Am J Physiol Endocrinol Metab 308: E1085–E1105, 2015.
First published April 21, 2015; doi:10.1152/ajpendo.00023.2015.

A stringent validation of mouse adipose tissue identity markers

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Submitted 15 January 2015; accepted in final form 15 April 2015

de Jong JM, Larsson O, Cannon B, Nedergaard J. A stringent validation of mouse adipose tissue identity markers. *Am J Physiol Endocrinol Metab* 308: E1085–E1105, 2015. First published April 21, 2015; doi:10.1152/ajpendo.00023.2015.—The nature of brown adipose tissue in humans is presently debated: whether it is classical brown or of brite/beige nature. The dissimilar developmental origins and proposed distinct functions of the brown and brite/beige tissues make it essential to ascertain the identity of human depots with the perspective of recruiting and activating them for the treatment of obesity and type 2 diabetes. For identification of the tissues, a number of marker genes have been proposed, but the validity of the markers has not been well documented. We used established brown (interscapular), brite (inguinal), and white (epididymal) mouse adipose tissues and corresponding primary cell cultures as validators and examined the informative value of a series of suggested markers earlier used in the discussion considering the nature of human brown adipose tissue. Most of these markers unexpectedly turned out to be noninformative concerning tissue classification (*Car4, Cited1, Ebf3, Eva1, Fbxo31, Fgf21, Lhx8, Hoxc8*, and *Hoxc9*). Only *Zic1* (brown), *Cd137, Epsti1, Tbx1, Tmem26* (brite), and *Tcf21* (white) proved to be informative in these three tissues. However, the expression of the brite markers was not maintained in cell culture. In a more extensive set of adipose depots, these validated markers provide new information about depot identity. Principal component analysis supported our single-gene conclusions. Furthermore, *Zic1, Hoxc8, Hoxc9*, and *Tcf21* displayed anteroposterior expression patterns, indicating a relationship between anatomic localization and adipose tissue identity (and possibly function). Together, the observed expression patterns of these validated marker genes necessitates reconsideration of adipose depot identity in mice and humans.

adipose tissue; brown; brite; beige; white

BOTH WHITE AND BROWN ADIPOCYTES are able to store energy in the form of triglycerides, but brown adipocytes possess the additional ability to dissipate energy in the form of heat via the action of the mitochondrial uncoupling protein UCP1 (3, 21). Brown and white adipocytes are generally localized to distinct brown and white adipose tissue depots (BAT and WAT). However, within certain white adipose depots, cells with some of the characteristics of brown adipocytes can be induced, a phenomenon first described some 30 years ago (51). These inducible adipocytes are now commonly called "brite" (brown-like in white) or "beige" cells (11, 28) (hereafter referred to as brite). Qualitatively, brite adipocytes are functionally very similar thermogenically to the brown adipocytes found in interscapular BAT (25, 38), but they have a smaller thermogenic capacity and possess distinct molecular and developmental characteristics.

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A number of earlier of studies indicated that brown adipocytes share a developmental origin with skeletal muscle cells (2, 14, 35, 43) but that white and brite adipocytes do not (35, 43). However, the developmental relationship between different adipocytes now appears to be much more complex than this (4, 18, 19, 32, 33, 39).

It became clear some years ago that adult humans possess brown adipose(-like) tissue (7, 23, 31, 45, 46, 52). Due to the possibility of utilizing BAT activity to ameliorate obesity and its comorbidities such as type 2 diabetes, there is presently a large interest in characterizing these BAT(-like) depots molecularly and developmentally. As lineage-tracing studies are impossible to perform in humans, there is a need for marker genes that allow distinction to be made between different classes of (human) adipocytes and adipose tissues. Such markers can thus be obtained only from animal studies, and it could be used to influence the development of strategies to promote recruitment of the tissues and activation of energy expenditure.

We have examined here the information value of a series of proposed marker genes for the brown/brite/white lineages. The currently used marker genes originally identified in screens using primary adipose cell cultures and clonal adipose cell lines (40, 43, 48). We found it important to verify the innate validity of these marker genes under strict premises. We would principally expect the expression of validated brown, brite, and white marker genes to be restricted to only one of those three tissues and the distinction ideally to be cell autonomous, i.e., to be retained in primary cell cultures derived from these tissues (although gene expression might be altered under culture conditions). An ideal marker would show "absolute" differences in expression between the different adipose tissues, not merely minor relative differences. The issues are, however, somewhat more complex than this. Thus, as brite adipose tissues may be considered to be a mixture of both brite and classical white adipocytes, a classical white marker would be expected to be detected in a brite adipose tissue. Additionally, white adipocytes in visceral and subcutaneous white adipose depots might have different marker gene expression profiles, in line with possible functional differences between these depots (reviewed in Ref. 42). A good marker for brown or brite would allow for determination of adipose identity even when the tissues/adipocytes are in a nonrecruited state and would not merely mirror UCP1 expression levels. Our results show that few, if any, of the proposed marker genes could be validated in such a stringent system.

MATERIALS AND METHODS

Animals. All experiments were approved by the Animal Ethics Committee of the North Stockholm region. For tissue analysis, male Naval Medical Research Institute (NMRI) mice (ordered from Scanbur, Sweden) were kept at room temperature until they were 6 wk old. They were then singly caged and exposed to either thermoneutrality

(30°C) or cold (4°C) for 3 wk. The mice had free access to chow food (Labfor R70; Lantmännen, Södertälje, Sweden) and water and were kept on a 12:12-h light-dark cycle. Animals were euthanized with CO_2 followed by cervical dislocation. After death, tissues were dissected and immediately snap-frozen in liquid nitrogen. Entire depots were taken unless otherwise stated (see description below).

Cell culture. For primary cell cultures, 4-wk-old outbred male NMRI mice were used. Mice were acclimated in our facility for at least 2 days before they were euthanized using CO_2 and cervical dislocation. Tissues were dissected and prepared for cell culture as described in Petrovic et al. (27). Cell cultures were performed for BAT (combined interscapular, cervical, and axillary depots) and inguinal and epididymal WAT. Culture medium was changed 24 h after seeding and every 48 h thereafter. Cells were treated with either ethanol (control) or 1 μM rosiglitazone maleate (Alexis Biochemicals, no. 350-103-M100) at the time of medium changes. After 7 days (or as specifically indicated), cells were treated for 2 h with water (control) with or without 1 μM norepinephrine (Sigma-Aldrich, A9512). Cells were harvested in Tri Reagent (Sigma-Aldrich, T9424) and kept at −80°C until further processing.

Dissection of adipose tissues. Adipose tissues are given in the order of how they were dissected; see also Fig. 7.

isWAT (interscapular WAT) and iBAT (interscapular BAT):
When the skin on the back is removed, a layer of WAT is visible at the level of the shoulder blades. This layer covers the two lobes of iBAT. We dissect these two tissues together and separate them further outside the body, as the two tissues are clearly distinguishable based on their color.

asWAT (anterior subcutaneous WAT):
At the level of the isWAT, at the flanks of the body on both sides, there is a layer of WAT positioned against the rib cage. This is a thin layer, readily distinguishable from the isWAT.

cBAT (cervical BAT):
Underneath the muscles between the scapulae and the head, two sausage-like lobes are located in a cavity formed by muscles in the neck. They run from the back of the head (cervix) to where the interscapular tissues are located.

aBAT (axillary BAT):
When the scapulae are folded outward, the axillary (or subscapular) BAT is exposed.

triWAT (triceps-associated WAT):
Aligned with the front leg at the level of the triceps muscle, a small depot of WAT is located running parallel with the muscle.

ingWAT (inguinal WAT):
The subcutaneous ingWAT is located posteriorly, running dorsally along the hindlimb to the thigh ventrally. As described (47), this depot can be divided into the dorsolumbar, inguinal, and gluteal portions. In this study, we dissected the central part of the depot, i.e., the ingWAT.

mWAT (mesenteric WAT):
Lining the intestines is a large vessel-rich adipose depot which is the mWAT.

eWAT (epididymal WAT):
Attached to each epididymis and testis is the large visceral eWAT. Epididymides and vas deferens were excluded from tissue samples.

prBAT (perirenal BAT):
The small prBAT is located at the site of the hilum of the kidney. In cold-acclimated mice, it is more easily visible and distinct from the perirenal WAT, but in most cases it can still be identified at thermoneutrality.

prWAT (perirenal WAT):
The prWAT is located on the other side of the renal capsule and posterior to the capsule.

rpWAT (retroperitoneal WAT):
The rpWAT is located against the dorsal body wall, anterior to the kidneys.

cWAT (cardiac WAT):
cWAT is a very small depot lining the left ventricle of the heart.

mBAT (mediastinal BAT):
The mBAT is a thin "strip" of adipose tissue located around the aorta within the thoracic cavity.

RNA isolation, cDNA synthesis, and real-time qPCR. Frozen tissues were homogenized in Tri Reagent. RNA was extracted using the chloroform-isopropanol method according to the manufacturer's instructions. RNA (500 ng) was reverse-transcribed using the High Capacity cDNA Kit (Life Technologies, no. 4368814) in a total volume of 20 μl. Gene-specific primers were premixed with 11 μl of SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, S4438) to a final concentration of 0.3 μM. cDNA was diluted 1:10, and aliquots of 2 μl per reaction were run in triplicate. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by melting curve analysis on a Bio-Rad CFX Connect Real-Time system. The ΔC_T method ($2^{-\Delta C_T}$) was used to calculate relative changes in mRNA abundance [i.e., C_T values for transcription factor IIB (TFIIB) were subtracted from the C_T value of each gene to adjust for variability in cDNA synthesis]. Primer sequences are listed in Table 1.

Principal component analysis. For principal component analysis, the mean log2-transformed ΔC_T values were used as they are presented in the bar graphs (only for tissue data, not cell culture). To allow for log2 transformation of all data points (including those with value 0), the minimum expression value obtained (0.001) was added to each data point. This was followed by gene mean centering to 0. The resulting data (without the TFIIB and *aP2* genes and the recently suggested cell surface marker genes *Asc1*, *Pat2*, and *P2rx5*) were used for principal component analysis in R (r-project.org) using the "prcomp" function. The scores for all samples in *component 1* and *component 2* were plotted. Euclidean distances between 30°C and 4°C conditions in the space of the first two components were calculated using the "dist" function in R.

RESULTS

To validate proposed brown, brite and white adipose tissue marker genes, we first investigated their expression in iBAT, ingWAT, and eWAT and in primary cell cultures derived from these tissues. iBAT is generally considered a "classical" brown fat depot. ingWAT is commonly regarded as the white adipose depot with the strongest browning (britening) capacity. eWAT has very low britening capacity and represents a "classical" white fat depot. These depots were thus selected as being the most representative of the functionally different adipose depots.

For tissue analysis, male outbred NMRI mice were acclimated for 3 wk at 30°C or 4°C to induce minimal and maximal physiological adrenergic stimulation (i.e., "recruitment" or "browning/britening/beigeing") (12). Because tissues are heterogeneous mixtures of different cell types, detected gene expression does not necessarily come from adipocytes or preadipocytes. Therefore, to examine whether expression remains present in in vitro differentiated adipocytes, we analyzed primary cell cultures differentiated from the stromal-vascular cells from BAT (combined interscapular, axillary, and cervical depots), from ingWAT, and from eWAT. To induce browning, we treated some of these cell cultures chronically with the PPARγ agonist rosiglitazone (27, 28, 40). Additionally, to observe responses to adrenergic stimulation, cells were acutely treated with norepinephrine (NE).

As a reference gene we chose TFIIB. TFIIB was essentially equally expressed throughout a large set of different adipose tissues, and expression levels were not influenced by cold

Table 1. *Primer sequences*

Gene	Forward (5′ - 3′)	Reverse (5′ - 3′)	Reference
aP2	CGCAGACGACAGGAAGGT	TTCCATCCCACTTCTGCAC	Franks et al., 2008
Asc1	GGGTGGCACTCAAGAAAGAG	AGTGTTCCAGGACACCCTTG	Ussar et al., 2014
Car4	AGGTGAACAAGGGCTTCCA	GGAAGCATGTCCTGCAAACT	Newly designed*
Cd137	CGTGCAGAACTCCTGTGATAAC	GTCCACCTATGCTGGAGAAGG	Wu et al., 2012
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG	Petrovic et al., 2010
Cited1	GAGGCCTGCACTTGATGTC	TGGAGTAGGCCAGAGAGTTCA	Newly designed*
Ebf3	CGAAAGGACCGCTTTTGTGG	AGTGAATGCCGTTGTTGGTTT	Wu et al., 2012
Epsti1	ACCCTGATAGCACCAAACGA	AGGTCTGCCAGTTCTTGCTC	Sharp et al., 2012
Eva1	CCACTTCTCCTGAGTTTACAGC	GCATTTTAACCGAACATCTGTCC	Wu et al., 2012
Fbxo31	TGGCGTTTGTGAGAACCTG	TGTGTCTGTATCGGTGAAGCA	Newly designed‡
Fgf21	AGATGGAGCTCTCTATGGATCG	GGGCTTCAGACTGGTACACAT	Newly designed*
Hoxc8	GTCTCCCAGCCTCATGTTTC	TCTGATACCGGCTGTAAGTTTGT	Waldén et al., 2012
Hoxc9	GCAGCAAGCACAAAGAGGAGAAG	GCGTCTGGTACTTGGTGTAGGG	Waldén et al., 2012
Lhx8	GAGCTCGGACCAGCTTCA	TTGTTGTCCTGAGCGAACTG	Waldén et al., 2012
Pat2	ACAGGGATCCTCGGACTTC	GAGGCCCATTACCAGCAAG	Newly designed†
P2rx5	CTGCAGCTCACCATCCTGT	CACTCTGCAGGGAAGTGTCA	Ussar et al., 2014
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG	Waldén et al., 2012
Tbx1	GGCAGGCAGACGAATGTTC	TTGTCATCTACGGGCACAAAG	Wu et al., 2012
Tcf21	CATTCACCCAGTCAACCTGA	TTCCTTCAGGTCATTCTCTGG	Waldén et al., 2012
TFIIB	TGGAGATTTGTCCACCATGA	GAATTGCCAAACTCATCAAAACT	Waldén et al., 2012
Tmem26	ACCCTGTCATCCCACAGAG	TGTTTGGTGGAGTCCTAAGGTC	Wu et al., 2012
Ucp1	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT	Waldén et al., 2012
Zic1	AACCTCAAGATCCACAAAAGGA	CCTCGAACTCGCACTTGAA	Waldén et al., 2012

*Primers from the original study (40) were tested but did not work properly under our conditions. †Primers from the original study (44) were tested but did not work properly under our conditions. ‡Primers from the original study (48) had an efficiency of 120%; hence, we designed a new set of primers which only had a slightly lower efficiency (111%).

acclimation. TFIIB expression levels were also minimally affected under the different cell culture conditions (see below).

