A Smooth Muscle-Like Origin for Beige Adipocytes

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http://dx.doi.org/10.1016/j.cmet.2014.03.025

SUMMARY

Thermogenic UCP1-positive cells, which include brown and beige adipocytes, transform chemical energy into heat and increase whole-body energy expenditure. Using a ribosomal profiling approach, we present a comprehensive molecular description of brown and beige gene expression from multiple fat depots in vivo. This UCP1-TRAP data set demonstrates striking similarities and important differences between these cell types, including a smooth muscle-like signature expressed by beige, not classical brown, adipocytes. In vivo fate mapping using either a constitutive or an inducible Myh11-driven Cre demonstrates that at least a subset of beige cells arise from a smooth muscle-like origin. Finally, ectopic expression of PRDM16 converts bona fide vascular smooth muscle cells into Ucp1-positive adipocytes in vitro. These results establish a portrait of brown and beige adipocyte gene expression in vivo and identify a smooth muscle-like origin for beige cells.

INTRODUCTION

Obesity and associated metabolic disorders, including diabetes, cardiovascular disease, and hypertension, are growing in prevalence worldwide. A promising and emerging avenue for obesity treatment is to increase energy expenditure by augmenting the number or the activity of thermogenic adipocytes (Nedergaard et al., 2011; Wu et al., 2013). These specialized cells are characterized by having multiple lipid droplets, high mitochondrial density, and the expression of uncoupling protein 1 (UCP1), an inner mitochondrial membrane protein that dissipates the proton gradient to uncouple fuel oxidation from ATP synthesis (Cannon and Nedergaard, 2004; Nicholls et al., 1978). In rodents, there are at least two distinct types of UCP1-positive cells (Wu et al., 2012). The classical brown fat is found both in the interscapular region as well as in the perirenal area. Beige adipocytes are found interspersed in various white fat depots, and their formation can be stimulated by cold exposure or by β-adrenergic receptor agonists (Cousin et al., 1992; Young et al., 1984). That brown or beige fat activity confers metabolic benefit is now well established in mice (Cederberg et al., 2001; Cohen et al., 2014; Feldmann et al., 2009; Harms and Seale, 2013; Seale et al., 2011). Imaging and histological analyses have confirmed the existence of UCP1-positive fat in both newborn and adult humans and indicate the presence of both classical brown fat and beige fat (Cypess et al., 2009, 2013; Lidell et al., 2013; Sharp et al., 2012; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

Classical brown and beige adipocytes both express UCP1 and share many morphological and biochemical characteristics, including a well-characterized β-adrenergic receptor/cAMP-dependent pathway that regulates thermogenic gene expression. However, multiple lines of evidence have demonstrated that brown and beige adipocytes are in fact distinct cell types. First, classical brown, but not beige, adipocytes arise from a Myf5/Pax7 skeletal muscle lineage, indicating that there are separate developmental origins for these two cell types (Lepper and Fan, 2010; Sanchez-Gurmaches et al., 2012; Seale et al., 2009). Second, classical brown and beige fat are differentially responsive to various hormonal stimuli or genetic manipulations (Bostrom et al., 2012; Harms and Seale, 2013). Third, classical brown, beige, and white adipocytes possess distinct gene expression signatures in cell culture (Wu et al., 2012). Despite this progress, a complete molecular description of UCP1-positive adipocytes in vivo is still lacking. This is in part because UCP1-positive adipocytes generally, and beige adipocytes in particular, occur within a complex, heterogeneous mixture of other cell types in adipose tissues.

To address the cellular heterogeneity issue, we have adapted translating ribosome affinity purification (TRAP) technology (Heiman et al., 2008; Sanz et al., 2009). This method, originally developed for neuroscience applications, allows for genetic tagging and isolation of polyosomes in a highly cell type-specific manner. Using this approach, we have selectively isolated polyosomes of UCP1-positive cells directly from whole adipose tissues in mice. Our studies demonstrate striking similarities and surprising differences between brown and beige cells in vivo, including a smooth muscle-like gene expression signature in beige, but not classical brown, cells. In vivo fate mapping and primary smooth muscle culture experiments provide additional evidence for a smooth muscle-like origin of some beige cells. These results reveal a hitherto unappreciated link between beige fat and smooth muscle-like cells and provide a powerful foundation for elucidating additional aspects of thermogenic adipocyte function in vivo.
RESULTS

Generation and Characterization of the UCP1-TRAP Mouse

To characterize UCP1-positive adipocytes in vivo, we first generated a TRAP mouse. This animal expresses a fusion protein of eGFP and the 60S ribosomal subunit L10a downstream of a lox-stop-lox cassette knocked into the Rosa26 locus. The TRAP mouse was then crossed to a BAC transgenic strain expressing Ucp1-driven Cre to produce UCP1-TRAP mice (Figure 1A). In this way, the TRAP protein (eGFP-L10a) is only produced in those cells that also possess sufficiently high levels of Ucp1 promoter activity to induce Cre expression. Because the recombination event is irreversible and heritable, recombination was minimized in the immediate postnatal period by breeding UCP1-TRAP mice at thermoneutrality and then weaning litters were housed at 4°C. Unexpectedly, the expression of UCP1 protein was detected in the mesenteric visceral WAT; Ax, axillary WAT; Pg, perigonadal WAT; Mes, mesenteric WAT.

(A) Schematic of the cross to generate UCP1-TRAP mice. A BAC transgenic expressing Ucp1-Cre was crossed with a TRAP mouse expressing a fusion protein of eGFP and the ribosomal subunit L10a (eGFP-L10a, referred to as the TRAP allele) under the control of a lox-stop-lox cassette in the rosa26 locus. (B) Western blot analysis of UCP1, GFP, and β-actin loading control from various fat depots in 6-week-old UCP1-TRAP mice at room temperature (−) or after 2 weeks cold exposure (+) at 4°C. Ing, inguinal WAT; Ax, axillary WAT; Pg, perigonadal WAT; Mes, mesenteric WAT.

(C) Representative H&E, UCP1, and GFP immunohistochemistry from 6-week-old male UCP1-TRAP (Cre positive, TRAP positive) or control mice (Cre negative, TRAP positive) after 2 weeks cold exposure at 4°C. (D) Relative mRNA expression of the indicated genes from various fat depots in UCP1-TRAP samples (left) or whole tissues (right). Tissues were harvested from 6-week-old female mice after 2 weeks cold exposure at 4°C. Data are presented as means ± standard error; n = 3–5/group. *p < 0.05 or < 0.01, respectively, in BAT versus pgWAT samples; ** p < 0.05 or < 0.01, respectively, in BAT versus iWAT samples. iWAT, inguinal WAT; pgWAT, perigonadal WAT; BAT, interscapular brown fat. Important findings include:

- Neither UCP1 nor eGFP-L10a protein was observed in the mesenteric visceral depot at any temperature, consistent with the known reduced prevalence of beige cells in visceral adipose depots.
- Immunohistochemical analysis of the inguinal depots from UCP1-TRAP mice is consistent with recombination occurring in a substantial portion of UCP1-positive cells in multiple fat depots (Figures 1C and S1A).
- Additionally, we observed colocalization of GFP and UCP1 protein at the cellular level (Figure S1B).
- Taken together, these data indicate that TRAP expression in the UCP1-TRAP mice recapitulates the endogenous expression of UCP1 in a substantial subset of cells.

