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An essential role for Tbx15 in the differentiation of brown and "brite" but not white adipocytes

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Gburcik V, Cawthorn WP, Nedergaard J, Timmons JA, Cannon B. An essential role for Tbx15 in the differentiation of brown and "brite" but not white adipocytes. *Am J Physiol Endocrinol Metab* 303: E1053–E1060, 2012. First published August 21, 2012; doi:10.1152/ajpendo.00104.2012.—The transcription factor Tbx15 is expressed predominantly in brown adipose tissue and in those white adipose depots that are capable of giving rise to brown-in-white ("brite"/"beige") adipocytes. Therefore, we have investigated a possible role here of Tbx15 in brown and brite adipocyte differentiation in vitro. Adipocyte precursors were isolated from interscapular and axilliary brown adipose tissues, inguinal white ("brite") adipose tissue, and epididymal white adipose tissue in 129/Sv mouse pups and differentiated in culture. Differentiation was enhanced by chronic treatment with the PPARγ agonist rosiglitazone plus the sympathetic neurotransmitter norepinephrine. Using short interfering RNAs (siRNA) directed toward Tbx15 in these primary adipocyte cultures, we decreased Tbx15 expression >90%. This resulted in reduced expression levels of adipogenesis markers (PPARγ, aP2). Importantly, Tbx15 knockdown reduced the expression of brown phenotypic marker genes (PRDM16, PGC-1α, Cox8b/Cox4, UCP1) in brown adipocytes and even more markedly in inguinal white adipocytes. In contrast, Tbx15 knockdown had no effect on white adipocytes originating from a depot that is not brite competent in vivo (epididymal). Therefore, Tbx15 may be essential for the development of the adipogenic and thermogenic programs in adipocytes/adipomyocytes capable of developing brown adipocyte features.

T-box 15; adipocyte differentiation; homeodomain transcription factors; brown fat; obesity

CLASSICALLY, TWO TYPES OF ADIPOSE TISSUE have been defined in mammals: white adipose tissue (WAT), adapted to store energy in the form of large lipid droplets, and brown adipose tissue (BAT), which oxidizes lipids to produce heat through the activity of uncoupling protein 1 (UCP1) found in the inner membrane of the numerous mitochondria in this tissue. However, today it is understood that these two tissues are not closely related. Rather, the classical brown adipocytes (the adipomyocytes) are derived from a common myogenic/brown adipocyte precursor and are found only in the classical BAT depots. The classical white adipocytes (that are not of the myogenic lineage) are now considered to be of two types: the "brite" (brown-in-white, "beige," "ectopic") adipocytes, which upon stimulation by cold acclimation/sympathetic agonists or with, e.g., a PPARγ agonist can differentiate into cells capable of expressing UCP1 [such cells are observed particularly in the subcutaneous inguinal adipose tissue depot (inguinal WAT) of the mouse], and the "true" white adipocytes, principally incapable of expressing UCP1 physiologically, found in visceral depots, e.g., epididymal white fat depots (epididymal WAT) of the mouse (10–12, 16, 18).

In our earlier analysis, in which we compared patterns of gene expression between classical brown and white adipocytes, we observed a series of genes that were expressed at significantly higher levels in the brown adipocytes than in the white adipocytes (16). Many of these genes were of myogenic character, and this allowed us to formulate that brown adipocytes were of a myogenic lineage (16). However, we identified several genes that were relatively highly expressed in brown adipocytes but not uniquely associated with the myogenic lineage, e.g., T-box 15 (Tbx15). Tbx15 belongs to the T-box family of homeodomain transcription factors that are highly conserved and essential for many developmental processes (8).

We subsequently analyzed gene expression patterns in nine defined adipose depots and observed that Tbx15 is expressed predominantly in BAT and in the inguinal white adipose depot (a brite-competent depot), but not in classical white adipose depots such as the epididymal depot (17). This raises the question of the role of Tbx15. Tbx15 could merely be a marker for brown and brite cells, just as the myogenic genes observed in brown adipocyte precursors would seem to be lineage markers, rather than being functionally important for the differentiation of brown adipocytes. However, an interesting possibility would be that Tbx15 is essentially involved in the differentiation process leading to the appearance of the brown phenotype. Therefore, here we have examined the involvement of Tbx15 in the differentiation process of the distinct adipocyte lineages. We conclude that Tbx15 is essential for complete differentiation of brown and brite but not white adipocytes.

METHODS

Animals, cell culture, and small interfering RNA transfection. We used the 129/Sv strain of mice as a source of adipocyte progenitors. In this strain, following cold exposure, UCP1 mRNA levels in subcutaneous adipose tissue approach those in interscapular brown fat (6). The 129/Sv mice (Harlan, UK) were bred at the Royal Veterinary College. Brown adipocyte (BA) precursors were isolated from the pooled interscapular and axillary BAT depots of 3-wk-old mice, and white adipocyte precursors [inguinal (iWA) and epididymal white adipocytes (eWA)] were isolated from inguinal and epididymal adipose depots. All isolated depots were processed as described previously (9). The pellet of precursor cells was suspended in culture medium, and the cells were cultured in six-well plates. Cells were cultured in Dulbecco's modified Eagle's medium with 10% (vol/vol)

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Table 1. *Target sequences for siRNAs used for Tbx15 knockdown*

siRNA	Target Sequence
J-055044-09	AAUGUGGGCUGUCGAGAAA
J-055044-10	GAGUCCACGUGAUCCGCAA
J-055044-11	ACUAUAUAGCAAUGGACAU
J-055044-12	ACUAAUAAUCAGCAGGCUA

siRNA, small interfering RNA; Tbx15, T-box 15.

newborn calf serum (Invitrogen), 2.4 nM insulin, 25 μg/ml sodium ascorbate, 10 mM HEPES, 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin supplemented or not (as indicated) with 1 μM rosiglitazone maleate (EnzoLifeSciences) and 1 μM norepinephrine (Sigma-Aldrich) from the first day in culture (10). The cells were grown at 37°C in an atmosphere of 5% CO_2 in air with 80% humidity. Where indicated, transfection was performed twice, 24 and 90 h after seeding, using 0.32% Lipofectamine 2000 (Invitrogen) and 20 nM small interfering RNA (siRNA) pool (Dharmacon) (Table 1) in 2.5 ml serum-containing cell culture medium (without antibiotics) according to the manufacturer's protocol. Control cells were treated with Lipofectamine. The cells were harvested on *day 6* in culture. Before harvesting, they were examined using phase contrast microscopy (on Leica DMIRB Inverted Microscope).