Brown and brite fat functional genes. To verify the adipose tissue nature of our sample collection of tissues and cell cultures, we first examined genes that are established to be expressed in classical brown (iBAT) and brite adipose tissue (ingWAT) and to a much lesser extent in white adipose tissue (eWAT).

Ucp1 was expressed in iBAT at thermoneutrality and fivefold increased upon cold acclimation (Fig. 1*A*). In ingWAT, *Ucp1* mRNA levels increased markedly at 4°C (27-fold), but the levels reached were still only in the same range as those in thermoneutral iBAT (Fig. 1*A*). *Ucp1* expression was also increased in eWAT but remained at much lower levels than in iBAT and ingWAT (Fig. 1*A*). In all the primary cell cultures [BA (brown adipocytes), ingWA (inguinal white adipocytes), and eWA (epididymal white adipocytes)], *Ucp1* expression was induced by NE and rosiglitazone treatment, in accord with earlier studies (Fig. 1*B*) (26, 28, 40). In cultured brown adipocytes (BA), *Ucp1* was increased to similar mRNA levels in the presence of acute NE or of chronic rosiglitazone (Fig. 1*B*). In ingWAT cultures, we observed a large difference in *Ucp1* induction between acute NE and chronic rosiglitazone treatment: acute NE treatment resulted in only a 40-fold induction of *Ucp1* expression, whereas chronic rosiglitazone treatment led to an 800-fold increase (Fig. 1*B*).

Prdm16 was originally described as the transcriptional regulator of the thermogenic gene program in brown and brite adipocytes (36, 37), but its significance is now considered to be much broader (10), with suggested developmental functions in other tissues (hematopoietic stem cells, pancreatic islets, etc.) (1, 41). *Prdm16* was expressed at the highest levels in iBAT, followed by ingWAT and with some expression in eWAT (Fig. 1*C*). Cold acclimation did not notably change its expression. In the cultures, *Prdm16* mRNA levels were highest in BA and decreased in the presence of rosiglitazone as observed earlier (Fig. 1*D*) (28). The treatments only slightly influenced *Prdm16* levels in ingWA (Fig. 1*D*).

Similarly, *Cidea* was expressed at markedly higher levels in iBAT than in ingWAT and eWAT (Fig. 1*E*). Cold acclimation induced *Cidea* mRNA levels only in ingWAT and eWAT, but levels were still lower than in iBAT. Rosiglitazone treatment induced *Cidea* expression in all three cell cultures, whereas acute NE treatment left *Cidea* mRNA levels unaffected (Fig. 1*F*).

To molecularly verify the adipose characteristics of the dissected tissues and to determine the differentiation efficiency in the cell cultures, we also measured the expression of *aP2* (also known as *Fabp4*). *aP2* was highly expressed at 30°C, and the expression increased at 4°C in all three tissues (Fig. 1*G*). In all three cultures, basal expression of *aP2* was somewhat higher in BA and ingWA than in eWA but was induced by rosiglitazone to similar levels (Fig. 1*H*). Thus, observation of other gene expression differences between the systems cannot be explained as being due to different degrees of general adipose differentiation.

The results above taken together demonstrate that the tissue and cell culture samples selected here provide a valid system for analysis of suggested brown, brite, and white marker genes.

Proposed (classical) brown fat markers. We therefore used this set of brown, brite, and white tissues and corresponding cell cultures to validate the expression of proposed marker genes. Although many marker genes have been suggested, we restricted our analysis here to the marker genes that have also been used in human studies (8, 13, 16, 40, 48). We will not discuss possible functional roles of these genes, only their validity as markers.

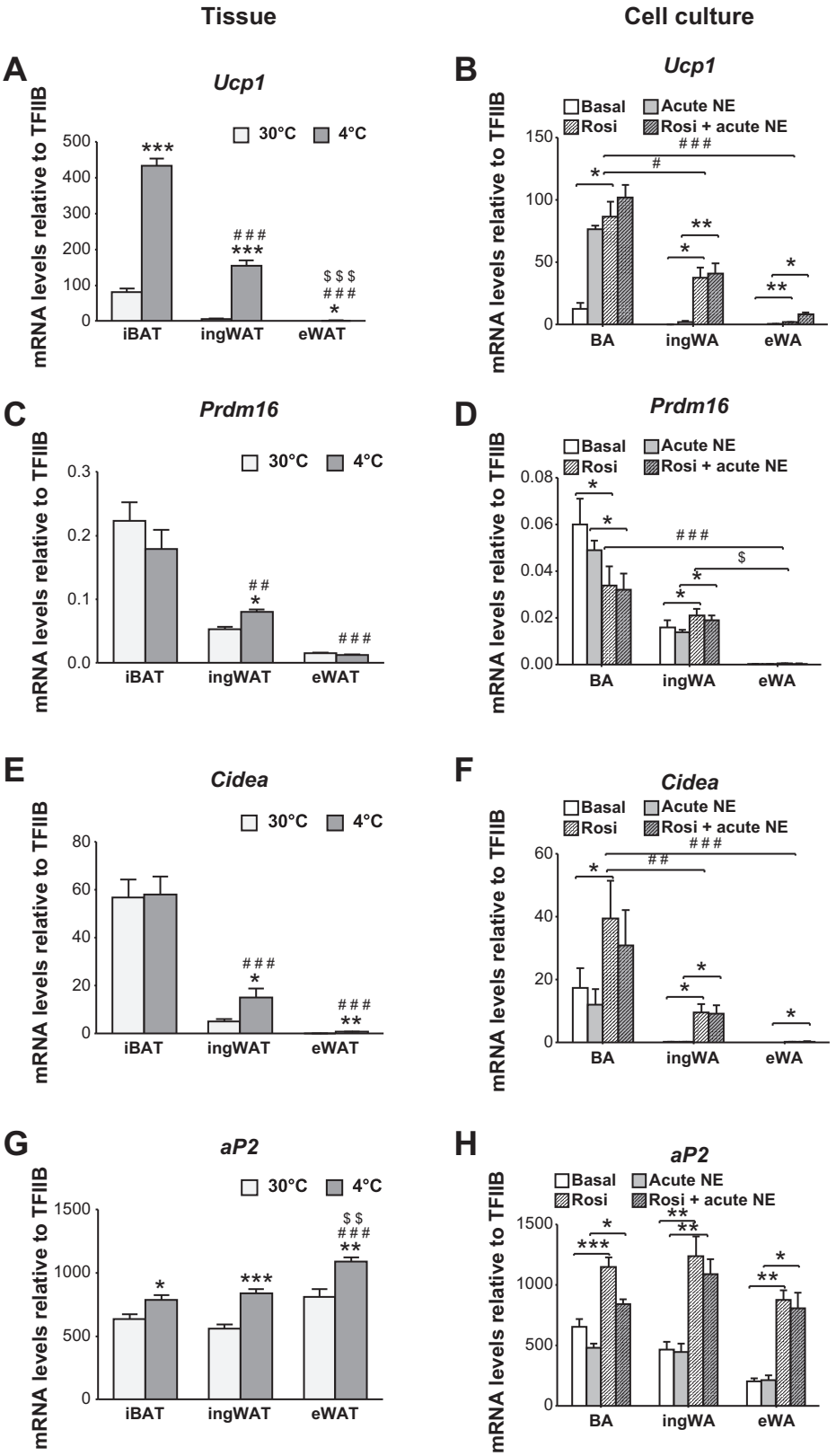

Fig. 1. Expression levels of brown adipose tissue (BAT) functional genes *Ucp1* (*A* and *B*), *Prdm16* (*C* and *D*), *Cidea* (*E* and *F*), and of *aP2* (*G* and *H*). See MATERIALS AND METHODS and Table 2 for definitions. Gene expression levels are shown for iBAT, ingWAT, and eWAT (*left*) from mice acclimated at 30°C (light bars, $n = 6$) or 4°C (dark bars, $n = 6$), and from differentiated primary cell cultures derived from BAT, ingWAT, and eWAT (*right*) treated for 7 days with ethanol (basal) or 1 μM rosiglitazone (Rosi, hatched bars) with or without 2 h of 1 μM norepinephrine (NE) prior to harvest. BAT cultures, $n = 4$; ingWAT cultures, $n = 5$; eWAT cultures, $n = 4$. Here and elsewhere, data are shown as means ± SE. Tissue: *Significance of cold effect was calculated with Student's unpaired *t*-test. Differences between tissues at 4°C were analyzed with a one-way ANOVA followed by Bonferroni multiple comparisons test. #Difference from iBAT; $difference between ingWAT and eWAT. Here and elsewhere, */#/$$P < 0.05$, **/##/$$P < 0.01$, ***/###/$$$P < 0.001$. Cell culture: *Significant effect of rosiglitazone in the presence or absence of NE, calculated using Student's paired *t*-test; differences between cultures from different tissues (in the presence of rosiglitazone only) were calculated using one-way ANOVA followed by Bonferroni multiple comparisons test. #Significant difference from BA; $significant difference between ingWA and eWA.

Initially, we looked at marker genes that have been suggested to distinguish (classical) brown fat from brite/white fat. Ideally, validated brown fat markers would be practically absent in ingWAT and eWAT and would be well expressed in iBAT, or at least be expressed at sufficiently different levels to allow a clear distinction, both in tissues and in primary cultures derived from them (i.e. cell autonomous). Their presence should also be independent of recruitment state. Suggested

specific marker genes for classical brown fat are examined below in alphabetical order.

Ebf3 was expressed at slightly higher levels in iBAT than in ingWAT and eWAT, (Fig. 2*A*). Similarly, in cell cultures, *Ebf3* was expressed at slightly higher levels in BA and ingWA than in eWA, but there was no difference in *Ebf3* levels between BA and ingWA (Fig. 2*B*). Thus, we do not consider *Ebf3* to be a suitable BAT marker gene.

Epsti1, initially suggested as a brown marker, was surprisingly expressed at much higher levels in ingWAT than in iBAT and decreased after cold acclimation. In cell culture, we additionally found *Epsti1* to be more highly expressed in eWA (Fig. 2*D*) than in ingWA. *Epsti1* could thus not be considered a brown but perhaps a brite adipose tissue marker, although it did not retain its expression in primary cell cultures.

We saw only modest differences in expression levels of *Eva1* between iBAT, ingWAT, and eWAT (Fig. 2*E*). *Eva1* expression increased upon cold acclimation in all three tissues (Fig. 2*E*), implying a possible functional role in cold acclimation in adipose tissue; in this respect it correlates with *Ucp1* gene expression (see also Figs. 9*C* and 11*D*). In cell culture, basal levels were similar in BA, ingWA, and eWA, with some tendency to a better recruitment induction in BA (Fig. 2*F*). These data would exclude *Eva1* as a good BAT marker gene. It is not more informative than only measuring *Ucp1*.

Fbxo31 was expressed at similar levels in iBAT and eWAT; the levels were approximately twofold higher than those in ingWAT (Fig. 2*G*). *Fbxo31 e*xpression levels were similar in all three cell cultures (Fig. 2*H*). Together, this shows that *Fbxo31* would not be a good indicator of BAT and brown adipocyte identity.

Lhx8 was absent in eWAT (both tissue and culture), but levels in iBAT and ingWAT were within the same range (Fig. 2*I*). *Lhx8* mRNA was downregulated after cold acclimation (Fig. 2*I*) and, in line with earlier observations (28), also in the presence of rosiglitazone in BA cultures (Fig. 2*J*). The same downregulation was observed in ingWA cultures (Fig. 2*J*). Although the difference in *Lhx8* expression was very clear between iBAT and eWAT, the detected levels in ingWAT implied that *Lhx8* expression could not clearly distinguish brown from brite tissue or brite cell culture.

The only gene tested that could be considered a clear brown fat marker was *Zic1*. *Zic1* expression was detectable only in iBAT, both in tissue and in cell culture, and it could therefore be considered a good marker gene for classical brown fat (Fig. 2, *K* and *L*). *Zic1* was unresponsive to temperature or to any of the treatments in cell culture (Fig. 2, *K* and *L*).

Taken together, these results indicate that, of the earlier proposed brown fat marker genes, only *Zic1* fulfilled the stringent requirements listed above.

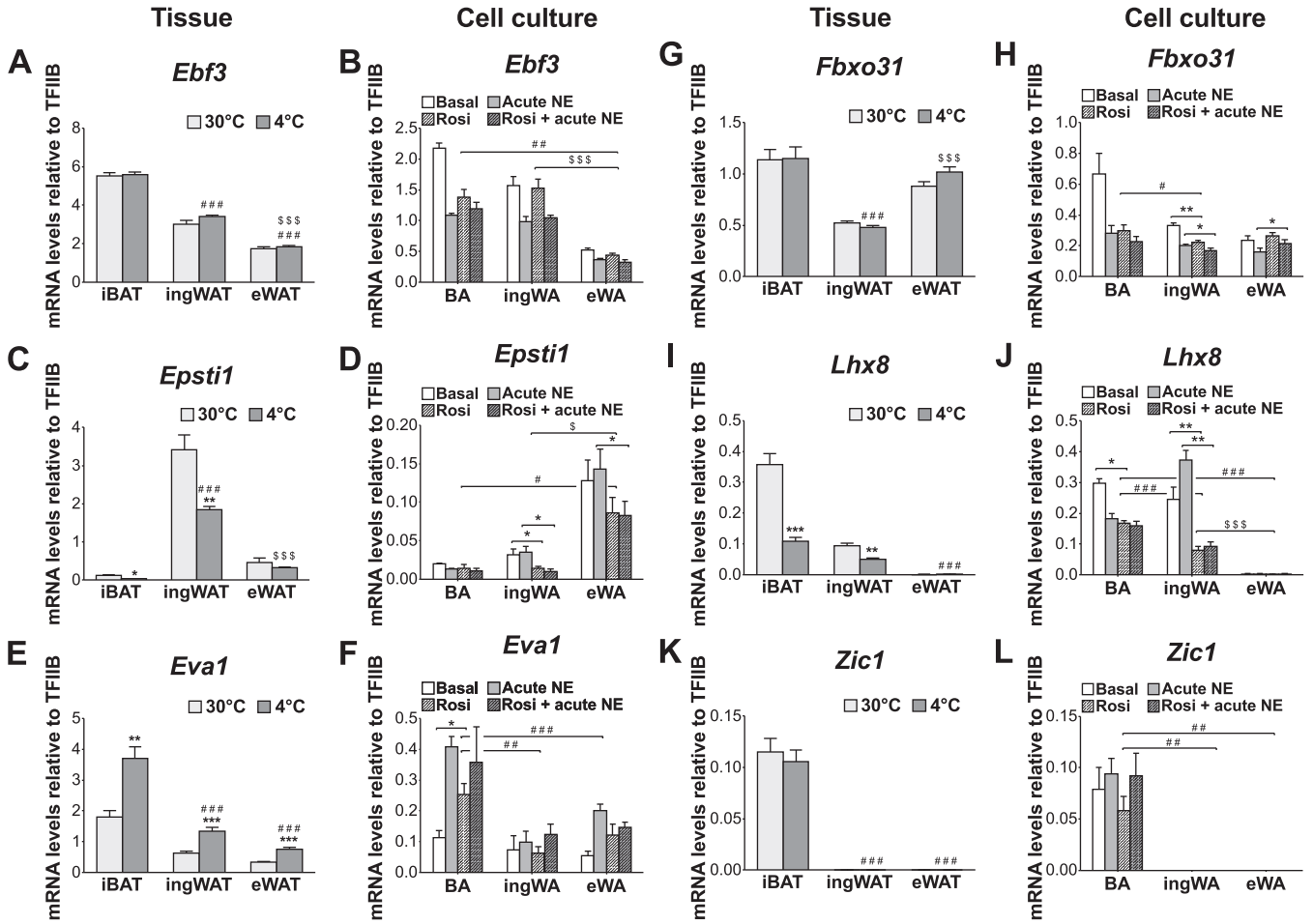

Fig. 2. Expression levels of genes proposed as brown adipose markers *Ebf3* (*A* and *B*), *Epsti1* (*C* and *D*), *Eva1* (*E* and *F*), *Fbxo31* (*G* and *H*), *Lhx8* (*I* and *J*), and *Zic1* (*K* and *L*) in tissue (*left*) and primary cell culture (*right*). See legend to Fig. 1 for details on tissue and culture conditions and statistics.

