

Ebf2 is a selective marker of brown and beige adipogenic precursor cells

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Brown adipocytes and muscle and dorsal dermis descend from precursor cells in the dermomyotome, but the factors that regulate commitment to the brown adipose lineage are unknown. Here, we prospectively isolated and determined the molecular profile of embryonic brown preadipose cells. Brown adipogenic precursor activity in embryos was confined to platelet-derived growth factor α^+ , myogenic factor 5^{Cre}-lineage-marked cells. RNA-sequence analysis identified early B-cell factor 2 (*Ebf2*) as one of the most selectively expressed genes in this cell fraction. Importantly, *Ebf2*-expressing cells purified from *Ebf2*^{GFP} embryos or brown fat tissue did not express myoblast or dermal cell markers and uniformly differentiated into brown adipocytes. Interestingly, *Ebf2*-expressing cells from white fat tissue in adult animals differentiated into brown-like (or beige) adipocytes. Loss of *Ebf2* in brown preadipose cells reduced the expression levels of brown preadipose-signature genes, whereas ectopic *Ebf2* expression in myoblasts activated brown preadipose-specific genes. Altogether, these results indicate that *Ebf2* specifically marks and regulates the molecular profile of brown preadipose cells.

beige adipocyte | brown adipose tissue

Brown adipose tissue (BAT) expends energy in the form of heat in response to various stimuli, including cold exposure and certain diets (1). The thermogenic activity of brown adipocytes is mediated by uncoupling protein 1 (Ucp1), a mitochondrial membrane protein. Ucp1, when activated, dissipates the electrochemical gradient that powers ATP synthesis, which allows for low rates of ATP production despite high levels of substrate oxidation (1). Thermogenically competent Ucp1-expressing brown-like adipocytes, called beige or brite (brown-in-white) cells, also develop in certain white adipose tissue (WAT) depots in response to cold exposure (reviewed in ref. 2). In mice, BAT and/or beige fat-mediated thermogenesis suppresses high-fat-diet-associated obesity and metabolic disease (2–8). High levels of activated BAT also correlate with reduced adiposity in people (9). There is thus great hope that brown/beige fat activity can be increased to reduce obesity and metabolic disease.

BAT depots mature during the embryonic and fetal stages of mouse development. In humans, the major BAT depot is located in the interscapular region of newborns, but this tissue regresses and is absent in adults (10). However, brown and beige adipocytes are found in other depots of adult humans, particularly in the supraclavicular area, neck, and along the spinal column (10–14). Recent lineage studies showed that brown adipocytes originate from precursor cells that express *myogenic factor 5* (*Myf5*), *paired box protein 7* (*Pax7*), *Pax3*, and *engrailed 1* (*En1*) in the somitic mesoderm (15–17). However, the factors that control brown preadipose cell fate in this “tripotent” compartment are unknown.

In the absence of molecular markers for committed brown preadipocytes, such cells can only be recognized retrospectively after their differentiation into Ucp1⁺ adipocytes. Here, we combined lineage and cell surface markers to prospectively isolate brown preadipose cells during embryogenesis. Global mRNA

transcriptomic analyses identified a brown-preadipose-specific gene signature, which included *early B-cell factor 2* (*Ebf2*), a critical transcriptional regulator in mature brown adipocytes (18). We found that brown/beige preadipose activity in embryos and adult fat depots was restricted to *Ebf2*-expressing cells. Furthermore, *Ebf2* deficiency in brown preadipose cells reduced the expression of nearly all brown preadipose signature genes, whereas ectopic expression of *Ebf2* in myoblasts activated brown preadipose-selective genes. Altogether, our study reveals that *Ebf2* is a specific marker of brown/beige preadipose cells and that *Ebf2* functions at this stage to control precursor identity.

Results

Prospective Isolation of *Myf5*^{Cre}-Lineage-Marked Brown Preadipose Cells. Specific marker genes for brown preadipose cells were previously unknown. To identify such factors, we isolated enriched populations of brown preadipose cells from mouse embryos. Brown adipocytes descend from a *Myf5*^{Cre}-expressing cell lineage that also gives rise to skeletal muscle cells (17). To enable the efficient purification of *Myf5*^{Cre}-lineage-marked cells by flow cytometry, we generated *Myf5*^{Cre}; *R26R-mTmG* mice, in which *Myf5*^{Cre}-expressing cells are genetically and heritably labeled by a membrane-targeted form of GFP [*Myf5*^{Cre}(GFP)] (19). We noted that GFP was readily detected in somites starting from embryonic day (E) ~E9.5 (Fig. S14). In agreement with previous findings, the majority of peroxisome proliferator-activated receptor

Significance

High levels of brown/beige fat activity protects animals against metabolic disease, but there has been little known about the precursor cells that mediate the expansion of brown or beige fat. We discovered that early B-cell factor 2 (*Ebf2*), a transcription factor, is selectively expressed in brown and beige fat cell precursors. Through purification of *Ebf2*⁺ cells, we identified a gene profile of brown fat precursors that can be used to distinguish these cells from other developmentally related cell types. Importantly, *Ebf2* was also found to regulate the gene expression profile of brown fat precursor cells. Taken together, this study identifies *Ebf2* as a highly specific marker of brown and beige preadipose cells and reveals that *Ebf2* functions to control brown preadipose cell identity.

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Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE60443).

See Commentary on page 14318.

