A stringent validation of mouse adipose tissue identity markers

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A number of earlier 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 UCPI 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

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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 CO2 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 CO2 and cervical dislocation. Tissues were dissected and prepared for cell culture as described in Petrovic et al. (27). 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.
- **cWAT (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.
- **trWAT (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.
- **pWAT (perirenal WAT):** The pWAT is located on the other side of the renal capsule and posterior to the capsule.
- **rpWAT (retroperitoneal WAT):** The rWAT 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.

**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 TFIB. TFIB was essentially equally expressed throughout a large set of different adipose tissues, and expression levels were not influenced by cold
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. 1A). 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. 1A). *Ucp1* expression was also increased in eWAT but remained at much lower levels than in iBAT and ingWAT (Fig. 1A). 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. 1B) (26, 28, 40). In cultured brown adipocytes (BA), *Ucp1* was increased to similar mRNA levels in the presence of acute NE or of chronic rosiglitzone (Fig. 1B). In ingWAT cultures, we observed a large difference in *Ucp1* induction between acute NE and chronic rosiglitzone treatment: acute NE treatment resulted in only a 40-fold induction whereas chronic rosiglitzone treatment led to an 800-fold increase (Fig. 1C). Cold acclimation did not notably change its expression. In the cultures, *Prdm16* mRNA levels were highest in BA and decreased in the presence of rosiglitzone as observed earlier (Fig. 1D) (28). The treatments only slightly influenced *Prdm16* levels in ingWA (Fig. 1D).

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

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. 1G). In all three cultures, basal expression of *ap2* was somewhat higher in BA and ingWA than in eWAT but was induced by rosiglitzone to similar levels (Fig. 1H). 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.

Table 1. Primer sequences

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*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%).
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. 2A). 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. 2B). 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. 2D) 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. 2E). *Eva1* expression increased upon cold acclimation in all three tissues (Fig. 2E), implying a possible functional role in cold acclimation in adipose tissue; in this respect it correlates with *Ucp1* gene expression (see also Figs. 9C and 11D). In cell culture, basal levels were similar in BA, ingWA, and eWA, with some tendency to a better recruitment induction in BA (Fig. 2F). 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. 2G). *Fbxo31* expression levels were similar in all three cell cultures (Fig. 2H). 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. 2I). *Lhx8* mRNA was downregulated after cold acclimation (Fig. 2I) and, in line with earlier observations (28), also in the presence of rosiglitazone in BA cultures (Fig. 2I). The same downregulation was observed in ingWA cultures (Fig. 2J). 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.
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. 3A). 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. 3B). However, the same response occurred in BA cultures (36-fold increase) and in eWA cultures (7-fold increase; Fig. 3B). 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. 3C). 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. 3D), 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-differentiated...
ated cells, the expression in brite cells was, if anything, even lower than in more-differentiated cells (Fig. 4A). 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. 3E). In all three types of culture, Cited1 expression tended to be increased by acute NE stimulation (Fig. 3F). Cited1 expression was also induced by rosiglitazone, but only in ingWA and eWA cultures (Fig. 3F). 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. 3G). In all three cell cultures, we saw a synergistic effect of rosiglitazone and acute NE treatment on Fgf21 mRNA levels (Fig. 3H). Fgf21 mRNA levels were highest in BAT cultures (Fig. 3H). 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. 3I). Similarly, Hoxc9 was expressed in both ingWA and eWA (Fig. 3J). 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. 3L), 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. 3N). 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. 4B). 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. 5A). 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. 5C). 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...
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 β3-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. 8B) as in tissues (Fig. 8A), 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. 9A). 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. 9A). 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. 9A). Among the white/brite adipose depots, there was a wide range of *Ucp1* expression levels and thus
arguably a wide range of “briteness” (Fig. 9A). ingWAT and triWAT had the highest $Ucp1$ expression levels (Fig. 9A). isWAT, rpWAT, and prWAT had slightly lower maximum $Ucp1$ levels (Fig. 9A). cWAT and asWAT had even lower $Ucp1$ expression levels in the cold, but these were still higher than those in eWAT and mWAT (Fig. 9A).

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. 9B). 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. 9B). The total RNA amounts in other depots were unaffected by temperature (Fig. 9B). Calculation of the total amount of $Ucp1$ mRNA in each depot (data in Fig. 9A multiplied by data in Fig. 9B) showed a cold acclimation-induced increase in all analyzed tissues (Fig. 9C). 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. 9E).

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
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, Epstl1, 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-
considered to be equally brite as ingWAT (Figs. 9A 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. 12D). 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. 12F). These tissues display different levels of Ucp1 expression (Fig. 9A). From the presence of Cd137, Epst11, 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. 12F).

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. 11C). 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.

Eval and Fg21 were the only two marker genes that were upregulated in almost all adipose depots under cold-acclimated conditions (Fig. 13, B and G). Eval expression levels displayed a clear correlation with Ucp1, similar to what was observed for Prdm16 and Cidea (Fig. 11D); thus, Eval 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. 11E). 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 the negative factor causing suppression of the transcriptional activation of the thermogenic program.