Proposed brite/beige fat markers. When analyzing iBAT, ingWAT, and eWAT, expression of a good brite marker gene would be expected to be restricted to ingWAT and to primary cultures derived from this tissue. Whether the expression of markers would be expected to be affected by recruitment enhancers (cold, rosiglitazone, NE) would depend on the view of the origin of the brite cells. If they are dormant cells only lacking, e.g., *Ucp1* gene expression, recruitment factors should not influence marker expression. However, if the brite cells proliferate during recruitment and become a larger fraction of all cells, the markers should also be recruitment dependent.

Although proposed as a brite marker gene, *Car4* was expressed at the highest levels in iBAT of warm-acclimated mice (Fig. 3*A*). *Car4* expression was similar in ingWAT and eWAT (and iBAT at 4°C). In agreement with earlier observations (40), *Car4* expression was increased (~5-fold) by rosiglitazone treatment in ingWA cultures (Fig. 3*B*). However, the same response occurred in BA cultures (36-fold increase) and in eWA cultures (7-fold increase; Fig. 3*B*). Thus, based on these data, *Car4* should not be considered a brite adipose marker, neither in tissue nor in cell culture. The clear response of *Car4* to rosiglitazone in cell cultures might be of interest for further investigation of possible functional roles of *Car4* in adipose biology.

A marker gene that did seem to be selective for brite adipose tissue was *Cd137*. In tissue, the smallest difference observed between ingWAT and iBAT or eWAT was 28-fold, with eWAT at 4°C (Fig. 3*C*). Somewhat unexpectedly for a brite marker, *Cd137* expression was decreased in ingWAT of cold-acclimated mice (and also in iBAT and eWAT). In the corresponding cell cultures, however, the difference in *Cd137* expression between ingWAT and iBAT or eWAT was not maintained (Fig. 3*D*), and the absolute levels were low. The earlier observation was that *Cd137* marks brite precursors (48), and our analyses here were in fully differentiated cells. However, when we examined *Cd137* gene expression in less-differenti-

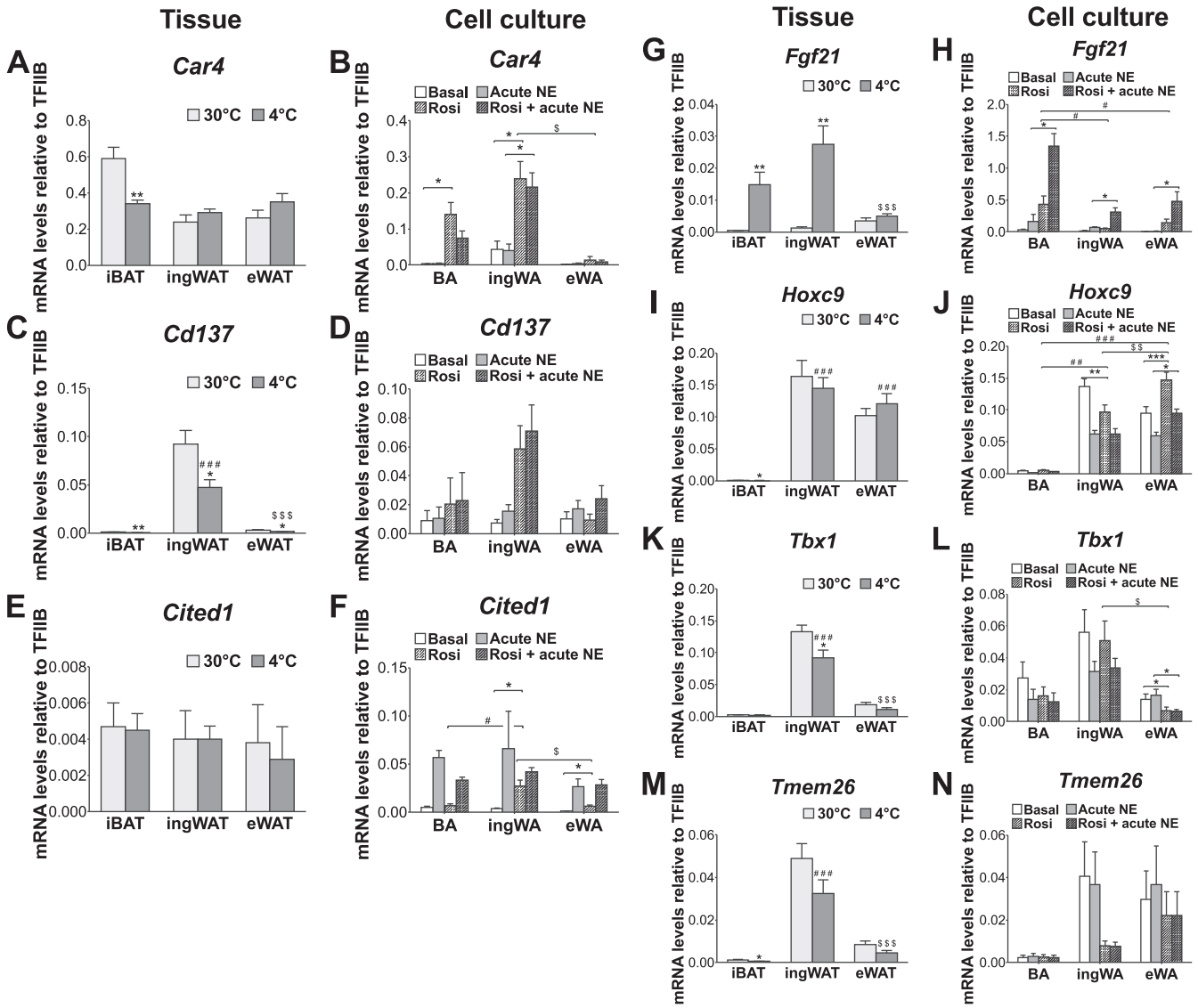

Fig. 3. Expression levels of genes proposed as brite/beige adipose markers *Car4* (*A* and *B*), *Cd137* (*C* and *D*), *Cited1* (*E* and *F*), *Fgf21* (*G* and *H*), *Hoxc9* (*I* and *J*), *Tbx1* (*K* and *L*), and *Tmem26* (*M* and *N*) in tissue (*left*) and primary cell culture (*right*). See legend to Fig. 1 for details on tissue and culture conditions and statistics.

ated cells, the expression in brite cells was, if anything, even lower than in more-differentiated cells (Fig. 4*A*). Thus, *Cd137* gene expression is not informative under these cell culture conditions. *Cd137* expression, both in precursors and mature cells, may instead be regulated by external factors. These results suggest that at the tissue level, *Cd137* could perhaps serve as a brite marker, but a low expression does not necessarily indicate that the tissue under investigation is not brite.

Cited1 was expressed at similar but extremely low levels in all three depots (Fig. 3*E*). In all three types of culture, *Cited1* expression tended to be increased by acute NE stimulation (Fig. 3*F*). *Cited1* expression was also induced by rosiglitazone, but only in ingWA and eWA cultures (Fig. 3*F*). Thus, *Cited1* does not distinguish between brite and brown or white, but its response to NE and rosiglitazone implies a possible functional role for *Cited1* in adipocytes.

At thermoneutrality, the proposed brite marker gene *Fgf21* showed highest expression in eWAT. In cold-acclimated mice, *Fgf21* expression was induced 30- and 23-fold in iBAT and ingWAT, respectively, but even the cold-induced *Fgf21* mRNA level in ingWAT was barely twofold higher than in iBAT (Fig. 3*G*). In all three cell cultures, we saw a synergistic effect of rosiglitazone and acute NE treatment on *Fgf21* mRNA levels (Fig. 3*H*). *Fgf21* mRNA levels were highest in BAT cultures (Fig. 3*H*). Together, our results show that *Fgf21* expression was inducible in tissues (iBAT and ingWAT) and in cell culture. However, *Fgf21* would not serve as a suitable brite marker gene, as its expression levels in the different tissues and cultures, for the same treatments, were always in the same range (Fig. 3, *G* and *H*).

Hoxc9 was virtually absent in iBAT but showed similar expression levels in ingWAT and eWAT, independent of temperature (Fig. 3*I*). Similarly, *Hoxc9* was expressed in both ingWA and eWA (Fig. 3*J*). Thus, although practically absent in iBAT and BAT-derived cultures, *Hoxc9* did not distinguish between brite and white adipose tissues and cells. However, *Hoxc9* expression did seem to discriminate between brown vs. brite/white adipose tissue and cells, and *Hoxc9* could thus be useful to make that distinction.

Two other genes that seem to be potential brite markers are *Tbx1* and *Tmem26*. Both genes were expressed at ~50-fold higher levels in ingWAT than in iBAT (Fig. 3, *K* and *M*). However, the differences between ingWAT and eWAT were much smaller, approximately sevenfold for both genes. In all three cell cultures, *Tbx1* was expressed at very similar levels (Fig. 3*L*), i.e., no qualitative difference between BA and both WA cultures. *Tmem26* expression was lower in BA cultures but similar in ingWA and eWA cultures (Fig. 3*N*). Similarly to *Cd137*, *Tmem26* is thought to mark brite precursor cells (48); but again, we did not see any tendency to a higher expression level in less differentiated brite cells (but unexpectedly in white precursor cells) (Fig. 4*B*). External regulation of expression of these genes, just as for *Cd137*, could apply to *Tbx1* and *Tmem26*. Thus, *Tbx1* and *Tmem26* may serve as brite markers in tissues but lose this capacity in culture.

Overall, at the tissue level, *Cd137* seemed to be a distinctive brite marker gene. *Tbx1* and *Tmem26* were somewhat questionable but could be taken into consideration. However, in primary cell cultures, none of the proposed brite markers was expressed solely in ingWAT cultures, and this makes even these genes less robust brite markers.

Proposed white fat markers. In the three adipose tissues analyzed here, the expression of a selective white marker gene should either be restricted to eWAT or, considering ingWAT to be a mixture of brite and white adipocytes, be most pronounced in eWAT and to a lesser extent detectable in ingWAT.

One of the few genes that is discussed as a white marker, *Hoxc8*, was expressed in both eWAT and ingWAT (and much lower in iBAT) (Fig. 5*A*). Both in tissue and in culture, *Hoxc8* was expressed at only two- to threefold lower levels in ingWAT than in eWAT (Fig. 5, *A* and *B*). Thus, *Hoxc8* does not distinguish between white and brite tissues, although it cannot be excluded that its lower expression in ingWAT reflects a mixture of brite and white adipocytes. However, like *Hoxc9*, *Hoxc8* does distinguish between brown and brite/white adipose tissues.

The other proposed white adipose marker, *Tcf21*, was much more restricted to eWAT (Fig. 5*C*). *Tcf21* was practically absent from BAT and ingWAT, both in tissue and in culture (Fig. 5, *C* and *D*). Thus, *Tcf21* seems to be a good marker gene

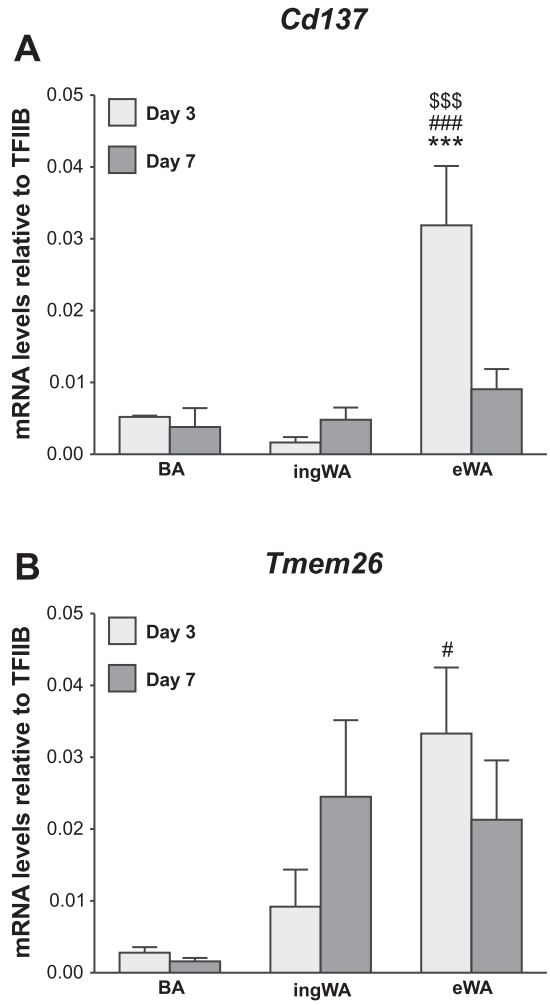

Fig. 4. Expression levels of suggested brite markers *Cd137* and *Tmem26* in undifferentiated and differentiated adipocyte cultures. Cells from different cell culture preparations were analyzed at *day 3* (n = 3–7) or at *day 7* (n = 7–11). Statistics were calculated using two-way ANOVA followed by Bonferroni multiple comparisons test. *Significant difference between *day 3* and *day 7*; #significant difference from BA; $significant difference between ingWA and eWA. #$P < 0.05$, ***/###/$$$$P < 0.001$.