RNA isolation and quantitative real-time PCR. Total RNA was isolated by TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was dissolved in 20 μl of RNAse-free water and quantified using a Nanodrop (NanoDrop Technologies). The yield was 3–7 μg of total RNA from one well of a six-well plate. For determination of mRNA levels, 1 μg of RNA was reverse-transcribed with a High Capacity cDNA kit (Applied Biosystems) in a total volume of 20 μl. Exon-spanning primers (Invitrogen; Table 2) were premixed with SYBR Green JumpStart *Taq* ReadyMix (Sigma-Aldrich), and aliquots of 10 μl were applied to 384-well optical plates (Applied Bio-Rad). cDNA was diluted 1:10, and aliquots of 2 μl were added. Thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 62°C, and 20 s at 72°C on a CFX384 Real-Time System (Bio-Rad). cDNA from 3T3-L1 adipocytes was obtained as described previously (1). For 18S measurements used for normalization, cDNA was diluted an additional 200 times, and the levels were measured using the Taqman assay according to the manufacturer's protocol. The 18S levels were not systematically influenced by the treatment of the cells with NE/Rosi or with siRNAs against Tbx15 (not shown). The ΔC_T method (gene of interest vs. 18S) was used to calculate relative changes in mRNA abundance. The PCR analyses were run in triplicate.

Western blot. Adipocytes treated as indicated were washed twice in ice-cold PBS and then harvested in a modified RIPA buffer [50 mM Tris·HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF (Complete-Mini; Roche Diagnostics), 1 mM Na_3VO_4, and 1 mM NaF]. Cells were lysed on ice for 15 min and then centrifuged at 14,000 *g* for 15 min. The concentration of proteins in the supernatant was determined by Nanodrop. An equal volume of reducing sample buffer [62.5 mM Tris·HCl, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 100 mM dithiothreitol, and 0.1% (wt/vol) bromphenol blue] was added to each sample. Proteins were separated by SDS-PAGE in a 12% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences) in 48 mM Tris·HCl, 39 mM glycine, 0.037 (wt/vol) SDS, and 20% (vol/vol) methanol, using an electrophoretic transfer cell (Bio-Rad Trans-Blot SD; Bio-Rad Laboratories) at 100 V for 60 min. After transfer, the membrane was stained with Ponceau S for examination of equal loading of proteins; no effect in this respect of siRNA treatment was observed (not shown). After the washing procedure, the membrane was blocked in 5% milk in Tris-buffered saline-0.2% Tween for 1 h at room temperature and then probed with the indicated antibodies for 1 h at 4°C. The immunoblots were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL kit; GE Healthcare Life Sciences). Antibodies used were UCP1 antibody (rabbit polyclonal, raised against COOH-terminal decapeptide) diluted 1:3,000, cytochrome *c* oxidase 4 (COX4) antibody (sc-58348; Santa Cruz Laboratories) diluted 1:1,000, aP2 [fatty acid-binding protein 4 (FABP4)] antibody (3544S; New England Biolabs) diluted 1:1,000, and β-actin antibody (4967S; Cell Signaling) diluted 1:2,000.

Statistical analysis. Expression levels of the genes were calculated using the ΔC_T method, where the threshold cycle (C_T) for the endogeneous control (18S) was subtracted from the C_T value of the target gene to adjust for variations in the efficiency of the cDNA synthesis process. The gene expression level relative to 18S was determined by the formula $2^{-(\Delta C_T)}$. Values on graphs are represented as relative expression levels where the expression level represented by the first ("control") bar was set in each experiment to 1.0, whereas the levels in the other cultures were expressed relative to this value in each individual experiment. The absolute level of expression relative to 18S is given in the figure legends as well as the variation between cell cultures (means ± SE). Statistical significance of siRNA treatment was determined in GraphPad Prism 6 using two-way repeated-measures ANOVA on the raw expression values (ΔC_T), where all of the genes constituted rows, different experimental conditions constituted columns, and independent experiments constituted subcolumns. The two-way ANOVA was followed by Newman-Keuls multiple comparison tests. Brown adipocytes and inguinal adipocytes were analyzed separately.

RESULTS

To establish the expression pattern of Tbx15 in cells derived from distinct adipose tissues, we cultured preadipocytes from pooled BAT depots (interscapular and axillary; the resulting cells here are called BA) and from two distinct white fat depots: the inguinal depot (which is a brite-competent depot in vivo; the cells are called iWA) and the epididymal depot (which is a brite-resistant depot in vivo; the cells are called eWA) (17). The cells were harvested on *day 6*. We determined the levels of Tbx15 mRNA with quantitative PCR (Fig. 1*A*).