As some of these 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. 14A). Hoxc9 had not earlier been detected in BAT. However, the more posterior prBAT showed pronounced Hoxc9 expression (Figs. 13I and 14A). 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. 14B). 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. 11E). 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. 14C). 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. 14H). 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
However, since \textit{Ucp1} expression levels are practically negligible, this does not affect the results in \textit{A}-\textit{C}. Tissues were ranked based on \textit{Ucp1} mRNA levels in the entire series of tissues analyzed (for abbreviations see Table 2). Tissues were ranked based on \textit{Ucp1} expression levels at 4°C. B: amounts of total RNA in μg isolated from each tissue. C: amounts of total \textit{Ucp1} mRNA per tissue (calculated by multiplying data for each mouse from \textit{chart A} with those from \textit{chart B}). D: amounts of total \textit{Ucp1} mRNA combined for all BATs and WATs (based on indicated nomenclature). E: data from \textit{D} given in %total \textit{Ucp1} mRNA in all analyzed tissues combined, at 30°C and 4°C. *Significance of cold effect in \textit{A–C} calculated with Student’s unpaired \textit{t}-test: \(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). For every tissue and condition, \(n = 6\). S8 and C: mWAT data are not presented because of large variation in RNA yield.

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.

\textit{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 \textit{Tmem26} was expressed at moderate levels in rpWAT, cWAT, and asWAT, but at levels lower than in ingWAT, triWAT, and mWAT (Fig. 12E). 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.

\textit{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. 15A, 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 (\textit{Cd137}, \textit{Eptsl1}, \textit{Tbx1}, and \textit{Tmem26}) were clustered together (Fig. 15A). The brite markers do not cluster with \textit{Ucp1}, as the brite markers are not expressed in the \textit{Ucp1}-rich classical brown depots. Furthermore, as expected, the classical brown marker \textit{Zic1} also does not cluster with \textit{Ucp1}, because \textit{Zic1} is not expressed in all \textit{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 (\textit{Cd137}, \textit{Eptsl1}, \textit{Tbx1}, and \textit{Tmem26}) provided similar information about tissue identity. The three genes that displayed posteriorly restricted expression, \textit{Hoxc9}, \textit{Hoxc8}, and \textit{Tcf21} (Fig. 14, \textit{A}, \textit{B}, and \textit{H}), also clustered together (Fig. 15A). 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 \textit{Ascl1}, \textit{Pat2}, and \textit{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 \textit{P2rx5} and \textit{Pat2}, as expected, were found in the noninformative cluster that does not distinguish between brown and brite, and \textit{Ascl1} was found on the whitish side, between the brite gene cluster and the white cluster.

In Fig. 15B, 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 \textit{Zic1} (sBAT, cBAT, and iBAT) were clustered together on the PCA plot, indicative of their similar gene expression profiles (Fig. 15B). Note that cold acclimation consistently moved each tissue in the same direction, although to different extents (Fig. 15B 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
**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), ing-WAT (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. 2C). 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. 2D). 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...
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. 12A) (47). However, Zic1 was also detected in isWAT (Fig. 12A). According to the moderate expression levels of the functionally characteristic brown fat genes, isWAT would not generally be considered to be (classical) BAT (Figs. 9A and 10, A and B). An explanation for this observation seems to be the positional expression pattern of Zic1 (Fig. 14C), 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
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. 14C). 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. 12A) (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. 9A, 10A 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. 12A). 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...
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. 2C), 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
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. 9A 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. 12E). 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. 9A).
Principal Component Analysis

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, Efβ3, Eva1, Fbxo31, Fgfl, and Prdm16. We have indicated with ovals the clustering of brite marker genes (Cd137, Epst1, 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 Fgfl 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. 12F). 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. 12F). This is 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. 14H), presumably explaining the detection of Tcf21 in these seemingly functionally different de- pots. 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, Epst1, 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

<table>
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<tr>
<th>Tissue</th>
<th>Distance</th>
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<tr>
<td>mWAT</td>
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<tr>
<td>cBAT</td>
<td>2.4</td>
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<tr>
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<td>3.1</td>
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<td>cWAT</td>
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<td>aBAT</td>
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<td>prWAT</td>
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<td>mBAT</td>
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<tr>
<td>triWAT</td>
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Table 3. (with Fig. 15B). Euclidian distances in the principal component analysis (PCA) space for each tissue at 4°C vs. 30°C as presented in Fig. 15B. 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 iSAT. This is the interpretation by Long et al. (19), but is of course based on the presumption that iSAT is really not brown, not even disguised (unrecruited) brown. This post hoc analysis thus leaves us without any brown fat markers.

Similarly, Cd137, Epstil, 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. 9A). In the pre hoc interpretation, this observation may suggest a potentially 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, Epstil, 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. 12A). Based on Ucp1, Prdm16, and Cidea mRNA levels (Figs. 9A 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. 9A), implying the presence of brite adipocytes. The absence of Cd137, Epstil, 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. 9A)]. 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 brite (Fig. 3C). 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. 3K). 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 Epstil 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.

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AUTHOR CONTRIBUTIONS


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