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γ (Ppar γ)-expressing adipocytes in the interscapular BAT as well as the Desmin-expressing skeletal myofibers in the trunk and limb were GFP⁺ at E16.5 (Fig. S1B) (17). GFP was also expressed in adipocytes within the axillary and cervical BAT (Fig. S1C) and in the majority of preadipocytes from postnatal BAT (Fig. S1D). GFP⁺ cells also contributed to the dorsal dermis (Fig. S1B). These data indicate that *Myf5*^{Cre} cells contribute to at least three different mesodermal tissue types in late stage embryos: BAT, skeletal muscle, and dorsal dermis. Consistent with recent studies, GFP was also found in some adipocytes in the retroperitoneal and back s.c. WAT of adult mice (20) (Fig. S1E). Thus, *Myf5*^{Cre} (GFP) is a selective but not specific marker of developing brown fat cells.

BAT forms prenatally in mice, but the embryonic stages associated with the morphological and molecular differentiation of brown adipocytes were unclear. To assess this differentiation, we performed hematoxylin and eosin (H&E) staining and immunohistochemistry on transverse sections of E13.5–E16.5 embryos. At E14, we detected distinctive clusters of *Myf5*^{Cre}(GFP)⁺ presumptive brown adipogenic cells that expressed Ppar γ (Fig. S2). Consistent with previous studies, BAT was easily recognized by H&E staining at E15.5 (21, 22) and contained cells that expressed the mature adipocyte marker perilipin (Fig. S2). This suggested that the dorsal interscapular region of E14 embryos is a rich source of pioneering brown adipose precursors.

We next considered whether particular cell surface markers could be combined with *Myf5*^{Cre}-lineage marking to provide additional selectivity for brown preadipose cells. Platelet-derived growth factor α (Pdgfra) is a cell surface marker of adipogenic precursors in adult WAT and muscle (23–26); this prompted us to examine Pdgfra expression during BAT development. Lineage tracing using a *Pdgfra*^{Cre} allele, previously shown to mark most/all white adipocytes in adult mice (23), showed that *Pdgfra*-expressing cells broadly contributed to somite-derived tissues, including BAT, skeletal muscle, and dermis (Fig. S3A). At E14.5, Pdgfra⁺ cells were abundant in the dorsal anterior region of embryos and the proportion of Pdgfra⁺ cells in this region declined by E15.5 as the number of perilipin-expressing adipocytes increased (Fig. S3B).

To examine the adipogenic potential of Pdgfra⁺ cells in the *Myf5*^{Cre}-expressing lineage, we dissociated cells from the dorsal body wall of E14.5 *Myf5*^{Cre} (GFP) embryos and used fluorescence-activated cell sorting (FACS) to fractionate cells into four populations: GFP⁻; Pdgfra⁻, GFP⁻; Pdgfra⁺, GFP⁺; Pdgfra⁻, and GFP⁺; Pdgfra⁺ (Fig. 1A). Integrin alpha-7 (Itga7), a marker of skeletal myoblasts (27), was used to select against the differentiated brown adipocytes found in the GFP⁺; Pdgfra⁻ fraction. The sorted populations were cultured and treated with standard adipogenic inducers to analyze their differentiation potential. Oil Red O staining for lipids revealed that a high proportion of both GFP⁻; Pdgfra⁺ and GFP⁺; Pdgfra⁺ cells differentiated into adipocytes, whereas GFP⁻; Pdgfra⁻; Itga7⁺ cells differentiated into multinucleated muscle cells (Fig. 1B).

Gene expression analysis was used to further analyze the phenotype of the differentiated cell cultures. The GFP⁻; Pdgfra⁺ and GFP⁺; Pdgfra⁺ cultures expressed high levels of general adipocyte genes compared with all Pdgfra⁻ cells. However, only GFP⁺; Pdgfra⁺ cells expressed brown-fat-selective genes and mitochondrial genes, and at levels that were comparable to or higher than their levels in genuine brown adipocytes from an established cell line (Fig. 1C and Fig. S4A). Immunofluorescence staining showed that ~70% of GFP⁺; Pdgfra⁺ cells differentiated into brown adipocytes that expressed the general adipocyte marker, Ppar γ , and the brown-selective factor, PR domain containing 16 (Prdm16) (Fig. 1D and Fig. S4B). GFP⁻; Pdgfra⁺ cells did not express muscle-selective genes [*myogenic differentiation 1* (*MyoD*) and *myogenin* (*MyoG*)] (Fig. S4C) and had lower levels of white-adipocyte-selective markers (*Agt* and *Retn*) relative to GFP⁻; Pdgfra⁺ cells (Fig. S4D). Taken together, these results indicate that cell surface expression of Pdgfra enriches for brown