Table 2. *Primers used for quantitative RT-PCR analysis*

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Tbx15	TTCCATGATATCGGAACAGAGA	ATGGCAGGAAACATCCTCCT
aP2	CGCAGACGACAGGAAGGT	TTCCATCCCACTTCTGCAC
UCP1	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT
PPARγ	GAAAGACAACGGACAAATCACC	GGGGGTGATATGTTTGAACTTG
PRDM16	ACAGGCAGGCTAAGAACCAG	CGTGGAGAGGAGTGTCTTCAG
PGC-1α	GAAAGGGCCAAACAGAGAGA	GTAAATCACACGGCGCTCTT
Cox8b	CCAGCCAAAACTCCCACTT	GAACCATGAAGCCAACGAC

UCP1, uncoupling protein 1; PPARγ, peroxisome proliferator-activated receptor-γ; PRDM16, PR domain-containing protein 16; PGC-1α, PPARγ coactivator-1α; Cox8b, cytochrome *c* oxidase 8b.

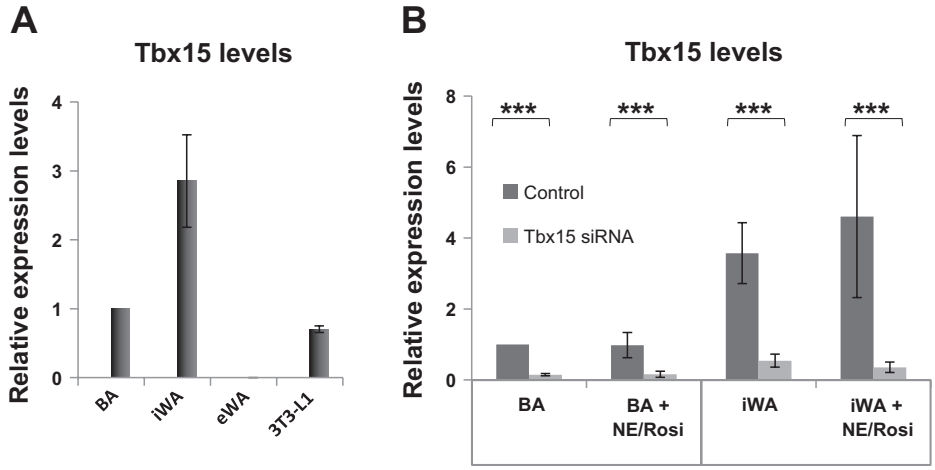

Fig. 1. Expression levels of T-box 15 (Tbx15) in different types of primary adipocytes. *A*: primary cultures of brown adipocytes (BA) and inguinal (iWA) and epididymal white adipocytes (eWA) were grown for 6 days in culture medium without additional stimulation. cDNA from 3T3-L1 cells was from cells *day 8* postinduction cultured under conditions published earlier (1). Tbx15 expression was analyzed by quantitative PCR (qPCR). The expression levels of Tbx15 were normalized to the 18S levels in each sample. The values represent means ± SE of 4 independent experiments. Expression levels in the BA cultures were set in each experiment to 1.0, whereas the levels in the other cultures were expressed relative to this value in each individual experiment. The 1.0 value corresponds to $5.0 \pm 1.0 \times 10^{-6}$ Tbx15 mRNA per 18S RNA. *B*: primary cultures of BA and iWA were grown for 6 days in the absence or chronic presence of norepinephrine/rosiglitazone (NE/Rosi). Where indicated, the cells had been transfected with the small interfering RNA (siRNA) pool against Tbx15. The values represent means ± SE of 5 independent experiments. Expression levels in the untreated BA cultures were set in each experiment to 1.0, whereas the levels in other conditions and cells were expressed relative to this value in each individual experiment. The 1.0 value corresponds to $5.8 \pm 3.0 \times 10^{-6}$ Tbx15 mRNA per 18S RNA. Statistical analysis of the siRNA effects was performed and indicated as described in METHODS. $***P < 0.001$.

Tbx15 was expressed robustly in cells derived from brown and inguinal adipose tissues (C_T values 27–29), but Tbx15 was not expressed at all in cells derived from epididymal tissue (apparent C_T values ~38). Thus, the qualitative difference in Tbx15 gene expression between the inguinal and the epididymal depots (17) is not secondary to their different anatomic locations, with accompanying differences in innervation and blood supply, but clearly reflects a cell-autonomous difference between the cell types [as indicated earlier (5)]. We also analyzed the expression of Tbx15 in 3T3-L1 adipocytes (*day 8* postinduction). The Tbx15 level in these cells was in the brown/brite cell range rather than being absent as in the eWA cultures (Fig. 1*A*).

Tbx15 knockdown in brown and brite adipocytes. For examination of the possible significance of Tbx15 for differentiation of primary brown and brite adipocytes, we used cultures as described above prepared from brown and inguinal white adipose tissue. To promote the differentiation and induction of a "brown-like" phenotype, some cells were grown with chronic treatment with the sympathetic neurotransmitter norepinephrine (NE) combined with the peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone (Rosi) (9, 10). The cultures were examined by phase contrast microscopy after 6 days of differentiation (see below). This indicated that >80% of the control cells and ~100% of the NE/Rosi-treated cells showed a mature cell morphology. Chronic NE/Rosi stimulation of BA or iWA did not modulate Tbx15 expression (Fig. 1*B*).

To evaluate the significance of Tbx15 for the differentiation process, we performed knockdown experiments. We used 20 nM of a pool of siRNA to transfect the preadipocyte cultures. The pool consisted of four different siRNAs directed at the Tbx15 sequence (Dharmacon; Table 1). On *day 6* after seeding, we harvested the cultures and determined the levels of Tbx15 by quantitative PCR (qPCR). siRNA knockdown resulted in an ~10-fold reduction in Tbx15 mRNA in both BA and iWA and in both control adipocytes and adipocytes treated chronically with NE and Rosi (Fig. 1*B*).

To examine whether Tbx15 knockdown visibly affected the differentiation status of the cells, we studied the cell cultures by phase contrast microscopy. Tbx15 knockdown resulted in a somewhat less pronounced level of differentiation in both BA (Fig. 2*A*) and iWA (Fig. 2*B*) judged by cell morphology. Chronic NE/Rosi treatment, as stated above, boosted the differentiation in both cell types; however, this was less pronounced in the absence of Tbx15.

