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Pref-1 Marks Very Early Mesenchymal Precursors Required for Adipose Tissue Development and Expansion

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SUMMARY

Pref-1 is an EGF-repeat containing protein that inhibits adipocyte differentiation. To better understand the origin and development of white adipose tissue (WAT), we generated transgenic mouse models for transient or permanent fluorescent labeling of cells using the Pref-1 promoter, facilitating inducible ablation. We show that Pref-1 marked cells retain proliferative capacity and are very early adipose precursors, prior to expression of Zfp423 or PPAR g. In addition, Pref-1 marked cells establish adipose precursors as mesenchymal, but not endothelial or pericyte in origin. During embryogenesis, Pref-1 marked cells first appear in the dorsal mesenteric region as early as E10.5. These cells become lipid-laden adipocytes at E17.5 in the subcutaneous region, whereas visceral WAT develops after birth. Finally, ablation of Pref-1 marked cells prevents not only embryonic WAT development but also later adult adipose expansion upon high fat feeding, demonstrating the requirement of Pref-1 cells for adipogenesis.

INTRODUCTION

The major function of white adipose tissue (WAT) is for triglyceride storage which is hydrolyzed and released as fatty acids in times of energy shortage. In modern times, obesity, characterized by excess WAT has become a disease of epidemic proportions. In humans,

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Supplemental methods and data can be found with this article.

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WAT has been described to emerge during late embryogenesis, whereas WAT in mice is typically thought to develop postnatally. Moreover, adipose tissue can increase in mass by hypertrophy and hyperplasia (Tang and Lane, 2012). However, the definitive origin and location of adipose precursors, especially early during embryonic development, have not been well defined. It has been proposed that within WAT, progenitors with adipogenic potential reside near the vasculature and they may be endothelial and/or pericyte in origin (Tang et al., 2008; Gupta et al., 2012). On the other hand, a mesenchymal stem cell population capable of adipogenesis has previously been isolated from the stromal vascular fraction (SVF) of adipose tissue, suggesting a mesenchymal origin of adipose progenitors (Gimble et al., 2011). Also, a subset of adipocytes has been reported to arise from neural crest cells (Billon et al., 2007). Thus, the definitive origin of adipocytes still remains controversial.

The role of PPARγ and C/EBPs, as well as other transcription factors in adipocyte differentiation, has been extensively studied (Gregoire et al., 1998; Farmer, 2006). Recently, Zfp423 was reported to be critical for the preadipocyte commitment process (Gupta et al., 2012). Many soluble factors also affect adipogenesis to respond to environmental cues. We originally identified Preadipocyte factor-1 (Pref-1, or Dlk1) as an inhibitor of adipocyte differentiation (Smas and Sul, 1993; Hudak and Sul, 2013). Pref-1 is synthesized as an EGF-repeat containing transmembrane protein upon cleavage by TACE, Pref-1 generates a soluble factor which activates MEK/ERK to upregulate Sox9 and affect adipogenesis (Lee et al., 2003; Wang et al., 2010). Adipose progenitors labeled via PPARγ-driven reporters were recently identified near the vasculature (Tang et al., 2008). However, expression of PPARγ, not only in preadipocytes but also in adipocytes, somewhat complicated the detection of adipose precursors. Pref-1 may be a better candidate for studying adipose lineage since Pref-1 is expressed only at the preadipocyte but not adipocyte stage of adipocyte differentiation.

Here, we show that cells marked by the Pref-1 promoter have proliferative capacity and represent very early adipose precursors. We also show that Pref-1 marked cells are not of endothelial or pericyte, but mesenchymal in origin and first appear as early as E10.5 in the dorsal mesenteric region. These precursors differentiate into lipid filled adipocytes detectable at E17.5, thus identifying very early adipose progenitors and their location during embryogenesis. By ablating Pref-1 cells using diphtheria toxin-A (DTA), we also show that Pref-1 cells are required for adipose tissue development during embryogenesis and its expansion in adults.

RESULTS

Generation of Transgenic Mouse Models for Labeling of Pref-1 Cells

Pref-1 provided us with a unique tool to study the origin and development of adipose tissue, as Pref-1 is expressed in preadipocytes but not found in mature adipocytes. Thus, to perform lineage tracing of Pref-1 cells, we created Pref-1 reverse tet transactivator (rtTA) transgenic mice expressing rtTA under the control of −6 kb of the Pref-1 promoter. We found a 9-fold increase in luciferase activity and GFP in Dox-treated 3T3-L1 cells (Figure S1A). For our Pref-1-GFP mouse model, Pref-1-rtTA mice were crossed with mice expressing a histone 2B

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GFP fusion protein (H2BGFP) under the control of TRE (Kanda et al., 1998), thus labeling Pref-1 cells with a nuclear-localized GFP reporter which is stable in postmitotic cells but lost upon cell division after the removal of Dox. To permanently label Pref-1 cells, we crossed our Pref-1-rtTA mice with TRE-Cre mice. The resulting Pref-1-rtTA-TRE-Cre mice were then crossed with ROSA26-flox-stop-flox-tdTomato mice (Perl *et al.*, 2002), where Dox treatment induces the cytoplasmic tdTomato reporter and is expressed in all progeny even after cell division (Figure 1A).

We compared the expression of the fluorescent reporters in adipose tissue of Pref-1-GFP and Pref-1-tdTomato mice. Laser scanning confocal (LSC) microscopy showed the presence of GFP positive cells only in mice treated with Dox in adipose tissue sections (Figure 1B), and whole mount adipose (Figure S1A) but not in control mice in the presence or absence of Dox. Similarly, TdTomato was detected in WAT sections from Pref-1-tdTomato mice upon Dox treatment, but not in untreated or control mice. We conclude these models would be useful for adipose lineage tracing since reporters were detected in an inducible manner in adipose tissue.

