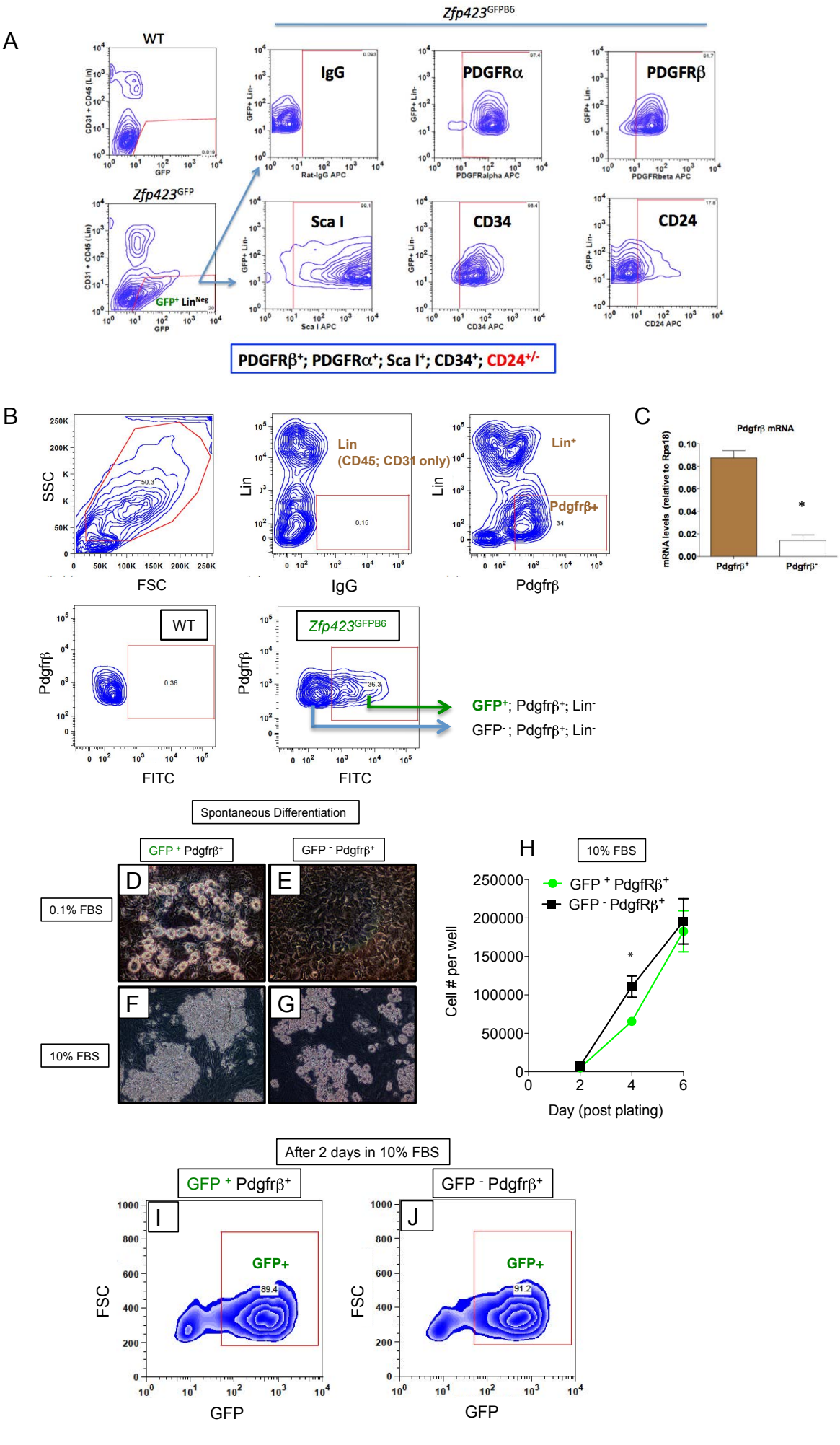

A
Zfp423GFPB6
WT
IgG
PDGFRα
PDGFRβ
Zfp423GFP
Sca I
CD34
CD24
GFP+ LinNeg
PDGFRβ+; PDGFRα+; Sca I+; CD34+; CD24+/-
B
SSC
FSC
Lin
IgG
Lin+
Pdgfrβ+
Pdgfrβ
WT
Zfp423GFPB6
FITC
GFP+; Pdgfrβ+; Lin-
GFP- ; Pdgfrβ+; Lin-
C
Pdgfrβ mRNA
mRNA levels (relative to Rps18)
Pdgfrβ+
Pdgfrβ-
Spontaneous Differentiation
GFP + Pdgfrβ+
GFP - Pdgfrβ+
0.1% FBS
10% FBS
D
E
F
G
H
10% FBS
GFP + PdgfRβ+
GFP - PdgfRβ+
Cell # per well
Day (post plating)
After 2 days in 10% FBS
I
J
GFP+
89.4
91.2
FSC
GFP