We further investigated the significance of Tbx15 for the differentiation process by analyzing the expression of general adipogenic genes (PPARγ and aP2), transcriptional regulators governing brown phenotype and mitochondriogenesis [PR domain-containing protein 16 (PRDM16) and PPARγ coactivator-1α (PGC-1α)], the general mitochondrial structural gene COX8b and, in particular, the brown-specific mitochondrial gene UCP1 in the different types of adipocytes.

Tbx15 knockdown impairs adipogenesis in primary brown and brite adipocytes. In the basal state, mRNA levels of PPARγ, one of the major regulators of adipocyte differentiation, were not significantly different between brown and brite adipocytes (Fig. 3*A*); the baseline C_T values were ≈23. The mRNA levels were not significantly affected by chronic treatment with NE/Rosi. PPARγ levels were reduced to about one-half upon Tbx15 knockdown in both cell types with and without NE/Rosi treatment (Fig. 3*A*).

More marked effects of both NE/Rosi and siRNA treatment were seen when we analyzed the levels of the general adipogenic marker aP2 (*FABP4*). Basal aP2 levels were generally high (C_T values ≈15), suggesting that the cells were robustly differentiated. The enhanced differentiation of the brown and brite adipocytes in the chronic presence of NE/Rosi was reflected by an increased expression of aP2 (Fig. 3*B*), as

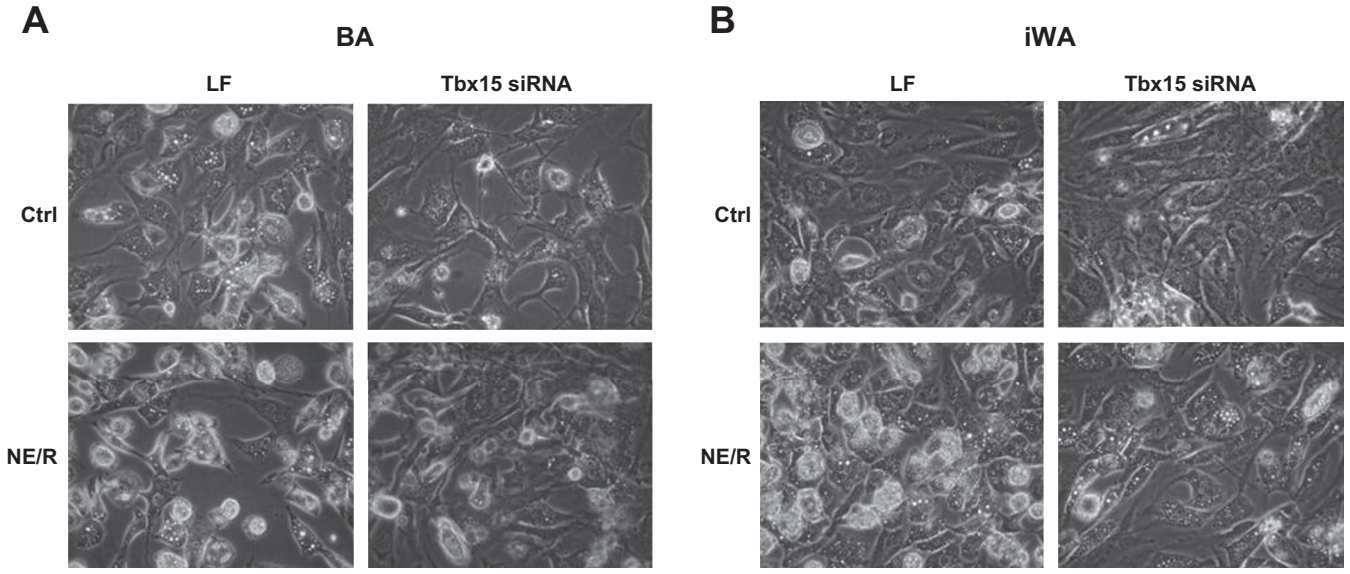

Fig. 2. Tbx15 knockdown leads to a less mature phenotype in BA and iWA. Cells in control and NE/Rosi (NE/R)-stimulated cultures of BA (*A*) and iWA (*B*) with and without Tbx15 knockdown were examined using phase contrast microscopy. LF, Lipofectamine; Ctrl, control.

expected (9, 10). Notably, Tbx15 knockdown caused a 50–70% reduction in the expression level of aP2 in both types of adipocytes, with or without the NE/Rosi treatment (Fig. 3*B*). Thus, based both on aP2 expression levels and on the visual appearance of the cells (Fig. 2), Tbx15 appears to be important for the full adipogenic process in these cells.

As pointed out above, epididymal adipocytes have no expression of Tbx15 (Fig. 1*A*). To confirm the specificity of the siRNA treatment, we transfected in some experiments epididymal primary cultures in parallel with inguinal cultures (Fig. 3, *C* and *D*). Reassuringly, the Tbx15 siRNA pool did not influence PPARγ (Fig. 3*C*) or aP2 levels (Fig. 3*D*) in the epididymal adipocytes, indicating an absence of off-target effects of the siRNA treatment.

The four independent siRNA sequences that constitute the Tbx15-targeting siRNA pool were each able to independently knock down Tbx15 (Fig. 4*A*), and critically, each led to a decreased expression of the adipogenesis marker aP2 in iWA (Fig. 4*B*) but not in eWA (Fig. 4*C*). This supports that the effects of the knockdown were not through off-target effects.

Tbx15 knockdown impairs mitochondrial gene expression in brown and brite adipocytes. Because our expression studies (17, 18), as well as the data in Fig. 1, indicated specifically that Tbx15 gene expression was associated with the mitochondria-rich brown and brite adipocytes, a critical issue was whether its presence or absence would affect the expression of genes associated with mitochondrial function in these cells.

