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Highly Selective In Vivo Labeling of Subcutaneous White Adipocyte Precursors with Prx1-Cre

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SUMMARY

The origins of individual fat depots are not well understood, and thus, the availability of tools useful for studying depot-specific adipose tissue development and function is limited. Cre drivers that selectively target only brown adipocyte, subcutaneous white adipocyte, or visceral white adipocyte precursors would have significant value because they could be used to selectively study individual depots without impacting the adipocyte precursors or intrinsic metabolic properties of the other depots. Here, we show that the majority of the precursor and mature subcutaneous white adipocytes in adult C57Bl/6 mice are labeled by Prx1-Cre. In sharp contrast, few to no brown adipocytes or visceral white adipocytes are marked by Prx1-Cre. This suggests that Prx1-Cre-mediated recombination may be useful for making depot-restricted genetic manipulations in subcutaneous white adipocyte precursor cells, particularly when targeting genes with fat-specific functions.

INTRODUCTION

Obesity is a risk factor for type 2 diabetes, dyslipidemia, cardiovascular disease, and many other conditions including cancer; however, not all fat depots are metabolically equal (Peirce et al., 2014; Tchkonia et al., 2013). For example, visceral obesity generally places individuals at a greater risk for metabolic complications compared with subcutaneous obesity. Exactly why depot-specific metabolic differences exist is a topic of great interest and importance. Adipose tissue function can be studied genetically in mice by a conditional gene knockout strategy with the adiponectin-Cre driver, which globally targets mature white and brown adipocytes (Berry and Rodeheffer, 2013; Eguchi et al., 2011; Lee et al., 2013; Sanchez-Gurmaches and Guertin, 2014). However, it has been more difficult to genetically study depot-specific adipose tissue functions, particularly in white fat, because tools to selectively manipulate gene expression in subcutaneous versus visceral white adipocytes are lacking.

An important advance in studying adipose tissue growth was establishing techniques to isolate adipocyte progenitor cells (APCs) from mice. APCs reside in the stromal vascular fractions (SVFs) and are separated from non-adipose precursors (e.g., endothelial progenitors, T cells, B cells, macrophages, etc.) using cell surface markers ($CD31^-$; $CD45^-$ (Lin^-); $CD29^+$; $CD34^+$; $SCA1^+$; Joe et al., 2009; Rodeheffer et al., 2008; Schulz et al., 2011). APCs can be further separated into a $CD24^+$ and $CD24^-$ population, both of which are adipogenic in vitro, but only the $CD24^+$ population reconstitutes a functional fat pad when transplanted into a lipodystrophic mouse (Rodeheffer et al., 2008), and thus it has been suggested that the $CD24^+$ subpopulation contains the adipocyte progenitors. Elucidating how APCs are regulated has important implications for understanding and treating obesity. Moreover, APCs from subcutaneous fat are an abundant source of stem cells for regenerative medicine, and thus the ability to purify, culture, and transplant them could lead to novel cell-based therapies. It would therefore be of significant value to identify Cre drivers that could direct genetic recombination in depot-specific manners.

We previously reported that precursor cells expressing the Myf5-Cre knockin driver give rise to a subset of white adipocytes in addition to brown adipocytes and skeletal muscle (Hung et al., 2014; Sanchez-Gurmaches and Guertin, 2014; Sanchez-Gurmaches et al., 2012; Tallquist et al., 2000). In young C57Bl/6 mice, nearly all of the anterior subcutaneous and retroperitoneal visceral white adipocytes are labeled with Myf5-Cre whereas all posterior subcutaneous white adipocytes and all mesenteric and perigonadal visceral white adipocytes are Myf5-Cre negative (Sanchez-Gurmaches and Guertin, 2014). Consistently, Myf5-Cre also labels many, but not all, of the APCs in the anterior subcutaneous and retroperitoneal depots. A similar pattern is recapitulated with Pax3-Cre in most depots with some notable exceptions including additional labeling of many perigonadal white adipocytes in male, but not female, mice (Sanchez-Gurmaches and Guertin, 2014). These findings reveal an unexpected degree of heterogeneity with respect to the Myf5-Cre and Pax3-Cre labeling in white fat; however, the utility of using the Myf5-Cre and Pax3-Cre drivers for in vivo genetic manipulation of white adipocyte precursors is limited because they neither uniformly nor selectively target any adipocyte class but rather appear to label adipocytes based on their anatomical location (i.e., dorsal-anterior) and in addition target some non-adipose tissues.