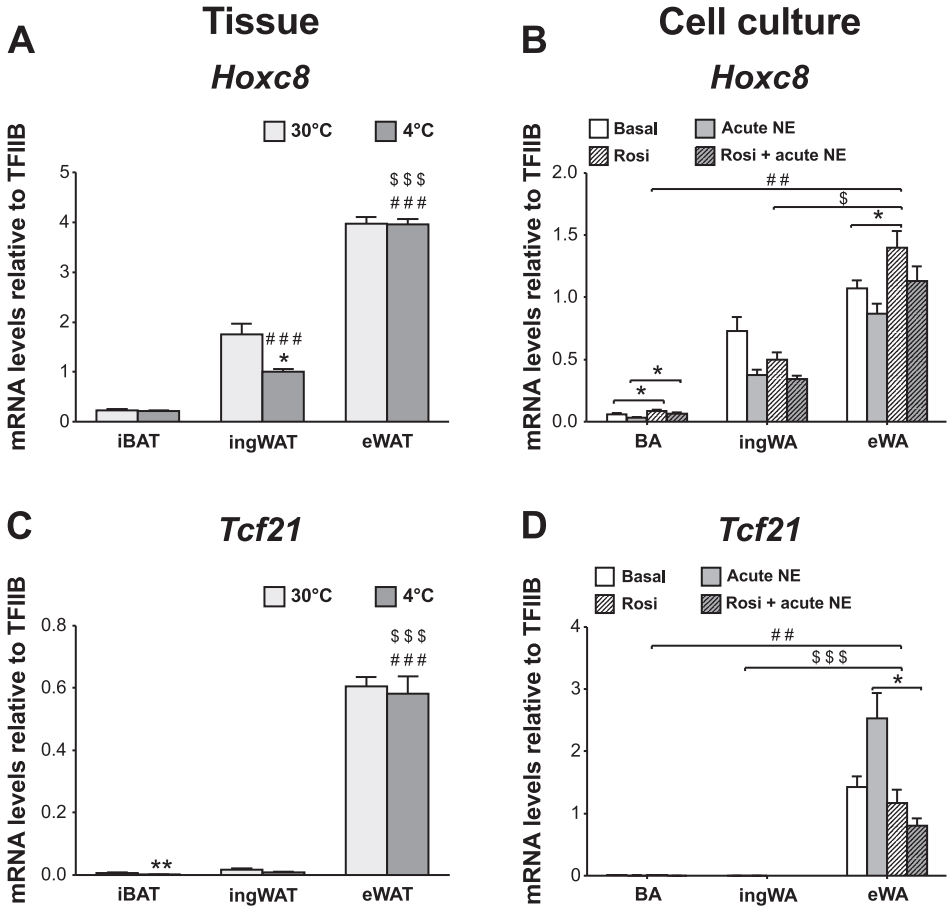

Fig. 5. Expression levels of genes proposed as white adipose markers *Hoxc8* (*A* and *B*) and *Tcf21* (*C* and *D*) in tissue (*left*) and primary cell culture (*right*). See legend to Fig. 1 for details on tissue and culture conditions and statistics.

for visceral WAT and corresponding primary cultures, a type of marker that has long been searched for.

Cell surface markers. To enable cell sorting, Ussar et al. (44) have proposed three genes as selective cell surface markers: *Asc1* (white marker), as well as *Pat2* and *P2rx5* (both brown/brite markers). As seen in Fig. 6, *A* and *B*, there is a tendency in the tissues that, in accord with the suggestions of Ussar et al., the expression levels of *Asc1* are somewhat higher in white than in brite (and brown) fat cells, and the reverse is true for *Pat2* and *P2rx5* (Fig. 6, *C* and *F*). Our expression data in this respect are similar to those of Ussar et al. Whether *Asc1* expression is excluded from brite (i.e., *Ucp1*-expressing) cells cannot be concluded based on our data.

Pat2 and *P2rx5* are clearly enriched in classical brown depots. Furthermore, in ingWAT and eWAT, expression of both *Pat2* and *P2rx5* is induced upon cold (our study) and in response to treatment with a β_3-adrenergic agonist (44), but this is not reflected in the response to adrenergic stimulation in cell cultures (Fig. 6, *D* and *F*). All in all, this means that these two markers might not be of use in determining the identity of adipocytes (classical brown vs. brite), as they mark both cell types. However, they might be useful as tools for selection of brown/brite adipocytes from tissues (44).

Establishing the nature of singular adipose depots based on validated markers. After examining the expression of proposed marker genes in parallel in iBAT, ingWAT, and eWAT, we concluded that only a few marker genes that had been proposed actually distinguished between the depots: *Zic1* (brown), *Cd137, Epsti1* (and arguably *Tbx1* and *Tmem26*) (brite, but not in cell culture), and *Tcf21* (white). Using these validated markers, we attempted to classify adipose tissue identity in a large set of adipose depots. To this end, we analyzed a total of 14 different depots (Fig. 7) (for abbreviations, see Table 2; for a description of the tissues and their dissection, see MATERIALS AND METHODS).

We validated the use of TFIIB as a reference gene for all these analyses (Fig. 8). As seen, neither depot nor acclimation state affected TFIIB levels [but the levels were double as high in cell cultures (Fig. 8*B*) as in tissues (Fig. 8*A*), mathematically explaining the general trend observed above to apparently lower gene expression levels in cell cultures than in tissues]. The significant but small effect of rosiglitazone on TFIIB gene expression in ingWA cultures does not affect the analyses presented here.

Ucp1 throughout the adipose organ. In all the depots, we first analyzed *Ucp1* expression (Fig. 9*A*). At 30°C, *Ucp1* expression was clearly most pronounced in the five adipose depots that are traditionally considered BAT (iBAT, mBAT, aBAT, prBAT, and cBAT; Fig. 9*A*). Cold acclimation significantly induced *Ucp1* mRNA expression in all depots analyzed, albeit to very different maximum levels (relative expression at 4°C ranges from 432 in iBAT to 0.4 in mWAT, i.e., a 1,000-fold difference). The five classical BAT depots showed the highest *Ucp1* mRNA levels not only at thermoneutrality but also in cold (Fig. 9*A*). Among the white/brite adipose depots, there was a wide range of *Ucp1* expression levels and thus

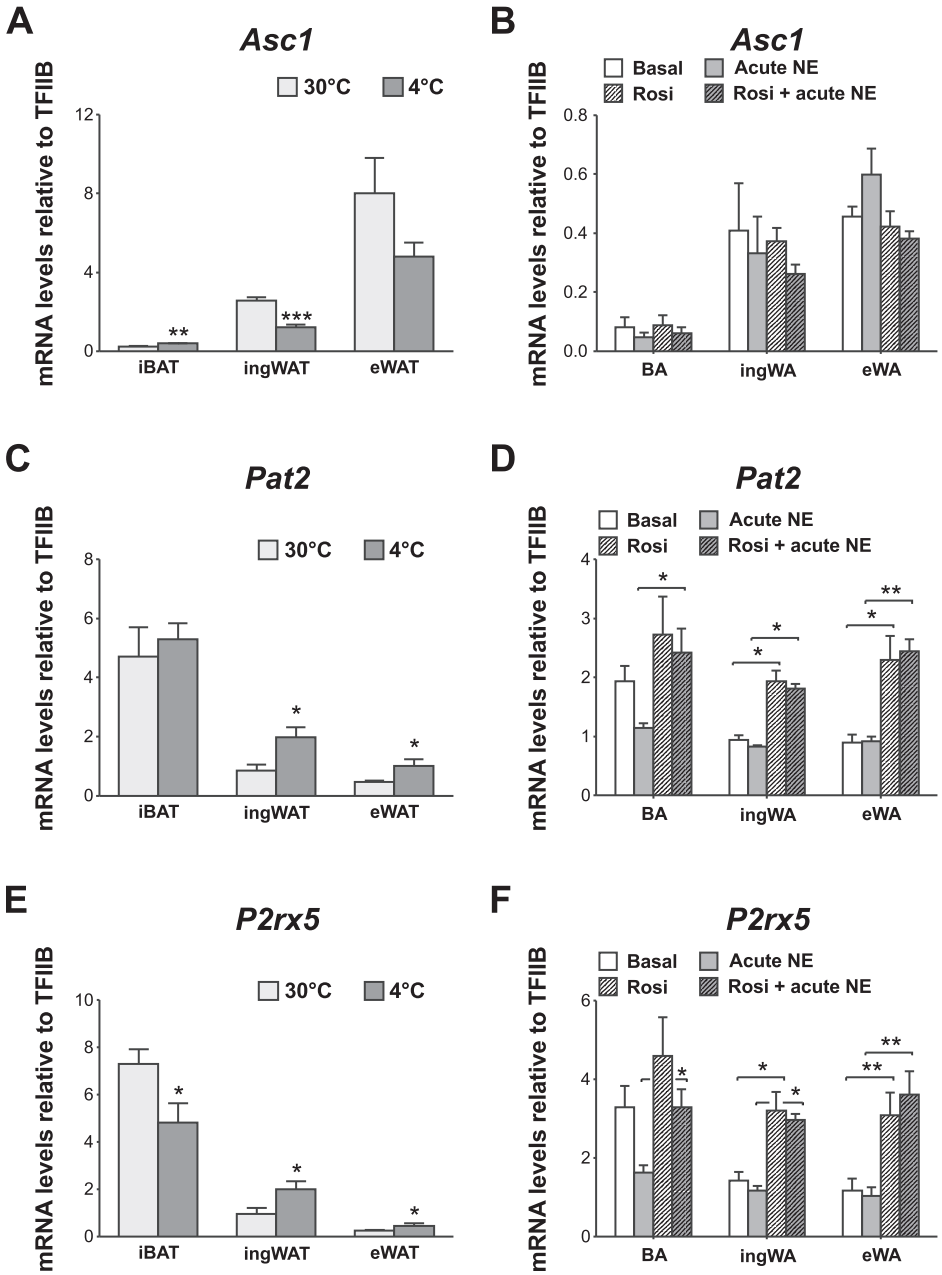

Fig. 6. Expression levels of proposed white cell surface marker *Asc1* (*A* and *B*) and brown/brite cell surface markers *Pat2* (*C* and *D*) and *P2rx5* (E and F) in tissue (*left*) and primary cell culture (*right*). See legend to Fig. 1 for details on tissue and culture conditions and statistics.

arguably a wide range of "briteness" (Fig. 9*A*). ingWAT and triWAT had the highest *Ucp1* expression levels (Fig. 9*A*). isWAT, rpWAT, and prWAT had slightly lower maximum *Ucp1* levels (Fig. 9*A*). cWAT and asWAT had even lower *Ucp1* expression levels in the cold, but these were still higher than those in eWAT and mWAT (Fig. 9*A*).

To estimate the relative contributions of the different depots to UCP1-mediated thermogenesis, we determined total *Ucp1* mRNA per depot following measurement of total tissue RNA. Total amounts of RNA showed the strongest cold-induced increase in iBAT (Fig. 9*B*). This increase in total RNA in iBAT was similar to what had been observed earlier (12). Significant cold-induced increases were also observed in mBAT, aBAT, cBAT, and ingWAT (Fig. 9*B*). The total RNA amounts in other depots were unaffected by temperature (Fig. 9*B*). Calculation of the total amount of *Ucp1* mRNA in each depot (data in Fig. 9*A* multiplied by data in Fig. 9*B*) showed a cold acclimation-induced increase in all analyzed tissues (Fig. 9*C*). Although the amount of *Ucp1*-expressing WAT depots was larger than the number of BAT depots, the combined total amount of *Ucp1* mRNA in the classical BAT depots after cold acclimation was still 80% of the combined total amount of *Ucp1* mRNA in all tissues analyzed (Fig. 9, *D* and *E*). The relative contribution of the WAT depots to the total amount of *Ucp1* increased upon cold acclimation from 8% to 20% (Fig. 9*E*).

Although it may be discussed whether *Ucp1* transcript levels are a good predictor of thermogenic capacity (24), these results do illustrate the transcriptional dynamic range for *Ucp1* in these tissues upon cold exposure. It may be noted that, in order for total UCP1 protein amount (total thermogenic capacity) in all white/brite adipose tissues to exceed that in all classical brown adipose tissue, the translation efficiency would have to

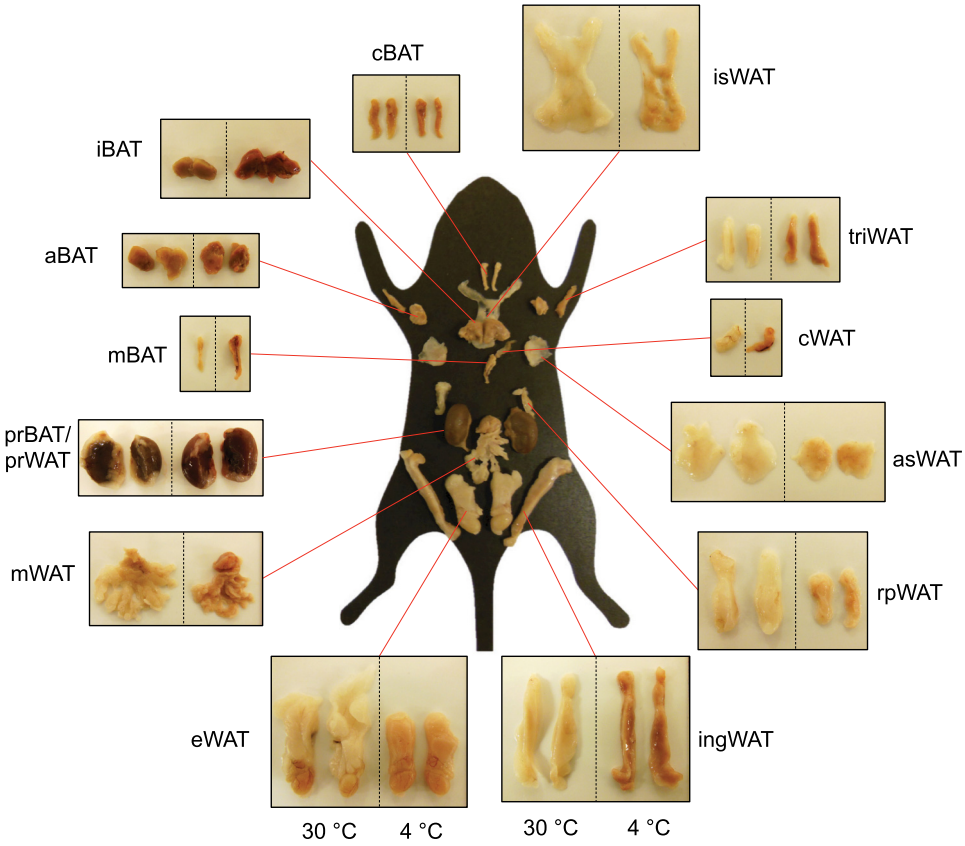

Fig. 7. Dissection of the adipose organ. In each frame, adipose depots from a warm (*left*) and cold-acclimated mouse are shown. Tissues in the mouse outline are from a cold-acclimated mouse. For tissue abbreviations see Table 2. Details on tissue localization and dissection are described in MATERIALS AND METHODS. For this illustration, tissues from warm- and cold-acclimated mice were kept in 4% paraformaldehyde in phosphate-buffered saline until all tissues were dissected. Pictures were taken with a regular camera.

be at least fivefold higher in brite/white tissues, which is perhaps rather unlikely. Furthermore, in a steady-state situation, the total *Ucp1* mRNA levels in the different tissues would seem to predict the total UCP1 protein levels (our unpublished observation) rather well, and the levels are also in good agreement with results estimating total UCP1-dependent thermogenic capacities of the tissues (38).