Similar to endogenous Pref-1 expression, rtTA was not detected or was very low in other tissues, but was highly expressed in WAT depots, demonstrating that expression of rtTA driven by the −6 kb Pref-1 promotor could mimic endogenous Pref-1 expression (Figure 1C). We examined GFP fluorescence in cryosections of WAT and detected nuclear GFP fluorescence in ~35% of cells in inguinal and epididymal WAT (Figure S1B), ~25% and ~5% in gluteal and renal WAT, and only a handful of labeled cells in BAT (which showed high background fluorescence). Using fluorescence activated cell sorting (FACS), we were able to detect ~1% of total cells labeled in the inguinal and epididymal depots (Figure S1C). Since the Pref-1 promoter is active only at the preadipocyte stage, we conclude that the GFP signal from transient labeling was specific to the preadipocyte population. We next compared the GFP expression between the SVF and adipocytes of inguinal WAT and found that Pref-1 mRNA was expressed only in the SVF, whereas FAS was expressed specifically in adipocytes (Figure 1D). More importantly, similar to the Pref-1 expression pattern, rtTA and GFP mRNA levels were much higher in the SVF compared to adipocytes. Overall, these data demonstrate that the −6 kb Pref-1 promoter is sufficient to drive tissue- and developmental pattern-specific expression of a reporter and that GFP is detected only in preadipocytes.

We next examined Pref-1 tdTomato mice for permanent labeling of Pref-1 cells (Figure 1B). Since Pref-1 expression is found in a variety of tissues during embryogenesis, we administered Dox starting at E10.5 when GFP positive cells first emerge (see Figure 4), which resulted in adipose specific labeling. Indeed, in inguinal and epididymal WAT, more than 90% of the cells, including adipocytes were tdTomato positive, representing a significantly higher percentage than in the Pref-1-GFP model. We also detected approximately 20% of cells that were labeled in BAT (Figure S1C), indicating that Pref-1 expressing cells are present in BAT. Furthermore, we detected GFP and tdTomato only in adipose depots, but not in other organs that we examined, such as liver, kidney, and muscle.

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Lastly, to confirm that the GFP reporter is directly labeling Pref-1 expressing cells, we employed single cell mRNA analysis. Cells from the SVF of Pref-1-GFP mice were plated in a microwell device so that each well contained a single cell (Figure S1D) (Dimov et al., 2014). Approximately 50% of the cells could be detected by GFP fluorescence (Figure 1D), and we detected Pref-1 mRNA in each GFP positive cell. Taken together, these results clearly demonstrate that reporter expression driven by the −6 kb Pref-1 promoter faithfully recapitulated endogenous Pref-1 expression and that our mouse models are valid for studying the origin and development of adipose tissue.

Pref-1 Marked Cells are of Mesenchymal but not Endothelial or Pericyte origin

To characterize Pref-1 cells, we analyzed expression of various endothelial/pericyte and mesenchymal markers. Using FACS, we isolated GFP positive cells of freshly collected SVF of WAT from Dox-treated Pref-1-GFP mice. GFP and Pref-1 mRNA levels were greatly higher in sorted GFP positive cells compared to GFP negative cells by RT-qPCR (Figure 2A). A commonly used marker of hematopoetic lineage, CD45, was almost undetectable in GFP positive cells, as were several markers of endothelial cells or pericytes. In contrast, expression of markers used in various combinations to identify mesenchymal stem cells or adipose stromal cells were greatly higher in GFP positive compared to negative cells. Also, Sox9, a mesenchymal fate determining factor that we reported to be upregulated by Pref-1 (Wang and Sul, 2009), was highly expressed in GFP positive cells (Figure S2A). GFP labeled Pref-1 cells were also found to colocalize with Pref-1 staining, but did not colocalize with endothelial markers, confirming our gene expression results. Nor did we detect the presence of a hematopoietic marker in GFP positive cells (Figure 2B, S2A). On the other hand, we detected colocalization of GFP positive cells with several markers of mesenchymal cells (Figure 2C). We also investigated the expression of previously described inhibitors of adipogenesis, Necdin and Wnt10a (MacDougald and Burant, 2005; Cawthorn *et al*., 2012), and we found that while Pref-1 expressing cells appear to also express Necdin, they do not colocalize with Wnt10a expression (Figure S2B). We quantified the percentage of GFP positive cells expressing these markers using imageJ software and found that >90% of the GFP positive cells expressed mesenchymal or adipose stromal cell markers. Overall, the expression of these markers in GFP positive cells indicates that Pref-1 expressing cells are not endothelial/pericyte, but mesenchymal in origin.

By generating Tie2-GFP-Pref-1-tdTomato mice for permanent dual labeling experiments, we examined if Pref-1 cells could originate from an endothelial lineage (in this model, endothelial cells are labeled with GFP while Pref-1 cells are permanently labeled with tdTomato) (Figure S2C). Confocal microscopy of inguinal WAT sections showed that Tie2 cells were clearly lining the vasculature, whereas tdTomato could be visualized throughout the adipose tissue (Figure S2D), indicating that Pref-1 labeled adipose precursors are not from an endothelial origin. Furthermore, the lack of tdTomato expression near the vasculature indicates that endothelial cells are not derived from Pref-1 expressing cells.

We next examined various adipogenic markers and detected much lower levels of PPARγ, a master regulator of adipogenesis. We did not detect Zfp423, a transcription factor recently reported to be critical for adipose commitment. However, GFP positive cells expressed high

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levels of Ki67, a marker of proliferative capacity, as well as CD24, which was shown to mark proliferative adipose progenitors (Rodeheffer *et al*., 2008). PDGFRα, a recently reported marker of adipose derived stem cells (Mohsen-Kanson et al., 2013), was also highly expressed in GFP positive cells (Figure 2D). We also found that GFP positive cells of Pref-1-GFP mice did not colocalize with PPARγ or Zfp423, confirming that GFP positive cells did not contain significant levels of these adipogenic transcription factors, however, some GFP positive cells were colocalized with CD24. We also found colocalization of some tdTomato positive cells with Ki67 in Dox-treated Pref-1-tdTomato mice (Figure 2E). Overall, transiently marked Pref-1 cells do not express early adipogenic transcription factors including Zfp423 or PPARγ, but have proliferative capacity. We also show that Pref-1 cells are not of endothelial, pericyte, or hematopoietic origin, but have characteristics of mesenchymal cells.