Following this 2 weeks cold exposure protocol, the expression of genes related to thermogenesis was compared from whole tissues and also from immunofluorescence purified mRNA samples (UCP1-TRAP samples). In principle, the UCP1-TRAP samples should provide a readout of gene expression only in the UCP1-positive cells from different fat pads. Consistent with previous reports (Seale et al., 2011), the thermogenesis-related genes Ucp1, Elvi3, and Ppargc1a (PGC-1α) were highly expressed in the BAT depot, at an intermediate level in the subcutaneous inguinal (iWAT) depot, and at the lowest levels in the perigonadal (pgWAT) depot (Figure 1D). Importantly, the expression of these genes from UCP1-TRAP samples was much more similar in magnitude across the depots (Figure 1D). These data strongly suggest that the basic thermogenic gene program of classical brown and beige cells is quite similar. Moreover, the differences observed in expression of these genes on the tissue level are likely because UCP1-positive cells are diluted by the presence of other cell types.
of other cell types, especially white adipocytes. Interestingly, not all adipocyte genes were "normalized" in the UCP1-TRAP samples. Resistin (Retn), a marker of white adipocytes, was highly expressed in white depots at a pad level as well as in the UCP1-TRAP samples (Figure 1D). These data indicate that UCP1-positive beige cells in vivo retain some white-like characteristics that are completely absent in UCP1-positive cells of the classical BAT. Finally, we surveyed the expression of a panel of gene-expression events that were previously annotated to be classical BAT. Finally, we surveyed the expression of a panel of gene-expression events that were previously annotated to be classical BAT.

Identification of the Common Gene-Expression Program from Brown and Beige Adipocytes In Vivo
These initial experiments suggest that the TRAP methodology provided a unique gene-expression signature of UCP1-positive cells that would be otherwise obscured by other large differences in whole fat pad gene expression. To obtain an unbiased portrait of the gene-expression pattern of brown and beige cells in vivo, UCP1-TRAP samples from I.WAT, pgWAT, and BAT were analyzed by RNA-Seq (Table S1). At a global level, the expression signature of UCP1-positive cells from these distinct fat pads was remarkably similar (Figure 2). This similarity was quantified by two different methods. First, pair-wise comparisons between UCP1-TRAP samples from distinct depots are shown in Figures 2A–2C. Genes that map along the diagonal are equally expressed in UCP1-positive cells between the two depots, whereas genes that fall off the diagonal are enriched in UCP1-positive cells from one depot versus the other. By this analysis, a majority of genes are coordinately expressed between depots (Pearson's $r > 0.85$). Second, we devised a "ratio" metric so that UCP1-TRAP gene expression could be evaluated from all three depots simultaneously (see Experimental Procedures and Table S2). A ratio greater than 3 indicates at least a 3-fold difference in the expression of a particular gene between the highest and lowest expressing depot in UCP1-TRAP samples. Using this metric, only 1601 of the 9015 detected genes (18%) are differentially expressed in UCP1-TRAP samples; the remaining 7414 genes (82%) are common and equivalently expressed by UCP1-positive cells, regardless of depot of origin (Figure 2D). As a specific example, the 100 most abundant genes in the UCP1-TRAP-Seq data set are shown in Figure 2E. Many of the highly abundant genes in the "equivalent" category include well-known genes of thermogenesis and mitochondria (i.e., Ucp1, Cox8b, and Atp5b), as well as genes critical for the function of all adipocytes (i.e., Fabp4) (Figures 2E and S2A). In contrast, Scd1 and Fasn were in a small group of genes that were differentially expressed owing to their enrichment in beige versus classical BAT cells (Figure S2B). Taken together, these two analyses demonstrate that >80% of the gene expression in classical brown and beige cells converge onto a common and shared thermogenesis program in vivo following cold exposure (Figure S2C, and for a more detailed analysis see Table S3 and Experimental Procedures).

Identification of the Common Gene-Expression Signature In Vivo
(A–C) The average signal intensities (FPKM) for genes in the UCP1-TRAP-Seq data set are pairwise compared between depots. Pearson's correlation coefficient ($r$) for each comparison is shown as an inset. $n = 2$ for pgWAT and $n = 3$ for I.WAT and BAT. UCP1-TRAP-Seq samples were obtained from 6-week-old female mice following 2 weeks cold exposure at 4°C. (D) Pie chart showing the fraction of total detected genes from UCP1-TRAP-Seq classified as either "equivalent" (ratio $\leq 3$) or "different" (ratio > 3) (also see Experimental Procedures). (E) Scatter plot showing the ratio metric versus average signal intensity for the top 100 most abundant UCP1-TRAP-Seq genes. Abundance was determined by total FPKM detected across all UCP1-TRAP-Seq samples.

UCP1-Positive Cells Have a Signature of the Anatomical Location in which They Reside
We next analyzed the differentially expressed genes from our UCP1-TRAP-Seq data set. Hierarchical clustering of the differentially expressed genes (ratio > 3) from the UCP1-TRAP-Seq gene set is shown in Figure 3A. Interestingly, the majority of differentially expressed genes are depot specific, as shown by low degree of overlapping red rows in the heat map. To further study these differences, the most abundant and selectively expressed genes from each depot of the UCP1-TRAP-Seq data
were identified (groups 1, 2, and 3 in Figure 3A; see also Experimental Procedures and Table S4) and their depot-selective expression was validated by qPCR in an independent cohort of UCP1-TRAP mice (Figure S3).

Initially, we hypothesized that these differentially expressed genes could directly serve as novel in vivo markers for distinct populations of UCP1-positive cells. However, a straightforward application of this simplistic logic proved difficult because many of these genes also convey information about the anatomical location. As a specific example, the most abundantly expressed and iWAT-selective genes from UCP1-TRAP samples (group 3 from Figure 3A) are also highly expressed at the whole tissue level in iWAT pads versus other fat pads (Figure 3B). Genes in this iWAT-enriched set include transcriptional components (e.g., Irx1) as well as cell-surface proteins (e.g., Ltb). This striking similarity implies that UCP1-positive cells share the gene expression of adipose pads in which they reside. Consequently, many differentially expressed UCP1-TRAP genes do not provide direct information regarding enrichment within the UCP1-positive versus UCP1-negative cells of a particular depot. Rather, the dissociation of anatomical gene-expression information from true markers of classical brown or beige cells required further refinements of the UCP1-TRAP gene signatures.