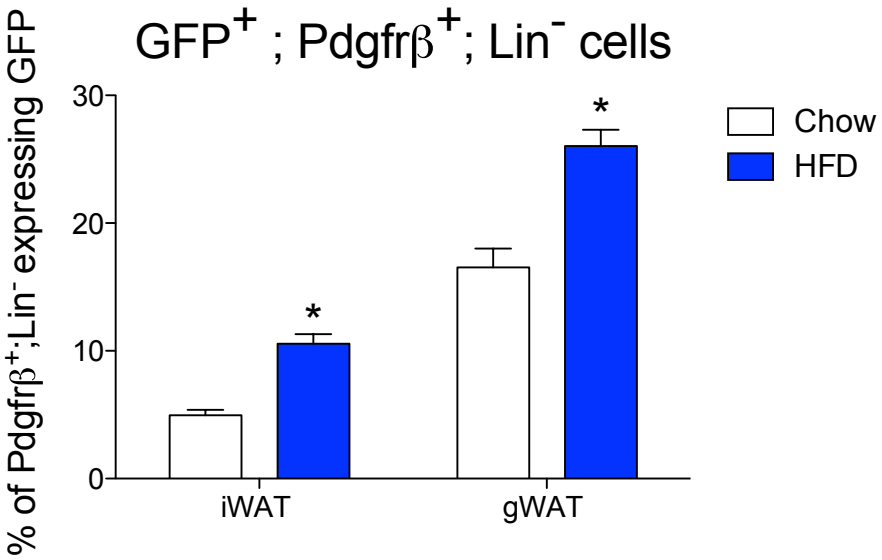
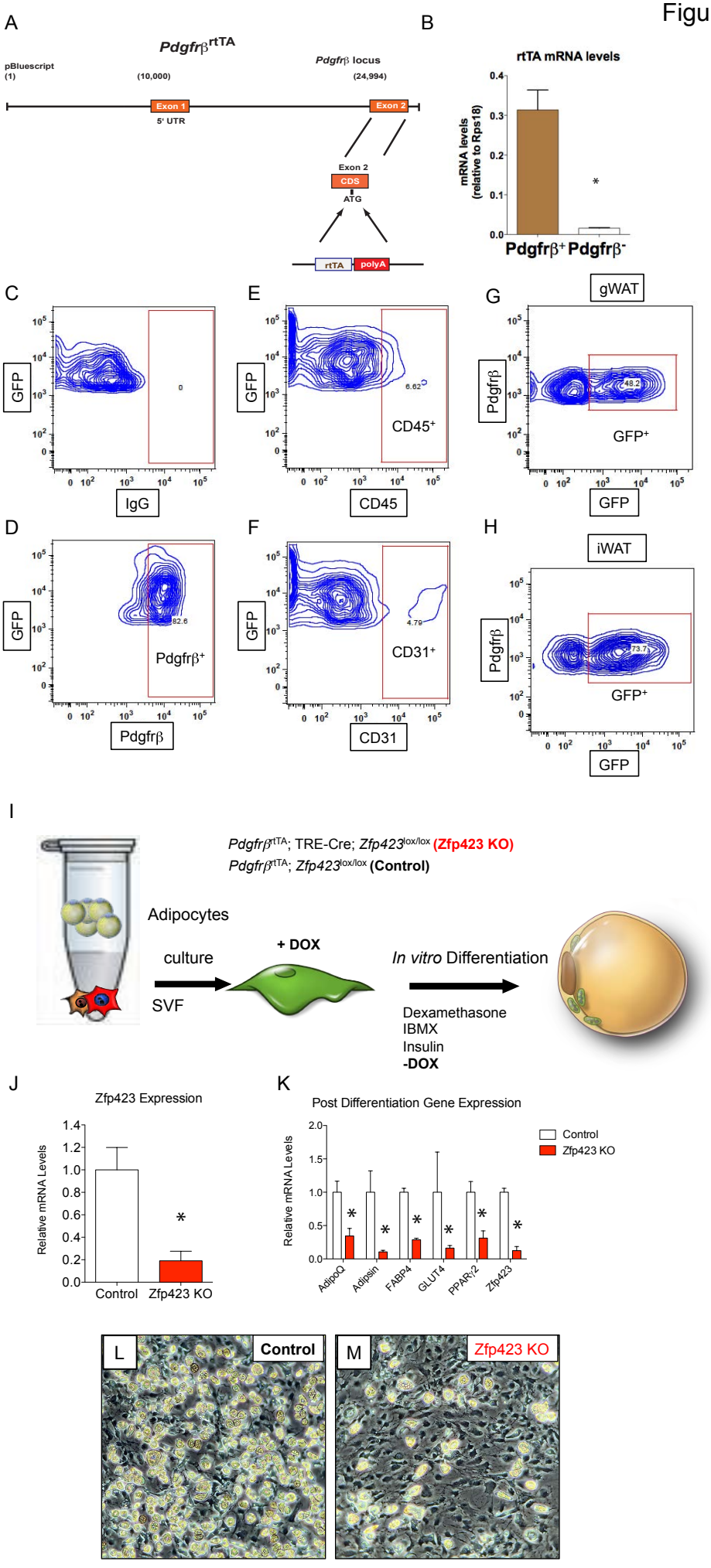