PRDM16 is a transcriptional regulator of brown adipocyte fate (13). In the present experiments, it was expressed at similar levels in all cells and conditions (baseline C_T values ≈28; Fig. 5*A*). PRDM16 expression was in general decreased by Tbx15 knockdown (Fig. 5*A*). As was the case for PPARγ, this effect was relatively small (about a halving of the expression level).

The mitochondriogenesis-related gene PGC-1α (Fig. 5*B*) was well expressed in brown adipocytes (baseline C_T values ≈25), i.e., about ninefold higher than in iWA. PGC-1α was strongly induced by NE/Rosi in iWA adipocytes (~10-fold), whereas in brown adipocytes its level was only doubled. PGC-1α levels were generally reduced by Tbx15 knockdown to ~25% in unstimulated BA and iWA and to ~50% in NE/Rosi-stimulated adipocytes. Thus, loss of Tbx15 compromises the expression of two key regulators of brown and brite adipocyte fate, PRDM16 and PGC-1α.

A marker of mitochondrial content, Cox8b, was about sevenfold more expressed in brown adipocytes than in iWA (Fig. 5*C*). Cox8b was not altered by NE/Rosi treatment in brown adipocytes, whereas NE/Rosi treatment in iWA induced Cox8b to the levels found in brown adipocytes. This is the same pattern of responses as that noted for PGC-1α and thus one that would be consistent with the proposed role of PGC-1α in mitochondrial biogenesis (3). Cox8b levels were reduced by Tbx15 knockdown in both types of adipocytes, showing highly significant reduction in BA and iWA + NE/Rosi, again in parallel with its effects on PGC-1α.

Baseline C_T values for the brown-specific gene UCP1 in brown adipocytes were ≈27. UCP1 was some sixfold more expressed in BA than in iWA (Fig. 5*D*). Upon chronic NE/Rosi treatment, UCP1 was strongly induced in both cell types (600-fold on average), as expected (note the logarithmic scale in Fig. 5*D*) (14), but the level in iWA remained markedly (≈5-fold) lower than in BA. Tbx15 knockdown did not have a significant impact on baseline UCP1 levels in untreated cells. However, in NE/Rosi-treated brown adipocytes the UCP1 mRNA levels were halved, and in NE/Rosi-treated inguinal white adipocytes Tbx15 knockdown markedly reduced the induction of UCP1 expression (by ~70%).

Tbx15 knockdown diminishes adipogenesis-related and mitochondrial protein levels in brown and brite adipocytes. As seen in Figs. 3 and 5, Tbx15 knockdown clearly altered the adipogenic and mitochondrial gene expression programs. To substantiate that these gene expression effects would impact on cell metabolism or function, we examined the effect of Tbx15 knockdown on adipogenesis-related protein (aP2) and mitochondrial protein levels (Cox4 and UCP1) in brown adipocytes and in inguinal white adipocytes. We performed Western blots