Pref-1 Expressing cells Represent Very Early Adipose Precursors

To better establish that Pref-1 marked cells are indeed adipose precursors *in vivo*, we employed our permanently labeled Pref-1-tdTomato mice. We treated Pref-1-tdTomato mice with Dox starting at E10.5 and examined cryosections of inguinal WAT for the presence of tdTomato labeled cells, lipid staining, and expression of adipocyte markers. Indeed, all lipidTox stained cells showed tdTomato fluorescence. In addition, tdTomato fluorescence colocalized with the adipocyte markers PPARγ and FABP4. We also found that tdTomato labeled cells in the adult WAT expressed ZFP423 (Figure 3A). Therefore, permanently labeled Pref-1 positive cells express ZFP423, PPARγ and FABP4. Following culture and adipogenic media treatment of SVF from WAT of Dox-treated Pref-1-tdTomato mice, differentiation was detected at day 5 when most of the tdTomato positive cells developed lipid droplets, but tdTomato negative cells did not show lipid accumulation (Figure 3B). We also cultured the SVF from WAT of Dox treated Pref-1-GFP mice and subjected them to adipogenic media. At Day 5, GFP positive cells showed lipid staining in the cytoplasm, clearly demonstrating adipogenic differentiation of these cells, whereas GFP negative cells did not show any lipid staining, which was further confirmed via Oil Red O staining (Figure 3C). Cells from Dox treated Pref-1 GFP mice expressed both GFP and Pref-1 at a high level prior to differentiation which decreased during the course of differentiation. In contrast, expression of ZFP423 or PPARγ was at a non-detectable or very low level but increased greatly during differentiation (Figure 4C, lower panel).

Next, to better investigate the adipogenic potential of Pref-1 expressing cells, we measured adipogenic marker expression in GFP positive and negative cells from the SVF of inguinal WAT. Unlike the GFP negative cells that were unable to undergo differentiation, bright field microscopy showed adipocyte morphology with multiple lipid droplets in GFP positive cells. At Day 5, mRNA levels of adipogenic markers were all significantly higher in GFP positive cells compared to negative cells. In contrast, the Pref-1 mRNA level was significantly lower upon differentiation (Figure 3D). These results show that transiently labeled GFP-positive cells are adipose precursors prior to expression of Zfp423 or PPARγ.

To further confirm the adipogenic potential of these precursors, we isolated permanently labeled (tdTomato) Pref-1 positive cells from 4 week-old Dox-treated Pref-1-tdTomato mice

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and subcutaneously injected the SVF into SCID mice. After two weeks, the cells differentiated into adipocytes as identified by Bodipy staining of lipid droplets, and immunostaining of the adipocyte marker, FABP4 (Figure S3A). Expression levels of the adipogenic markers, PPARγ and FABP4, were found to be greatly higher in the transplanted cells, compared to the undifferentiated SVF and control 3T3-L1 cells (Figure S3B). Taken together, we conclude that Pref-1 expressing cells appear very early during embryogenesis and differentiate into adipocytes, thus representing very early adipose precursors.

Spatial and Temporal Pattern of Embryonic WAT Development

We employed our mouse models to examine embryonic WAT development *in vivo* by providing Dox water to mice at mating. Whole embryo cryosections were examined by LSC microscopy and lipidTox staining. At E8.5, no obvious GFP positive cells or lipid staining were detected. At E10.5 however, GFP positive cells were readily detected in two distinct groups in the dorsal mesenteric layer between the vertebrae and the skin, poised for adipose development at the presumptive inguinal and dorsal subcutaneous depots (Figure 4A, upper). We believe that this is the earliest time point when marked adipose precursors have been detected. At E13.5, GFP positive cells formed a distinct line at the dorsal edge of the embryo close to the skin, but lipid staining was not detected (Figure 4A, middle left). We also detected GFP positive cells that expressed Ki67, indicating that these cells were proliferating (Figure 4A, upper right). Finally, at E17.5, lipid staining was clearly detected in this region representing early WAT, and GFP positive cells were localized near the outer edge of this early WAT (Figure 4A, middle left). At E19.5, the number of lipid staining cells were increased by 2.5 fold compared to E17.5, indicating hyperplasia of WAT during embryogenesis (Figure 4A, lower left). To trace the development of adipose tissue, we examined our Pref-1-tdTomato mice. At E17.5, we could detect Pref-1 marked cells in the same region where we detected GFP labeled Pref-1 expressing cells. At E19.5, permanently labeled tdTomato positive cells were found in the same region where Oil red O positive cells were detected, overall indicating that Pref-1 labeled cells became adipocytes (Figure 4A, lower). At birth, GFP positive Pref-1 cells were detected at the edge of the subcutaneous WAT identified by the presence of lipid droplets by Oil Red O staining and rounded adipocyte morphology. Altogether, these results show that very early adipose precursors are first detectable as early as E10.5 at a distinct region of the mesenteric dorsal edge, which become subcutaneous WAT at E17.5 during embryogenesis.

In contrast to subcutaneous WAT, we could not detect any GFP labeling or lipid droplets the presumed visceral adipose depots during embryogenesis. And, it was not until 6 days after birth that we could detect epididymal WAT where GFP positive cells were localized mostly around the outer edge (Figure 4A, lower), indicating that while subcutaneous depots develop early during embryogenesis, visceral adipose depots appear postnatally. In this regard, permanently labeled Pref-1 cells were detected throughout the P21 epididymal WAT, identifiable by their large rounded morphology (Figure 4A, lower panels). When we examined the epididymal WAT of adult Pref-1-GFP mice, GFP positive cells were found in clusters and also dispersed throughout WAT. Furthermore, when we examined the BAT of adult Pref-1-tdTomato mice, we found a small population of tdTomato labeled cells, indicating that a subset of brown adipocytes may stem from Pref-1 expressing precursors

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(Figure S4A). Overall, these results demonstrate that subcutaneous WAT develops prenatally, while visceral WAT develops postnatally and that inguinal, epididymal, gluteal and renal WAT and some interscapular BAT is derived from Pref-1 expressing precursors.