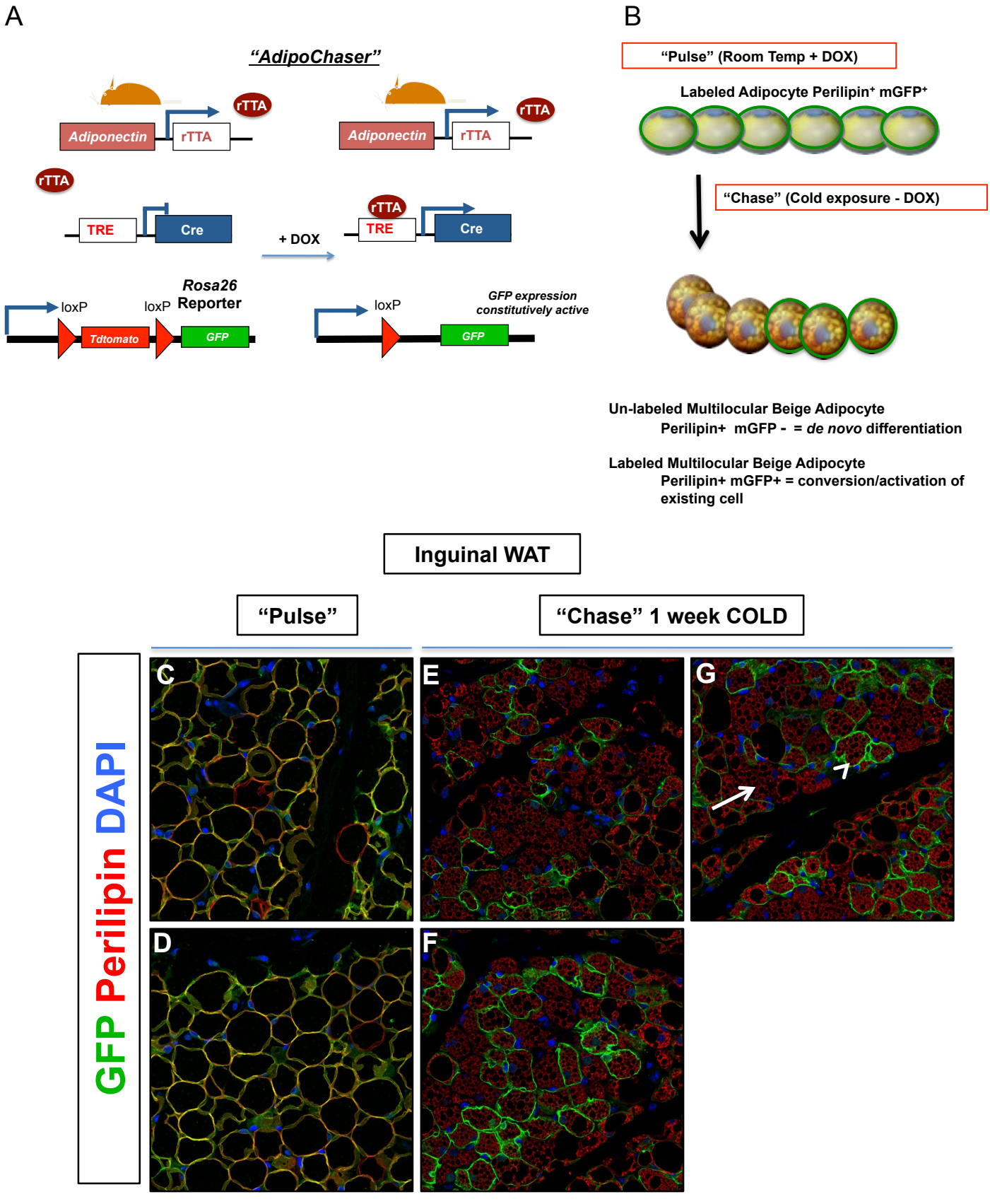