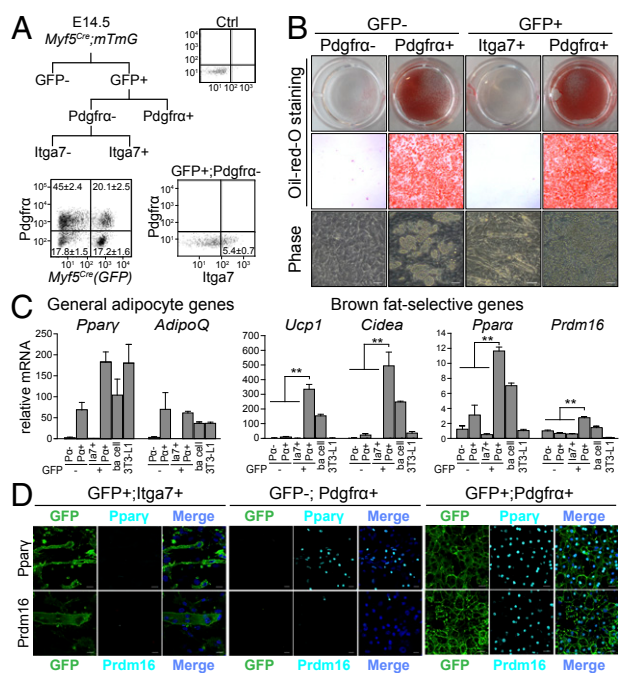


Fig. 1. *Myf5*^{Cre}-lineage-derived brown adipogenic precursors are Pdgfra⁺. (A) Cells from E14.5 *Myf5*^{Cre}; *mTmG* embryos were fractionated based on expression of GFP, Pdgfra, and Itga7. The percentage of each cell fraction (of total cell number) is reported as mean \pm SD, $n = 3$. (B) FACS-purified fractions were induced to differentiate into adipocytes and stained with Oil Red O. (C) mRNA levels of general adipogenic genes, brown-fat-selective markers in indicated cell cultures. P α , Pdgfra; la7, Itga7; ba, brown adipocyte. Values are mean \pm SD, $n = 3$; * $P < 0.05$, ** $P < 0.01$. (D) Brown adipogenic conversion of sorted precursor populations was evaluated by immunofluorescent staining of Ppar γ and Prdm16.

adipose precursors in a heterogeneous population of embryonic *Myf5*^{Cre} (GFP)⁺ cells.

Ebf2 Expression Identifies Brown Preadipose Cells During Development.

We then focused our analysis on freshly isolated brown adipogenic *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells. Using flow cytometry, we found that the *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ fraction contained only 2.6% CD31⁺ endothelial, 0.1% CD45⁺ hematopoietic, and 0.1% Itga7⁺ cells (Fig. S5A). Notably, 43.4% of *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells were CD34⁺, and 89.6% were CD24⁺ (Fig. S5A), showing that this population expresses markers that are also found on white-fat-derived precursors (23, 25). However, most embryonic *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells were negative for Sca1 (Fig. S5A), whereas preadipose cells in postnatal BAT and WAT were Sca1⁺ (Fig. S5B) (28, 29). Quantitative PCR (qPCR) analysis revealed that *Pparγ* and *Zfp423*, key adipocyte-lineage genes (30, 31), were expressed at higher levels in *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells relative to other sorted fractions (Fig. S5C). *MyoD*, the master regulator of skeletal muscle differentiation, was undetectable in *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells (Fig. S5C), whereas *Dermo1* (also called *Twist2*), a dermis lineage marker (32), was highly expressed in *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells (Fig. S5C). These data suggest that the *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ fraction contains brown fat and dermal precursor cells.

To search for specific markers of brown adipogenic cells within the *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ fraction, we used RNA-sequencing (RNA-seq) to globally compare gene expression between *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ and *Myf5*^{Cre}(GFP)⁺; Pdgfra⁻; Itga7⁺ cells. This led to the identification of *Ebf2* as one of the most selectively expressed transcription factors in *Myf5*^{Cre}(GFP)⁺; Pdgfra⁺ cells (Fig. S5D). *Ebf2* cooperates with Ppar γ in adipocytes to regulate the expression of thermogenic genes (18), but a function

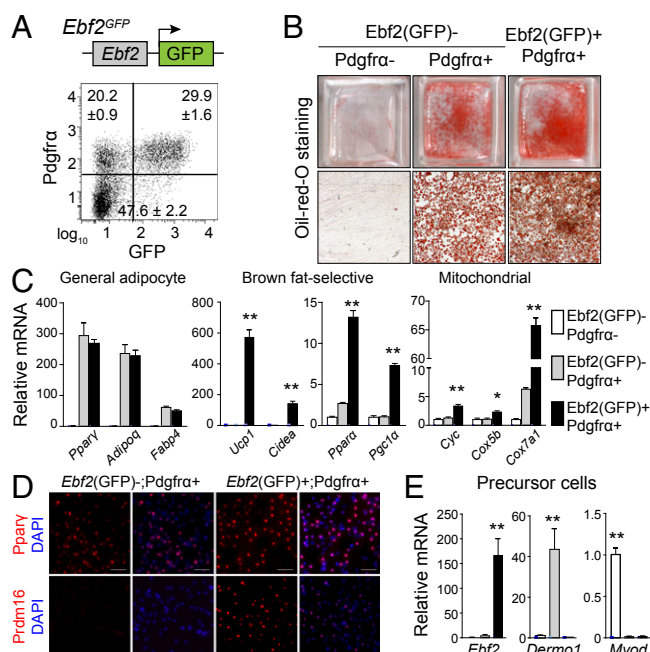


Fig. 2. Prospective identification of *Ebf2*⁺ brown adipogenic precursors. (A) Cells from E14.5 *Ebf2*^{GFP} embryos were fractionated based on GFP and *Pdgfra* expression. The percentages of each population are reported as mean \pm SD, $n = 3$. (B) Flow-sorted cell populations (from A) were induced to undergo adipocyte differentiation and stained with Oil Red O. (Scale bar, 100 μ m.) (C) mRNA levels of general adipocyte markers, brown-fat-selective genes, and mitochondrial genes in indicated cultures. Values are mean \pm SD, $n = 3$; * $P < 0.05$, ** $P < 0.01$. (D) Immunofluorescence staining of *Ppar* γ and *Prdm16* in indicated cell cultures. (Scale bar, 50 μ m.) (E) mRNA levels of *Ebf2*, *Dermo1*, and *Myod* in freshly sorted cell fractions. Values are mean \pm SD, $n = 3$; * $P < 0.05$, ** $P < 0.01$.

for *Ebf2* in precursor cells was unknown. Immunofluorescence analysis showed that *Ebf2* protein was present in $\sim 70\%$ of sorted *Myf5*^{Cre}(GFP)⁺; *Pdgfra*⁺ cells both before and after adipogenic conversion and was only very rarely found in *Pdgfra*⁻ cells (<1%) (Fig. S5E).