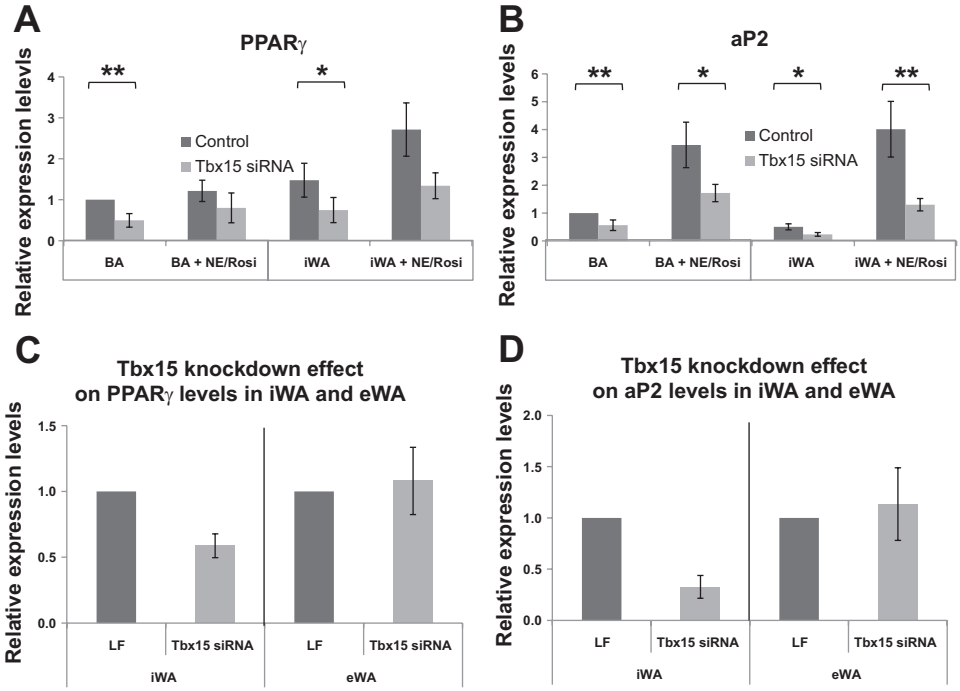

Fig. 3. Tbx15 knockdown specifically impairs adipogenesis in BA and iWA vs. eWA. Expression levels of adipogenic marker genes in control and NE/Rosi-stimulated cultures of BA and iWA were measured with and without Tbx15 knockdown. The same cDNA samples as those in Fig. 1*B* were examined. Peroxisome proliferator-activated receptor-γ (PPARγ; *A*) and aP2 levels (*B*) in control and NE/Rosi-treated BA and iWA with and without Tbx15 knockdown are shown. Expression levels in the BA cultures were set in each experiment to 1.0, whereas the levels in the other cultures were expressed relative to this value in each individual experiment (the 1.0 level corresponds to 0.0015 ± 0.0009 mRNA per 18S for PPARγ and to 0.14 ± 0.07 mRNA per 18S for aP2). The values represent means ± SE of 5 independent experiments. Statistical analysis of the siRNA effects was performed and indicated as described in METHODS. Effect of Tbx15 knockdown on PPARγ (*C*) and aP2 levels (*D*) was tested in parallel in iWA and eWA. Expression levels in the LF-treated inguinal or epididymal adipocytes were set in each experiment to 1.0, whereas the levels in the cultures with Tbx15 knockdown were expressed relative to this value in each individual experiment. The 1.0 value corresponds to 0.018 ± 0.004 for PPARγ in iWA, 0.0013 ± 0.04 for PPARγ in eWA, 0.12 ± 0.04 mRNA per 18S for aP2 in iWA, and 0.10 ± 0.03 for aP2 in eWA. $^{**}P < 0.01$; $^{*}P < 0.05$.

on cell lysates made from 6-day-old cultures (Fig. 6*A*). The results were congruent with the qPCR results. aP2 protein levels (Fig. 6, *A* and *B*) were reduced, in agreement with the qPCR results on aP2 mRNA levels (Fig. 3*B*). Also, Cox4 protein levels (Fig. 6, *A* and *C*) were markedly reduced, indicating that the diminished levels of Cox8b mRNA observed (Fig. 5*C*) reflected a general reduction in mitochondriogenesis.

UCP1 protein was undetectable in cells that were not treated with NE/Rosi (Fig. 6, *A* and *D*). This was expected due the very low mRNA levels in the untreated cells (Fig. 5*D*). In accord with the qPCR results, UCP1 protein abundance in

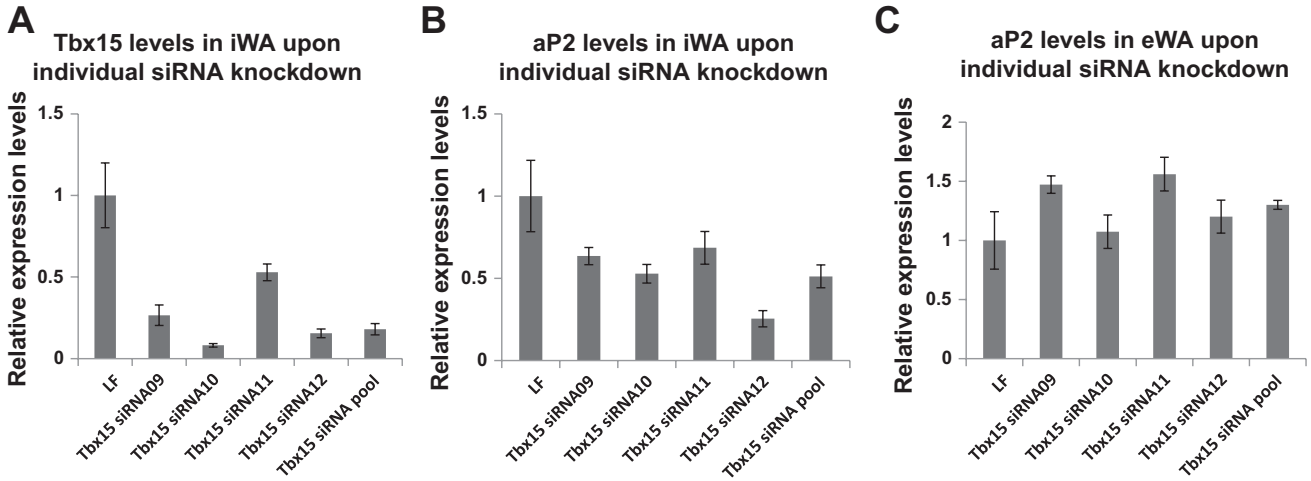

Fig. 4. Effects of siRNA pool components. Tbx15 levels were measured in iWA (*A*), and aP2 levels were measured in iWA (*B*) and eWA (*C*) using individual siRNAs from the siRNA pool as well as the siRNA pool. Values represent the means ± SE of the qPCR triplicates from a single experiment. The expression levels of the genes were normalized to the 18S levels in each sample. The mean expression level in the LF-treated wells was set to 1.0, whereas the levels in the other wells were expressed relative to this value.