Brown fat functional genes in a broad range of adipose tissues. In addition to *Ucp1*, we analyzed *Prdm16* and *Cidea* expression in the entire set of dissected adipose tissues. Both *Prdm16* and *Cidea* mRNA levels correlated very well with those of *Ucp1* along the range of tissues (Figs. 10, *A* and *B*, and 11, *A* and *B*). The correlation between *Prdm16* and *Ucp1* over all tissues is in line with the observation that *Prdm16* has an important role in the regulation of thermogenic function of both brown and brite adipose depots (6, 10, 36). These correlations of *Cidea* and *Prdm16* thus show that their expression does not provide more information about tissue identity than *Ucp1* expression itself (although they are more robust measures at thermoneutrality).

aP2 was expressed within the same range in all tissues analyzed (Fig. 10*C*). *aP2* mRNA was increased significantly but modestly in almost all depots in cold-acclimated mice (Fig. 10*C*). Thus, *aP2* expression confirms the adipose nature of all tissues dissected.

Identity of a broad range of adipose tissues. We used the marker genes that we positively validated in iBAT, ingWAT, and eWAT to classify tissue identity throughout the adipose organ. *Zic1*, the main classical BAT marker, was expressed in only three of five tissues that have traditionally been considered classical BAT: iBAT, axillary (aBAT), and cervical brown adipose tissue (cBAT) (Fig. 12*A*). Perirenal BAT (prBAT) and mediastinal BAT (mBAT) did not express *Zic1*, although they did express *Ucp1*, *Cidea*, and *Prdm16* at levels similar to those of the other BAT depots. *Zic1* was also expressed in interscapular WAT (isWAT), a depot with clearly lower *Ucp1*, *Cidea*, and *Prdm16* mRNA levels than any of the BAT depots (see DISCUSSION).

The brite marker genes *Cd137, Epsti1, Tbx1*, and *Tmem26* displayed similar expression patterns. All four brite markers were expressed, in addition to in ingWAT, in the WAT located along the triceps muscle (triWAT; Fig. 12, *B–E*). Based on *Ucp1*, *Prdm16*, and *Cidea* expression, triWAT could be con-

Table 2. *Tissue abbreviations*

Abbreviation	Full Tissue Name
iBAT	Interscapular brown adipose tissue
mBAT	Mediastinal brown adipose tissue
aBAT	Axillary brown adipose tissue
prBAT	Perirenal brown adipose tissue
cBAT	Cervical brown adipose tissue
ingWAT	Inguinal white adipose tissue
triWAT	Triceps white adipose tissue
isWAT	Interscapular white adipose tissue
prWAT	Perirenal white adipose tissue
rpWAT	Retroperitoneal white adipose tissue
cWAT	Cardiac white adipose tissue
asWAT	Anterior subcutaneous adipose tissue
eWAT	Epididymal white adipose tissue
mWAT	Mesenteric white adipose tissue

Table 2. Overview of abbreviations of dissected adipose tissues presented in Figs. 7–10 and 12–15. See MATERIALS AND METHODS for details on tissue localization.

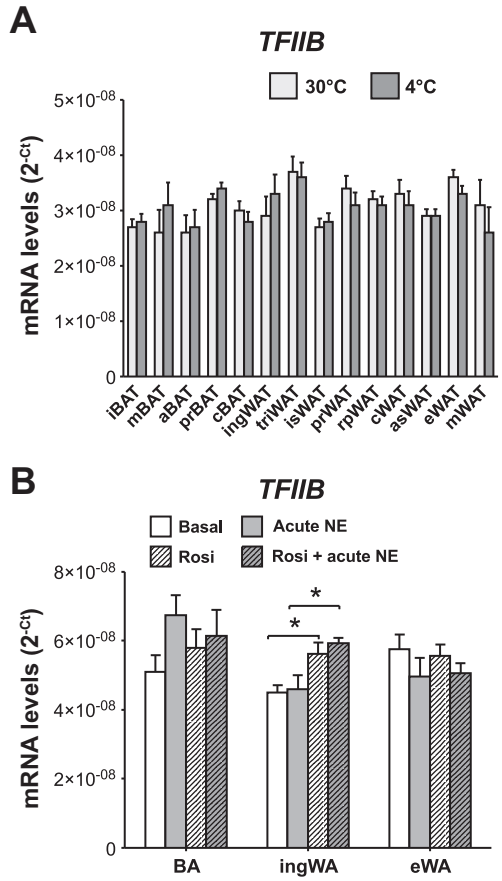

Fig. 8. Expression levels of reference gene transcription factor IIB (TFIIB) in tissues (*A*) and cell cultures (*B*). For tissue abbreviations see Table 2. Values presented were calculated as $2^{-\Delta C_T}$. Data are presented as means ± SE. *Significant effect of rosiglitazone, calculated using Student's paired *t*-test ($P < 0.05$). It may be noted that TFIIB expression in cell cultures is ~2-fold higher than in tissues.

sidered to be equally brite as ingWAT (Figs. 9*A* and 10, *A* and *B*). However, each of these brite markers also marked the "whitest" tissue of all (mWAT) (see DISCUSSION).

Additionally, *Tbx1* was also detected in mBAT (Fig. 12*D*). As the absence of *Zic1* from mBAT called into question its identity as a classical brown depot, detection of *Tbx1* strengthened that idea. However, the absence of the other brite markers might imply that true brite adipose tissues are indicated by the combined presence of these four marker genes.

Tcf21, in addition to being found in eWAT, was also detected in mWAT and in both perirenal adipose depots (prBAT and prWAT; Fig. 12*F*). These tissues display different levels of *Ucp1* expression (Fig. 9*A*). From the presence of *Cd137, Epsti1, Tbx1*, and *Tmem26*, mWAT could be defined as a brite, albeit nonthermogenic, depot. Since brite adipose tissues are generally considered to contain a mixture of white and brite adipocytes, the presence of the white adipose marker *Tcf21* would not be unexpected. However, the absence of *Tcf21* in ingWAT and triWAT would imply that white adipocytes with different gene expression profiles exist in different depots (Fig. 12*F*).

Negatively validated markers in a broad range of adipose tissue. The marker genes that were found not to allow distinction between iBAT, ingWAT, and eWAT were also analyzed for completeness throughout the entire set of adipose tissues. Several of these genes (*Ebf3, Fbxo31, Car4*, and *Cited1*) were expressed at rather similar levels in all adipose depots (Fig. 13, *A, C, E*, and *F*). *Ebf3*, as the only one of these four genes, showed a positive correlation with *Ucp1* throughout the set of tissues (Fig. 11*C*). However, the differences in *Ebf3* expression between the tissues are not considered to be of identification value. Thus, the expression of *Ebf3, Fbxo31, Car4*, and *Cited1* is a feature shared by all adipose depots analyzed here.

Eva1 and *Fgf21* were the only two marker genes that were upregulated in almost all adipose depots under cold-acclimated conditions (Fig. 13, *B* and *G*). *Eva1* expression levels displayed a clear correlation with *Ucp1*, similar to what was observed for *Prdm16* and *Cidea* (Fig. 11*D*); thus, *Eva1* is essentially not more informative about tissue identity than *Ucp1* itself.

Lhx8, *Hoxc8*, and *Hoxc9* were expressed throughout the set of adipose tissues and did not show restrictions to brown, brite, or white adipose tissues (Fig. 13, *D, H*, and *I*). However, at 4°C, *Hoxc8* expression showed a tendency to correlate negatively with *Ucp1* expression in tissues (with only few exceptions) (Fig. 11*E*). This is in line with the observations of *Hoxc8* functioning as a negative regulator of the adipocyte browning process (22). Two tissues had both low *Hoxc8* and low *Ucp1* levels (cWAT and mWAT), implying that in these tissues *Hoxc8* is not the factor causing suppression of the transcriptional activation of the thermogenic program.

As some of these here negatively validated markers have previously been used in human studies, the conclusions based on them should be carefully reconsidered.

Positional expression patterns. As the expression of *Hox* genes is spatially regulated (15), we asked whether their expression levels in adipose depots displayed a similar positional pattern. To this end, we reordered the expression data in a simple, anatomically defined anteroposterior manner. This showed that *Hoxc9* expression is restricted to the posterior depots (Fig. 14*A*). *Hoxc9* had not earlier been detected in BAT. However, the more posterior prBAT showed pronounced *Hoxc9* expression (Figs. 13*I* and 14*A*). The posterior restriction of *Hoxc9* explains why it had not been observed in BAT before, as previously analyzed BAT depots were located anteriorly (aBAT, cBAT, mBAT, and iBAT) (47).

For *Hoxc8*, we also found an anteroposterior expression pattern but with some exceptions (Fig. 14*B*). This may, in part, explain the lower *Ucp1* levels in more posterior WAT depots, as *Hoxc8* expression has been shown to decrease *Ucp1* expression (22). Indeed, *Hoxc8* expression correlated negatively with *Ucp1* expression levels (Fig. 11*E*). *Hoxc8* may thus provide an example that positionally restricted gene expression might directly affect tissue functionality.

Strikingly, *Zic1* expression is completely restricted to the most anterior adipose tissues (Fig. 14*C*). This is not completely unexpected, as *Zic1* expression during embryonic development is restricted to the most anterior somites (26). Indeed, there is a sharp boundary between neural crest-derived and mesoderm-derived tissues at the level of the clavicle (20), and *Zic1* expression may thus be related to this difference in origin of the tissues. In contrast, *Tcf21* expression seemed to be restricted to the more posterior tissues (Fig. 14*H*). It would thus seem that these genes are more related to the anatomic positioning of a certain tissue rather than to its function, something that had been suggested previously for the *Hox* genes and other

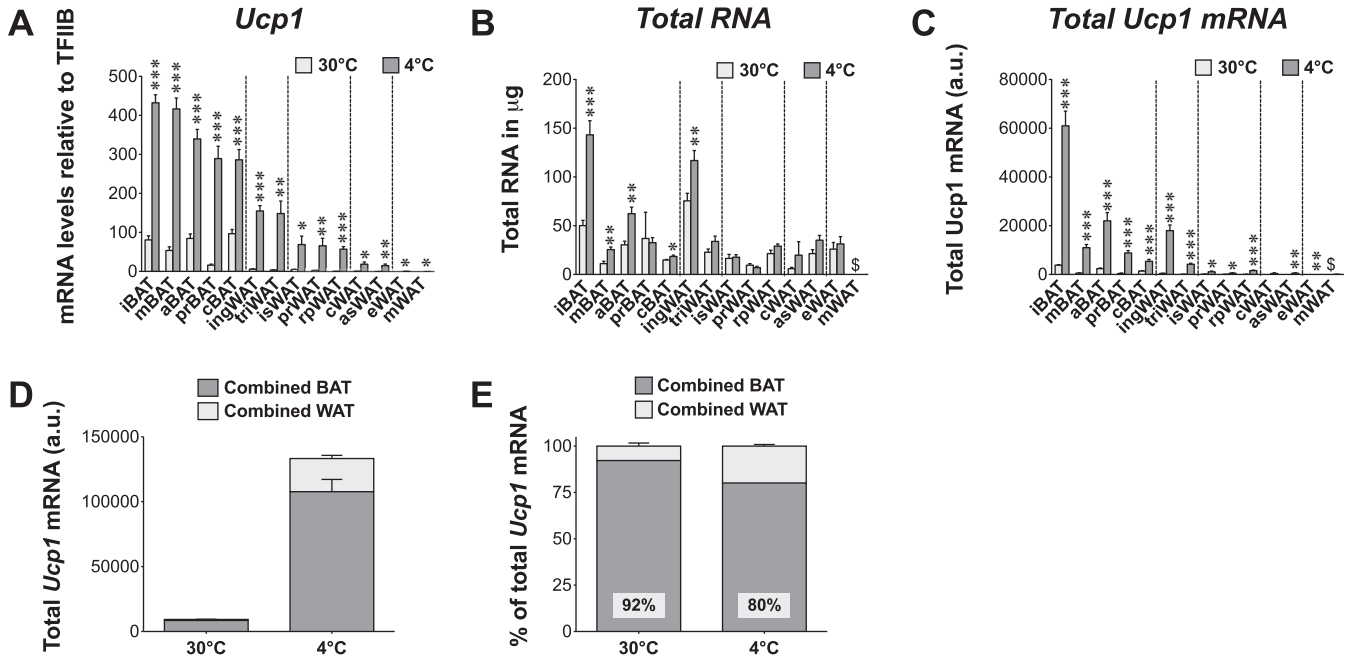

Fig. 9. *A*: relative *Ucp1* mRNA levels in the entire series of tissues analyzed (for abbreviations see Table 2). Tissues were ranked based on *Ucp1* expression levels at 4°C. *B*: amounts of total RNA in μg isolated from each tissue. *C*: amounts of total *Ucp1* mRNA per tissue (calculated by multiplying data for each mouse from *chart A* with those from *chart B*). *D*: amounts of total *Ucp1* mRNA combined for all BATs and WATs (based on indicated nomenclature). *E*: data from *D* given in %total *Ucp1* mRNA in all analyzed tissues combined, at 30°C and 4°C. *Significance of cold effect in *A–C* calculated with Student's unpaired *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. For every tissue and condition, $n = 6$. $*B* and *C*: mWAT data are not presented because of large variation in RNA yield. However, since *Ucp1* mRNA levels are practically negligible, this does not affect the results in *C, D*, and *E*.

developmental genes in adipose depots (9, 50). However, it may also be considered that the anatomic localization of a (adipose) tissue could be determinative for its identity and functionality, and these genes will provide this information to the cell. No dorsoventral patterns of gene expression could be observed.

Orphan tissues. While most tissues displayed pronounced expression of at least one of the positively validated marker genes, there were three tissues that did not. Based on the positively validated markers, no clear identity could be ascribed to rpWAT, cWAT, and asWAT. Only *Tmem26* was expressed at moderate levels in rpWAT, cWAT, and asWAT, but at levels lower than in ingWAT, triWAT, and mWAT (Fig. 12*E*). The lack of pronounced expression of any of the marker genes implies that these tissues represent a class different from any of the other commonly studied depots. This furthermore demonstrates the heterogeneous nature of the adipose organ, but also the need for additional means (markers) to classify these tissues.

A global view. To approach the analysis by a global rather than a single-gene approach, we performed principal component analysis (PCA) both of the ability of the entire group of markers to determine tissue classification, and of the nature of the tissues, based on all markers.