We isolated and examined the differentiation potential of *Ebf2*⁺ cells purified from the dorsal anterior region of E14.5 *Ebf2*-GFP (*Ebf2*^{Gfp/+}) knockin mice. Virtually all *Ebf2* (GFP)⁺ cells were also *Pdgfra*⁺ and expressed high levels of *Ebf2* mRNA (Fig. 2A and E). Under adipogenic conditions, *Ebf2*(GFP)⁺; *Pdgfra*⁺ and *Ebf2*(GFP)⁻; *Pdgfra*⁺ cells differentiated into Oil Red O-stained adipocytes that expressed similar levels of general adipocyte genes (Fig. 2B and C). However, only *Ebf2*(GFP)⁺ cells expressed brown-fat-selective genes and high levels of mitochondrial genes (Fig. 2C). Immunofluorescence staining for *Ppar* γ and *Prdm16* showed that nearly all *Ebf2*-expressing cells differentiated into (*Ppar* γ ⁺; *Prdm16*⁺) brown adipocytes (Fig. 2D). *Ebf2* (GFP)⁺ cells isolated from adult BAT also underwent adipogenic differentiation much more efficiently than *Ebf2* (GFP)⁻ cells (Fig. S6). Importantly, *Ebf2*⁺ precursor cells did not express either *MyoD* or *Dermo1*, which mark muscle and dermal precursor cells, respectively (Fig. 2E). *MyoD* was uniquely expressed in *Ebf2* (GFP)⁻; *Pdgfra*⁻ cells, whereas *Dermo1* was selectively expressed in *Ebf2*(GFP)⁻; *Pdgfra*⁺ cells (Fig. 2E). These results indicate that *Ebf2* expression identifies precursor cells that are competent to undergo brown adipogenesis.

***Ebf2* Marks Beige Adipogenic Precursor Cells in WAT.** Beige adipocytes that develop in s.c. WAT do not originate from *Myf5*^{Cre}-lineage⁺ precursor cells (2, 14, 17, 20, 24, 29). Our immunofluorescence experiments showed that *Ebf2* was present in a subpopulation of

embryonic *Myf5*^{Cre} (GFP)⁻ cells (Fig. S5E). To quantify the proportion of *Ebf2*-expressing cells that belong to the *Myf5*^{Cre}-marked brown fat lineage, we incorporated a *Myf5*^{Cre}-driven *dTomato* lineage reporter gene into *Ebf2*^{GFP} mice (Fig. S7A). Flow cytometric analysis showed that $\sim 50\%$ of the *Ebf2* (GFP)⁺; *Pdgfra*⁺ cells from the dorsal region of embryos were *Myf5*^{Cre} (*dTomato*)⁺ (Fig. S7A). All sorted populations of *Pdgfra*⁺ cells, whether or not they expressed *Myf5*^{Cre} (*dTomato*) or *Ebf2* (GFP), robustly differentiated into adipocytes that expressed general adipocyte genes (Fig. S7B and C). However, only the *Ebf2*⁺ cells [both *Myf5*^{Cre} (*dTomato*)⁺ and *Myf5*^{Cre} (*dTomato*)⁻], activated brown-fat-selective genes (Fig. S7C). Moreover, rosiglitazone, a PPAR activator, increased the expression of brown-fat-specific genes in both populations of *Ebf2*⁺ cells. Brown versus beige fat markers (*Eva1*, *Pdk4*, and *Oplah*) (14) were expressed at higher levels in *Myf5*^{Cre} (*dTomato*)⁺ relative to *Myf5*^{Cre} (*dTomato*)⁻ adipocytes (Fig. S7D). Conversely, beige-versus brown-fat cell-selective gene markers (*Tmem26* and *Cd137*) (14) were expressed at higher levels in *Myf5*^{Cre} (*dTomato*)⁻ relative to *Myf5*^{Cre} (*dTomato*)⁺ adipocytes (Fig. S7D). These results suggest that *Ebf2* expression marks brown and beige adipogenic cells regardless of their developmental origin.

The next question was whether *Ebf2* is a specific marker for beige adipogenesis in adult WAT. We used flow cytometry to analyze the SVF from the inguinal (s.c.) WAT (iWAT) of *Ebf2*-GFP^{TG} mice. At thermoneutrality, $5.2 \pm 0.6\%$ of the *Pdgfra*⁺ cells in the SVF were *Ebf2* (GFP)⁺ (Fig. 3A). Exposure of mice to the cold for 3 d, which stimulates beige fat development, increased the proportion of *Ebf2* (GFP)⁺ cells to $12.7 \pm 0.8\%$ (Fig. 3A). FACS-purified populations of *Ebf2*⁺; *Pdgfra*⁺ and *Ebf2*⁻; *Pdgfra*⁺ cells from iWAT efficiently differentiated into adipocytes that expressed very similar levels of general adipogenic genes (Fig. 3B and C). Remarkably, however, only *Ebf2* (GFP)⁺ cells differentiated into adipocytes that expressed brown-fat-specific genes, including *Ucp1*, *Cidea*, and *Ppara* (Fig. 3D). These data strongly suggest that *Ebf2* is a specific marker for the beige adipogenic precursor cells in WAT that are