To identify Cre drivers that might be useful for targeting different adipocytes populations, we scanned the literature for well-characterized Cre drivers that have propensity to label adipocyte precursors and tested their ability to activate a reporter in different fat depots. One candidate was paired related homeobox transcription factor 1 Cre (Prx1-Cre) because it reportedly expresses in the mesenchymal precursors of the limbs and head (Logan et al., 2002), was recently reported to express in precursors of both brown and white adipocytes (Calo et al., 2010), and because PRX1 itself reportedly functions in adipogenesis (Du et al., 2013). A very recent report by Krueger and colleagues also indicates that Prx1-Cre labels inguinal subcutaneous adipocyte precursors (Krueger et al., 2014). Here, we provide a detailed analysis of Prx1-Cre labeling in fat and other tissues and show that Prx1-Cre labeling is largely restricted among the fat depots to the precursor and mature subcutaneous white adipocytes. Furthermore, we show that Prx1-Cre labeling of subcutaneous WAT is stable and that Prx1-Cre also labels brite/beige adipocytes in subcutaneous WAT. Finally, the utility of using Prx1-Cre and other Cre drivers for lineage tracing, cell marking, and conditional gene deletion in adipose tissue is discussed.

RESULTS

Prx1-Cre Labeling of Adipocyte Precursors

Prx1-Cre mice were crossed with the *R26R-mTmG* reporter (Logan et al., 2002; Muzumdar et al., 2007), which is a dual color labeling system that utilizes a membrane-targeted eGFP reporter downstream of a floxed membrane-targeted tdTomato reporter and stop cassette. This reporter is particularly useful for labeling mature adipocytes because they are largely composed of lipid and have very little cytoplasm, making them less amenable to labeling with cytoplasmic reporters (Berry and Rodeheffer, 2013; Sanchez-Gurmaches and Guertin, 2014). Previous studies using adiponectin-Cre to drive *R26R-mTmG* reporter expression indicate the reporter has high efficiency and specificity (Berry and Rodeheffer, 2013; Jeffery et al., 2014; Sanchez-Gurmaches and Guertin, 2014). In *Prx1-Cre;R26R-mTmG* mice, all Cre^{+} cells and their descendants express mGFP whereas all Cre^{neg} cells and their descendants express tdTomato. Using these mice, we scanned for reporter expression in precursor and mature adipocyte pools in all major fat depots. The depots we examine are abbreviated as follows: for white adipose tissues (WATs), anterior subcutaneous (asWAT), posterior subcutaneous (psWAT), retroperitoneal (rWAT), perigonadal (pgWAT), and mesenteric (mWAT), and for brown adipose tissues (BATs), interscapular (iBAT), subscapular (sBAT), cervical (cBAT), perirenal (prBAT), and periaortic (paBAT).

Using 6-week-old *Prx1-Cre;R26R-mTmG* male and female mice, we first asked whether mGFP reporter activity is detectable in the APCs of any depots. We fractionated the SVFs from the mature adipocytes in several depots and, from the SVF fractions, isolated $CD31^{-}$;$CD45^{-}$(Lin^{-}); $CD29^{+}$;$CD34^{+}$;$SCA1^{+}$ cells (Figure 1A), which are highly enriched for APCs (Rodeheffer et al., 2008; Sanchez-Gurmaches et al., 2012). In males, 12.5% of the APCs in asWAT are $mGFP^{+}$, and similarly, the $CD24^{+}$ and $CD24^{-}$ populations in this depot are 10.5% and 10.9% $mGFP^{+}$, respectively, whereas no $mGFP^{+}$ signal was detectable in the Lin^{+} pool (Figure 1B). In psWAT, the number of $mGFP^{+}$ precursors is significantly greater, reaching nearly 75% in the APC, $CD24^{+}$, and $CD24^{-}$ pools again with no $mGFP^{+}$ signal detectable in the Lin^{+} pool (Figure 1C). Representative dot plots and distribution plots of the APC populations are shown in Figure S1. In contrast, few $mGFP^{+}$ cells are detectable in male visceral WAT depots (rWAT and pgWAT) or BAT depots (iBAT and sBAT; Figures 1D–1G). In female mice, the Prx1-Cre labeling pattern in the subcutaneous WAT APC pool is similar to that in males: in asWAT, the $mGFP^{+}$ population is slightly higher in the females at 21.4% and in psWAT nearly identical to the males at 75.8%, with the $CD24^{+}$ and $CD24^{-}$ populations showing similar percentages in each depot and all Lin^{+} cells being $tdTomato^{+}$ (Figures 1B and 1C). The labeling pattern in female mice is also largely similar to the males in visceral depots, except in pgWAT, in which a small percentage of APCs are $mGFP^{+}$ (Figure 1E). Thus, in both genders, Prx1-Cre activates reporter gene expression in most white adipocyte progenitors in psWAT.