**Identification of “Anatomy-Independent” Molecular Markers for Brown and Beige Adipocytes In Vivo**

We devised two different methods for analyzing UCP1-positive and UCP1-negative cells within a single anatomical depot. As demonstrated below, these methods can identify anatomy-independent markers for brown or beige cells in vivo. First, we posited that true BAT-enriched genes should also be more highly expressed in the interscapular BAT compared to the neighboring interscapular white fat. UCP1-TRAP-Seq BAT-selective genes (group 1 in Figure 3A) not enriched in this manner may simply be more highly expressed in the interscapular region compared to other parts of the body. Such a comparison for BAT-selective UCP1-TRAP genes is shown in Figure 4A. As expected, the known thermogenic genes Ucp1, Ppargc1a, and Pmdm16 were 2- to 8-fold enriched in the interscapular BAT versus interscapular WAT (Figure 4A). The UCP1-TRAP-Seq BAT-selective gene set (group 1) was roughly equally distributed between enrichment in interscapular BAT or enrichment in interscapular WAT [median log2(fold change) = −0.1]. This distribution confirms that anatomical information is a significant contributor to the expression signature of UCP1-positive cells within the classical BAT. Of the BAT-selective UCP1-TRAP (group 1) genes analyzed by qPCR, only Gmnt and Arhgdig were enriched in the expected manner for a true BAT marker (Figure 4A). Thus, these two genes may serve as novel molecular markers that distinguish classical brown from beige cells in mice in an anatomy-independent manner. Gmnt and Arhgdig encode for an N-methyltransferase and a GDP dissociation inhibitor, respectively. Whether these proteins contribute to classical BAT-selective functions remains to be determined.

For the iWAT and pgWAT depots, dissection of neighboring UCP1-positive and UCP1-negative cells is not technically feasible as all beige cells are embedded within white fat tissues. As an alternative strategy, we compared UCP1-positive cells of the iWAT or pgWAT with all adipocytes in the corresponding pad. Anatomical markers should be equally expressed between these two groups, whereas true iWAT- or pgWAT-specific markers of UCP1-positive cells should be appropriately enriched. To obtain the proper comparison group, the TRAP mice were crossed with Adiponectin-Cre to generate Adipoq-TRAP mice (Eguchi et al., 2011); in these animals TRAP protein is expressed in mature adipocytes, irrespective of Ucp1 expression (Figure S4). Immunopurified RNA from cold-acclimated Adipoq-TRAP mice was then used in a direct comparison with UCP1-TRAP samples (Figures 4B and 4C). The expression of Ucp1 was ~6-fold or ~80-fold enriched in UCP1-TRAP versus Adipoq-TRAP in the iWAT and pgWAT depots, respectively (Figures 4B and 4C). Of the UCP1-TRAP pgWAT-selective genes (group 2), only Prune2 was also significantly enriched in UCP1-positive cells versus all adipocytes of the pgWAT (Figure 4B). Prune2 is also highly expressed in the central nervous system (Li et al., 2011), but its function remains poorly characterized, and a role for Prune2 in adipocytes has not been previously described. In contrast to both the BAT and pgWAT comparisons, we found many more UCP1-TRAP-Seq iWAT-selective genes (group 3) enriched in UCP1-TRAP versus Adipoq-TRAP (Figure 4C; also see Table S5 for a full list). Similar to the BAT and pgWAT, many of these iWAT-enriched genes (e.g., Serinc2) are poorly characterized and have not been previously linked to adipocyte function.

**Beige but Not Brown Adipocytes Express a “Smooth Muscle-like” Signature**

In addition to the large-scale analyses presented above, the UCP1-TRAP data set also provides a powerful platform for investigating novel aspects of brown or beige adipocyte biology in vivo. Toward this end, we focused on the unusual constellation of enriched UCP1-TRAP genes (Figure 4C; Acta2, Tagln, Myh11, Myb, and Cnn1) that have classically been associated with smooth muscle cells (SMCs) (Miano et al., 1994; Owens et al., 2004) or...
smooth muscle-like cells (Lazard et al., 1993; Li et al., 2006) (Figure 4C). This smooth muscle-like signature is enriched in UCP1-TRAP samples from both the inguinal and axillary subcutaneous depots versus BAT (Figure S5A), indicating that this signature is not specific to the inguinal fat pad alone. Lastly, endothelial cells and pericyte markers such as Cspg4, Pdgfb, and Cdh5 were equally expressed in UCP1-TRAP across all the depots, demonstrating a specificity for the smooth muscle-like expression pattern versus other vasculature cells (Figure S5B).

Beige Cells Can Arise from a Smooth Muscle-like Origin

To date, the cellular origins of beige cells in vivo remain enigmatic, though skeletal muscle precursors (Seale et al., 2008) and existing adipocytes (Wang et al., 2013) have been excluded as sources. Based on the UCP1-TRAP expression signature, we investigated the possibility that the precursors for some beige cells might be smooth muscle-like cells. To do this, two genetic fate mapping approaches were employed. The tracing of SMCs in vivo is generally difficult owing to the heterogeneity of this population and the lack of single genetic markers that can precisely and concurrently differentiate SMCs (e.g., vascular SMCs), related smooth muscle-like cells (e.g., myoepithelial cells), and non-SMCs (e.g., pericytes). In the first approach, we selected a constitutive Myh11 promoter-driven Cre because Myh11 is generally considered to be the most selective marker for bona fide mature smooth muscle and other smooth muscle-like cells (Miano et al., 1994) and the specificity of this transgenic line had been previously validated by lacZ tracing (Xin et al., 2002). Transgenic mice expressing a bicistronic Cre recombinase and eGFP cassette under the control of the Myh11 promoter were crossed with reporter mice expressing a lox-stop-lox tdTomato cassette (Figure 5A). The Myh11-GFP/tdTomato mouse model provides independent fluorescence signals for both recombination (tdTomato) and Myh11 expression (GFP). Thus, cells currently expressing high levels of Myh11 (GFP positive; tdTomato positive) can be distinguished from cells that have arisen from SMCs that had once expressed Myh11 (GFP negative; tdTomato positive).

