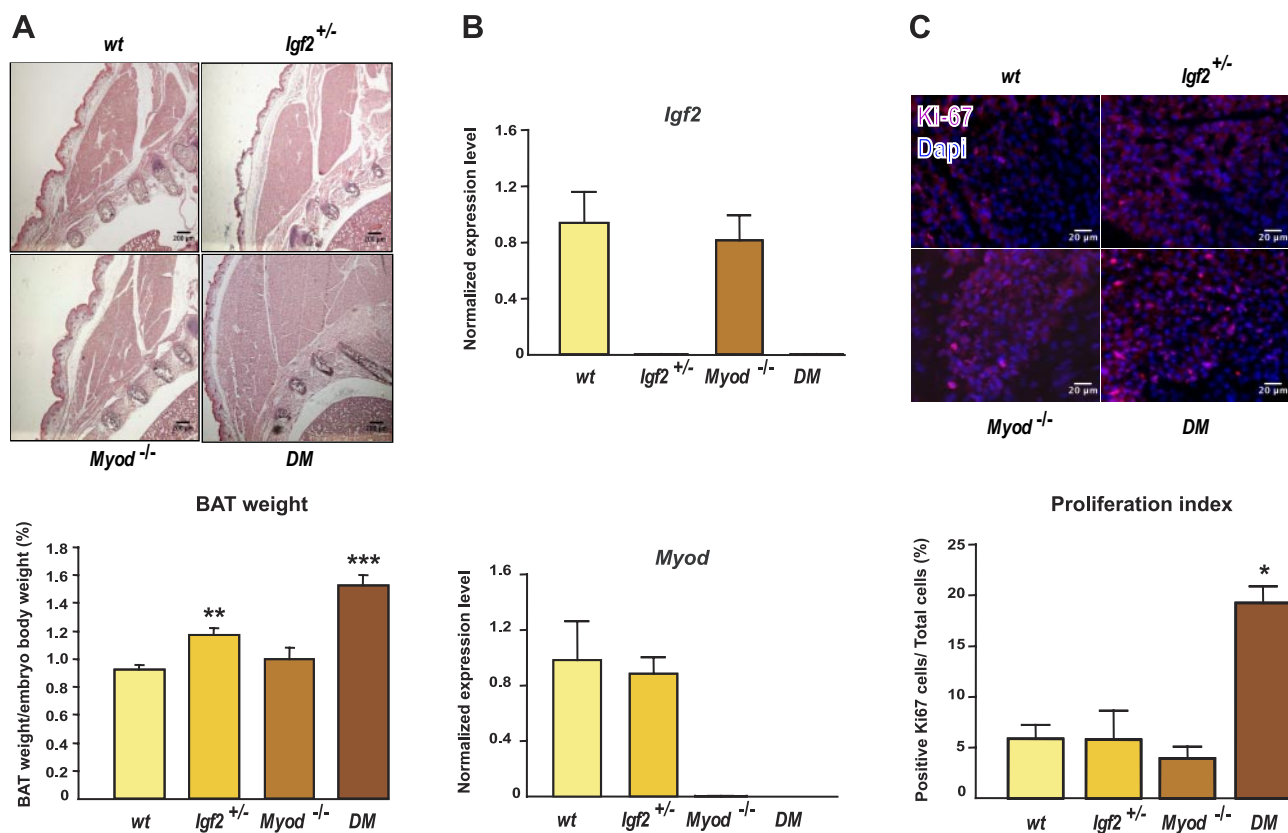


Figure 1. BAT hypertrophy in E18.5 WT and mutants. A) Sections stained with hematoxylin and eosin are shown for WT, *Igf2* SM ($Igf2^{+/-}$), *Myod* SM ($Myod^{-/-}$), and DM ($Myod^{-/-}Igf2^{+/-}$) BAT ($n=5$ /genotype). BAT was dissected from the embryos and weighed. Graph represents the ratio of BAT weight to embryo body weight. Numbers of BAT samples are 20 for WT, 17 for $Igf2^{+/-}$, 6 for $Myod^{-/-}$, and 10 for DM. B) RT-qPCR was performed on E18.5 BAT from WT, SM, and DM mice ($n=5$ /genotype) using primers for the *Igf2* gene (top panel) and *Myod* gene (bottom panel). C) Nuclei of BAT sections stained with antibodies to Ki67 (red) and DAPI (blue) for WT, *Igf2* SM, *Myod* SM, and DM mice ($n=4$ /genotype). Graph presents the ratio of Ki67-positive cells to total cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. WT.

results indicate a lack of genetic compensation in this tissue.