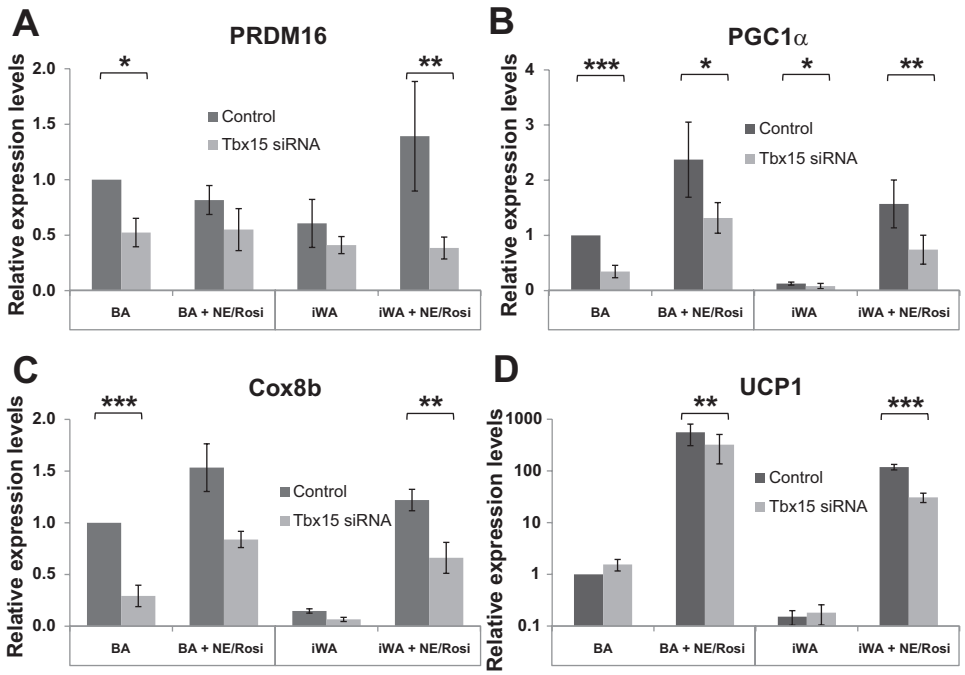

Fig. 5. Tbx15 knockdown impairs mitochondrial biogenesis in brown and "brite" adipocytes and diminishes the brown phenotype. Expression levels of thermogenic marker genes in control and NE/Rosi-stimulated cultures of BA and iWA were measured with and without Tbx15 knockdown. The same cDNA as that in Figs. 1*B* and 3, *A* and *B*, was analyzed. PR domain-containing protein 16 (PRDM16; *A*), PPARγ coactivator-1α (PGC-1α; *B*), cytochrome *c* oxidase 8b (Cox8b; *C*), and uncoupling protein 1 (UCP1; note the logarithmic *y*-axes) levels (*D*) are shown in control and NE/Rosi-treated BA and iWA with and without Tbx15 knockdown. Expression levels in the BA cultures were set in each experiment to 1.0, whereas the levels in the other cultures were expressed relative to this value in each individual experiment. Levels corresponding to 1.0 were $8.7 \pm 3.5 \times 10^{-6}$ PRDM16 mRNA, $7.9 \pm 3.1 \times 10^{-5}$ PGC-1α mRNA, 0.0004 ± 0.0001 Cox8b mRNA, and $7.6 \pm 5.6 \times 10^{-5}$ UCP1 mRNA per 18S RNA. The values represent means ± SE of 5 independent experiments. Statistical analysis of the siRNA effects was performed and indicated as described in METHODS. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

NE/Rosi-treated inguinal adipocytes was increased, but it was still lower than in the NE/Rosi-treated brown adipocytes. Tbx15 siRNA clearly downregulated UCP1 protein levels in both cell types. These results thus confirm the conclusions obtained from the mRNA measurements, that cells in which Tbx15 is knocked down have a diminished ability to carry out the brown and brite adipocyte molecular differentiation program.

DISCUSSION

We observed earlier that the transcription factor Tbx15 is well expressed in brown adipocytes in culture (but not in white adipocytes in culture) (16) and is also well expressed in vivo in brown and brite adipose depots but not in white depots (17) [the difference between inguinal and epididymal Tbx15 expression levels has been observed earlier (5, 19), but the similarity between brown and inguinal depots is not consistent between observations (19)]. Therefore, we have examined here the expression of Tbx15 in adipocytes of different lineages as well as the functional significance of Tbx15 for full differentiation of brown and brite adipocytes.

We find that the expression of Tbx15 is cell autonomously restricted to the brown and the inguinal, i.e., typically brite, adipocyte lineages. We demonstrate that knockdown of Tbx15 in brown and brite primary adipocytes results in a marked reduction in the expression levels of several important adipogenic and thermogenic markers. This occurs in untreated brown and brite adipocytes but is particularly noteworthy in adipocytes promoted to differentiate through chronic treatment with rosiglitazone and norepinephrine. The results in brown and brite adipocytes are in clear contrast to the results in epididymal white adipocytes, where no effect of Tbx15 knockdown is seen (in accord with the absence of Tbx15 gene expression in these adipocytes). The data point to Tbx15 as being an important or perhaps even essential factor in the differentiation of those cells that are of or can adopt a brown or brite phenotype.

The significance of Tbx15. We examined the significance of Tbx15 for the differentiation process in the different types of adipocytes by assessing the effect of Tbx15 knockdown on key gene expressions. As is often the case in siRNA knockdown studies, the effect of the knockdown was only partial, resulting in an ~10-fold decrease in Tbx15 mRNA levels but not in a complete elimination of Tbx15 mRNA. The effect of the siRNA treatment on gene expression was also only partial, with the siRNA treatment resulting in a decrease in the expression of regulatory genes by nearly 50% and of structural genes by about 75%. Thus it may be discussed whether Tbx15 is only partly required for the progression of the differentiation process or whether Tbx15 is essential for the process. Because it is likely that Tbx15 is expressed in excess and that the binding of Tbx15 to its targets follows normal kinetics, the apparent quantitative discrepancy between Tbx15 knockdown and gene expression reduction may be understandable, even if Tbx15 is indispensable for the differentiation process. We suggest that this is the case; i.e., the adipocytes will not enter the browning pathway in its absolute absence. The tendency to a higher effect of the knockdown on the structural than on the regula-