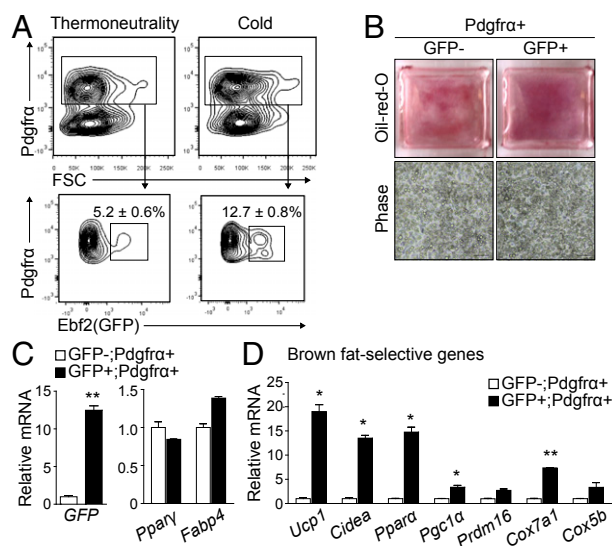


Fig. 3. *Ebf2* marks beige adipogenic precursors in WAT. (A) FACS plots and percentage of stromal cells expressing *Ebf2*-GFP in iWAT from mice housed at thermoneutrality (30 °C) or cold (4 °C) for 3 d. FSC, forward scatter. The percentage is mean \pm SD, $n = 3$ (six mice per experiment). (B) Sort-purified cell populations from iWAT were induced to undergo adipocyte differentiation and stained with Oil Red O. (C and D) mRNA levels of *Gfp*, general adipogenic genes, and (D) brown-fat-selective genes in cell cultures from B. Values are mean \pm SD, $n = 3$; * $P < 0.05$, ** $P < 0.01$.

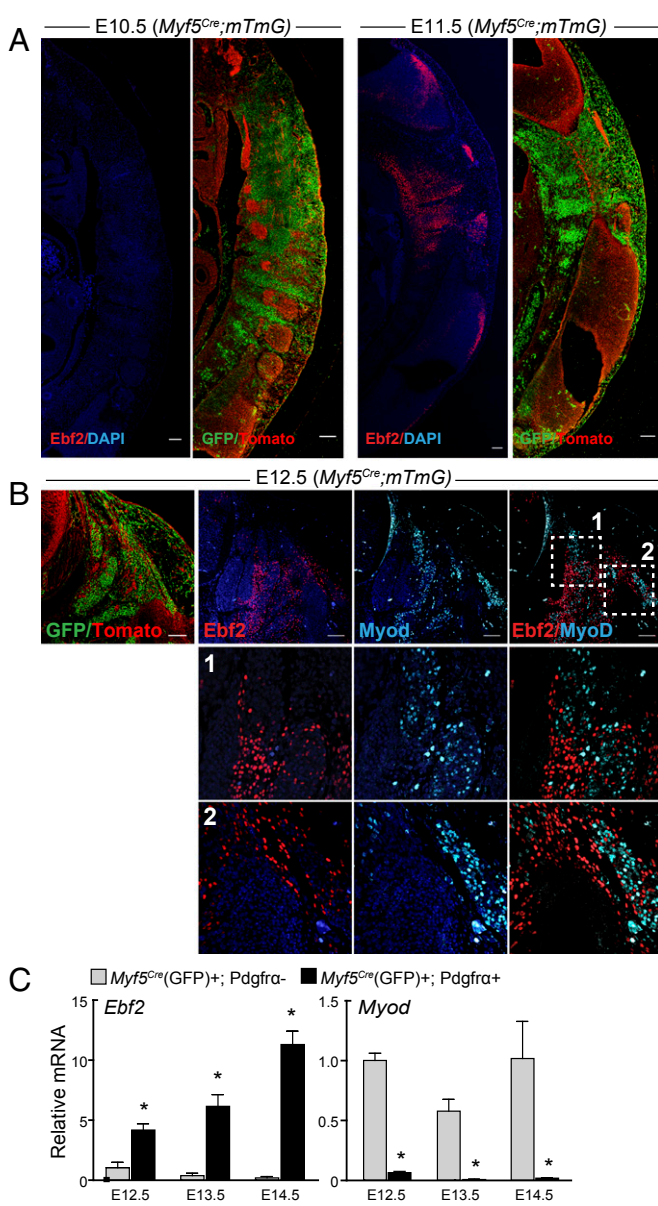


Fig. 4. Ebf2 and MyoD are expressed in distinct cells within the somitic mesoderm. (A) Immunofluorescence staining of tdTomato, GFP (*Myf5^{Cre}*), and Ebf2 (red) on adjacent sagittal sections of E10.5 and E11.5 *Myf5^{Cre}; mTmG* embryos. (B) Costaining of Ebf2 (red) and MyoD (cyan) on transverse sections of anterior somites of E12.5 *Myf5^{Cre}; mTmG* embryos. GFP (*Myf5^{Cre}*) tdTomato double staining is shown (far Left). White dashed boxes show magnified regions 1 and 2. (C) mRNA levels of *Ebf2* and *MyoD* in freshly sorted cells from E12.5, E13.5, and E14.5 embryos. Values are mean \pm SD, $n = 3$ experiments; * $P < 0.05$, ** $P < 0.01$.

competent to activate a brown-fat-selective gene program in response to cold.