GFP+ ; Pdgfrβ+; Lin- cells
% of Pdgfrβ+;Lin- expressing GFP
30
20
10
0
*
*
iWAT
gWAT
Chow
HFD

Figure S3: Supplement to Figure 3




A
"AdipoChaser"
Adiponectin
rTTA
rTTA
Adiponectin
rTTA
rTTA
rTTA
TRE
Cre
+ DOX
rTTA
TRE
Cre
Rosa26
Reporter
loxP
loxP
Tdtomato
GFP
loxP
GFP
GFP expression
constitutively active
B
"Pulse" (Room Temp + DOX)
Labeled Adipocyte Perilipin+ mGFP+
"Chase" (Cold exposure - DOX)
Un-labeled Multilocular Beige Adipocyte
Perilipin+ mGFP - = de novo differentiation
Labeled Multilocular Beige Adipocyte
Perilipin+ mGFP+ = conversion/activation of existing cell
Inguinal WAT
"Pulse"
"Chase" 1 week COLD
GFP Perilipin DAPI
C
D
E
F
G

Supplemental Figure Legends

Figure S1: Supplement to Figure 1
Cell surface phenotype of Zfp423/GFP$^+$; Lin$^-$ adipose stromal cells; representative FACS collection gates; effect of fetal bovine serum (FBS) on adipogenic potential of GFP$^+$ and GFP$^-$ mural cells.
(A) Flow cytometry analysis reveals that Zfp423/GFP$^+$;Lin$^-$ SVF cells from gonadal WAT of *Zfp423*^{GFPB6} mice express accepted adipose stem cell markers such as Sca1, CD34, and Pdgfrα. Most of these cells are CD24$^-$, suggesting that they are among the "preadipocyte" population recently described by Berry and Rodeheffer. Zfp423/GFP$^+$;Lin$^-$ SVF cells also express Pdgfrβ, consistent with their mural phenotype. **(B)** Representative FACS collection gates for the isolation of Pdgfrβ^+; Lin$^-$ cells and/or GFP$^+$ and GFP$^-$ Pdgfrβ^+; Lin$^-$ cells from SVF cells of gonadal WAT from *Zfp423*^{GFPB6} mice. **(C)** mRNA levels of *Pdgfrβ* in freshly sorted Pdgfrβ^+ and Pdgfrβ^- adipose SVF cells, confirming the selectivity of the antibody. Bars represent mean + sem. * denotes $p< 0.05$ in student's t-test **(D-G)** Freshly isolated GFP$^+$ and GFP$^-$ mural cells were cultured in growth medium containing 1% ITS supplement and either 0.1% or 10% FBS. Spontaneous adipocyte differentiation appears in small clusters of GFP$^+$ (D) but not GFP$^-$ (E) mural cell cultures when cells are grown and maintained in 0.1% FBS. With 10% FBS, both populations differentiate to a similar degree (F-G). With 10% FBS, GFP$^-$ proliferate at a slightly greater rate **(H)** (* denotes $p<0.05$), suggesting that the high degree of adipogenesis in cultures initially GFP$^-$ is unlikely due to a growth advantage and selection of GFP$^+$ cells. Flow cytometry indicates that GFP expression is induced in GFP$^-$ cultures within 2 days of initial plating in cultures dishes **(I,J)**. These data suggest that Zfp423/GFP in adipose mural cells is unstable in medium containing 10% FBS.