In Fig. 15*A*, we show components 1 and 2 for the genes analyzed, covering 38% and 15% of total variance, respectively. Further components covered less than 10% of total variance. The genes that clustered centrally are the ones that did not distinguish between different adipose depots. The four genes in this PCA that could be considered brite marker genes (*Cd137, Epsti1, Tbx1*, and *Tmem26*) were clustered together (Fig. 15*A*). The brite markers do not cluster with *Ucp1*, as the brite markers are not expressed in the *Ucp1*-rich classical brown depots. Furthermore, as expected, the classical brown marker *Zic1* also does not cluster with *Ucp1*, because *Zic1* is not expressed in all *Ucp1*-expressing depots. Together, the PCA results of the marker genes therefore confirmed that several analyzed markers were noninformative in adipose tissues and that the four brite markers (*Cd137, Epsti1, Tbx1*, and *Tmem26*) provided similar information about tissue identity. The three genes that displayed posteriorly restricted expression, *Hoxc9, Hoxc8*, and *Tcf21* (Fig. 14, *A, B*, and *H*), also clustered together (Fig. 15*A*). It is noteworthy that the results obtained with PCA are in line with the conclusions made for the single-gene analysis.

In the analysis above, the three cell surface markers *Asc1, Pat2*, and *P2rx5* were not included, as they have not been used to distinguish between brown and brite identity of human adipose tissues (44). Reanalysis including these genes (not shown) revealed that *P2rx5* and *Pat2*, as expected, were found in the noninformative cluster that does not distinguish between brown and brite, and *Asc1* was found on the whitish side, between the brite gene cluster and the white cluster.

In Fig. 15*B*, PCA of the different tissues is presented. Components 1 and 2 cover 59% and 23% of total variance, respectively. The three tissues that are considered classical BAT and that express *Zic1* (aBAT, cBAT, and iBAT) were clustered together on the PCA plot, indicative of their similar gene expression profiles (Fig. 15*B*). Note that cold acclimation consistently moved each tissue in the same direction, although to different extents (Fig. 15*B* and Table 3), indicating their responsiveness to cold acclimation. As can be seen, the classical brown and white depots displayed smaller overall responses in gene expression than did the more brite depots. Of

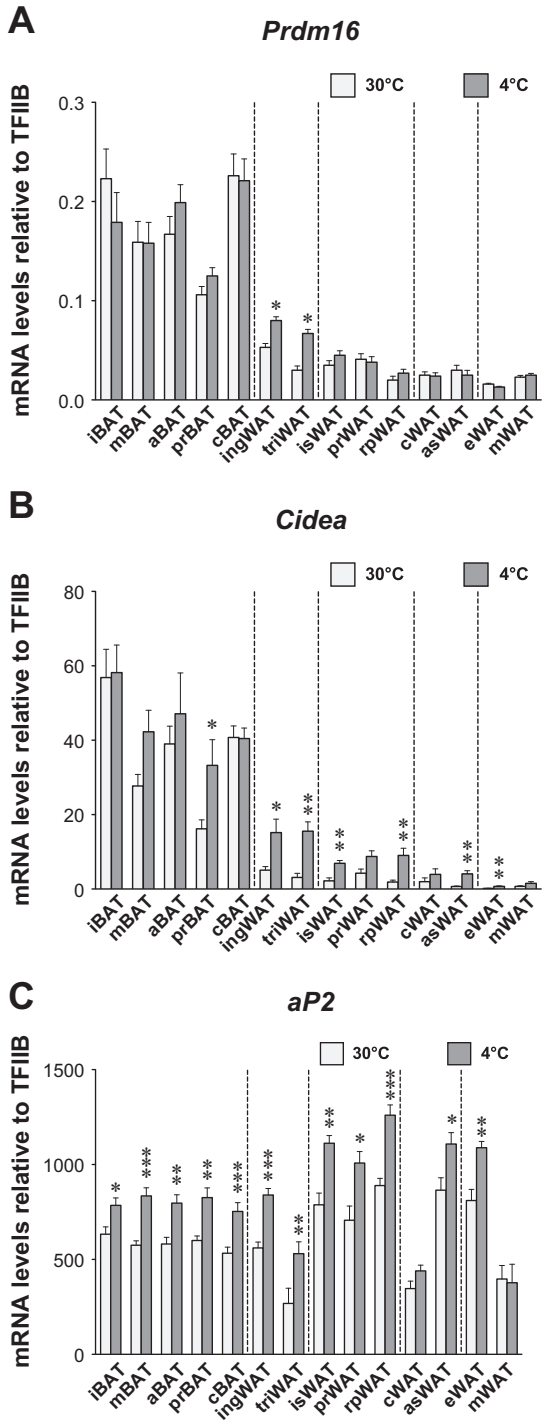

Fig. 10. Expression levels of brown fat characteristic genes [*Prdm16* (*A*) and *Cidea* (*B*)] and *aP2* (*C*) in the entire series of adipose tissues analyzed. The order of tissues is the same as in Fig. 9*A*. For abbreviations see Table 2. Effect of temperature was calculated with unpaired Student's *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. See Fig. 11 for correlations with *Ucp1* gene expression.

the orphan tissues (asWAT, cWAT, and rpWAT), only asWAT and cWAT were localized together in the PCA plot (Fig. 15*B*). This indicates that, although they were similar in terms of validated marker genes (Fig. 12), the whole set of analyzed genes indicated that asWAT and cWAT are more closely related to each other than to rpWAT.

DISCUSSION

In the present study, we evaluated the expression of recently proposed adipose marker genes that have been used to attempt to determine the identity of human adipose tissues. By making direct comparisons in classically defined iBAT (brown), ingWAT (brite), and eWAT (white) tissues, we invalidated the majority of proposed markers, as they did not unambiguously identify one of the three types of adipose tissues in mice. However, expression of some positively validated marker genes sheds new light on the identity of some of the adipose tissues analyzed. Furthermore, we demonstrate anteroposterior expression patterns for some markers.

Thus, the data presented here provide new insights into the ongoing discussion about adipose tissue identity in mice and humans and open new approaches to understanding and controlling adipose tissue development.

Few markers distinguish between iBAT, ingWAT, and eWAT. On the basis of the many studies that have looked at functional, molecular and developmental characterizations, we can assume that iBAT, ingWAT, and eWAT represent characteristic brown, brite, and white adipose depots, respectively. When we examined the ability of the proposed marker genes to identify these three tissues, we saw that only a few marker genes withstood this test. However, we were able to positively validate that some of the marker genes were able to distinguish between iBAT, ingWAT, and eWAT. *Zic1* (brown), *Cd137, Tbx1, Tmem26* (brite), and *Tcf21* (white) proved to have distinguishing ability at the tissue level. *Epsti1*, originally proposed as a classical brown marker (40), surprisingly appeared as a brite adipose tissue marker (Fig. 2*C*). In primary cell culture, only *Zic1* and *Tcf21* maintained their absolute distinctive ability.

Limitations of the present observations. Some of our results are not entirely in line with previous observations. For this, several explanations exist. For any study, choices concerning, e.g., mouse strain, cell culture conditions, etc., have to be made, and this may influence the outcomes.

Most strikingly, as pointed out above, we validated *Epsti1* to be a brite adipose tissue marker gene, whereas it was previously proposed as a classical brown adipose marker based on its expression in primary cell cultures (40). One explanation could be the use of different mouse strains (C57BL/6 vs. NMRI). However, at the tissue level, *Epsti1* displayed very similar expression patterns in iBAT, ingWAT, and eWAT of C57BL/6 and NMRI mice (not shown). Thus, our conclusion is not strain dependent.

Alternatively, the different observations regarding *Epsti1* (and also *Lhx8* and *Car4*) in culture could be explained by the differences in culture conditions. In our cell cultures, *Epsti1* was not more highly expressed in brown cells than in ingWA or eWA cells (Fig. 2*D*). Sharp et al. (40) made use of an adipogenic differentiation cocktail, whereas we obtained full differentiation in the absence of such a cocktail (28). It cannot be said which of the two systems is more correct.

Determination of tissue identity using the validated markers. Determination of adipocyte and adipose tissue identity, both in humans and in mice, is still an ongoing discussion. Whether brite cells differ from white adipocytes already at the precursor level, or if they arise through transdifferentiation of mature white adipocytes, is still under debate (29). Furthermore, since

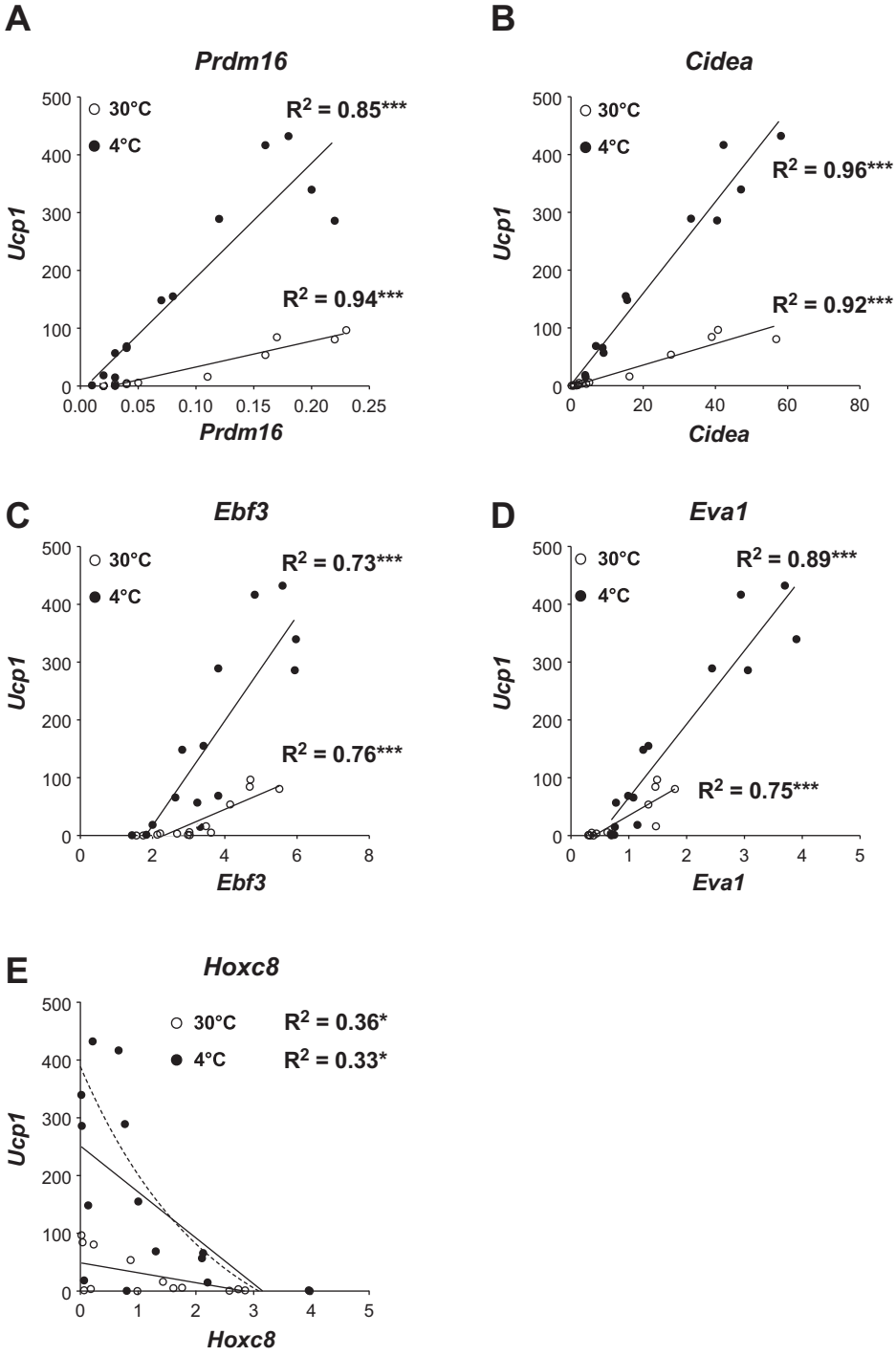

Fig. 11. Expression correlation with *Ucp1*. For *Prdm16* (*A*), *Cidea* (*B*), *Ebf3* (*C*), *Eva1* (*D*), and *Hoxc8* (*E*), correlation of mRNA levels with those of *Ucp1* is shown at both 30°C and 4°C. Based on data in Fig. 10 (*Prdm16* and *Cidea*) and Fig. 13 (*Ebf3, Eva1*, and *Hoxc8*), each point represents the average of 6 mice for one tissue at 30°C (○) or 4°C (●). None of the other genes analyzed in this study showed similar correlation with *Ucp1*. For *Hoxc8* (*E*), curve shows nonlinear fit R^2. Linear regression and nonlinear fit (*Hoxc8*) were calculated in GraphPad Prism v. 5.0f. $*P < 0.05$, $***P < 0.0001$. Encircled points were excluded from the analysis.

not all white adipocytes are functionally equal (i.e., subcutaneous vs. visceral; reviewed in Ref. 42), brown adipocytes in different BAT depots may also possess certain different characteristics. Most studies on adipose identity have been limited to only a few adipose depots (most often iBAT, ingWAT, and eWAT). Using the markers that we validated in these three tissues, we shed light here on differences in adipose tissue identity in a large set of adipose depots.

BAT according to Zic1. Zic1, the proposed classical brown adipose marker, was expressed in iBAT, aBAT, and cBAT at similar levels, as observed earlier (Fig. 12*A*) (47). However, *Zic1* was also detected in isWAT (Fig. 12*A*). According to the moderate expression levels of the functionally characteristic brown fat genes, isWAT would not generally be considered to be (classical) BAT (Figs. 9*A* and 10, *A* and *B*). An explanation for this observation seems to be the positional expression pattern of *Zic1* (Fig. 14*C*), something that has been suggested recently (19). Long et al. excluded *Zic1* as a classical brown marker because of its presence in isWAT.