Following a 2 weeks cold exposure protocol, robust tdTomato immunofluorescence signal was observed in the iWAT of Cre-positive, but not Cre-negative mice (Figure 5B). In addition to tdTomato labeling of SMCs in the vessels, strong tdTomato signal was also observed in many cells that displayed morphological characteristics similar to multilocular adipocytes (Figure 5C). While the Myh11-positive cells surrounding lymph vessels expressed high levels of GFP, the tdTomato-positive extralymphatic cells were GFP negative, indicating that they arise from a once Myh11-expressing precursor. These data suggest that the smooth muscle-like expression signature in beige cells, while detectable, is still significantly lower compared to vascular SMCs or smooth muscle-like cells. Costaining with the markers perilipin and UCP1 unambiguously established the identity of these extralymphatic cells as mature, thermogenic adipocytes (Figures 5C and 5D). Lastly, we quantified the number of the double-positive population (tdTomato positive; UCP1 positive) cells relative to all UCP1-positive cells in the iWAT and BAT depots. Though we can detect a small number of double-positive adipocytes in the BAT, these cells constitute a much higher fraction of total UCP1-positive in the iWAT (Figure 5E and 5F).

In the second approach, we sought to pulse-chase existing Myh11-positive cells in adult mice and map their fate upon cold exposure. Toward this goal, we crossed transgenic mice expressing an inducible Myh11-driven Cre (Myh11-CreERT2) with GFP (ROSA26R.TdTomato) reporter mice (Figure 5G). Myh11-CreERT2/GFP mice were treated with tamoxifen daily for 4 days to permanently mark Myh11-expressing smooth muscle and smooth muscle-like cells. After a 1 week tamoxifen washout period, mice were either left at room temperature or housed at 4°C (Figure 5H). By stopping tamoxifen prior to the cold exposure, only smooth muscle and smooth muscle-like cells at the time of the injection, as well as their progeny, are labeled with GFP throughout the experiment. Immunohistochemical staining for GFP in the iWAT depot at room temperature indicated that recombination and labeling occurred only in the SMCs but not in the existing beige adipocytes (Figure 5I). Cold exposure
Some evidence indicates that a stem cell population in adipose tissue possesses a thermogenic gene signature. Initially, in the classical BAT depot we observed GFP positivity only in the vasculature surrounding the brown adipocytes but not in the brown adipocytes themselves (Figure 5J). Taken together, these fate-mapping experiments with the UCP1-TRAP data strongly suggest that mature Myh11-expressing cells suppress, but do not extinguish, their smooth muscle gene signature during differentiation into mature beige adipocytes.

Lastly, it is now well established that the many adipose precursors in vivo reside near the vasculature (Gupta et al., 2012; Tang et al., 2008). These populations have been characterized by both genetic (Berry and Rodeheffer, 2013; Gupta et al., 2012; Lee et al., 2012, 2013; Sanchez-Gurmaches et al., 2012) or prospective (Rodeheffer et al., 2008; Schulz et al., 2011) methods. To better understand the relationship between these previously identified adipocyte precursors and the smooth muscle-like population identified here, we analyzed Tomato-positive and Tomato-negative cells from the stromal vascular fraction of Myh11-GFP/tdTmTomato mice. Tomato-positive cells were de-enriched in markers for pericytes, endothelial cells, and hematopoietic cells, and enriched in some (e.g., Sca1, Pdgfra, Zfp423) but not all (e.g., Pparg) of the previously identified preadipocyte markers (Figure S6). Thus, the smooth muscle lineage may constitute a portion of previously uncharacterized preadipocyte populations.

**In Vitro Conversion of SMCs into Adipocytes with a Thermogenic Gene Signature**

Some evidence indicates that a stem cell population in adipose tissues can differentiate into either SMCs or adipocytes (Rodriguez et al., 2006; Yin et al., 2012), but there are no investigations of a direct phenotypic switch between SMCs or SMC-like cells into adipocytes. Since the development of a beige phenotype requires the coregulatory protein PRDM16 (Cohen et al., 2014), we asked whether this factor might promote adipogenesis with a thermogenic signature, even in mature SMCs. Ectopic expression of PRDM16 markedly suppressed smooth muscle gene expression in primary murine aortic SMCs compared to GFP control, including the expression of the master smooth muscle transcription factor Srf and a principal noncardiac coactivator, Mkl1 (MRTF-A) (Figure 6A). Subjecting these cells to the adipogenic cocktail for 2 days, followed by a longer-term treatment with insulin, rosiglitazone, and triiodothyronine, produced lipid droplet-containing adipocytes in PRDM16-overexpressing SMCs that were UCP1 positive (Figures 6B–6D). Notably, in these SMCs-derived adipocytes, the thermogenic genes Ucp1 and Ppargc1a were responsive to the cAMP-signaling agent forskolin, indicating that these cells are bona fide UCP1-positive adipocytes (Figure 6E). Taken together, these data indicate that mature SMCs can give rise to adipocytes with a thermogenic gene-expression signature, at least under these experimental conditions.

**DISCUSSION**

Here we provide a comprehensive and detailed molecular description of brown and beige adipocytes in vivo. Several insights of potential importance emerge: first, a large fraction of the gene expression shared by brown and beige fat cells represents a common and core thermogenesis gene signature, independent of anatomical location and cell type. The equivalent thermogenic potential of beige and classical brown adipocytes has been suggested in cell culture (Wu et al., 2012) and in humans (Lidell et al., 2013), and here we provide direct evidence for this hypothesis in rodents in vivo. By virtue of their coexpression with well-established thermogenic factors, the poorly annotated genes within this “core” signature are likely to have some type of functional role in the adaptive thermogenesis. These factors include transcriptional components (e.g., Zfp768), potential secreted proteins (e.g., BC028528), and cell-surface receptors (e.g., Iil7rc).

Second, many differentially expressed genes between UCP1-positive adipocytes reflect the anatomical location in which they reside. We can also identify anatomy-independent markers that distinguish brown and beige fat in mice. These data suggest that the beige adipocyte population is perhaps more heterogeneous than previously recognized and could provide a rational for why some genetic models show preferential browning in either the subcutaneous (Seale et al., 2011) or visceral (Kiefer et al., 2012) depots. Regarding the gene-expression events previously identified as being brown or beige specific, the analyses presented here point to limitations of our approach and also potential reinterpretation of the existing literature. For some genes (e.g., Zic1 [Seale et al., 2007; Timmons et al., 2007]), anatomical information simply cannot be dissociated from differential expression in classical brown versus beige fat cells. Our inability to detect others (e.g., Tmem26 [Wu et al., 2012]) suggests that actively translating mRNAs do not completely reflect total mRNAs in brown or beige cells. Regardless, in light of these observations, it is of paramount importance for future human studies that comparisons are done from neighboring white and brown/beige regions in approximately the same anatomical location.