Prx1-Cre Labeling of Mature Adipocytes

To quantify the extent to which Prx1-Cre-labeled APCs contribute to the mature adipocyte population, we examined each depot by confocal microscopy. In the asWAT, the labeling pattern is heterogeneous (Figures 2A and 2B), consistent with the mixed $mGFP^{+}$ and $tdTomato^{+}$ APC labeling in this depot (Figure 1B). Notably, the labeling pattern in asWAT varies with anatomical location; for example, the dorsal region of the depot is $tdTomato^{+}$ (Figure 2A), whereas the ventral region of the depot contains a variegated mixture of $mGFP^{+}$ and $tdTomato^{+}$ adipocytes (Figure 2B). On the other hand, 96% of the adipocytes in psWAT are $mGFP^{+}$ (Figure 2C), which is consistent with the high percentage of $mGFP^{+}$ APCs in this depot (Figure 1C). However, because psWAT is roughly 1.5–2.0 times the mass of asWAT, these results indicate that most of the subcutaneous white adipocytes are mGFP in *Prx1-Cre;R26R-mTmG* mice.

In contrast to the subcutaneous depots, 100% of the male visceral white adipocytes in mWAT, rWAT, and pgWAT are $tdTomato^{+}$, indicating Prx1-Cre did not express in their ancestry (Figures 2D–2F). Similarly, in females, 100% of