Figure S2: Supplement to Figure 2.
Frequency of *Zfp423*-expressing adipose mural cells after eight weeks of chow or high-fat diet feeding.
Percentage of Pdgfrβ^+;Lin$^-$ cells expressing GFP (GFP$^+$; Pdgfrβ^+; Lin$^-$) in WAT depots of *Zfp423*^{GFPB6} male mice fed chow or high fat diet (HFD) for eight weeks following weaning. * denotes $p <0.05$ from student's t-test. Bars represent mean $\pm$ SEM. *n*= 5-6 animals for each group.

Figure S3: Supplement to Figure 3.
Generation and characterization of *Pdgfrβ*^{rtTA} transgenic mice; inactivation of Zfp423 in cultured Pdgfrβ+ SVF cells.
(A) The *Pdgfrβ*^{rtTA} transgene is a ~25 kilobase genomic region of the mouse *Pdgfrβ* locus containing 10 kilobases of sequence upstream of Exon 1 (encoding UTR) and a total of ~25 kilobases of sequence upstream of the initiation ATG in Exon 2. rtTA along with a polyA tail was inserted by BAC recombineering into Exon 2, replacing the

initiating ATG sequence. **(B)** rtTA expression in the adipose tissue SVF is largely confined to Pdgfrβ+ cells isolated by FACS with Pdgfrβ antibodies. Bars represent mean + sem. * denotes p< 0.05 in student's t-test. mRNA levels normalized to levels of Rps18 housekeeping gene. **(C-F)** Cell surface profile of mGFP+ SVF cells from inguinal WAT of doxycycline-treated *Pdgfrβ*^{rtTA}; Tre-Cre; Rosa$^{mT/mG}$ male mice. mGFP+ cells express Pdgfrβ (D) but are largely negative for CD45 (hematopoietic marker) (E) and CD31 (endothelial marker) (F). Similar results were observed in the gonadal WAT depot of the same mice. **(G-H)** Percentage of resident Pdgfrb+ mural cells expressing GFP in Pdgfrβ^{rtTA}; Tre-Cre; Rosa$^{mT/mG}$ male mice following pulse-labeling (DOX for 9 days). Labeling efficiency is ~50% in the gonadal WAT (gWAT) (G) and ~70% in the inguinal WAT (iWAT) (H). All plots shown are representative of 3-4 independent experiments. **(I)** SVF was isolated from inguinal WAT depots of *Pdgfrβ*^{rtTA}; Tre-Cre; *Zfp423*$^{loxP/loxP}$ male mice (Zfp423 KO) and control animals (*Pdgfrβ*^{rtTA}; *Zfp423*$^{loxP/loxP}$) and expanded in growth medium containing high-glucose DMEM with 10% FBS. Proliferating SV cultures were then treated with DOX for five days in order to induce Cre-mediated recombination of the *Zfp423* locus in Pdgfrβ+ SV cells. Confluent cultures were then induced to differentiate into adipocytes in medium containing dexamethasone, IBMX, and insulin (without DOX) as previously described (Gupta et al., 2012). **(J)** *Zfp423* mRNA in SV cultures 5 days after DOX treatment i*n vitro*. **(K)** qPCR analysis of adipocyte selective gene expression at 5 days post induction of differentiation. Bars represent mean + sem. * denotes p< 0.05 in student's t-test *n*=3 **(L-M)** Phase-contrast images of differentiated control and Zfp423 KO SV cultures.