Third, and perhaps most importantly, we provide evidence for a close relationship between smooth muscle-like cells and beige fat cells but not classical brown adipocytes. Three independent lines of evidence support this hypothesis: (1) UCP1-TRAP mice identified a smooth muscle-like gene expression signature in beige cells from multiple fat depots; (2) two independent fate-mapping approaches showed that beige adipocytes arise from Myh11-expressing, smooth muscle-like cells in vivo; and (3) ectopic expression of PRDM16 converted primary murine aortic SMCs into UCP1-positive adipocytes in vitro. Differentiated SMCs, unlike skeletal or cardiac muscle cells, are known to possess a remarkable degree of phenotypic plasticity even in their mature state. For instance, during vascular calcification, SMCs of the aorta transdifferentiate into osteochondrogenic precursors and chondrocyte-like cells (Speer et al., 2009). In cell culture, cholesterol-loaded SMCs lose their SMC features and adopt a gene expression and phenotype that is characteristic of macrophages (Rong et al., 2003). The data presented here suggest that a SMC-to-adipocyte conversion is also possible in vivo, and ectopic expression of PRDM16 can promote this conversion in vitro. This hypothesis is consistent with the observation that smooth muscle-specific deletion of Pparg ablates the perivascular fat depot and, furthermore, suggests that perivascular UCP1-positive adipocytes are in fact beige fat cells.
cells (Chang et al., 2012). Combined with the previous recognition of at least eight distinct cellular lineages that give rise to various SMC or smooth muscle-like populations (Majesky, 2007), these data imply a potential mosaic of developmental origins for beige adipocytes in vivo.

In summary, the UCP1-TRAP mouse and corresponding UCP1-TRAP data set provides a powerful platform for elucidating the function of brown and beige fat in the in vivo context.

Figure 6. Conversion of Primary Aortic SMCs into Thermogenic Adipocytes

(A and B) Relative expression of the indicated genes in primary murine aortic SMCs. SMCs were transduced with retrovirus overexpressing PRDM16 or GFP control and then selected with puromycin. Day 0 indicates the gene expression before differentiation. Day 6 indicates gene expression after 2 days of an adipogenic cocktail followed by 5 days of insulin, rosiglitazone, and triiodothyronine. Data are presented as means ± standard error; n = 3–5/group. *p < 0.05 or < 0.01, respectively, in GFP- versus PRDM16-overexpressing samples at the same time point.

(C) Western blot analysis of ACTA2, PPARG, UCP1, and β-actin loading control from primary aortic murine SMCs at the indicated times. G, GFP; PR, PRDM16.

(D) Oil red O staining of primary murine aortic SMCs with the indicated overexpressing construct and at the indicated times.

(E) Relative expression of the indicated genes in primary murine aortic SMCs at day 6, after 4 hr treatment with DMSO or forskolin (10 μM). Data are presented as means ± standard error; n = 3–5/group. *p < 0.05 or < 0.01, respectively, in forskolin-treated versus DMSO-treated within the same overexpressing group.

Projecting forward, functional interrogation of the common or differentially expressed gene sets, as well as the metabolic characterization of mice with perturbed or enhanced smooth muscle function, should afford further insights into the “browning” process in vivo. These studies may also illuminate novel pharmacologically tractable pathways for manipulating thermogenic adipocytes in humans.

EXPERIMENTAL PROCEDURES

General Animal Information

Animal experiments were performed according to procedures approved by the Dana-Farber Cancer Institute IACUC. Myh11-Cre (stock #007742), Myh11-CreERT2 (stock #019079), tdTomato reporter mice (stock #007914).

Figure 5. Smooth Muscle-like Origin of Beige Cells

(A) Schematic of the cross to generate Myh11-GFP/tdTomato reporter mice. Transgenic mice expressing a bicistronic transgene consisting of Cre and eGFP under the control of 16 kb of the Myh11 promoter were crossed with tdTomato reporter mice.

(B) GFP immunofluorescence and endogenous tomato fluorescence in the iWAT pad. Representative reporter mice (Cre positive, tomato positive) and control mice (Cre negative, tomato positive) are shown. (C–E) Perilipin (C) or UCP1 (D and E) immunofluorescence and endogenous Tomato fluorescence in iWAT (C and D) or BAT (E).

(F) Quantification of double tdTomato positive; UCP1-positive cells as a percentage of total UCP1-positive cells from iWAT and BAT of Myh11-GFP/tdTomato reporter mice. Data are shown as means ± standard error; n = 3 mice per group. *p < 0.05 for iWAT versus BAT.

(G) Schematic of the cross to generate Myh11-CreERT2/GFP reporter mice. BAC transgenic mice expressing a CreERT2 allele under the control of the Myh11 promoter were crossed to GFP (ROSAmT/mG) reporter mice.

(H) Cartoon depicting the time course for tamoxifen injections, the washout period, and cold exposure. Tamoxifen was injected at 2 mg/mouse/day for 4 consecutive days prior to the washout period.

(I and J) Immunohistochemical staining of tissues from day 25 for UCP1 (left panels) or GFP (right panels) in the iWAT (I) or BAT (J) of Myh11-CreERT2/GFP reporter mice. For both reporter experiments, mice were heterozygous for Cre and heterozygous for the reporter gene. For (I) and (J), representative images were taken in 6-week-old female Myh11-GFP/tdTomato reporter mice following 2 weeks cold exposure at 4°C. For (I) and (J), representative images were taken in 8-week-old male Myh11-CreERT2/GFP reporter mice following the treatment scheme indicated in (H).
and ROSA<sup>Utxtm</sup> reporter mice (stock #007676) were obtained from Jackson Laboratories. The generation of TRAP and Ucp1-Cre transgenic mice is described in detail in the Supplemental Experimental Procedures. UCP1-TRAP breeding pairs were housed at 30°C, and litters were weaned to room temperature at 3 weeks. Unless otherwise stated, for cold-exposure experiments 4-week-old female UCP1-TRAP mice were individually housed at 4°C for 2 weeks.

**Molecular Studies**

qPCR and western blotting were done according to standard methods. For qPCR from whole tissues or TRAP samples, all values were normalized by the ΔΔCT method to Tbp or to Actb, respectively. RNA-Seq was performed by the Dana-Farber Cancer Institute Center for Cancer Computational Biology Sequencing Facility (see Supplemental Experimental Procedures for details). The following antibodies were used: UCP1 (catalog #ab10983, Abcam), GFP (catalog #ab290, Abcam, for immunoaffinity purifications and western blotting), GFP (catalog #NB-100-1678, Novus, for immunohistochemistry), perilipin (catalog #ab61682, Abcam) β-actin (catalog #ab20272, Abcam), and PPARγ (catalog #22435, Cell Signaling).

**TRAP RNA Isolation**

Immunofluinity purification of TRAP RNA from adipose tissues was carried out similar to the protocol previously described (Sanz et al., 2009) but with some minor modifications as described in the Supplemental Experimental Procedures.