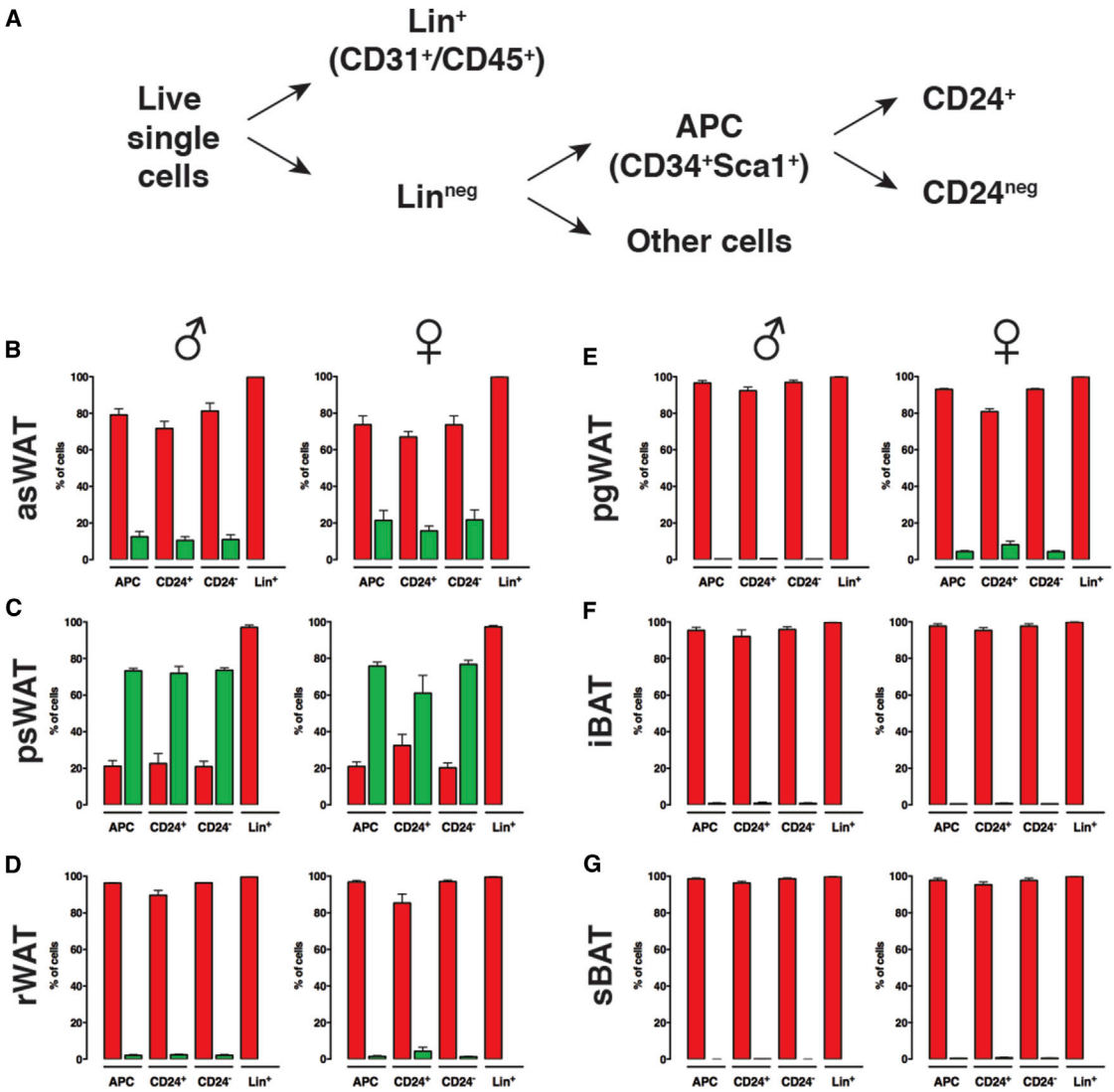

Figure 1. Prx1-Cre Labels Adipocyte Precursors in Subcutaneous White Fats

(A) SVF populations analyzed in the present study by flow.

(B–G) Number of tdTomato$^+$ and mGFP$^+$ APCs isolated from each of the indicated depots from 6-week-old *Prx1-cre;R26R-mTmG* males and females (n = 3 per each sex; mean + SEM).

See also Figure S1.

the mWAT and rWAT adipocytes are also tdTomato$^+$. However, in female pgWAT, a few mature adipocytes (4.7%) are mGFP$^+$, which reflects the small number of Prx1-Cre-labeled APCs that we also detect in this depot (Figure 1E). Nevertheless, these results indicate that, among the major white fat depots, Prx1-Cre recombinase activity drives reporter expression in adipocytes that are largely restricted to subcutaneous white fat.

Given that almost no brown APCs in iBAT and sBAT express mGFP in *Prx1-Cre;R26R-mTmG* mice, we expected few if any mature brown adipocytes would be mGFP$^+$. Indeed, no mGFP$^+$ brown adipocytes are detectable in iBAT, sBAT, cBAT, or prBAT in either male or female *Prx1-Cre;R26R-mTmG* (Figures 3A–3D). However, in paBAT, mGFP is detectable in 64.3% and 62.2% of the cells in males and females, respectively (Figure 3E). Thus, in most major BAT depots, the brown adipocytes are not labeled with Prx1-Cre; however, in one depot (paBAT), Prx1-Cre marks the majority of the brown adipocytes.

Utility of Prx1-Cre for Targeting Subcutaneous Fat

To gauge the potential utility of using Prx1-Cre to selectively target subcutaneous white adipocytes, we compared the mRNA expression of *mGFP* between adipose tissues and