Figure S4: Supplement to Figure 4

***De novo* differentiation of beige adipocytes in iWAT occurs upon cold exposure**

(A) The "AdipoChaser" lineage tracing system is generated by breeding the Adiponectin^{rtTA} transgenic mice to mice expressing Cre recombinase under the control of the tet-response element (TRE-Cre) and carrying the Rosa26-mT/mG reporter allele. In the presence of doxycycline (DOX) rtTA activates Cre expression. Cre excises the loxP-flanked tdtomato cassette allowing activation of constitutive mGFP expression. **(B)** Cartoon depicting "Pulse-Chase" lineage tracing approach. DOX-containing food is administered to AdipoChaser mice for 14 days at room temperature to label mature adipocytes ("Pulse"). Animals are then switched to cold chambers (6 C) and fed a standard chow diet devoid of DOX ("Chase"). **(C,D)** GFP and perilipin staining of inguinal WAT (iWAT) after 14 days of DOX treatment at room temperature **(E,F,G)** GFP and perilipin staining of iWAT following 1 week cold exposure (without DOX). GFP-perilipin+ adipocytes represent de novo differentiated beige cells formed during the 1 week cold exposure period (arrow). GFP+ perilipin+ cells represent beige cells formed from pre-existing cells present during the pulse labeling (arrowhead).

Supplemental Tables

Table S1: Related to Figure 1
Genes With Elevated Expression in Zfp423/GFP^{+} Mural Cells

Table S2: Related to Figure 1
Genes With Elevated Expression in Zfp423/GFP^{-} Mural Cells

Table S3: Related to Figure 1
Top 20 Gene sets Enriched in Zfp423/GFP^{+} Mural Cells

Table S4: Related to Figure 1
Top 20 Gene Sets Enriched in Zfp423/GFP^{-} Mural Cells

Supplemental Experimental Procedures

Derivation of Transgenic animals. The previously described *Zfp423*^{GFP} BAC DNA (Gupta et al., 2012) was microinjected into fertilized C57BL/6 embryos by the UT Southwestern Transgenic Core Facility using standard pronuclear injection techniques. Founder *Zfp423*^{GFPB6} reporter mice were genotyped as previously described (Gupta et al., 2012). The transgenic construct (~25 kilobases) expressing rtTA under the control of the *Pdgfrβ* promoter was generated by BAC recombineering (detailed below). Transgenic animals were also generated by the UT Southwestern Transgenic Core Facility. In total, four founders were identified by PCR and one founder was chosen for further study based on faithful expression pattern (see Figure S3). Genotyping of founders and all subsequent animals was performed by PCR using the following primers:

rtTAF- 5'-ATTCCGCTGTGCTCTCCTCTC

rtTAR-5'-GTACAGTGCGTTCTCCAGGGA

Zfp423$^{loxP/loxP}$ animals were a kind gift of Dr. S. Warming (Genentech, Inc.) (Warming et al., 2006).

BAC Recombineering to Derive *Pdgfrβ*^{rtTA} transgene.

Generation of the targeting cassette for recombineering:

Pdgfrβ homology arms corresponding to the first 50 bp upstream and the first 50 bp immediately downstream of the initiation ATG in Exon 2 were added to the forward and reverse primers, respectively, that amplify the rtTA-FRT-NEO-FRT sequence in plasmid pL451-rtTA-FRT-Neo/Kan-FRT. The resulting PCR product was electroporated into SW105 cells carrying BAC RP23-304O21 that contains the *Pdgfrβ* locus. Homologous recombination of the resulting kanamycin/chloramphenicol resistant clones was confirmed by PCR amplification using the following primers: Pdgfrβ-rtTAF1: GATGTCTTCCCAGCCTTGAGA, Pdgfrβ-rtTAR1: AGCGAGTTTCCTTGTCGTCAG

Retrieval of 25 kb region of *Pdgfrβ* locus:

Since BAC RP23-304O21 contains several distinct loci, we retrieved a 25,621 bp fragment depicted in Figure S3 by BAC retrieval methods (Warming et al., 2005). Homology arms located 10 kb upstream of Exon 1 (5' UTR) (chr18:61194804+61195324 (mm9)) and 150 bp downstream of Exon 2 (containing ATG) chr18:61219975-61220424) were derived by PCR and cloned into pBluescript. The 5' homology arm (*Pdgfrβ* arm1) was a 521-bp fragment created using forward and reverse primers: 5'-AGAGTCCTGGGGAGGCTAGGT-3', 5'-GACCCTTCTCTCCCGTGGGAG-3'. A BamHI site was added to the 5' end of the forward primer, and an SpeI site was added to the 5' end of the reverse primer. The 3' homology arm (*Pdgfrβ* arm 2) was a 450-bp product derived using the forward primer 5'-TTGGGAGGTGGTCTGTGGAGG -3', with an SpeI site added to the 5' end, and the reverse primer 5'- CCCCTGGGGGCTTGAAGGACA -3' with an Xba I site added to the 5' end.