To evaluate the important BAT hypertrophy in DM embryos, Ki67 labeling was used to assess cell proliferation status in E18.5 embryos (Fig. 1C). The ratio of Ki67-positive nuclei over total cell number showed no difference between WT and SMs. In contrast, in absence of both *Myod* and *Igf2*, cell proliferation was increased 4-fold. This high proliferation index thus constitutes a key feature of the DM hypertrophy. The number of adipocytes per field was also evaluated at high magnification and showed no difference among samples (Supplemental Fig. S1). Therefore, the adipocyte size is probably not modified between the different genotypes, suggesting that their number may be greatly increased in the DM embryos. Taken together with the increased mass of the DM BAT, we can conclude that the double knockout of both *Myod* and *Igf2* genes results in hyperplasia of the brown adipocytes.

Expression of MRFs in SM and DM BAT

It has been well established that the absence of MYOD in skeletal muscle is rescued by overexpression of the other MRFs, especially MYF5 (19, 20). Because a common origin has been proposed for myoblasts and brown adipocytes, we investigated whether such genetic compensation also occurs in BAT. The expression of *Myf5*, *Myogenin*, and *Mrf4* was evaluated by RT-qPCR in E18.5 BAT from WT, *Igf2*^{+/-}, *Myod*^{-/-}, and DM mice (Fig. 2). Interestingly, *Myf5* and *Myogenin* genes are overexpressed in the absence of *Myod*. Surprisingly, this genetic compensation of myogenic regulators in the BAT was not observed in DM mice (Fig. 2A, B), indicating that IGF2 is required for BAT *Myf5* and *Myogenin* overexpression in *Myod*^{-/-} mice. In sharp contrast, *Mrf4* expression was comparable between *Igf2*^{+/-}, *Myod*^{-/-}, and WT newborns, whereas it was significantly down-regulated (-65%) in the absence of

both *Myod* and *Igf2*. This finding suggests that *Myod* and *Igf2* act coordinately according to parallel independent pathways to activate *Mrf4*.

Prdm16 and *Ucp1* expression is strongly increased in DM BAT

To decipher the molecular mechanisms implicated in BAT hypertrophy, we evaluated expression levels of specific markers of BAT differentiation by RT-qPCR on BAT samples. Comparable levels of *C/ebpβ* and *Pparγ2* were observed in WT, SM, or DM BAT, suggesting that brown adipocyte commitment is not affected in the DM embryos (Fig. 3A, B).

We then focused on the *Prdm16* and *Ucp1* genes, which are, respectively, early and mature specific brown fat markers. In striking contrast, a 4-fold increase in *Prdm16* expression was observed in DM BAT compared with the other genotypes (Fig. 3C). This observation is in accordance with the major role of *Prdm16* in the brown adipogenesis pathway. A 3-fold increase of the *Ucp1* gene expression was also detected in DM BAT, compared with WT and SM BAT (Fig. 3D). This result confirms that combined expression of both *Myod* and *Igf2* is essential to control BAT proliferation.

Down-regulation of *Myod* and *Igf2* transcripts leads to an acceleration in brown preadipocyte differentiation

To exclude potential systemic counterregulatory mechanisms and to confirm the direct involvement of *Myod* and *Igf2* in the regulation of BAT development, we used the brown adipocyte T37i cell line (16) and knocked down either *Myod* or *Igf2* or both by a siRNA strategy. This cell line is a preadipocyte model for which differentiation into mature brown adipocytes can be triggered by addition of insulin and triiodothyronine. Efficiency of the knockdown of *Igf2* and *Myod* was evaluated by RT-qPCR (Fig. 4A, B). At 3 d after siRNA treatment, a reduction of ~90% of the *Igf2* transcript level in the presence of the si-Igf2 (Fig. 4A) and 85% of