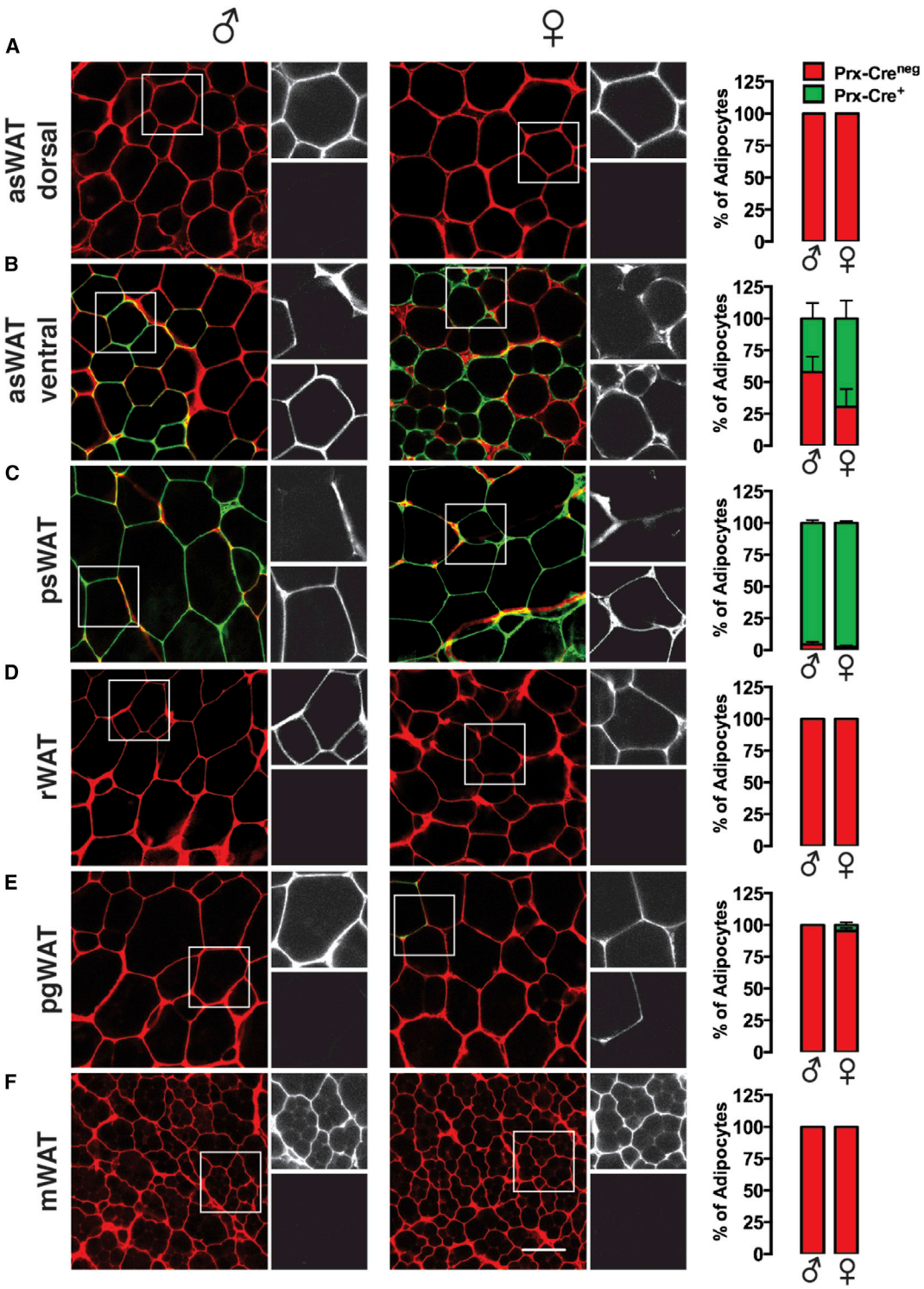

Figure 2. Prx1-Cre Labels Mature Subcutaneous White Adipocytes

(A–F) Representative images of the indicated WATs of 6-week-old *Prx1-Cre;R26R-mTmG* male and female mice (top insert, tdTomato; bottom insert, mGFP). The number of tdTomato$^+$ and mGFP$^+$ mature white adipocytes in each depot is indicated in a graph at the left side of each row (n = 3 per each sex; mean + SEM). For all panels, scale bar represents 50 μm.

See also Figure S2.