Requirement of Pref-1 cells for embryonic WAT Development and for WAT Expansion in Adults

We subjected Pref-1-GFP mice to a high fat diet and BrdU to examine accelerated expansion of WAT. After three weeks, we could clearly detect BrdU in GFP positive cells indicating proliferation. Quantification by cell sorting showed that the number of GFP positive cells was decreased in epididymal and inguinal WAT by 90% and 75%, respectively. We detected an increase in the number of BrdU positive cells and a corresponding decrease in the number of GFP positive cells upon high fat diet feeding (Figure 5A). A similar experiment employing Pref-1-tdTomato mice indicated a larger adipocyte size, especially in inguinal WAT (Figure S5A), indicating that Pref-1 expressing adipose precursors contribute to WAT expansion via hyperplasia.

To further examine the role of Pref-1 expressing cells in adipose development and adipose expansion in adults, we crossed our Pref-1-rtTA mice with the TRE-DTA mouse line to create a Pref-1-DTA model, whereby expression of DTA ablates Pref-1 expressing cells (Figure 5B). First, we deleted Pref-1 expressing cells at E10.5 and examined the embryos for lipid staining at E17.5, we found >70% reduction in the number of lipid containing cells in Pref-1-DTA embryos (Figure 5B); demonstrating the requirement of Pref-1 expressing cells for the generation of adipocytes during embryogenesis. We did not detect any gross morphological or phenotypic effects in any other tissues. To examine the postnatal development of WAT, Pref-1-DTA mice were given Dox from P1-P21 to ablate adipose precursors. Both epididymal and inguinal WAT showed drastically decreased lipid staining compared to control mice, to an almost undetectable level in the epididymal depot, and the number of lipid containing cells were decreased by >95% and 60% and in the epididymal and inguinal WAT depots, respectively (Figure 5C). Some remaining cells in adipose tissue contained small lipid droplets, probably due to differentiation of residual Pref-1 cells that were not ablated due to incomplete penetrance of DTA expression, as has been demonstrated previously (Nir et al., 2007). We also found a significant decrease (65% and 40%) in the WAT mass of epididymal and inguinal fat depots, amd total triglyceride content (Figure 7D). In contrast, lipid content in the liver was increased (data not shown), indicating potential ectopic deposition of triglycerides, resembling lipodystrophy mouse models (Moitra et al., 1998). We next performed a glucose tolerance test and found that Pref-1-DTA mice were glucose intolerant compared to control mice (Figure 5D). Overall, these data show that Pref-1 expressing cells are required for normal development of adipose tissue, adipose tissue function, and adipose expansion in adults.

DISCUSSION

The obesity epidemic and its related disorders make it critical to understand the developmental process of adipose tissue. In obesity, not only do adipocytes enlarge, the number of adipocytes increases by differentiation of precursors into adipocytes, presumably

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recruited from the stromal vascular fraction of adipose tissue. However, the early adipose precursors and the developmental pattern of adipogenesis have not been well studied via lineage tracing. A critical aspect of lineage tracing requires the expression of a unique marker by a specific subset of cells. In this case, except certain neuroendocrine type of cells, Pref-1 expression in adults is restricted mainly to adipose tissue. Furthermore, in adipose tissue, Pref-1 is found only in preadipocytes and its expression is extinguished upon differentiation into adipocytes. This Pref-1 expression pattern provides a unique tool to mark adipose precursors prior to differentiation. Here, by labeling Pref-1 expressing cells transiently and permanently *in vivo* in an inducible manner, we show that these cells become adipocytes and represent adipose precursors. We also detected Pref-1 expressing cells as early as E10.5 in the dorsal mesenteric region, which subsequently become adipocytes by E17.5 during embryonic development. To our knowledge, this is the earliest adipose precursor population that has been identified. We demonstrate the depot-specific differences in WAT development as evidenced by the lack of Pref-1 marked cells in visceral areas before birth. With lineage tracing, we show that WAT is derived from cells that are labeled by Pref-1 promoter activation. Furthermore, by employing our Pref-1-GFP, Pref-1-tdTomato and Pref-1-DTA mouse models, we unequivocally demonstrate the requirement of Pref-1 marked cells for adipose tissue formation. We also show that Pref-1 adipose precursors proliferate during adipose expansion upon high fat feeding, and deletion of Pref-1 cells markedly decreases expansion of WAT in adults. We believe that this is the first evidence that deleting a specific adipose precursor population results in an impairment of WAT development.

Here we show that cells that are indelibly marked by the Pref-1 promoter become adipocytes that express PPARγ and ZFP423. Since transiently marked Pref-1 cells do not significantly express Zfp423 or PPARγ, these cells must represent very early adipose progenitors prior to expression. Moreover, GFP positive cells express the proliferation marker, Ki67 and can incorporate BrdU, showing that Pref-1 expressing cells are proliferative adipose progenitors, and can undergo cell division during adipose expansion. Since those GFP positive cells later become adipocytes, we can conclude that Pref-1 marked adipose precursors undergo cell division prior to differentiation into adipocytes, a property of preadipocytes that has been shown *in vitro* (Tang and Lane, 2012). We conclude that Pref-1 marks very early adipose precursors prior to Zfp423 or PPARγ expression, and that these cells have proliferative capacity.

Adipose progenitors have been reported to be of endothelial and/or pericyte origin (Olson and Soriano, 2011). However, Pref-1 marked cells do not express endothelial markers or pericyte markers, indicating that these adipose precursors are not of endothelial or pericyte origin. We also did not detect clear localization of Pref-1 marked cells to the vasculature. Moreover, the Tie2-GFP-Pref-1-tdTomato mice we generated showed that Pref-1 marked cells were not labeled by Tie2, demonstrating that the Pref-1 cells are not endothelial cells. One remaining possibility is that endothelial cells later become Pref-1 positive. However, this cannot be the case since others have used an endothelial promoter which was activated after Pref-1 positive cells emerged in their adipose lineage tracing study, and these cells did not express Pref-1. In addition, Zfp423 has been detected in adipose precursors localized in

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endothelial and perivascular cells but these cells did not express Pref-1, adding to the discrepancy in defining the origin of adipose precursors. Our present work shows a mesenchymal origin of Pref-1 marked cells due to the expression of several mesenchymal markers. Various other proteins including CD24 and PDGFRα have been reported to be markers of adipose precursors, however, these markers are expressed in many other tissues. Furthermore, even in WAT, not all the cells labeled by CD24 or PDGFRα became adipocytes. It should be noted that virtually all Pref-1 labeled cells become adipocytes in the WAT depots we examined, clearly demonstrating that Pref-1 can be used as an exclusive marker for adipose precursors in several WAT depots.