Mutually Exclusive Expression of Ebf2 and MyoD in Developing Somites by E12.5. We performed immunofluorescence studies in *Myf5^{Cre}; R26R-mTmG* embryos to analyze the timing and pattern of Ebf2 expression during brown fat development. At E11.5, but not at E10.5, we detected Ebf2 protein expression in a subpopulation of *Myf5^{Cre}* (GFP)⁺ cells in anterior somites (Fig. 4A). At E12.5, there was a dramatic expansion of Ebf2-expressing *Myf5^{Cre}* (GFP)⁺ cells (Fig. 4B). Already at this early stage, Ebf2 and MyoD were expressed in a mutually exclusive manner in the

same *Myf5^{Cre}*-lineage-marked compartment (Fig. 4B). qPCR analysis of FACS-purified cells confirmed that *Ebf2* and *MyoD* expression was enriched in different embryonic cell populations at E12.5, E13.5, and E14.5. *Ebf2* levels increased from E12.5 to E14.5 in *Myf5^{Cre}* (GFP)⁺; Pdgfra⁺ cells, whereas *MyoD* levels remained relatively constant in Pdgfra⁻ cells over the same interval (Fig. 4C). These results suggest that brown adipocyte and myogenic precursors have undergone lineage commitment by the time that Ebf2 protein expression is detected in *Myf5^{Cre}*-lineage⁺ cells.

Ebf2 Regulates the Molecular Identity of Brown Preadipose Cells. Ebf2 is required in mature (or differentiating) adipocytes to promote or maintain the expression of terminal brown-fat-specific genes (18). Our results above suggested that Ebf2 may also play a functional role in brown fat precursor cells. Because there were no molecular markers that could be used to examine the role of Ebf2 at the preadipose stage, we first sought to establish a brown-preadipose-specific gene signature that could be used to monitor cell identity. Global transcriptomic analyses identified 58 genes whose mRNA levels were enriched by twofold or more in (i) *Ebf2* (GFP)⁺ versus *Ebf2* (GFP)⁻ cells and in (ii) *Myf5^{Cre}* (GFP)⁺; Pdgfra⁺ versus *Myf5^{Cre}* (GFP)⁺; Pdgfra⁻ cells (Fig. 5A). As a secondary filter, we profiled the expression of these 58 genes in nonadipogenic and adipogenic (brown and white) cell lines. This led us to identify 21 genes that were very selectively expressed

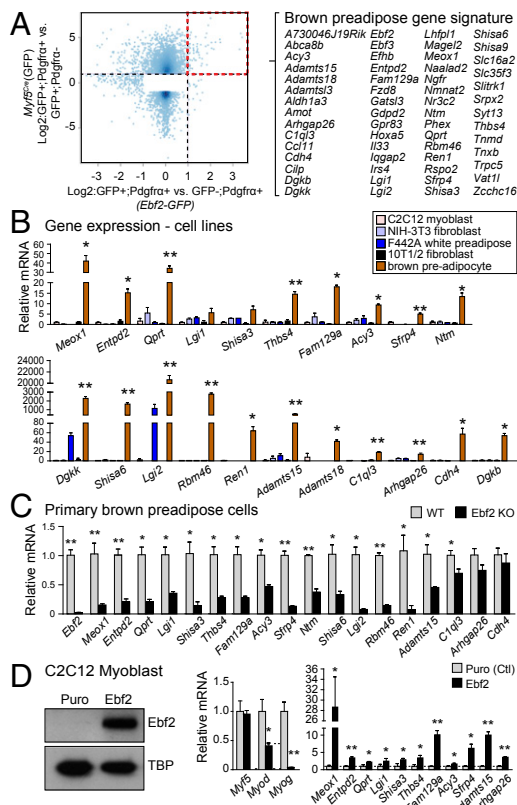


Fig. 5. Ebf2 regulates the brown-preadipose-specific gene signature. (A) Scatter plot showing global gene expression in freshly sorted *Ebf2⁺* versus *Ebf2⁻* (all Pdgfra⁺) precursor cells (x axis) and Pdgfra⁺ versus Pdgfra⁻ [all *Myf5^{Cre}*(GFP)⁺] cells (y axis). (B) mRNA levels of select signature genes in established cell lines. (C) mRNA levels of signature genes in wild-type (WT) and Ebf2-knockout (KO) brown preadipose cells. (D) C2C12 myoblasts were transfected with control (ctl) puromycin (Puro)- or Ebf2-expressing retrovirus. Western blot analysis of Ebf2 protein levels (Left). mRNA levels of muscle-specific genes and brown-preadipose-selective genes (Right). Values are mean \pm SD, $n = 3$; * $P < 0.05$, ** $P < 0.01$.

in brown preadipose cells relative to fibroblasts (NIH 3T3, 10T1/2), white preadipose (3T3-F442A) and muscle precursor cells (C2C12) (Fig. 5B and Fig. S84). The majority of these genes were expressed at high levels in preadipose cells and were decreased in expression during the process of adipocyte differentiation (Fig. S8B). Moreover, most of these genes had the same expression pattern as *Ebf2* in that they were selectively enriched in *Myf5^{Cre}* (GFP)⁺; *Pdgfra*⁺ cells relative to other cell fractions isolated from the dorsal region of embryos starting from E11.5 of development (Fig. S8C).

To determine whether *Ebf2* was genetically required to establish brown preadipose cell identity, we analyzed the expression of the 21 brown preadipose signature genes in primary preadipose cells isolated from WT and *Ebf2*-knockout (KO) BAT. Strikingly, the expression of 16/21 of these genes was significantly diminished by *Ebf2* deficiency, including a 70–80% reduction in the levels of *Meox1*, *Enpd2*, *Qprt*, *Shisa3*, *Thbs4*, *Fam129a*, *Sfrp4*, *Lgi2*, *Rbm46*, and *Ren1* (Fig. 5C and Fig. S8D). By contrast, genetic deletion of *Pparγ*, the master regulator of differentiation in all types of adipocytes, did not affect the expression levels of any brown preadipose signature genes (Fig. S8E). These results show that *Ebf2* functions independently from *Pparγ* in establishing and/or maintaining the gene program of brown adipose precursors.