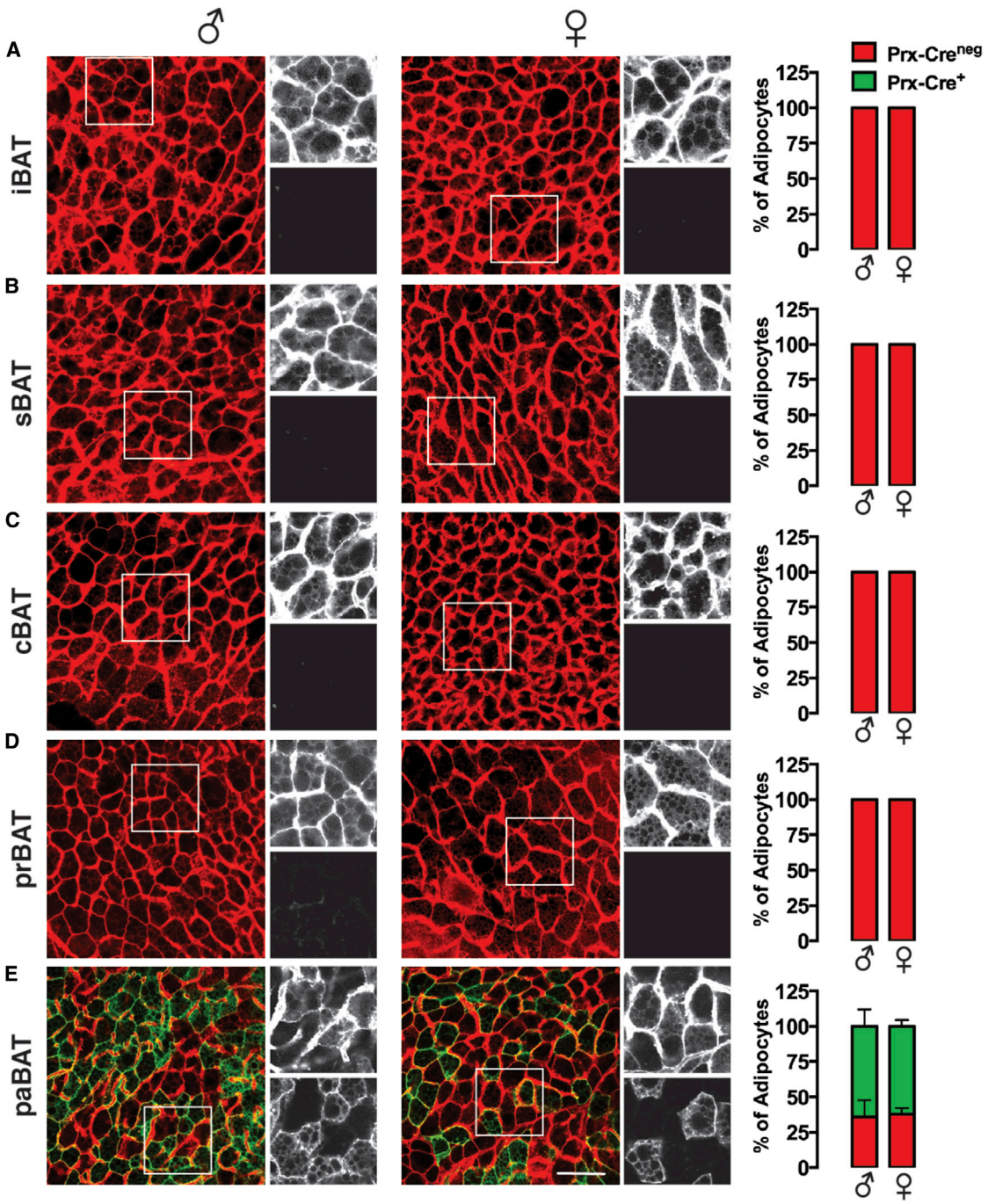

Figure 3. Most Brown Adipocytes Are Not Labeled by Prx1-Cre

(A–E) Representative images of the indicated BATs of 6-week-old *Prx1-Cre;R26R-mTmG* male and female mice (top insert, tdTomato; bottom insert, mGFP). The number of tdTomato$^+$ and mGFP$^+$ mature brown adipocytes in each depot is indicated in a graph at the left side of each row (n = 3 per each sex; mean + SEM). For all panels, scale bar represents 50 μm.

See also Figure S2.

non-adipose tissues as a measure of past or present Prx1-Cre recombinase activity. Consistent with the imaging results, *mGFP* reporter expression among the fat depots is highest in psWAT, with only asWAT and paBAT, as predicted, showing significantly higher expression above background (Figure 4A). Among skeletal muscles, the level