Overall, we show here that Pref-1 marked cells are very early adipose precursors prior to the expression of Zfp423 or PPARγ, which first appear as early as E10.5 during embryogenesis at the dorsal edge of the mesenteric region and become lipid-filled adipocytes at E17.5. We also show that these adipose progenitors marked by Pref-1 are not endothelial or pericyte in origin, but are mesenchymal. Furthermore, Pref-1 marked cells are proliferative and are required for embryonic adipose WAT development and expansion later in adults upon high fat feeding.

EXPERIMENTAL PROCEDURES

Animals

The Pref-1-rtTA mouse was generated via insertion of the reverse tetracycline transactivator protein (rtTA) directly downstream of −6.0 kb of the Pref-1 promoter sequence (see Supplementary Methods for full description). All other mice were purchased from the Jackson Laboratory. All animal studies were performed under the guidance of UC Berkeley ACUC and OLAC regulations.

Cell Culture

For adipocyte differentiation, 2 day post-confluent cells were treated with differentiation media containing 1 μM DEX, 0.5 mM MIX and 1.67 μM insulin in DMEM with 10% FBS and maintained for 2 days, after which cells were cultured in DMEM with 10% FBS for further experiments. For Dox treatment, 1 μg/mL Dox was added to the media.

RT-qPCR

Total RNA prepared with Trizol (Invitrogen) was reverse transcribed using ImpromII reverse transcriptase (Promega). The cDNAs were mixed with Maxima SYBR Green mastermix (Fisher), specific primers for the genes indicated, and analyzed using ABI7900 (Applied Biosystems), normalized 18S RNA by the ΔΔCT method. The mean CT was converted to relative expression value by the equation, 2-ΔΔCt, and the range was calculated by the equation, 2^-(ΔΔCT +stdevΔΔCT).

Immunostaining

Whole embryos or tissues were flash frozen in O.C.T. (Sakura) and sectioned on a Leica CM3050S Cryostat in 10 or 12 μm thick sections, and collected on Superfrost + coated glass slides (Fisher). Frozen sections were stained following standard protocol.

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Microscopy

Laser Scanning Confocal Microscopy was performed using a Zeiss LSM710 running Zen 2010 software equipped with 40X, 63X and 100X oil immersion objectives using standard excitation wavelengths for DAPI, GFP (or Alexafluor 488), tdTomato (or Alexafluor 594). Raw data was processed using NIH imageJ software (http://imagej.nih.gov/ij/, 1997-2012).

Glucose Tolerance Test

Mice were fasted overnight and tail blood was taken in the morning prior to glucose injection. Mice were injected with 2 mg/kg BW glucose in saline. Glucose levels were measured by an Accu-chek glucometer (Roche) at the indicated timepoints.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Berry R, Rodeheffer MS. Characterization of the adipocyte cellular lineage in vivo. Nat. Cell Biol. 2013; 15:302–308. [PubMed: 23434825]

Billon N, Dani C. The generation of adipocytes by the neural crest. Stem Cell Rev. 2012; 8:55–66. [PubMed: 21365256]

Dimov IK, Rong L, Lee EP, Seita J, Sahoo D, Park SM, Wiessman IL, Lee LP. Discriminating cellular heterogeneity using microwell-based RNA cytometry. Nature Comm. 2014; 5:3451.

Cawthorn WP, Bree AJ, Yao Y, Du B, Hemati N, Martinez-Santibanez G, MacDougald OA. Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a beta-catenin-dependent mechanism. Bone. 2012; 15(2):477–489. [PubMed: 21872687]

Farmer SR. Transcriptional control of adipocyte formation. Cell Metab. 2006; 4:263–273. [PubMed: 17011499]

Gimble JM, Bunnell BA, Floyd ZE. Prospecting for adipose progenitor cell biomarkers: biopanning for gold with in vivo phage display. Cell Stem Cell. 2011; 9:1–2. [PubMed: 21683669]

Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol. Rev. 1998; 78:783–809. [PubMed: 9674695]

Gupta RK, Mepani RJ, Kleiner S, Lo JC, Khandekar MJ, Cohen P, Frontini A, Bhowmick DC, Ye L, Cinti S, et al. Zfp423 Expression Identifies Committed Preadipocytes and Localizes to Adipose Endothelial and Perivascular Cells. Cell Metab. 2012; 15:230–239. [PubMed: 22326224]

Hudak CS, Sul HS. Pref-1: A gatekeeper of adipogenesis. Front Endocrinol. 2013; 4

Kanda T, Sullivan KF, Wahl GM. Histone–GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. Curr Biol. 1998; 8:377–385. [PubMed: 9545195]

Lee K, Villena JA, Moon YS, Kim K-H, Lee S, Kang C, Sul HS. Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). J. Clin. Invest. 2003; 111:453–461. [PubMed: 12588883]

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MacDougald OA, Burant CF. Fickle factor foils fat fate. Nat Cell Biol. 2005; 7:543–545. [PubMed: 15928698]

Mohsen-Kanson T, Hafner A-L, Wdziekonski B, Villageois P, Chignon-Sicard B, Dani C. Expression of cell surface markers during self-renewal and differentiation of human adipose-derived stem cells. Biochem. Biophys. Res. Commun. 2013; 430:871–875. [PubMed: 23268339]

Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, et al. Life without white fat: a transgenic mouse. Genes Dev. 1998; 12:3168–3181. [PubMed: 9784492]

Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest. 2007; 117:2553–2561. [PubMed: 17786244]

Olson LE, Soriano P. PDGFRβ signaling regulates mural cell plasticity and inhibits fat development. Dev Cell. 2011; 20:815–826. [PubMed: 21664579]

Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA. Early restriction of peripheral and proximal cell lineages during formation of the lung. Proc Natl Acad Sci. 2002; 99:10482–10487. [PubMed: 12145322]

Rodeheffer MS, Birsoy K, Friedman JM. Identification of White Adipocyte Progenitor Cells In Vivo. Cell. 2008; 135:240–249. [PubMed: 18835024]