**Bioinformatic Analyses**

The “ratio” metric (Figure 2 and Table S2) was calculated as follows. In the UCP1-TRAP-Seq data set, a detected gene was defined by FPKM > 1 in at least two out of the eight samples; this filtering yields 9015 detected genes. For each gene from this list, the average FPKM for each depot was calculated. The “ratio” for a given gene is then the quotient of the depot with the highest average expression to the depot with the lowest average expression. In this manner, the ratio metric is a measure of the normalized range for a particular gene and is always greater than 1. As a cutoff, genes with ratios > 3 or ≤ 3 were identified as differentially or equivalently expressed, respectively. Hierarchical clustering (Figure 3A) was performed using GENE-E software (Broad Institute). One minus Pearson correlation was used as the distance metric for clustering of rows and columns.

For the identification of the differently expressed genes from each UCP1-TRAP depot (groups 1, 2, and 3 in Figure 3A), slightly more generous initial criteria were used to minimize false negatives. For the identification of iWAT-selective genes, the filtering criteria were as follows: 10-fold enrichment in the average iWAT signal versus the combined average of the pgWAT and BAT signals, iWAT p value < 0.2 versus the average signal from the pgWAT and BAT, and iWAT average FPKM > 0.5. For the identification of pgWAT-selective genes, the filtering criteria were as follows: 5-fold enrichment in the average pgWAT signal versus the combined average of the iWAT and BAT signals, pgWAT p value < 0.2 versus the average signal from the pgWAT and BAT, and pgWAT average FPKM > 0.5. For the identification of BAT-selective genes, the filtering criteria were 5-fold enrichment in the average BAT signal versus the combined average iWAT and pgWAT signals, BAT p value < 0.2 versus the average signal from the pgWAT and BAT, and BAT average FPKM > 0.2.

Details regarding the analysis of the “core” thermogenesis set can be found in the Supplemental Experimental Procedures.

**Immunofluorescence and Immunohistochemistry**

Immunofluorescence and immunohistochemical stainings were done according to standard protocols. Details can be found in the Supplemental Experimental Procedures. ImageJ software was used for quantification of tdTomato or UCP1 positivity. Briefly, the percentage colocalized (tdTomato positive; UCP1 positive) was calculated by calculating the area of colocalization divided by the total UCP1-positive area.

**Cell Culture**

Retrovirus production and infection from pMSCV-PRDM16 or pMSCV-GFP was performed as described previously (Seale et al., 2008). To generate stable cells, primary murine aortic SMCs were infected with the indicated retroviruses and selected with 3 μg/ml puromycin. Adipogenic differentiation was induced by addition of a hormone cocktail in media for 2 days (isobutylmethylxanthine [Sigma], 0.5 mM; dexamethasone [Sigma], 5 μM; rosiglitazone [Cayman], 1 μM; insulin [Sigma], 5 μM; triiodothyronine [Sigma], 1 nM; indomethacin [Sigma], 125 μM) followed by maintenance media until time of harvest (rosiglitazone [Cayman], 1 μM; insulin [Sigma], 5 μM; triiodothyronine [Sigma]). Where indicated, cells were treated with forskolin (10 μM, Sigma) for 4 hr. A detailed protocol for the isolation of primary aortic SMCs can be found in the Supplemental Experimental Procedures.

**Statistics**

The Student’s t test was used for pair-wise comparisons. Unless otherwise specified, statistical significance was set at p < 0.05.

**ACCESSION NUMBERS**

The GEO accession number for the UCP1-TRAP-Seq data reported in this paper is GSE56248.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.03.025.

**ACKNOWLEDGMENTS**

We thank members of the Spiegelman and Rosen laboratories, S. Nallamshetty, J. Plutzky, L. Chang, and Y.E. Chen for helpful discussions. We are grateful for technical assistance from the Rodent Histopathology Core at the Dana-Farber/Harvard Cancer Center and the Histology Core at the Beth Israel Deaconess Medical Center. Imaging was performed at the Nikon Imaging Center of Harvard Medical School. J.Z.L. is supported by a joint postdoctoral fellowship from the American and Canadian Diabetes Associations (PF-3-12-3927-JL). K.J.S. is supported by the Swedish Research Council international fellowship from the American and Canadian Diabetes Associations (PF-3-12-3927-JL). K.J.S. is supported by the Swedish Research Council international postdoctoral fellowship. This work was supported by NIH grants DK085171 and DK017690 (to E.D.R.) and by NIH grant DK031405 (B.M.S.). B.M.S. is a shareholder in and consultant to Ember Therapeutics.

Received: February 19, 2014
Revised: March 13, 2014
Accepted: March 21, 2014
Published: April 4, 2014

**REFERENCES**

beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. Cell 156, 304–316.


Supplemental Information

A Smooth Muscle-Like Origin for Beige Adipocytes

SUPPLEMENTAL FIGURES AND LEGENDS

Fig. S1, related to Fig. 1. Additional characterization of UCP1-TRAP mice. (A) Immunohistochemistry staining for UCP1 and GFP from the classical BAT (A) or immunofluorescence of UCP1, GFP, and DAPI in iWAT (B) of 6-week old female UCP1-TRAP mice after two weeks cold exposure at 4°C. (C, D) Relative mRNA expression of indicated genes from UCP1-TRAP (C) or whole tissue (D) samples. For (C) and (D), data are presented as means ± standard error; n = 3-5/group. *, ** p < 0.05 or < 0.01, respectively, in BAT versus pgWAT samples; #, ## p < 0.05 or < 0.01, respectively, in BAT versus iWAT samples.

Fig. S2, related to Fig. 2. Additional analysis of the common and differentially expressed UCP1-TRAP genes. (A) DAVID gene ontology analysis of the 1000 most abundant “equivalent” genes from the UCP1-TRAP dataset. (B) Relative expression of equivalently (Fabp4, Ucp1) or differentially (Fasn, Scd1) expressed genes. These data are presented as means ± standard error and are taken directly from the UCP1-TRAP-Seq data; n = 2 for iWAT and n = 3 for pgWAT and BAT. (C) Venn diagram showing the overlap of core thermogenesis genes and general adipocyte genes. For this analysis (also see Supplemental Experimental Procedures), core thermogenesis genes (blue circle) were identified using the UCP1-TRAP-Seq dataset by FPKM > 1 in all depots and ratio between high and low depot ≤ 3; general adipocyte genes (red circle) were identified using whole adipose tissues by Affymetrix probe signal > 200 and ratio between high and low depot ≤ 3.
Fig. S3, related to Fig. 3. Validation of depot-selective UCP1-TRAP gene expression. UCP1-TRAP-Seq genes that were identified to be selective for each depot (Groups 1, 2, and 3) from Fig. 3A were validated by qPCR in an independent cohort of UCP1-TRAP mice. Data are presented as means; n = 3-4/group. Color coding was performed for each table where the relative high and low values are indicated by red and blue, respectively.

Fig. S4, related to Fig. 4. Characterization of Adiponectin-TRAP mice. (A) Western blotting for GFP and β-actin loading control from various fat depots in Adipoq-TRAP or control (Cre-negative, TRAP-positive) mice. (B) Relative expression of Ucp1 from immunopurified RNA (TRAP) samples of Adipoq-TRAP mice. Data are presented as means ± standard error; n = 3-5/group.