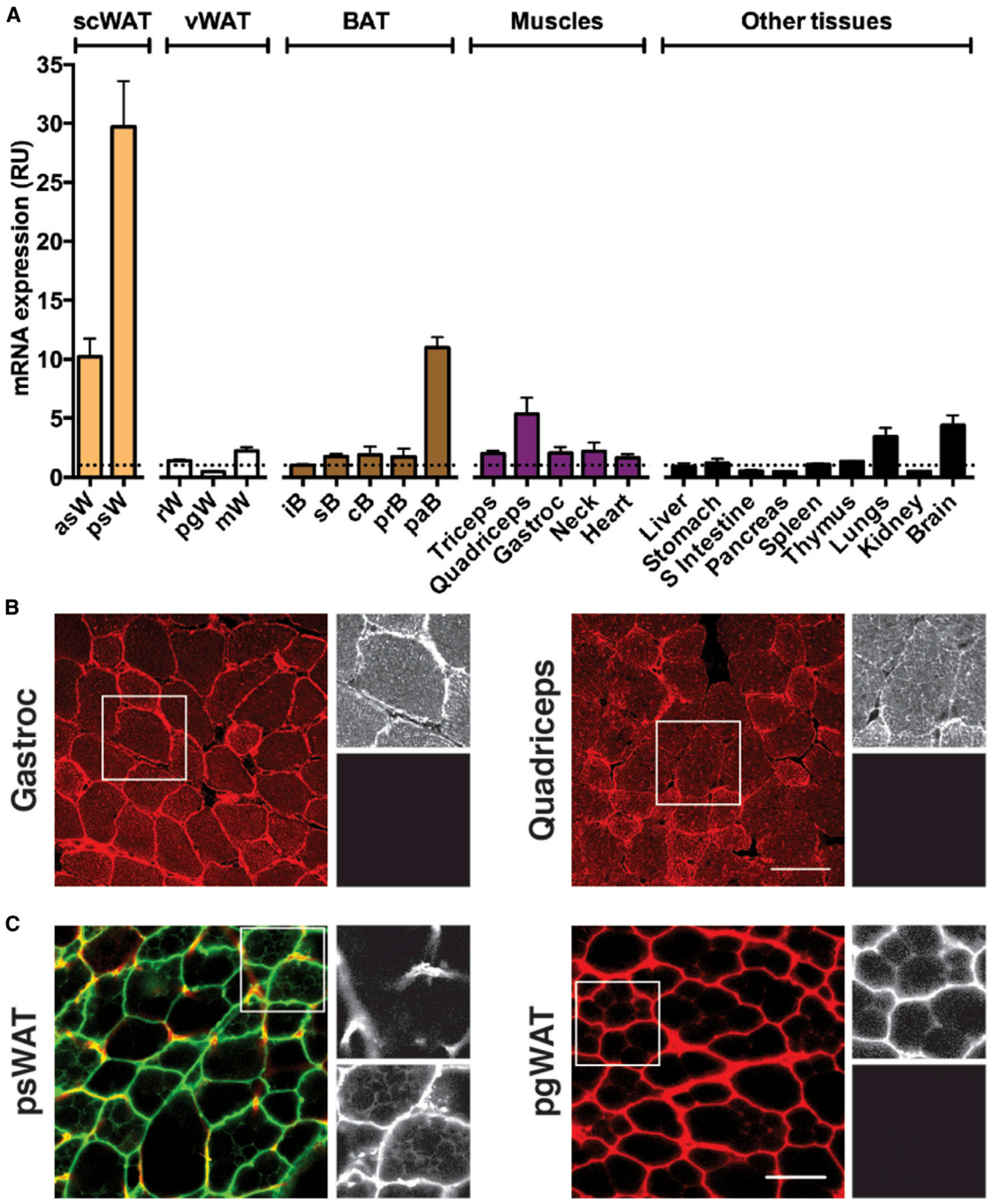

Figure 4. Prx1-Cre Targeting Shows a High Degree of Selectivity to Subcutaneous WAT

(A) *mGFP* mRNA expression in the indicated tissues. Dotted line indicates the background level of mGFP expression (defined as the level of expression of mGFP in no-Cre mice; n = 3 mice; mean + SEM).

(B) Representative images of the indicated skeletal muscles from *Prx1-cre;R26R-mTmG* mice. For all panels, scale bar represents 50 μm.

(C) Representative images of the indicated WATs of 6-week-old *Prx1-Cre;R26R-mTmG* male mice treated with CL316,243 for 1 week (top insert, tdTomato; bottom insert, mGFP; n = 3 mice; mean + SEM). For all panels, scale bar represents 50 μm.