Smas CM, Sul HS. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell. 1993; 73:725–734. [PubMed: 8500166]

Smas CM, Chen L, Sul HS. Cleavage of membrane-associated Pref-1 generates a soluble inhibitor of adipocyte differentiation. Mol Cell Biol. 1997; 17:977–988. [PubMed: 9001251]

Tang QQ, Lane MD. Adipogenesis: From stem cell to adipocyte. Ann Rev Biochem. 2012; 81:715–736. [PubMed: 22463691]

Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, Tallquist MD, Graff JM. White Fat Progenitor Cells Reside in the Adipose Vasculature. Science. 2008; 322:583–586. [PubMed: 18801968]

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. Defining the epithelial stem cell niche in skin. Science. 2004; 303:359–363. [PubMed: 14671312]

Wang Y, Sul HS. Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. Cell Metab. 2009; 9:287–302. [PubMed: 19254573]

Wang Y, Zhao L, Smas C, Sul HS. Pref-1 interacts with fibronectin to inhibit adipocyte differentiation. Mol Cell Biol. 2010; 30:3480–3492. [PubMed: 20457810]

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Highlights

- Pref-1 expressing cells are adipose precursors
- Adipose precursors are mesenchymal in origin
- Subcutaneous adipose tissue develops embryonically, visceral develops postnatally
- Ablation of Pref-1 expressing precursors prevents normal adipose tissue development

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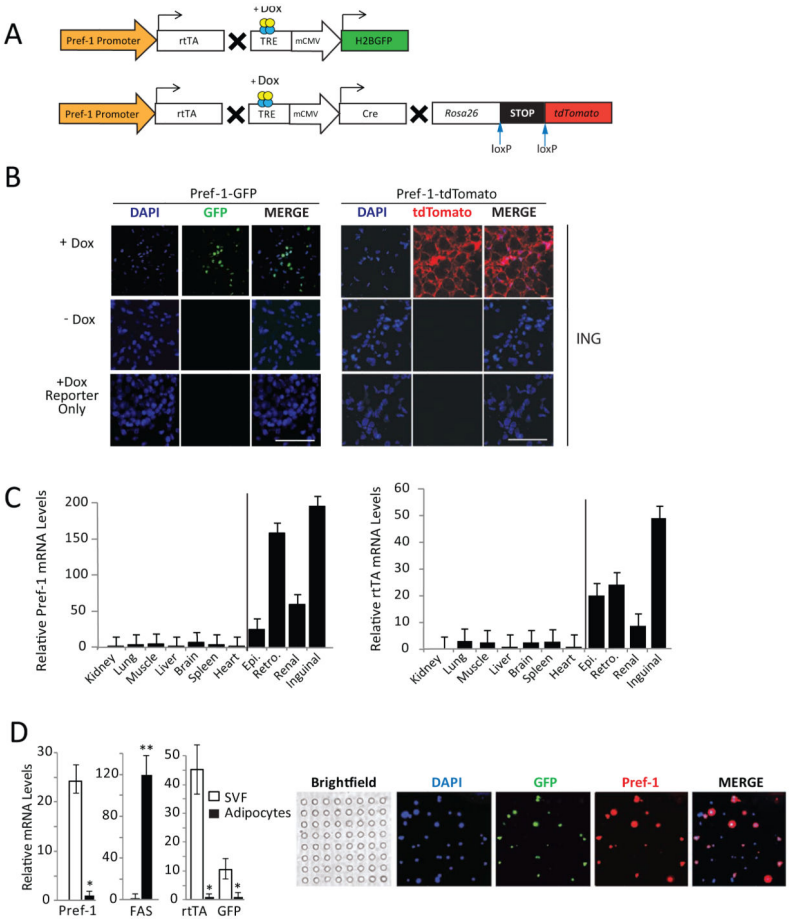

Figure 1. Labeling of Pref-1 Expressing Cells Is Specific to Adipose Tissue

(A) Pref-1-GFP and Pref-1-tdTomato mice were generated by inserting rtTA (Dox-On) directly following −6 kb of the Pref-1 promoter, whereby subsequent crossing allows fluorescent labeling (see supplemental) **(B)** Cryosections of inguinal WAT from Pref-1-GFP mice or Pref-1-tdTomato mice were treated with or without Dox (P0-21). Scale bars = 200 μm. **(C)** RT-qPCR of total mRNA from various tissues of Pref-1-rtTA mice. **(D)** Total mRNA from the SVF and adipocyte fractions of age-matched male Pref-1-GFP mice. Analysis of Pref-1 mRNA at the single cell level. Values are ± SEM of 5 mice per group. Paired t test, *p< 0.05, **p< 0.01.

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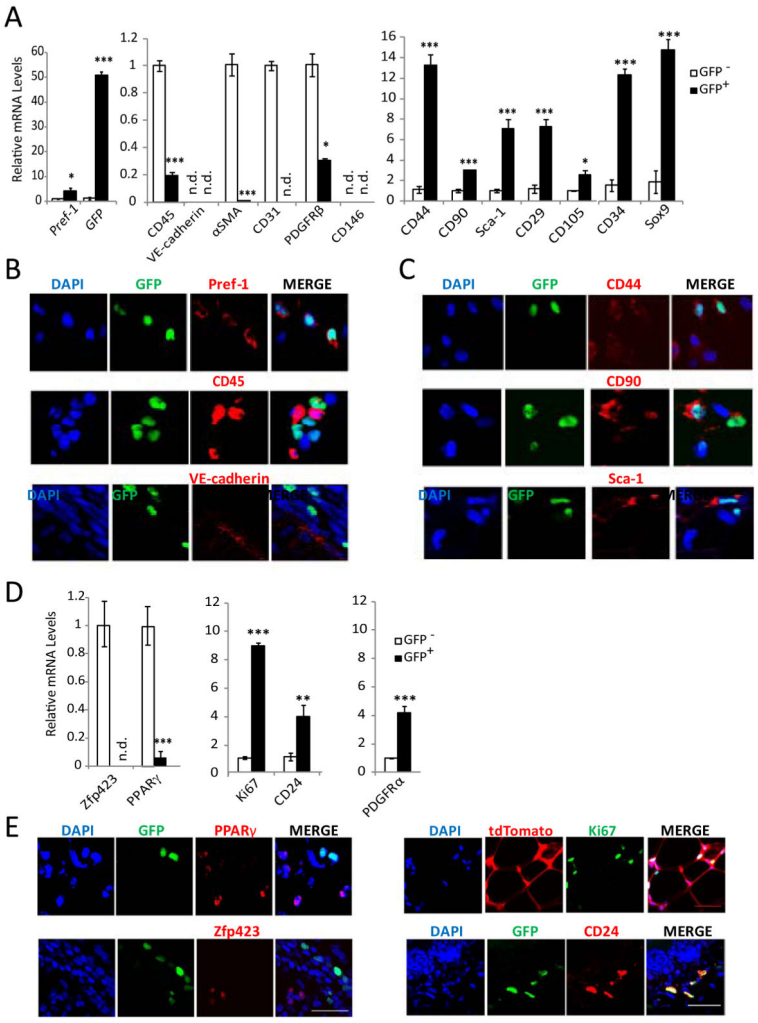