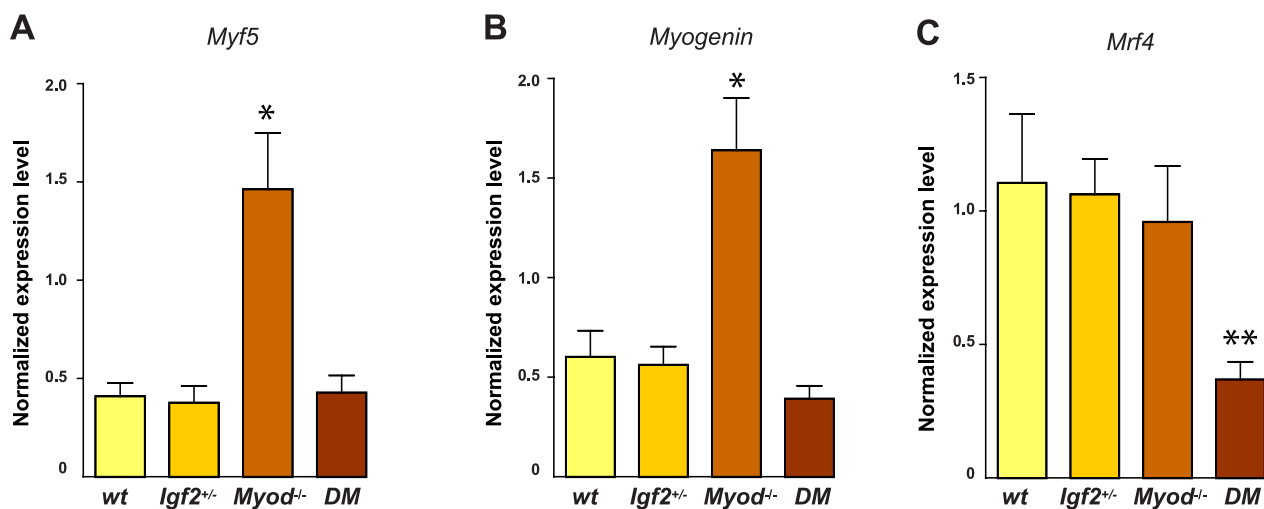


Figure 2. Expression levels of specific muscle genes. RT-qPCR was performed on E18.5 BAT from WT, SM, and DM mice ($n=5$ /genotype) using primers for the *Myf5* gene (A), *Myogenin* gene (B) and *Mrf4* gene (C). * $P < 0.05$, ** $P < 0.01$ vs. WT.

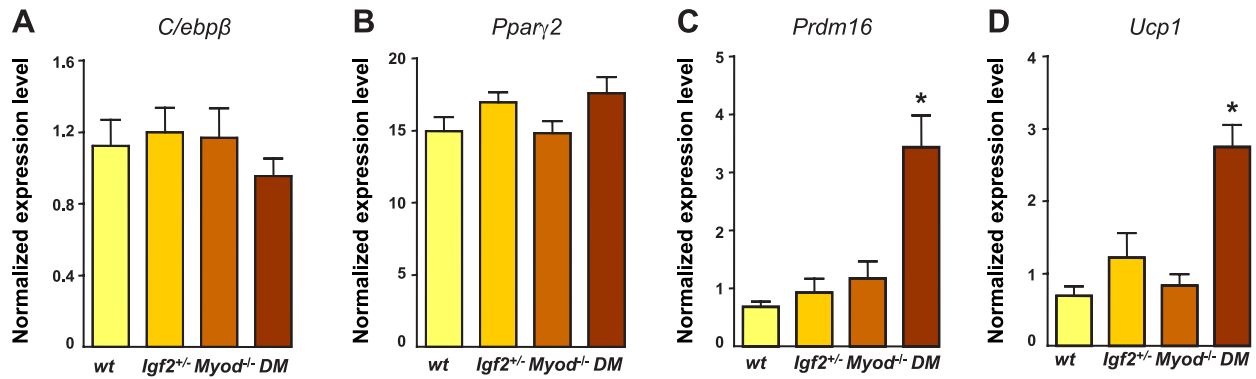


Figure 3. Expression levels of brown adipocyte genes in BAT. RT-qPCR was performed on E18.5 BAT from WT, SM, and DM mice ($n=5$ /genotype) using primers for the *C/ebpβ* gene (A), *Pparγ2* gene (B), *Prdm16* gene (C), and *Ucp1* gene (D). * $P < 0.05$ vs. WT.

the *Myod* transcript level in the presence of si-Myod (Fig. 4B) was observed. Interestingly, *Myod* and *Igf2* expressions are not modified in the absence of each other. This absence of compensation mirrors the *in vivo* situation.