pBluescript was first digested with BamHI and SpeI and ligated to *Pdgfrβ* arm1. The subsequent plasmid was then digested with SpeI and XbaI and ligated to *Pdgfrβ* arm2. The resulting pBluescript plasmid (pBS-Pdgfrβ homology) containing both homology arms was then linearized by SpeI digestion. SW105 cells carrying the

targeted *Pdgfrβ*^{rtTA} containing BAC were electroporated with linearized pBS-Pdgfrβ homology, and homologous recombined positive clones were selected with kanamycin and ampicillin. The Neo cassette was removed by L-arabinose induction of flp recombinase, resulting in bacteria carrying the final *Pdgfrβ*^{rtTA} plasmid. Homologous recombination was confirmed by sequencing and by PCR using the primers above.

Microarray and Gene Set Enrichment Analysis. For all gene expression profiling, total RNA was extracted from the Zfp423/GFP^{+} (n=3) and Zfp423/GFP^{-} (n=3) perivascular cell populations using the RNAqueous Micro Kit (Life Technologies AM1931) following manufacturer's guidelines. Each of the three microarray samples represent Zfp423/GFP^{+} or Zfp423/GFP^{-} mural cells isolated from pooled gonadal WAT of 6-7 males mice (age 5 weeks). Linear amplification, hybridization to Illumina MouseWG-6 V2 BeadChip arrays, and scanning were performed by the UT Southwestern Medical Center Genomics and Microarray Core Facility according to established methods https://microarray.swmed.edu/. The array data were normalized and analyzed using the open source software Bioconductor (Gentleman et al., 2004). Biological insights concerning the differentially expressed genes were explored via Gene Set Enrichment Analysis (GSEA). The analysis was performed with the GSEA software (Subramanian et al., 2005) (version 2.1.0) using the c2 (version 5) gene set database. Genes were first ranked based on real value using the weighted signal-to-noise metric. P-values and the false discovery rate (FDR) for the enrichment score of each gene set were calculated based on 1000 gene set permutations. Gene sets related to inflammation that were significantly enriched in the Zfp423/GFP^{-} mural cell samples were selected for leading edge analysis using the GSEA software to identify the common overlap between the significant gene sets. All microarray data has been deposited to Gene Expression Omnibus (accession number GSE70789).

FACS analysis. FACS experiments were carried out on a FACSAria^{TM} flow cytometer (UT Southwestern Medical Center Flow Cytometry Core Facility). Antibodies used for flow cytometry analysis can be found in Supplementary Experimental Procedures.

Flow cytometry. Samples of 10^6 SVF cells from either inguinal or epididymal WAT depots (isolated as described in Isolation of adipose SVF cells section) were first incubated on ice for 20 minutes in 200 uL of 2% FBS/PBS containing anti-mouse CD16/CD32 Fc Block (clone 2.4G2) (1:200). Cells were then incubated with primary antibody (anti-CD31 clone 390 1:200, anti-CD45 clone 30-F11 1:200, anti-CD140b clone APB5 3:200, CD140a clone APA5 1:100, anti-Ly-6A/E (ScaI) clone D7 1:200, anti-CD24 clone M1/69 1:200, anti-CD34 clone HM34, anti-CD11b clone M1/70 1:200) and were incubated rotating at 4°C for 30 minutes. They were then washed three times with 2% FBS/PBS, and either analyzed using a FACSCantoII^{TM} flow cytometer or sorted by a FACSAria^{TM} flow cytometer (UT Southwestern Medical Center Flow Cytometry Core Facility). For sorting, cells were initially selected by size, on the basis of forward scatter (FSC) and side scatter (SSC). Live cells were then gated on both SSC and FSC-Width singlets, ensuring that individual cells were analyzed. SVF cells isolated from wild-type mice, along with fluorescent-minus-one (FMO) controls, were used to

determine background fluorescence levels. The populations were gated as shown in Supplement to Figure 1. Cells were sorted into FBS and then either cultured (see Mural cell culture methods) or prepared for total RNA extraction. All flow cytometry antibodies were rat-derived and conjugated to APC or PE. All antibodies were obtained commercially from BioLegend (San Diego, CA USA).