Figure 2. Characterization of Pref-1 Marked Cells Reveals a Mesenchymal Origin of Adipose Precursors

(A and D) GFP positive and negative cells were sorted via FACS from freshly isolated SVF from WAT of Dox treated Pref-1-GFP mice, total mRNA was extracted and expression levels of various endothelial, pericyte, hematopoietic and mesenchymal markers were measured. Age-matched two week old Pref-1 GFP male mice were used. Paired t test; *p< 0.05, **p< 0.01, ***p< 0.005, N=5. **(B,C and E)** Confocal images of Dox treated (from E0) Pref-1 GFP mouse embryos (E19.5). Scale bar = 150 μm.

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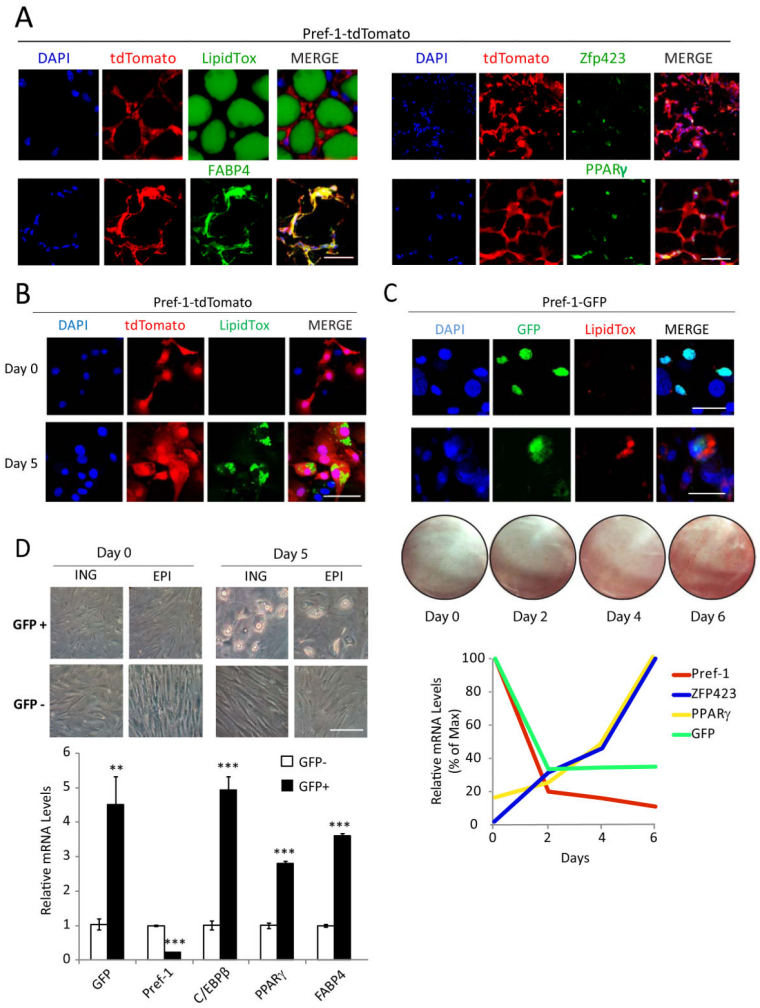

Figure 3. Pref-1 Labeled Cells Differentiate into Adipocytes

(A) Confocal images of cryosectioned WAT from Dox treated Pref-1-tdTomato mice. **(B)** Cultured SVF from Dox treated Pref-1-tdTomato mice treated with adipogenic cocktail **(C)** Cultured SVF from Dox treated Pref-1-GFP mice treated with adipogenic cocktail; Oil Red O staining; and RT-qPCR of adipogenic genes from total mRNA of cultured SVF throughout differentiation. **(D)** Brightfield microscopy of cultured sorted GFP positive and negative cells in adipogenic media. RT-qPCR of adipogenic genes from total mRNA of cultured sorted GFP positive and negative cells. Values are ± SD of biological duplicate and technical triplicates. t test, *p< 0.05, **p< 0.01, ***p< 0.005. Scale bars: (A, B) 100 μm, (C) 150 μm, (D) 250 μm.