As shown in Fig. 4C, D, *Myod* or *Igf2* gene inactivation alone did not significantly modify T37i cell differentiation programming, as evidenced by quantification of Oil Red O staining compared with that of scrambled siRNA-treated cells. In sharp contrast, the combination of *Myod* and *Igf2* siRNA led to a drastic acceleration of brown adipocyte differentiation, with marked lipid accumulation as early as d 5 after exposure to the induction cocktail. We next quantified gene expression of early and late adipocyte

markers along with the differentiation process of T37i cells transfected with either scramble siRNA, *Myod*, or *Igf2* siRNA, or both siRNAs (Fig. 5). After 1 d of differentiation (Fig. 5, D1), a significant increase in the expression of early genes, such as *C/ebpβ*, *Pparγ2*, and *Prdm16*, was observed in double-knockdown T37i cells, consistent with a premature adipocyte commitment. Accordingly, 4 d later (Fig. 5, D4), *Prdm16* and *Ucp1* expression was markedly enhanced in double-knockdown T37i cells compared with that in controls, indicating that simultaneous lack of MYOD and IGF2 promotes early brown adipogenesis. These results strongly support the *in vivo* observations. Taken together, these findings provide evidence for the involvement of both *Myod* and *Igf2*

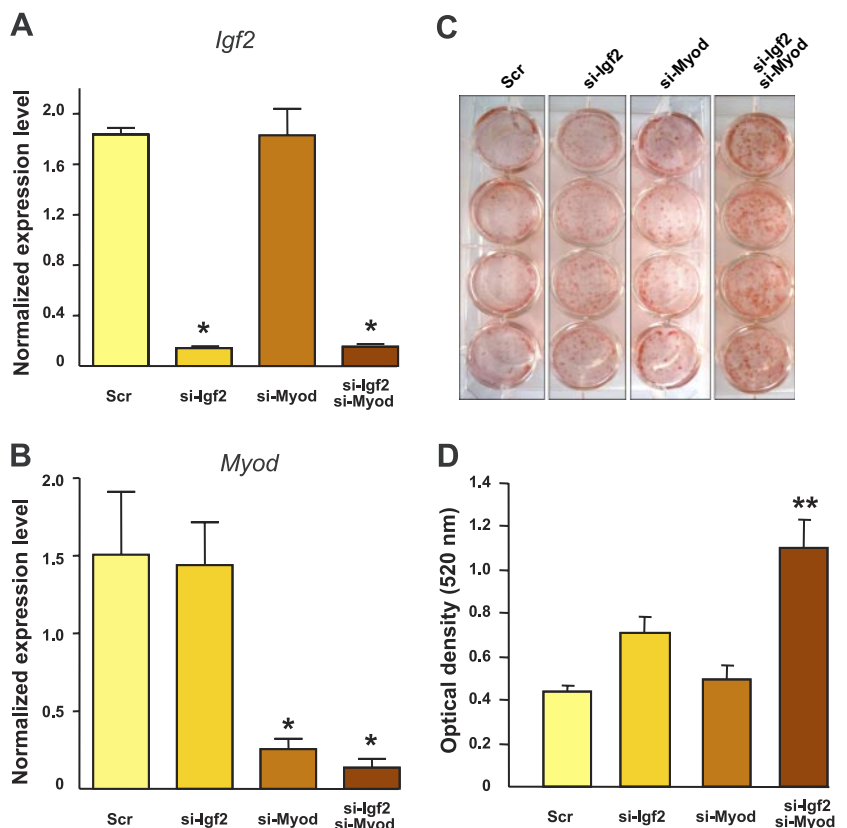


Figure 4. Knockdown of *Myod* and *Igf2* enhances brown adipocyte differentiation *in vitro*. A, B) Efficiency of knockdown was evaluated by RT-qPCR performed on 4 samples from scramble (Scr), si-Igf2, si-Myod, and si-Igf2 si-Myod T37i cells after 1 d of differentiation. Expression of the *Igf2* gene (A) and *Myod* gene (B) was analyzed. C) Adipocyte differentiation was evaluated by Oil Red O staining on Scr, si-Igf2, si-Myod, and si-Igf2 si-Myod T37i cells after 5 d of differentiation ($n=4$ /cell type). D) Optical density was measured at 520 nm and is represented as a graph. * $P < 0.05$, ** $P < 0.01$ vs. Scr.