Thus, among the adipose depots, *Zic1* is completely restricted to the most anterior depots and not only to the classical brown depots, as was initially thought (47). This anterior

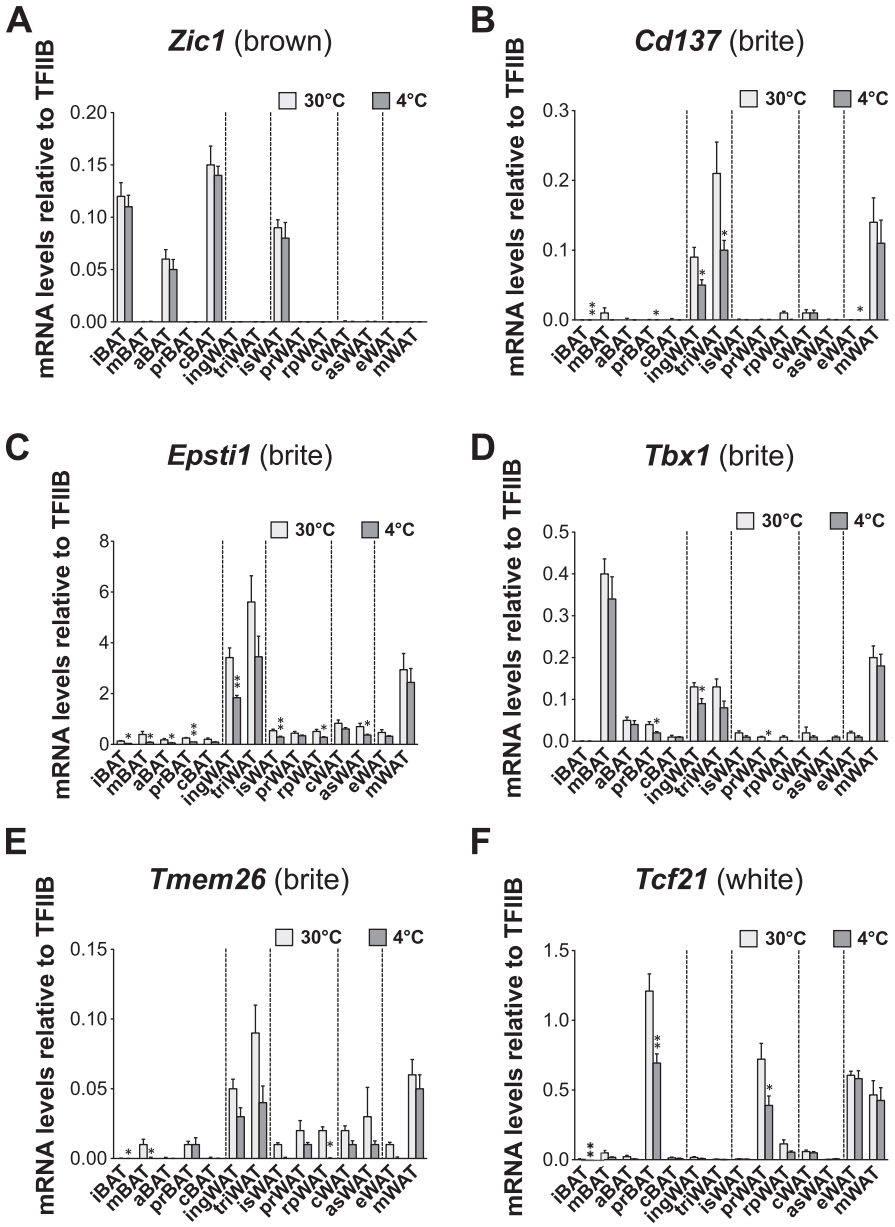

Fig. 12. Gene expression levels of validated marker genes *Zic1* (*A*), *Cd137* (*B*), *Epsti1* (*C*), *Tbx1* (*D*), *Tmem26* (*E*), and *Tcf21* (*F*) in the whole series of adipose tissues analyzed. For abbreviations see Table 2; see legend to Fig. 10 for statistics.

expression of *Zic1* is in line with its expression during embryonic development, which is restricted to the most anterior somites (26). This anatomic restriction of *Zic1* expression can also explain its absence in mBAT and prBAT, as these are the most posterior BAT(-like) depots (Fig. 14*C*). The absence of *Zic1* expression indicates that these tissues are different in origin from the other three classical BAT depots (iBAT, cBAT, and aBAT).

However, a trait that is associated with classical BAT identity is its origin from *Myf5*-expressing muscle precursors (35, 43), although significant amounts of *Myf5*-derived adipocytes have later been observed in isWAT and rpWAT (32, 33). The presence of *Zic1* in isWAT thus coincides with the presence of *Myf5*-derived cells in this tissue (Fig. 12*A*) (32, 33). It could thus be that isWAT represents an inherently classical BAT depot (i.e., *Myf5*-derived, expressing *Zic1*) that has lost the classical brown functionality, possibly due to limited adrenergic innervation.

Thus, it remains to be shown that *Myf5*-derived, *Zic1*-expressing (pre)adipocytes from the isWAT depot cannot potentially form functional brown adipocytes (when exposed to sufficient stimulation).

The absence of *Zic1* in mBAT and prBAT (generally considered classical BAT depots) coincides with the observation that adipocytes in these depots are not derived from the *Myf5* lineage (32). Thus, although having similar appearances as the other classical BAT depots (Figs. 9*A*, 10, *A* and *B*), mBAT and prBAT are clearly not classical BAT in the sense of *Myf5* and *Zic1*.

Although rpWAT is mostly derived from the *Myf5*-positive lineage (32), *Zic1* was not expressed in this tissue, at least not at the time investigated here (Fig. 12*A*). Interestingly, rpWAT displays a brown-like phenotype during a short period of time early in life but then reverts to a white-like tissue (49). Additionally, the contribution of *Myf5*-derived adipocytes decreased to about one-half within 6 mo (32). rpWAT thus

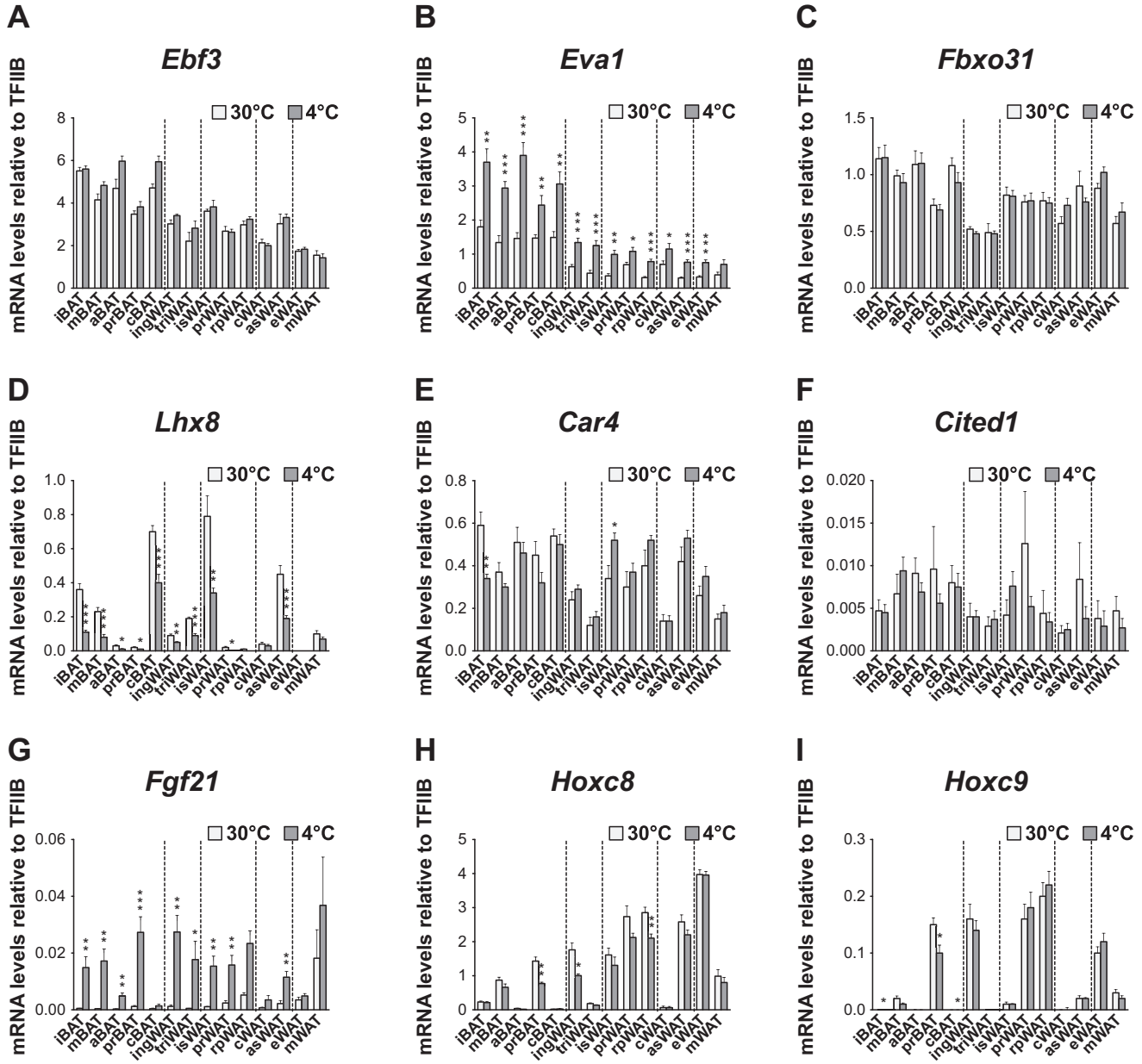

Fig. 13. Gene expression levels in the entire adipose tissues series of proposed markers that were excluded after analysis in iBAT, ingWAT, and eWAT (see Figs. 1–4). These genes include the proposed brown adipose markers *Ebf3* (*A*), *Eva1* (*B*), *Fbxo31* (*C*), and *Lhx8* (*D*), the proposed brite/beige markers *Car4* (*E*), *Cited1* (*F*), *Fgf21* (*G*), and *Hoxc8* (*H*) and *Hoxc9* (*I*). For abbreviations see Table 2; see legend to Fig. 10 for statistics.

proves to be a developmentally dynamic tissue. The possibility exists that *Zic1* expression in rpWAT might be lost over time with the shift to non-*Myf5*-derived adipocytes.

Adipose identity according to the brite markers. Of the proposed brite markers, only three (*Cd137*, *Tbx1*, and *Tmem26*) enabled distinction between ingWAT, iBAT, and eWAT. *Epsti1*, initially proposed as a classical brown marker (40), rather appeared to be a valid brite tissue marker, as it was expressed specifically in ingWAT compared with iBAT and eWAT (Fig. 2*C*), in both C57BL/6 and NMRI strains. This seemingly contrasting result of *Epsti1* could thus result from differential expression in tissue and under certain culture conditions. This example thus highlights the importance of analyzing marker expression both in tissue and in cell culture.

Notably, these four markers all lost their ability to distinguish between tissue origins in the cell cultures derived from iBAT, ingWAT, and eWAT, even when the cultured cells were stimulated with NE or rosiglitazone.

Each of these four brite markers is expressed in triWAT at levels similar to those in ingWAT (Fig. 12, *B–E*). Notably, with regard to all genes analyzed in this study, triWAT shows a striking resemblance to ingWAT. The two *Hox* genes are the only exceptions in this respect, which is in line with their anteroposterior expression pattern (Fig. 14, *A* and *B*). On the basis of this remarkably similar gene expression profile, we

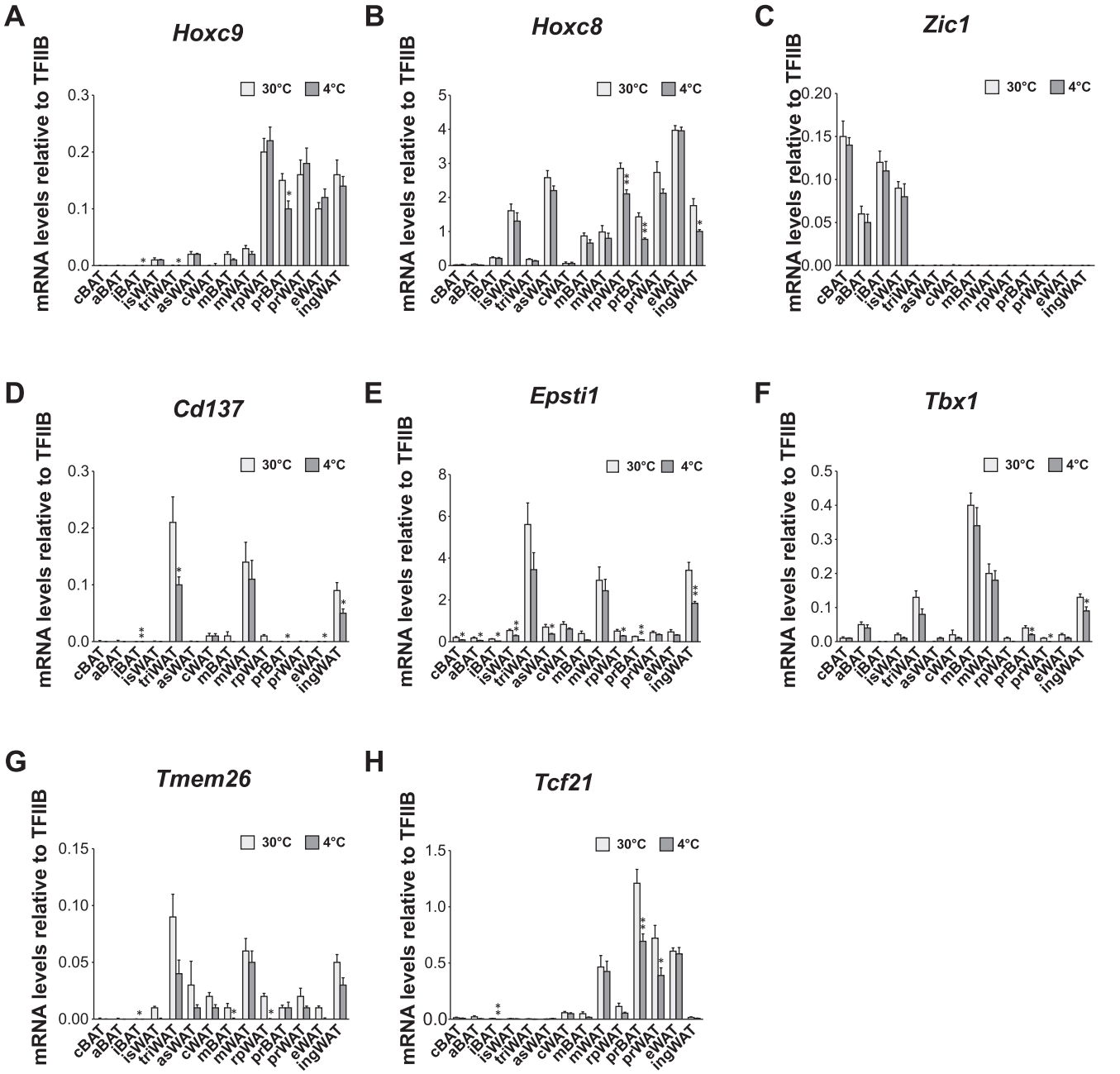

Fig. 14. Anterior to posterior expression patterns of *Hoxc9* (*A*), *Hoxc8* (*B*), *Zic1* (*C*), *Cd137* (*D*), *Epsti1* (*E*), *Tbx1* (*F*), *Tmem26* (*G*), and *Tcf21* (*H*). Tissues were ordered as they are anatomically localized in an anteroposterior manner in mice.

would conclude that triWAT is a brite depot with similar characteristics to ingWAT.