Colony-forming unit assays. Colony-forming unit potential was determined by plating replicate samples of cells at a low density (450 cells/10cm plate) and maintaining in growth media containing 2% FBS for 2 weeks at 10% CO_2. Cells were fixed for 5 minutes in 10% Formalin, washed then stained for 30 minutes with 0.05% Crystal Violet in ddH_2O. After staining, the cells were washed with ddH_2O three times, and then quantified under light microscopy. Eight replicate plates were quantified for each population. Statistical significance were evaluated using a student's t-test.

Indirect Immunofluorescence. The following antibodies and concentrations were used: chicken anti-GFP 1:700 (Abcam ab13970); rat anti-CD31 1:10 (BD Pharmingen 550274); guinea pig anti-perilipin 1:1500 (Fitzgerald 20R-PP004); goat anti-chicken IgG 1:200 (Invitrogen); donkey anti-rabbit Alexa 647 1:200 (Invitrogen); goat anti-guinea pig Alexa 647 1:200 (Invitrogen); goat anti-rat IgG 1:200 (Invitrogen). Briefly, cryosections were thawed for 5 minutes in PBS containing 0.1% Triton X-100 (PBS-T), and then blocked/permeabilized for 30 minutes in PBS containing 10% normal goat serum and 0.5% Triton X-100. Primary antibodies were then diluted in PBS containing 10% normal goat serum and 0.1% Triton X-100 and added to cryosections overnight at 4°C. Following washing in PBS-T, slides were then incubated with secondary antibodies diluted in PBS containing 10% normal goat serum and 0.1% Triton X-100 for 1 hour at 37°C. Washed slides were then mounted with Prolong Anti-Fade mounting medium containing DAPI (Invitrogen).

Paraffin sections were dewaxed and hydrated in Xylene and 100%-95%-80%-70%-50% Ethanol and ddH_2O. Slides were placed in chambers containing 1% R-Buffer Buffer A pH 6.0 solution and antigen retrieval was done using Antigen Retriever 2100 (Electron Microscopy Sciences) for 2 hours. Following PBS wash for 5 minutes, Fx Signal Enhancer (Invitrogen) was added to the slides for 30 minutes at room temperature. Slides were then blocked for 30 minutes in PBS containing 10% normal goat serum at room temperature. Primary antibodies were then diluted in PBS containing 10% normal goat serum and added to paraffin sections overnight at 4°C. Following washes in PBS, slides were then incubated with secondary antibodies diluted in PBS containing 10% normal goat serum for 2 hours at room temperature. Washed slides were then mounted with Prolong Anti-Fade mounting medium containing DAPI (Invitrogen). All Images were obtained using Leica SP5 scanning confocal microscope (Live cell Imaging Facility, UT Southwestern Medical Center).

Lineage tracing analysis. For high-fat diet studies, 8 weeks-old male animals were fed doxycycline-containing chow (600 mg/kg pellets, BIO-SERV S4107) for 9 days (diet was replenished every 5 days due to light sensitivity of doxycycline) to pulse label Pdgfrβ+ mural cells. Feed was then switched to standard rodent chow diet or HFD (60 kcal% fat, Research Diets D12492i) and mice were maintained on these diets for 8 weeks. For

cold exposure studies, 5 week-old single housed male mice were fed doxycycline-containing chow for 9 days as above. Diet was then switched to standard rodent chow and mice were placed in the 6°C cold chambers for 1 or 2 weeks.

For quantification of adipocyte hyperplasia, paraffin sections of inguinal and gonadal WAT from chow and HFD fed MuralChaser mice were stained for Perilipin and GFP using standard indirect immunofluorescence protocols described above. 16-26 random 10x magnification fields were photographed from duplicate sections of stained WAT depots from four animals of each group. The frequency of GFP^+; $perilipin^+$ adipocytes from each image was manually quantified and analyzed using GraphPad Prism. Statistical significance was evaluated using a student's t-test.

Statistical Analysis. All bars represent mean $\pm$ sem, unless otherwise indicated in corresponding figure legends. Unless otherwise indicated, statistical significance was evaluated using a student's t-test, with significance defined as $p<0.05$. With the exception of microarray analyses, GraphPad Prism or Excel was used for plotting data and performing t-tests.

Supplemental References

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