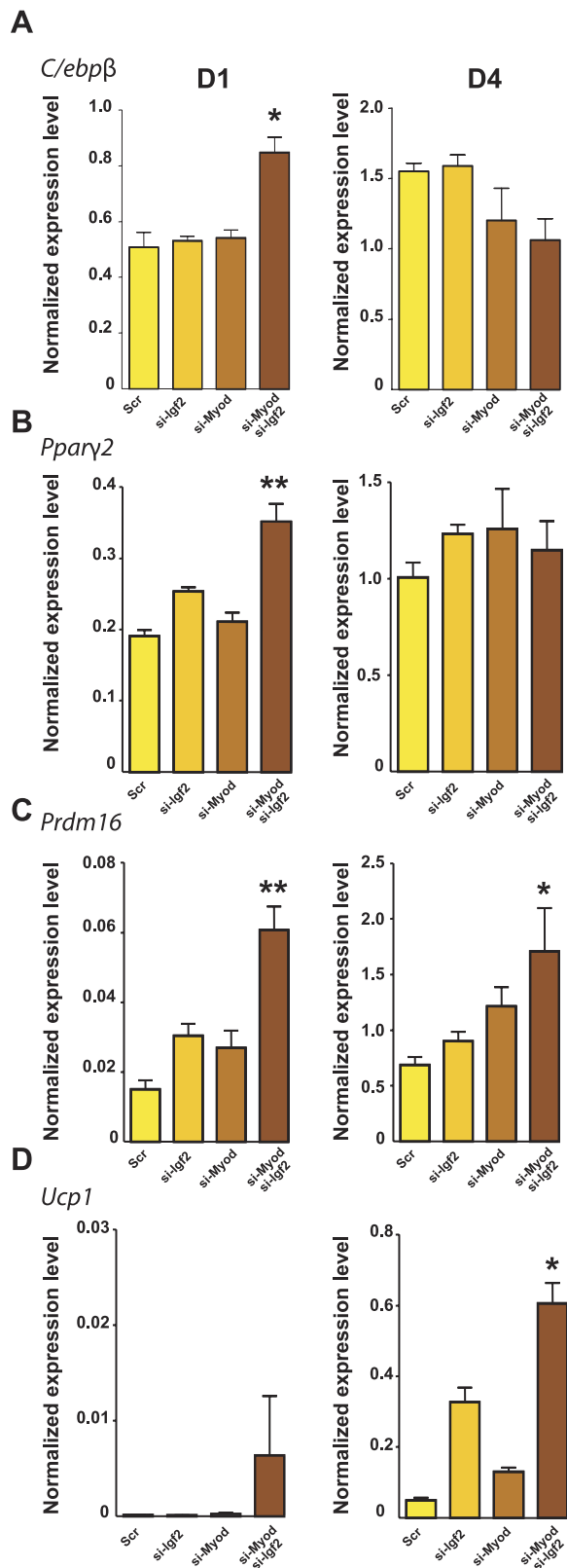


Figure 5. Knockdown of *Myod* and *Igf2* leads to early expression of brown adipocyte genes. RT-qPCR was performed on 4 samples from scramble (Scr), si-Igf2, si-Myod, and si-Igf2 si-Myod T37i cells after 1 and 4 d of differentiation. Expression of the *C/ebpβ* gene (A), *Pparγ2* gene (B), *Prdm16* gene (C), and *Ucp1* gene (D) was analyzed. * $P < 0.05$, ** $P < 0.01$ vs. Scr.

signaling pathways in the regulatory mechanisms governing brown adipocyte differentiation.