Ectopic expression of *Ebf2* in white fat or muscle precursor cells cooperates with a mixture of adipogenic inducers to drive a brown-fat cell-selective differentiation program (18). We wondered whether *Ebf2* reprograms precursor cell identity before adipogenic induction. To assess this question, we expressed *Ebf2* or a control retroviral vector in C2C12 myoblasts and measured the expression of brown preadipose signature genes. *Ebf2* expression was sufficient to activate the expression levels of many brown preadipose selective genes, including a 28-fold increase in *Meox1* and 6- to 10-fold increases in *Fam129a*, *Sfrp4*, and *Adamts15* (Fig. 5D). *Ebf2* expression also led to reduced levels of muscle-specific genes such as *MyoD* and *MyoG* in myoblasts but had no effect on *Myf5* mRNA levels (Fig. 5D). These data suggest that *Ebf2* expression reprograms myoblasts into a brown-preadipose-like state.

Discussion

Brown and beige fat cells dissipate chemical energy to protect animals against obesity and insulin resistance. We discovered that *Ebf2* is a selective marker of brown and beige preadipose cells—the lineage-committed cells that mediate brown and beige fat expansion. The ability to detect and isolate these precursor cells will open up new opportunities to identify the mechanisms that control brown/beige preadipose commitment, proliferation, and differentiation.

Brown adipocytes, muscle, and dorsal dermis arise from *Pax7*- and *En1*-expressing cells in the central dermomyotome (15, 16). Our analyses indicate that *Myf5^{Cre}* labels these same three differentiated cell types in late-stage embryos. Within this *Myf5^{Cre}*-marked lineage, *Ebf2* was only expressed in brown adipogenic precursor cells and was absent from both muscle and dermal cells. Indeed, embryonic brown adipogenic cells could be purified by their selective expression of *Ebf2*-GFP. *Ebf2* was expressed as early as E11.5 in *Myf5^{Cre}*-lineage-marked cells and was not detected in *MyoD*⁺ muscle cells, suggesting that brown adipogenic cells are committed by the time that *Ebf2* is activated.

The stable committed state of brown preadipose cells is well established (33). These cells can be isolated and propagated *ex vivo* without losing their brown-specific differentiation potential. However, whereas much is known about the factors that promote brown fat-selective transcription in mature adipocytes, no transcription factors that function at the preadipose stage had been identified. Our results indicate that *Ebf2* acts at the precursor cell stage to instruct and/or maintain preadipose cell fate. Future

studies will examine the function of *Ebf2*-regulated preadipose genes in brown fat lineage commitment and differentiation. It will also be important to identify the cues that activate *Ebf2* during brown fat induction. BMP signaling, which is critically required for early BAT development (22), is one such candidate for regulating *Ebf2* expression/activity.

Ebf2-deficient mice develop dysmorphic fatty tissue devoid of brown-fat-specific characteristics in place of BAT (18). Thus, whereas *Ebf2* is required to induce the brown-fat cell-selective gene program, it is dispensable for adipogenesis *per se*. We speculate that other members of the *Ebf* family act to support adipogenesis in the absence of *Ebf2*. In line with this speculation, *Ebf1* and *Ebf3* can stimulate adipocyte differentiation when expressed in fibroblasts (34). It is also interesting to note that *Ebf3* was identified here (Fig. 5) and previously as a brown-adipose-enriched factor (14).

Beige adipocytes in WAT express many brown-fat-selective genes and seem to function much like classical brown fat cells (35). The induction of beige adipocytes is strongly associated with a lean and healthy phenotype in mice (2). In iWAT, beige adipocytes are thought to arise *in situ* from the *de novo* differentiation of resident precursor cells (14, 24, 29, 36). We found that *Ebf2* is specifically expressed by adipogenic cells in WAT that are competent to activate a brown/beige fat-selective differentiation program. Elegant studies by the Granneman group showed that *Pdgfra*-expressing cells in gonadal WAT can differentiate into both brown/beige and white adipocytes (24). The relationship between these bipotent *Pdgfra*⁺ cells and the *Ebf2*⁺ cells identified here is unclear. One possibility is that the *Pdgfra*⁺ cell population isolated by Lee et al. are upstream progenitor/stem cells that give rise to more committed populations of *Ebf2*⁺ beige and *Ebf2*⁻ white preadipose cells in response to different cues. However, while our data strongly suggest that *Ebf2*⁺ cells are beige-specific precursors, we cannot exclude the possibility that these cells also give rise to white adipocytes *in vivo* under certain conditions. Finally, it is also possible that *Ebf2*⁺ cells are uniquely found in iWAT to provide that depot with a more robust capacity to undergo browning.

In conclusion, we report a critical role for *Ebf2* in the control of brown preadipose cell fate and identify *Ebf2* and other gene markers for committed brown/beige preadipose cells. This study opens up opportunities to examine the molecular pathways that control brown preadipose cell development and function. Gaining a better understanding of brown/beige committed precursors will be crucial for the design and implementation of brown and/or beige fat-targeted therapies.