Fig. S5, related to Fig. 5. Additional characterization of the SMC-like signature in UCP1-TRAP samples. (A) Relative expression in UCP1-TRAP samples of the indicated genes from the iWAT, axWAT (axillary), or BAT depots. Data are presented as means ± standard error; n = 3-5/group. (B) Relative expression of the indicated genes from the UCP1-TRAP-Seq dataset. Units are in FPKM. Individual replicates for each of the depots are shown; n = 2 for pgWAT and n = 3 for each iWAT and BAT. Red and blue indicate relative high and low expression, respectively, for the row.

Fig. S6, related to Fig. 6. Gene expression of Myh11-labeled and unlabeled cells in the stromal vascular fraction. Relative expression of the indicated genes from RFP-
positive and RFP-negative cells in the iWAT stromal vascular fraction (SVF) of Myh11-tdTomato reporter mice. After harvesting SVF and passaging once, cells were sorted by RFP fluorescence and replated, and total mRNA was collected one day later. Data are presented as means ± standard error; n = 3-5/group. *, ** p < 0.05 or < 0.01, respectively, for RFP-positive versus RFP-negative comparisons. Ref. 1-5 are indicated below:


**Table S1, related to Fig. 1. The UCP1-TRAP-Seq dataset.** This Excel sheet provides the raw UCP1-TRAP-Seq dataset, organized by gene name and the relative expression (in
FKPM) of the genes across UCP1-TRAP samples from the iWAT (n = 3), pgWAT (n = 2), or BAT (n = 3). Tissues were harvested from 6-week old UCP1-TRAP females after two weeks cold exposure at 4°C.

**Table S2, related to Fig. 2. Analysis of equivalent or differentially expressed genes in the UCP1-TRAP-Seq dataset.** This Excel sheet provides an initial equivalent/different filter to the raw UCP1-TRAP-Seq dataset. First, genes were identified as detected if they were present (FKPM > 1) in at least two samples from the UCP1-TRAP-Seq. Next, these 9015 detected genes were classified as either equivalently or differentially expressed between the UCP1-TRAP samples as described in the Experimental Procedures. This gene list is ordered from most to least abundant genes, as determined by the sum of the signals (total FPKM) across all UCP1-TRAP samples.

**Table S3, related to Fig. 2. Identification of the UCP1-TRAP core thermogenesis gene set.** This Excel sheet provides the individual gene names for the common thermogenesis and general adipocyte gene sets (see Experimental Procedures). This gene list is ordered from most to least abundant genes in UCP1-TRAP samples from each depot.

**Table S4, related to Fig. 3. Identification of differentially expressed genes from the UCP1-TRAP dataset.** The initial identification of depot-specific genes (Groups 1, 2, and 3 in Fig. 3A) from the UCP1-TRAP dataset is shown as three individual tabs in this Excel
sheet. The criteria for generating these gene lists are provided in the Experimental Procedures.

Table S5, related to Fig. 4. Genes analyzed for the identification of anatomy-independent markers of brown and beige cells. This Excel sheet provides the fold change and p-values of all UCP1-TRAP depot-specific genes evaluated between interscapular BAT versus interscapular WAT, or between UCP1-TRAP versus Adipoq-TRAP mice. Data are presented as means; n = 4-6/group.

Table S6, related to Experimental Procedures. qPCR primers for all genes used in this study.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of TRAP mice

The EGFP-L10a portion was PCR amplified from a construct provided by David Olson (Heiman et al., 2008) and subcloned between the Bsu36I and SexAI sites of a pCAG-HS-mG (hH2B-mCherry-2A-EGFP-GPI) plasmid kindly provided by Dr. Richard Behringer (Stewart et al., 2009). This transgene was further subcloned into a modified Rosa26-pCAG-LSL-WPRE-bGHpA targeting vector generously provided by Hongkui Zeng at the Fsel site between the LSL and WPRE sequences (Madisen et al., 2010). LSL sequence contains specifically loxP- Stop codons - 3x SV40 polyA – loxP. The targeting vectors were linearized (KpnI) and transfected into the 129/B6 F1 ES cell line G4. G418-resistant ES clones were first screened by PCR using primers spanning the 1.1 kb 5’ genomic arm (forward primer: 5’-GCCAAGTGGGAGGTACGTTACG-3’, reverse primer: 5’-TAGGTAGGGATCGGGACTCT-3’), and then confirmed using primers spanning the 4.4 kb 3’ genomic arm (forward primer: 5’-GCCAGCTCATGCTGCCAC-3’, reverse primer: 5’-GGCATGGAATGTTCAGCAG-3’). Positive ES clones were injected into C57BL/6J blastocysts to obtain chimeric mice following standard procedures. Chimeric mice were bred with C57BL/6J mice to obtain germline transmitted F1 mice. The TRAP mice were backcrossed at least six generations prior to use in the experiments.

Generation of Ucp1-Cre transgenic mice
A C57BL/6 mouse bacterial artificial chromosome (148M1) containing the Ucp1 gene was transformed into the recombinogenic EL250 bacteria cells and homologous recombination was performed. The Cre-FRT-Kan-FRT cassette was transformed into the Ucp1 BAC host EL250 cells and recombined to insert the Cre ATG into the Ucp1 ATG. Ucp1-Cre-FRT-Kan-FRT BAC host EL250 clones were identified by PCR screening. The FRT-Kan-FRT cassette was removed, and a Ucp1-Cre BAC host EL250 clone without mutation in the Cre coding sequence was obtained. The loxP site present in the vector sequence of the Ucp1-Cre BAC was removed. The transgenic construct was microinjected into pronuclei of fertilized one-cell stage embryos of FVB mice (Jackson Laboratories) via standard methods. The Ucp1-Cre founder was backcrossed at least eight generations onto C57BL/6J prior to use in the experiments.

**UCP1-TRAP RNA Sequencing**

RNA-Seq was performed by the Dana-Farber Cancer Institute Center for Cancer Computational Biology Sequencing Facility. Total RNA was first put through quality control using the Qubit (Life Tech) and the Bioanalyzer (Agilent). RNA quantity was determined on the Qubit using the Qubit RNA Assay Kit (Life Tech) and RNA quality was determined on the Bioanalyzer using the RNA Pico Kit (Agilent). Using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB), 100 ng of total RNA was converted into a DNA library following the manufacturer’s protocol, with no modifications. Following library construction, DNA libraries were then put through quality control. Library quantity was determined using the Qubit High Sensitivity DNA Kit (Life Tech) and library size was determined using the Bioanalyzer High Sensitivity
Chip Kit (Agilent). Finally, libraries were put through qPCR using the Universal Library Quantification Kit for Illumina (Kapa Biosystems) and run on the 7900HT Fast qPCR machine (ABI). Libraries passing quality control were diluted to 2 nM using sterile water and then sequenced on the HiSeq 2000 (Illumina) at a final concentration of 12 pM on a single read flowcell with 50 sequencing cycles, following all manufacturer protocols.