DISCUSSION

Increasing evidence suggests the existence of a developmental switch between myogenic and adipogenic commitment. Until recently, brown and white fat adipocytes were thought to be derived from the same precursor cell. There is now clear evidence that brown fat adipocytes arise from a separate and distinct population of progenitor cells (4, 7). Interestingly, this progenitor population gives rise to both myocytes and brown adipocytes, originating from a common *Myf5*-expressing precursor. The relationship between myogenic and adipogenic commitment was evidenced many years ago by phenotypes observed in mouse mutants invalidated for the MRFs. *Myod* and *Myf5* gene invalidation leads to a loss of muscle development replaced with adipose tissue (21). *Myogenin* gene invalidation results in nonviable embryos in which a large BAT depot was observed compared with that in control embryos (22).

Preadipocytes express these MRFs, such as MYF5, MYOD, and MYOGENIN, which are down-regulated on differentiation into adipocytes (23). The PRDM16 transcription factor is thought to be the key factor for adipogenesis because its expression in myogenic precursor cells induces their transformation into brown adipocytes. PRDM16 has been proposed as the regulator of the brown adipocyte fate by inhibiting expression of the MRFs and activating specific brown fat genes (13). How important is it to control the expression of myogenic factors to induce the brown fat pathway?

By investigating the effect of the two myogenic factors IGF2 and MYOD *in vivo*, we have been able to show their major role in controlling BAT development. Our study has revealed that newborn mice lacking both *Igf2* and *Myod* genes display an unexpected hypertrophy of BAT compared with that in WT and SM newborns, although *Igf2*^{+/-} mice already display a slight increase in BAT mass. The combined expression of these two genes therefore appears to play a major role in the control of the commitment toward brown adipogenesis.

Cell proliferation is strongly increased in DM BAT as shown by the Ki67 index. The presence of markers such as PPAR γ 2 and C/EBP β indicates that this hypertrophic mass is of adipogenic origin and the high level of UCP1 confirms its brown fat origin. We propose that *Igf2* and *Myod* play an important role in controlling the development of BAT by reducing cell proliferation. Whether the observed enlargement of DM BAT is due to recruitment of new cells or expansion of preexisting progenitors would require analysis of earlier embryonic stages. However, because the levels of *Myf5*, considered as a marker of the mesenchymal progenitors at the origin of brown adipocytes (12, 14), are similar in WT and DM BAT, expansion of preexisting progenitors is the most likely hypothesis.

Because of the proposed function of the *Prdm16* gene in controlling brown adipocyte fate, we investigated the level of expression of this gene in WT, SM, and DM

BAT. Its strong increase in expression in DM newborns suggests that this gene is under the control of both *Igf2* and *Myod* genes. Interestingly, only the combination of the two factors leads to this increased expression. Because the SMs do not display this strong hypertrophy of BAT nor increased expression of *Prdm16*, it can be concluded that IGF2 and MYOD act according to parallel independent pathways to control BAT size.

Based on published chromatin immunoprecipitation sequencing data (24), we searched for MYOD binding sites but found no direct binding either on the promoter or on the gene body of *Prdm16*. These data were available in myotubes in which *Prdm16* expression is repressed. We cannot, however, exclude either an indirect repressive effect of MYOD or binding on a long-range regulatory element of *Prdm16* in adipocytes.

Interestingly, whereas a general decrease in expression of all MRFs was observed in differentiating adipocytes, our data in *Myod*^{-/-} BAT now suggest that the reduction in expression of *Myf5* and *Myogenin* are under the control of MYOD. Furthermore, whereas *Myf5* and *Myogenin* expression levels are barely affected in DM BAT, *Mrf4* expression seems to be strongly reduced. Whether this reduction is a cause or a consequence of BAT hypertrophy remains unknown. This leads to two possibilities: either *Mrf4* expression is tightly regulated by the combined expression of *Igf2* and *Myod*, or *Mrf4* is under the direct control of the *Prdm16* gene. Nevertheless, both *Mrf4* and *Prdm16* are involved in the control of BAT determination. This finding also emphasizes the need to repress *Mrf4* gene expression to engage in adipogenesis (Fig. 6).

To our knowledge, very little is known about the influence of *Igf2* on BAT development. IGF2 is a fetal growth factor, which is repressed after birth and replaced by IGF1 (25, 26). A mouse model in which *Igf2* expression is reduced but not absent displayed decreased BAT development (27). We must therefore assume that other factors, such as IGF1, take over at birth and participate in BAT development.