Materials and Methods

Animals. All procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. The following mouse lines were obtained from The Jackson Laboratory: *Myf5^{Cre}* (B6.129S4-*Myf5^{tm3(cre)SorJ}*), stock no. 010529; *Pdgfra-Cre* (C57BL/6-Tg(*Pdgfra-cre*)1ClclJ), stock no. 013148; mTmG (B6.129(Cg)-*Gt(ROSA)26Sortm4 (ACTB-tdTomato,EGFP)LoxJ*), stock no. 007676; *Pparγ^{lox}* (B6.129-Ppargtm2RevJ), stock no. 004584; CAGG^{CreERT} (B6.Cg-Tg(CAG-cre/Esr1*)5AmcJ), stock no. 004682; and Rosa-dTomato (B6.Cg-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)HzeJ}*), stock no. 007909. *Ebf2^{GFP}* knock-in mice and *Ebf2-GFP^G* BAC (bacterial artificial chromosome)-transgenic mice were described before (37, 38). *Ebf2-GFP^G* mice were developed by the Mutant Mouse Regional Resource Center.

Cell Preparation and Flow Cytometry. For embryonic fibroblasts, excised tissue was digested in 6.1 mg·mL⁻¹ collagenase (Roche; 1108882001) and 2.4 mg·mL⁻¹ dispase (Roche; 04 942 078 001) in DMEM for 40 min at 37 °C in a shaking water bath. For SVF from iWAT, we performed two cycles of digestion. Digested tissue was successively filtered through 100-μm and 40-μm cell strainers (BD Biosciences). Cells were suspended in DMEM with 5% (vol/vol) FBS, and stained with antibodies for 30 min at 4 °C in the dark. Antibodies and secondary antibodies used for staining were: *Pdgfra* APC 1:200 (Biolegend; 135907), Itga7-PE 1:200 (AbLab; University of British Columbia), CD31-PE 1:200 (BD Biosciences; 553373), CD45-PE 1:200 (BD Biosciences; 553081),

Sca1-PE 1:200 (eBioscience; 12-5981-81), CD24-biotin 1:400 (eBioscience; 13-0242-81), CD34-biotin 1:400 (eBioscience; 13-0341-82), and streptavidin-PE 1:500 (Biolegend; 405204). Stained cells were analyzed by BD LSR II or sorted with BD FACS Aria. Debris and dead cells were excluded by forward scatter, side scatter, and DAPI gating. Data analysis was performed using FlowJo. Pparg-knockout cells were obtained by treating primary SVF cells from the P1 BAT of CAGG^{CreERT}, Pparg^{FloxFlox} mice with 1 μ M 4-hydroxytamoxifen (Sigma) for 3–5 d.

Cell Culture. Freshly sorted cells were cultured on 8-well chamber slides (Ibidi; 80826) or collagen-coated 24-well plates (BD Biosciences; 354408) in DMEM with 10% (vol/vol) FBS, 10 ng/mL bFGF for 3 d. After confluence, cells were exposed to brown adipogenic induction medium [DMEM with 10% (vol/vol) FBS, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 μ M dexamethasone, 20 nM insulin, and 1 nM T₃]. After 48 h, cells were switched to maintenance medium [DMEM with 10% (vol/vol) FBS, 1 nM T₃, 20 nM insulin] for 4 d before harvesting. To induce adipogenesis in WAT-derived precursors, 0.5 μ M rosiglitazone was included in the induction and maintenance medium.

RNA Extraction and Real-Time PCR. Total RNA was extracted from cultured cells or 5 \times 10⁴ freshly sorted cells using the TRIzol method (Invitrogen) combined with Qiagen RNeasy Micro Column and then reverse transcribed into cDNA using an ABI high-capacity cDNA synthesis kit. Real-time PCR was performed on an ABI7900HT PCR machine using SYBR green fluorescent dye (ABI).

Cytochemistry, Immunohistochemistry, and Oil Red O Staining. Freshly sorted cells were collected on chamber slides and fixed with 4% (wt/vol) paraformaldehyde (PFA) for 10 min. Primary and secondary antibodies used for staining are: Pparg 1:250 (Thermo Scientific; MA5-14889), Ebf2 5 ng·mL⁻¹ (R&D; AF7006), Prdm16 (in house) 1:200, Alexa Fluor 647 chicken anti-rabbit IgG 1:500 (Invitrogen), Alexa Fluor 647 donkey anti-sheep IgG 1:500 (Invitrogen). Tissues were fixed in 2% (wt/vol) PFA overnight. Paraffin-embedded sections were subjected to citrate-based antigen retrieval. Primary

antibodies used for immunohistochemistry are: Pparg 1:500 (Thermo Scientific; MA5-14889), perilipin 1:200 (Cell Signaling; 3470), Pdgfra 1:50 (R&D; AF1062), GFP 1:500 (Abcam; AB6673), Ebf2 15 ng·mL⁻¹ (R&D; AF7006), and MyoD 1:20 (Novocastra; NCL-MyoD). To stain lipid, cells were fixed in 10% (vol/vol) formalin, rinsed with PBS and 60% (vol/vol) isopropanol, incubated with Oil Red O in 60% isopropanol, and then rinsed in PBS.

Whole-Mount Confocal Microscopy. Whole-mount confocal microscopy was performed as previously described (23). Briefly, 4-wk-old male mice were perfused transcardially with 1% PFA for 10 min. The 5-mm³ pieces of adipose depots were dissected and fixed in 1% PFA for another 20 min. Images were captured on a Leica TCS SP8 confocal microscope.

RNA-Seq and cDNA Microarray. RNA-seq libraries were generated with the Illumina Truseq RNA Sample Preparation kit. High-throughput sequencing was performed using a HiSeq 2000. Expression values of mRNA were called by TopHat/Cufflink pipeline. For microarray analysis, total RNA was purified from three biological replicates per cell type. Array hybridization and scanning were performed by the Penn Molecular Profiling Facility using Affymetrix GeneChip Mouse Genome 2.0 arrays.

Statistical Analysis. Error bars show SD. Statistical significance was assessed by the Student *t* test, **P* < 0.05, ***P* < 0.01.

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