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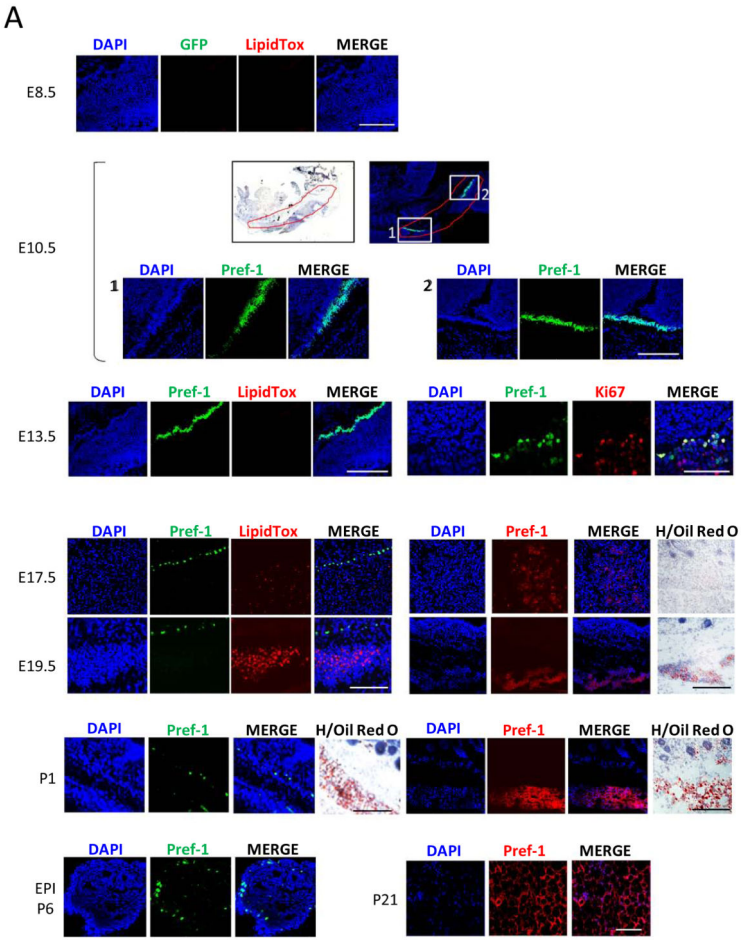

Figure 4. Pref-1 Adipose Precursors are Required for Embryonic Adipose Development and Expansion in Adults

(A) Female Pref-1-GFP mice were treated with Dox at mating and whole embryos were cryosectioned throughout embryogenesis, GFP, DAPI and lipidTox staining were examined. Whole embryo cryosection at E8.5 (top); at E10.5 (second); at E13.5 (third); at E17.5, some lipid droplets were stained by lipidTox, permanently labeled Pref-1 cells overlapped with lipid identified by Oil Red O staining (fourth); at E19.5, permanently labeled Pref-1 cells are developing into lipid containing cells as shown by Oil red O staining (fifth); at birth, GFP positive cells are located near the edge of the developing adipose tissue as shown by overlay with hematoxylin and Oil Red O staining, permanently labeled Pref-1 cells are developing into adipocytes shown by Oil red O staining in the same region (sixth). Direct fluorescence of GFP in epididymal WAT from P6 mice, GFP positive cells are located around the outer edge of the developing tissue (bottom). TdTomato labeled adipocytes are detected in P21 epididymal WAT. Scale bars= 250 μm, images are representative ≥ 5 mice.

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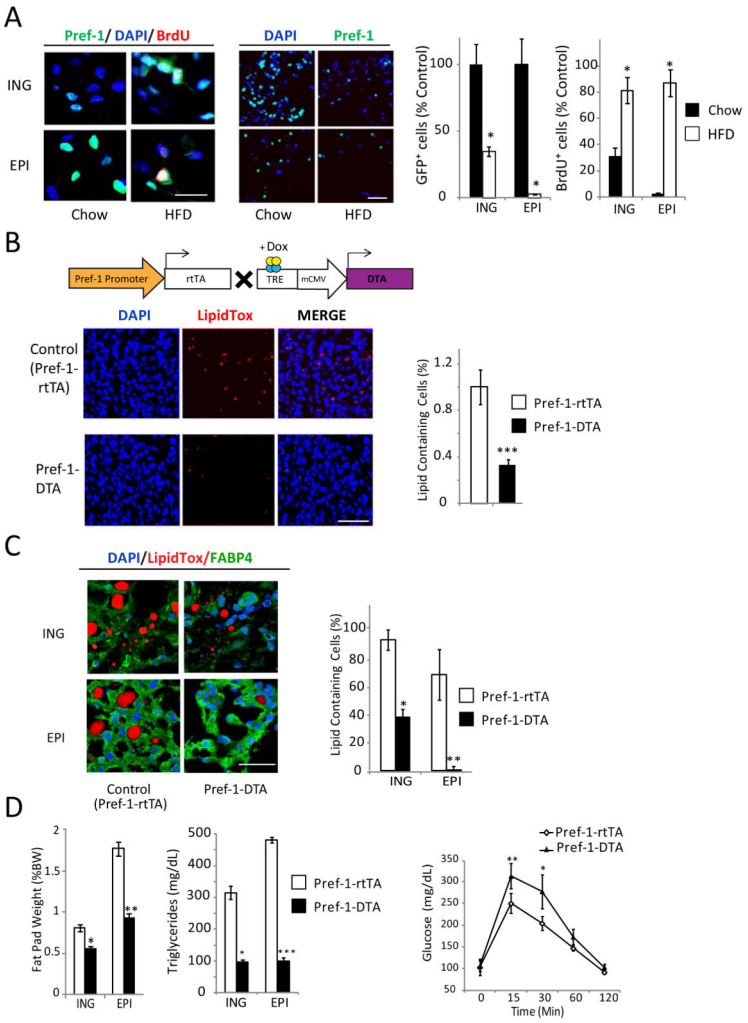

Figure 5. Deletion of Pref-1 Adipose Precursors Prevents Adipose Tissue Development and Expansion

(A) Confocal microscopy of cryosectioned inguinal and epididymal adipose tissue from Dox-treated Pref-1-GFP mice after 3 weeks of high fat feeding; Quantification of BrdU positive and GFP positive cells. Values are ±SEM of ≥ 5 mice. **(B)** Pref-1-DTA mice were generated by inserting rtTA directly following −6 kb of the Pref-1 promoter; Confocal microscopy of whole embryos at E17.5 following depletion of Pref-1 cells; Quantification of lipid containing cells in control and Pref-1 depleted inguinal and epididymal WAT. **(C)** Cryosections of inguinal and epididymal WAT from Dox treated Pref-1-rtTA control and Pref-1-DTA mice; Quantification of lipid containing cells in control and Pref-1 depleted inguinal and epididymal WAT. **(D)** Fat pad mass; Triglyceride content of inguinal and epididymal WAT from Pref-1-rtTA and Pref-1-DTA mice; Glucose tolerance test of Dox treated Pref-1-rtTA control and Pref-1-DTA mice. Values are ±SEM of 5 mice per group. t test, p=0.01,*p<0.05. Scale bars: (A, B, C) 200 μm.