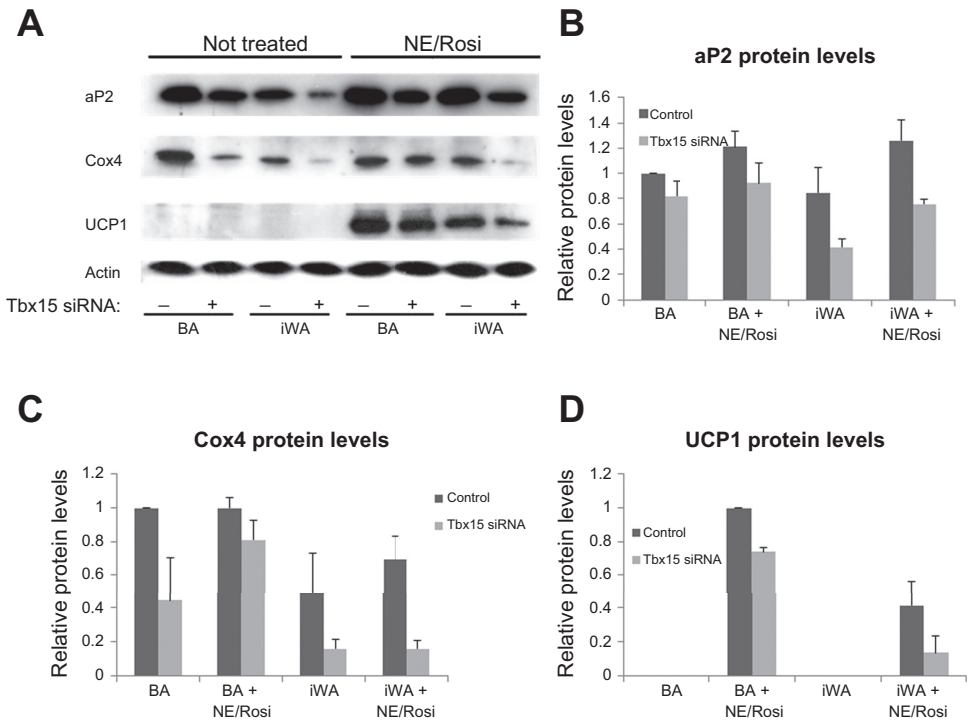

Fig. 6. Protein profiling of mitochondrial and adipogenesis markers. Primary cultures of adipocytes were grown as shown in Fig. 1*B*, and protein levels were analyzed by Western blot. *A*: representative Western blots are shown for aP2, Cox4, UCP1, and β-actin. aP2 (*B*), Cox4 (*C*), and UCP1 protein levels (*D*) are shown upon semiquantitative analysis of the Western blots of these proteins from 2 independent experiments. The data were arranged as shown in the proceeding figures to visualize the effect of Tbx15 knockdown in each condition; means ± SE (because results are from 2 experiments, the error bars are equivalent to the ingoing results from each experiment).

tory proteins would seem to be in accord with the effect of Tbx15 primarily being on the regulatory genes. It is particularly noteworthy that Tbx15 knockdown leads to a reduction in PRDM16 gene expression, because this places Tbx15 very early in the developmental process of these cells.

Cell-type specific effects of Tbx15 gene expression. It is clear from the data presented here that the effect of Tbx15 knockdown is dependent on the cell type studied. We see marked negative effects on differentiation in brown and brite adipocytes but no effect in eWA (which was unsurprising given that Tbx15 is not expressed in these cells). Notably, overexpression of Tbx15 in a nominally white model system, 3T3-L1 cells, has been reported to result in decreased adipogenesis and mitochondriogenesis (4). This would imply that Tbx15 plays opposing roles in different adipose tissue depots, depending on their propensity to become brown like.

Tbx15 and human adipose tissue. The data presented here examine Tbx15 gene expression and Tbx15 function only in murine cells. However, there are some indications that Tbx15 may also be of interest in human contexts.

A genome-wide association study (7) has demonstrated that a single nucleotide polymorphism (SNP) in the Tbx15 locus may explain ≈0.1% of the variation in waist/hip ratio among the subjects studied, and this SNP also correlated with the body mass index of these subjects.

Tbx15 gene expression levels in human brown adipose tissue are presently unknown. Concerning Tbx15 gene expression in brite (subcutaneous) vs. white (abdominal) adipose tissue in humans, available data are inconsistent. In children, expression of Tbx15 in subcutaneous adipose tissue (exact site not defined) is ≈30-fold higher than in visceral fat (14), i.e., in the same direction (but less dramatic) as that described here and earlier (5, 19) for mice. In adult humans, a similar but small tendency (+35%) toward a higher subcutaneous (gluteal) than abdominal level has been observed (7), but other observations surprisingly indicate the opposite pattern, i.e., a much higher (30-fold) expression in visceral depots than in subcutaneous depots (5). When this ≈1,000-fold shift in expression balance between children and adults would occur is unknown, but the high visceral levels are principally found only in lean adult humans (5), and overweight and obese persons would seem to express Tbx15 at nearly similar levels in the two tissues (5). The correlation between decreased Tbx15 expression in abdominal white adipose tissue and increased obesity would be in accord with the inhibitory effect of Tbx15 on white adipocyte differentiation demonstrated earlier (4), i.e., that only upon decreased Tbx15 expression can this white depot expand. Correspondingly, the tendency toward increased Tbx15 level in subcutaneous adipose tissue with increasing obesity (5) would be in accord with a tendency to recruit more brite cells in this depot. This interpretation would also be in accord with observed increased levels of UCP1 gene expression in subcutaneous depots in correlation with increased obesity (2, 15), perhaps in an attempt to protect the subjects against even further obesity.

Conclusion and perspectives. Here, we identify Tbx15 as a transcription factor that seems to be essential for the enhancement of the brown phenotype in both brite and brown adipocytes, i.e., in adipocytes of very different lineages. The identification of this role for Tbx15 may promote detailed studies of both the regulation of Tbx15 expression in itself and the molecular mechanisms through which it affects the browning program. In addition to the general significance this may have for understanding adipocyte differentiation, an insight into the mechanism of action of Tbx15 may reveal ways of promoting the browning phenotype in brown and brite adipose depots in humans with perspectives for altering metabolic efficiencies and thus counteracting the development of obesity-related morbidities.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.G., J.N., J.A.T., and B.C. did the conception and design of the research; V.G. and W.P.C. performed the experiments; V.G., J.N., and J.A.T. analyzed the data; V.G., J.N., J.A.T., and B.C. interpreted the results of the experiments; V.G. prepared the figures; V.G. drafted the manuscript; V.G., W.P.C., J.N., J.A.T., and B.C. approved the final version of the manuscript; W.P.C., J.N., J.A.T., and B.C. edited and revised the manuscript.

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