We also found *Cd137*, *Epsti1, Tbx1*, and *Tmem26* expressed in mWAT (Fig. 12, *B–E*), the depot with the lowest expression levels of *Ucp1* and very low *Prdm16* and *Cidea* expression (Fig. 10, *A* and *B*). This could imply that mWAT contains potentially brite (precursor) cells that under normal physiological recruiting conditions (i.e., cold exposure) do not become functionally brite (i.e., *Ucp1* expressing), again possibly due to limited adrenergic innervation. Otherwise, the presence of these four brite markers in a functionally white depot would argue against these genes as brite adipose tissue markers.

Furthermore, *Tbx1* is most highly expressed in mBAT, one of the tissues with high expression of the functional brown genes (Figs. 9*A* and 10, *A* and *B*). But since mBAT lacks expression of *Zic1*, its classification can be debated.

Tmem26 mRNA, in addition to being found in ingWAT, triWAT, and mWAT, is detected in all of the adipose tissues with the WAT nomenclature, at approximately one-half the level of ingWAT (Fig. 12*E*). Since the expressions of these four brite markers only overlap in ingWAT, triWAT, and mWAT, it is difficult to draw any conclusions about brite identity within the other adipose depots, which seem to contain brite adipocytes based on cold-induced *Ucp1* expression (Fig. 9*A*).

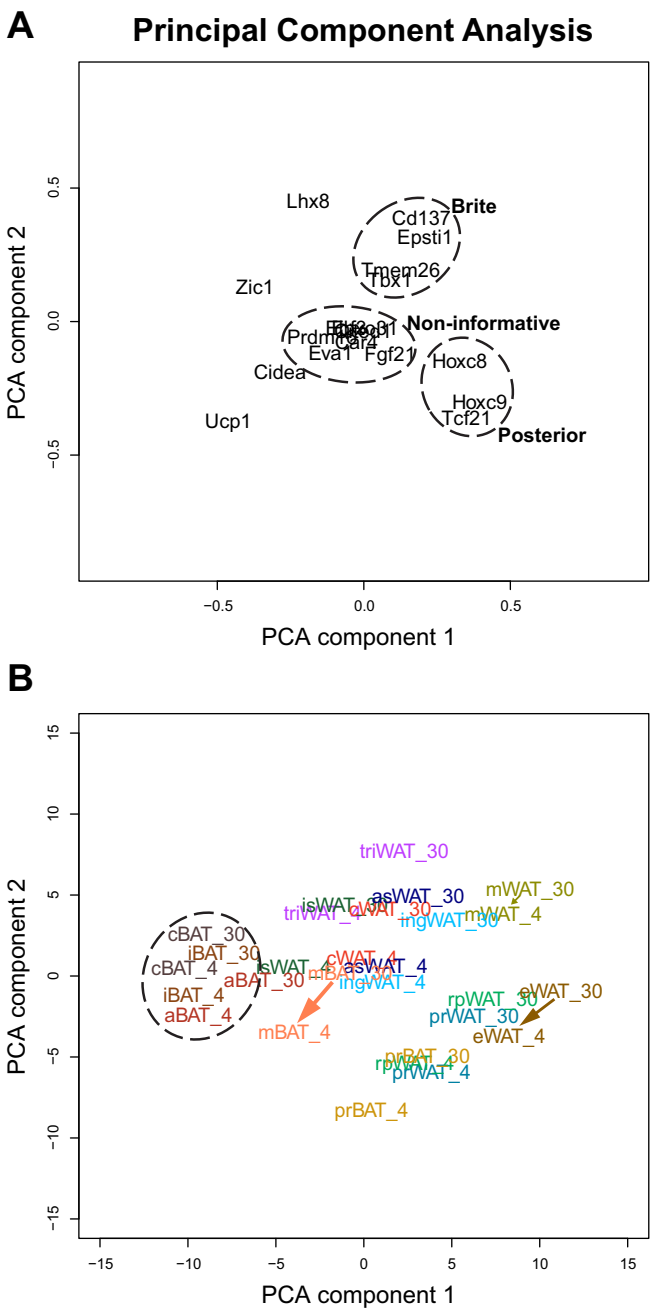

Fig. 15. Principal component analysis (PCA) plots for the analyzed genes (*A*) and tissues (*B*). *A*: genes clustered in the oval in the center (indicating that they are noninformative) are *Car4, Cited1, Ebf3, Eva1, Fbxo31, Fgf21*, and *Prdm16*. We have indicated with ovals the clustering of brite marker genes (*Cd137, Epsti1, Tbx1*, and *Tmem26*) and posteriorly expressed genes (*Hoxc8, Hoxc9*, and *Tcf21*). *B*: oval shows clustering of *Zic1*-expressing classical BAT depots (aBAT, Cbat, and iBAT). Note the consistent shift in (south-)southwest direction resulting from cold acclimation (indicated with arrows for a few tissues). For the values of this shift for each tissue see Table 3. Biplot analysis of the shift indicated that it was primarily due to *Ucp1, Cidea, Eva1*, and *Prdm16*. Although *Fgf21* expression is also strongly induced at 4°C, it did not "pull" tissues in the (south-)southwest direction.

WAT according to Tcf21. In addition to being present in eWAT, the proposed WAT marker *Tcf21* was detected in mWAT and in the two perirenal adipose depots (prBAT and prWAT) (Fig. 12*F*). As mWAT displayed expression of presumed brite markers, the additional detection of a white marker might result from a mixture of brite and white cells in this particular depot. According to the absence of *Tcf21* expression in ingWAT and triWAT, the nature of white adipocytes in these depots is seemingly different from those in mWAT (and eWAT) (Fig. 12*F*). This is in line with their different developmental origins (4).

The absence of *Zic1* in prBAT put the classical BAT identity of this depot into question, and the presence of *Tcf21* in the depot adds to this notion. This at least demonstrates the existence of a depot that expresses high levels of *Ucp1* but also a marker associated with white adipose identity. Additionally, analysis of the positional expression implied that *Tcf21* might only be expressed at this particular anatomic area where eWAT, mWAT, prBAT, and prWAT reside (Fig. 14*H*), presumably explaining the detection of *Tcf21* in these seemingly functionally different depots. However, again, the anatomic location of a particular adipose depot may also determine its functionality.

Alternative interpretations of the marker data. In the present approach, certain genes were validated as good markers based on their differential expression in iBAT, ingWAT, and eWAT. However, analysis in a broader set of tissues resulted in data that can be interpreted in alternative ways. The pre hoc interpretation, which is principally the one used here, is to determine tissue identity based on this initial validation of markers. The post hoc interpretation is to redetermine the informative value of the markers based on their expression throughout the adipose organ in relation to *Ucp1* expression levels.

Upon validation in the three characteristic brown (iBAT), brite (ingWAT), and white (eWAT) adipose depots, we concluded, as stated above, that *Zic1* (brown), *Cd137, Epsti1, Tbx1, Tmem26* (brite), and *Tcf21* (white) should be considered valid adipose tissue markers.

However, *Zic1* was also expressed in isWAT. This observation can be interpreted in two ways. We have considered the possibility that *Zic1* marks inherently brown adipose cells in isWAT. However, the condition of prolonged cold exposure has not resulted in formation of functionally brown adipocytes as indicated by *Ucp1* expression levels; but this may, e.g., be due to lack of nerve stimulation.

Table 3. *Tissue shifts*

Tissue	Distance
mWAT	2.1
cBAT	2.4
iBAT	3.1
cWAT	3.4
aBAT	3.7
eWAT	4.0
prWAT	4.2
prBAT	4.5
isWAT	4.7
mBAT	4.8
asWAT	5.0
ingWAT	5.3
rpWAT	5.9
triWAT	6.0

Table 3. (with Fig. 15*B*). Euclidian distances in the principal component analysis (PCA) space for each tissue at 4°C vs. 30°C as presented in Fig. 15*B*. Each of the values here indicates a shift in the (south-)southwest direction. These values may be said to indicate the general shift in marker gene expression associated with cold acclimation.

Alternatively, in the post hoc interpretation, it could be argued that *Zic1* should not be considered a proper brown marker exactly because of its presence in isWAT. This is the interpretation by Long et al. (19), but is of course based on the presumption that isWAT is really not brown, not even disguised (unrecruited) brown. This post hoc analysis thus leaves us without any brown fat markers.

Similarly, *Cd137, Epsti1, Tbx1*, and *Tmem26* (all here validated as brite markers) all were expressed in mWAT (Fig. 12, *B–E*), which has the lowest *Ucp1* expression levels (Fig. 9*A*). In the pre hoc interpretation, this observation may suggest a possibly dormant brite capacity of mesenteric WAT. Although physiological stimulation (3 wk of cold exposure) does not induce high levels of *Ucp1* mRNA, the marker data could potentially imply the presence of inherently brite cells, but again not physiologically evoked.

In contrast, the post hoc interpretation would argue that *Cd137*, *Epsti1*, *Tbx1*, and *Tmem26* turn out to be noninformative markers exactly because they are expressed in a white adipose tissue (mWAT). That interpretation leaves us with no brite/beige markers.

In addition to interpretation of the presence of proposed marker genes, the absence of their expression also can be interpreted in two ways. For example, *Zic1* is not expressed in mBAT and prBAT (Fig. 12*A*). Based on *Ucp1, Prdm16*, and *Cidea* mRNA levels (Figs. 9*A* and 10, *A* and *B*), these depots could be considered functionally brown. However, the absence of *Zic1* expression in the pre hoc interpretation implies that they are inherently different from the other classical brown adipose depots. The post hoc interpretation would again conclude that *Zic1* therefore is not a brown marker.

Similarly, most of the WATs have the capacity to express, at least to some extent, *Ucp1* (Fig. 9*A*), implying the presence of brite adipocytes. The absence of *Cd137, Espti1, Tbx1*, and *Tmem26* in most of these WAT depots implies that they are inherently different from ingWAT, triWAT, and mWAT. The post hoc interpretation would again conclude that these four genes are not brite markers, because they do not mark all depots that contain brite adipocytes.

One can argue for either way of interpretation of the marker data. It should be pointed out that acceptance of the post hoc interpretations would mean that currently we are totally devoid of any valid marker. It would, therefore, based on the post hoc interpretation, not be possible to state, e.g., whether a given depot of human *UCP1*-expressing tissue is of brown or brite/beige nature.

Relevance for human studies. Despite the fact that a post hoc interpretation would eliminate all until now suggested markers, we believe that with the information obtained here about marker genes it is now possible and important to reevaluate the conclusions from human studies that have been based on the expression of marker genes (5, 8, 13, 16, 17, 30, 34, 40, 48). As most of the marker genes used proved to be uninformative in our study, the revised results of the human studies now comes to rely on the observations of very few marker genes. An important open issue is whether marker genes identified in murine systems can be translated to humans, but, of course, if this is not the case we lack tools to identify the nature of the human depots. Given this prerequisite, we have reanalyzed the relevant papers for their observations on the validated markers, i.e., *ZIC1* for brown and *CD137*, *TBX1*, *TMEM26*, and *EPSTI1* for brite. A positive identification of brown or brite would be the case if these markers are enriched compared with white adipose tissue (mostly being defined as adipose depots with much lower *UCP1* expression levels).

ZIC1 is enriched in (deep) neck depots (8, 13) and in the interscapular depot (in babies) (16). In epicardial adipose tissue it is not enriched (30), although *UCP1* is enriched; the nature of this depot is therefore uncertain [we did not find *Ucp1* in the cardiac (white) adipose depot (Fig. 9*A*)]. Similarly, *ZIC1* was not enriched in the mediastinal adipose tissue depot, but here *UCP1* was only very marginally enriched (5). Due to the way gene expression was analyzed in Sharp et al. (40) (as relative to all other genes expressed in all tissues examined), it can only be concluded from the data of Sharp et al. that the expression levels of *ZIC1* in all examined human tissues were lower than that of, e.g., *UCP1*, but nothing can be said concerning the relative expression in different tissues. Thus, even with stringent validation, human brown fat is classical brown fat in the (deep) neck and in the interscapular area. Concerning any other depot, it can be neither concluded nor excluded that the tissue is classical brown.

From the stringent analyses here, the brite/beige marker gene *CD137* (or *TNFRSF9*) should be expressed at a level 10 times higher than that in white fat to qualify a given depot as being brite (Fig. 3*C*). *CD137* has been detected at higher levels in tissues designated as human BAT than in WAT control tissues (8, 40, 48), but the enrichment has generally been modest in, e.g., the neck depots (8, 48) and very varied in different depots (40). Many subjects did not show *CD137* enrichment (8, 40). Thus, the presence of brite cells in the neck and elsewhere is not fully verifiable by *CD137*.

Similarly, the brite marker *TBX1* should be expressed at 5–10 times higher levels in an examined depot compared with WAT to make it evident that the depot is brite (Fig. 3*K*). *TBX1* was expressed at modestly higher levels in human neck depots than in WAT controls in some studies [(48) ≈2-fold; (13) ≈3-fold], but not in all neck depot studies (8); the data in Ref. 40 are also here difficult to evaluate. Thus, the presence of brite cells in the neck and elsewhere is not fully verifiable by *TBX1* either.

A similar conclusion can be drawn from the results obtained for *TMEM26* expression in human BAT. In some studies, *TMEM26* appears in human BAT (40, 48), whereas other studies see no differences in *TMEM26* expression between BAT and WAT (8, 13).

EPSTI1 was originally analyzed as a brown-selective marker and was expressed at lower levels in human tissues identified as BAT than in control WAT (40). However, since we validated *Epsti1* in mice to be a brite marker, its low expression in human BAT would instead argue *against* a brite identity.

Thus, looking individually on the validated brite markers, the evidence that human BAT is (also) brite is not compelling. It may, however, be said that a tendency for all of these markers to be somewhat enriched in the examined depots collectively supports the coexistence of brite and classical brown adipose tissue in adult humans.

CONCLUSIONS

The nature of the marker gene expression profiles presented here indicates that the issue of tissue identity is more complex than previously assumed. Our results show that observations

performed in one or two (mouse) depots should not be extrapolated to other depots either in mice or in humans. The validation performed here of certain markers should enable better classification of the different human depots and through this allow for determination of the origin of the depots. Understanding of the developmental origin could provide more insight into the innate and acquired adipocyte diversity and how this affects adipose function in mice and humans.

ACKNOWLEDGMENTS

We thank Natasa Petrovic for constructive discussions.

GRANTS

These studies were supported by the Swedish Research Council, the European Union Collaborative Project DIABAT, and the Knut and Alice Wallenberg Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). B. Cannon is on the Scientific and Medical Advisory Board of Metabolic Solutions Development Corporation.

AUTHOR CONTRIBUTIONS

Author contributions: J.M.d.J., B.C., and J.N. conception and design of research; J.M.d.J. performed experiments; J.M.d.J., O.L., B.C., and J.N. analyzed data; J.M.d.J., B.C., and J.N. interpreted results of experiments; J.M.d.J. and O.L. prepared figures; J.M.d.J. and J.N. drafted manuscript; J.M.d.J., O.L., B.C., and J.N. edited and revised manuscript; J.M.d.J. and J.N. approved final version of manuscript.

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