Interestingly, it was recently shown that insulin receptor substrate-1 (IRS1) is involved in the maturation of adipocytes, implicating the AKT/PI3K pathway (28). IRS1 is a downstream target of IGF1/INS1 receptors. Because these receptors also bind IGF2, this could explain how IGF2 is involved in adipogenesis during the fetal period and how it could act as an upstream regulator of this AKT/PI3K pathway. Because IGF2 plays a dual role in proliferation and differentiation, it was not clear whether the BAT hypertrophy was due only to increased proliferation or also to altered differentiation. We therefore set up an *in vitro* model to follow brown adipocyte differentiation in preadipocyte cells. Interestingly, *Myod* and *Igf2* knockdown by siRNA shows a phenotype similar to that of the *in vivo* model. Si-Igf2 and si-Myod preadipocytes present a higher number of lipidic droplets after 5 d of culture compared with scramble and single siRNA samples, as well as an increase in *Prdm16* and *Ucp1* expression. Furthermore, *in vitro* differentiation revealed an earlier expression of differentiation genes such as *C/ebpβ* and *Pparγ2* only in the double-siRNA-treated cells.

In summary, our *in vivo* and *in vitro* studies have revealed a combined role of *Myod* and *Igf2* genes during

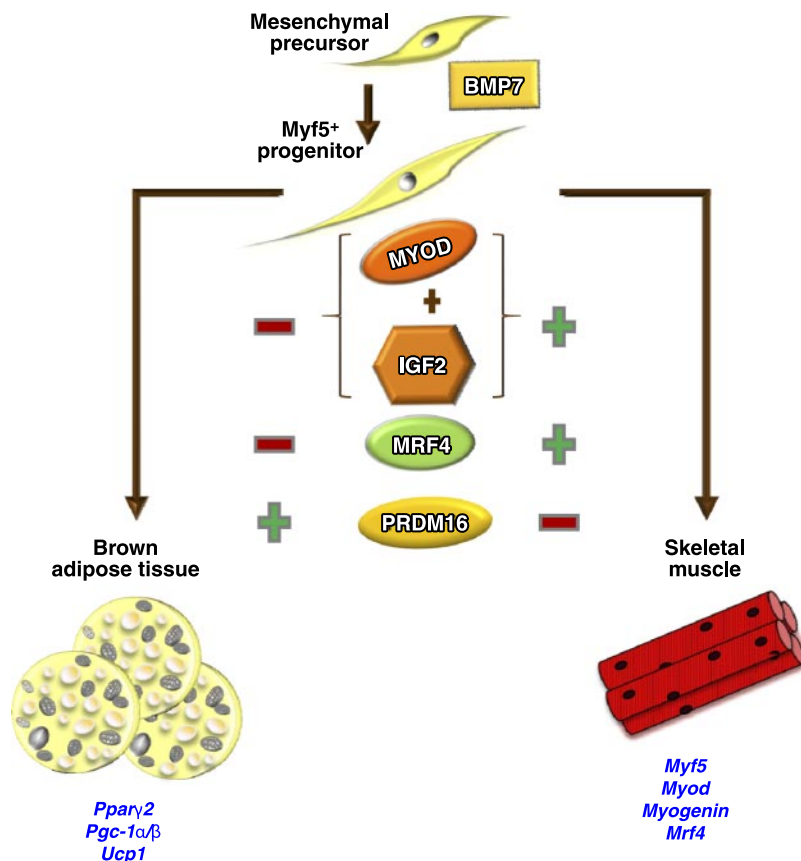


Figure 6. Model of brown adipocyte pathway under the control of the *Prdm16* regulator. MYOD and IGF2 act coordinately to repress PRDM16 in BAT formation and activate the skeletal muscle pathway.

brown adipocyte proliferation and differentiation. These two genes act coordinately to repress BAT development as shown both in the *in vivo* DM model and in the *in vitro* knockdown cell culture system. Because the expression of the *Prdm16* “brown master gene” is strongly up-regulated in the absence of these two genes, we propose that MYOD and IGF2 act as upstream regulators of PRDM16 to control proliferation of BAT during development.

BAT has been shown to regulate energy balance and to play a role in fighting against obesity in rodents. It may also play a significant role in the physiology of human adults. Better knowledge of the brown lineage is therefore an essential step in the control of obesity. Investigating mechanisms that control the timing and specificity of *Prdm16* expression during development is therefore an important health issue. FJ

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