**TRAP RNA isolation**

For the preparation of anti-GFP-conjugated dynabeads, for each sample 50 µl protein G dynabeads (Life Technologies) was first washed with PBS-T and then incubated with 2 µl anti-GFP antibody (Abcam, ab290) in a total volume of 200 ul PBS-T. After incubating for at least 20 min at room temperature, the PBS-T was removed and the tissue lysates were immediately added to the beads, as described below. Each tissue was harvested, briefly minced with a razor, and then manually dounce homogenized in 3 ml (iWAT, pgWAT) or 1 ml (BAT) of IP buffer [50 mM Tris, pH 7.5; 12 mM MgCl₂; 1% NP-40; 100 µg/ml cycloheximide (Sigma); 0.5 mM DTT; 100 mM KCl; 1x HALT protease inhibitor EDTA-free (Thermo); 1 mg/ml sodium heparin (Sigma); 0.2 units/µl RNasin (Promega)]. Following vortexing and centrifugation (12,000 x g for 10 min), the lipid layer was removed and the remaining supernatant was incubated with the anti-GFP-conjugated dynabeads. After 1 h at 4°C, dynabeads were separated and washed thrice with 1 ml high salt buffer [50 mM Tris, pH 7.5; 12 mM MgCl₂; 1% NP-40; 100 µg/ml cycloheximide (Sigma); 0.5 mM DTT; 300 mM KCl]. Following the last wash, TRIzol (500 µl, Life Technologies) was immediately added to the beads and RNA was purified using RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions.
Bioinformatic analyses

For analysis of the “core” thermogenesis set (Fig. S2C and Table S3), the approach was to first define a common gene set to the UCP1-TRAP dataset and then to remove genes that are common to adipocytes generally (e.g., Gapdh or Fabp4). The UCP1-TRAP common gene set was defined using the UCP1-TRAP-Seq dataset by FPKM > 1 in all depots and ratio ≤ 3. The adipocyte common gene set was defined using Affymetrix data GSE53307 (Cohen et al., 2014) from whole adipose tissues by Affymetrix probe signal > 200 and ratio ≤ 3. Genes that were not detected due to technical limitations (e.g., missing probes) were excluded from this analysis.

Immunofluorescence and immunohistochemistry

For immunohistochemical stainings, adipose tissues excised, fixed in 10% formalin overnight at 4°C, washed in PBS, embedded in paraffin, and cut into 5 µm of sections. Haematoxylin and eosin staining was performed according to standard protocols. Slide sections were deparaffinized and hydrated, antigen retrieval was performed using sodium citrate, and endogenous peroxidase activity was blocked with hydrogen peroxide. Then sections were incubated with 5% normal donkey serum (JacksonImmunoResearch Lab Inc, West Grove PA) for an hour at room temperature. Slides were then incubated with anti-GFP antibody (1:500) (Novus NB-100-1678) or anti-UCP-1 antibody (1:1000) (Abcam ab10983) overnight at 4°C. The slides were then washed and incubated with biotinylated donkey anti-goat secondary antibody (1:500) or biotinylated donkey anti-rabbit secondary antibody (1:500) for an hour at room temperature. Samples were
enhanced with Elite Vectashield ABC kit (Vector Lab), developed in DAB (Diaminobenzidine) metal enhanced kit (Vector lab) and counter stained with hematoxylin.

For immunofluorescence stainings, tissues were fixed in 4% PFA for 24 h and 20% sucrose for 24 h before embedding and cryosectioning at 50 µm thickness. Slides were blocked in PBS-T supplemented with 10% fetal bovine serum (FBS) for 30 min before incubation with either rabbit polyclonal UCP1 antibody (abcam, ab10983) at 2 µg/ml or goat polyclonal perilipin (Abcam, ab61682) over night at 4°C in PBS-T/ 1% FBS. Slides were washed three times in PBS-T and incubated with Alexa Flour conjugated antibodies and nuclei was stained with Hoechst 33342 (Invitrogen). Immunofluorescence stainings were observed with a Nikon Ti w/ A1R confocal laser scanning microscope using 10× and 20× objective lenses and Nikon Elements acquisition software.

**Isolation of primary aortic smooth muscle cells**

Primary murine aortic SMCs were obtained as follows: aortas from six female mice were obtained steriley and placed into a 100 mm dish containing media. The adventia was cleaned off and the aorta was cut into 1-2 mm pieces (horizontally). After placing into a collagen Biocoat plate (Fisher, catalog #08-772-69), the pieces were allowed to briefly dessicate and then cell culture media was added carefully. The next day, additional culture media was added if necessary. Explants were grown out in 3-7 days and removed. The next day, wells were washed (PBS), trypsinized, and passaged until P5, at which point they were used for experiments.
Figure S1

A. UCP1

B. UCP1, GFP, DAPI, MERGE

C. Ucp1-TRAP

D. Whole tissue

Relative expression

- Tbx1
- Tbx15
- Sfrp2
- Hoxc10
- Shox2
- Zic1

- iWAT
- pgWAT
- BAT

*, **, *** indicate statistical significance.
Figure S2

A

Cellular Compartment

Molecular Function

Biological Process

- mitochondrion
- mitochondrial part
- mitochondrial envelope
- mitochondrial membrane
- mitochondrial inner membrane
- organelle inner membrane
- ribosome
- structural constituent of ribosome
- structural molecule activity
- monovalent inorganic cation transmembrane transport activity
- hydrogen ion transmembrane transporter activity
- inorganic cation transmembrane transporter activity
- NADH dehydrogenase (ubiquinone) activity
- NADH dehydrogenase (quinone) activity

- generation of precursor metabolites and energy
- translation
- electron transport chain
- oxidation reduction
- energy derivation by oxidation of organic compounds
- cellular respiration
- oxidative phosphorylation

B

Relative expression

iWAT
pgWAT
BAT

C

1337 3959 1176

Adipocyte
UCP1-specific
Figure S3

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Figure S5

### A

![Bar chart](chart.png)

### B

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- **Pericyte markers**
  - Cagg4
  - Pdgfb
  - Pecam1
- **Endothelial cell markers**
  - Cdh5
  - Thbd
  - Vwf
- **SMC markers**
  - Acta2
  - Myh11
  - Myh9
  - Ctn1
  - Tagln

- **Cell types**
  - iWAT
  - pgWAT
  - BAT
Figure S6

- Pericyte
- Endothelial
- Hematopoietic
- Ref. 1
- Ref. 2
- Ref. 3
- Ref. 4
- Ref. 5

Relative expression

- RFP+
- RFP-