See also Figures S3 and S4.

of *mGFP* expression is generally low (Figure 4A); mGFP reporter fluorescence was not detectable in any mature muscle fibers in the quadriceps or gastrocnemius muscles by our direct imaging strategy (Figure 4B). Slightly higher than baseline levels of *mGFP* expression were detected in lung and brain tissue, indicating a few cells in these tissues might also be marked by Prx1-Cre. Notably, we did not detect significant Cre mRNA expression in any whole fat depot (not shown), suggesting that Prx1-Cre recombinase activity may be active in only a small number of cells or that it was active before 6 weeks of age. We verified fat depot identity by analyzing *Ucp1* and *Perilipin1* mRNA expression. *Ucp1* most highly expresses in BAT depots with only a very low level of expression in asWAT and psWAT and little if any in the visceral WAT (Figure S2A). *Perilipin1* highly expresses in all of the fat depots (Figure S2B).

We also analyzed the mRNA expression of both splicing variants of *Prx1* (Prx1a and Prx1b) and the levels of *Prx2*, the second member of the family of paired-related homeobox proteins. *Prx1a* highly expresses in subcutaneous WAT (asWAT and psWAT) and in paBAT compared to visceral WAT and the other BAT depots (Figure S3A). The expression level of *Prx1b* is also higher in subcutaneous WAT than in the visceral depots (Figure S3B). *Prx1a* and *Prx1b* also express more highly in skeletal and cardiac muscle, even though we cannot find evidence of Prx1-Cre-recombinase-driven reporter expression in muscle or cardiac cells. *Prx2* expresses at similar levels in both subcutaneous and visceral white fats (Figure S3C). Thus, *Prx1* mRNA does not precisely overlap with *mGFP* mRNA expression, which could reflect differences in promoter or mRNA regulatory elements (see Discussion). Nevertheless, Prx1-Cre-mediated recombination is highly selective to subcutaneous white adipocyte precursors and mature adipocytes.

Finally, we asked whether the Prx1-Cre labeling pattern is stable or changes with age (6 months) or in mice treated for 1 week with the beta-adrenergic agonist CL316,243, which induces browning. Neither age nor CL316,243 treatment changes the percentage of $mGFP^+$ versus $tdTomato^+$ APCs or mature adipocytes in any depot we examined (Figures 4C and S4; not shown). Notably, the multilocular cells that form in the psWAT of mice treated with CL316,243 express UCP1 and are labeled with mGFP in the *Prx1-Cre;R26R-mTmG* mice, indicating that Prx1-Cre does not distinguish between white and brite/beige adipocytes in this depot (Figure 4C). Thus, the contribution of the Prx1-Cre-marked adipocytes to the adipocyte population appears to be stable.

DISCUSSION

Recently, Krueger et al. (2014) reported that Prx1-Cre labels inguinal (posterior subcutaneous) white adipocyte precursors. Taken together, our studies provide complimentary evidence that, in the mouse, Prx1-Cre transgene activity labels adipocyte progenitors of subcutaneous white adipocytes. In further support, a recent study using Prx1-Cre to delete *Ptpn11*, which encodes SHP-2 phosphatase, notes that subcutaneous adipose tissue is absent in *Prx1-Cre;Ptpn11* floxed mice (Lapinski et al., 2013), which is consistent with another recent study reporting a role for SHP-2 in adipogenesis (He et al., 2013). Thus, Prx1-Cre may be useful for conditional gene knockout studies in scWAT, especially when targeting genes with adipocyte-specific functions. However, it is essential that any study employing Prx1-Cre to target subcutaneous adipocytes pay careful attention to osteoblasts and chondrocytes (Logan et al., 2002). Of note, another recent study finds that Wt1-Cre selectively labels progenitors of visceral white adipocytes (Chau et al., 2014). Thus, comparative studies using Wt1-Cre and Prx1-Cre, when used to manipulate genes encoding fat-specific regulators, could provide valuable insight about visceral versus subcutaneous fat function.

It was previously reported that Prx1-Cre expresses in the precursors of all brown and white adipocytes in 4- to 6-week-old mice (Calo et al., 2010). This report did not indicate which depots were examined, so we could not do a perfect comparison. Nevertheless, neither our study nor Krueger et al. (2014) detect broad labeling of precursor or mature brown adipocytes. The differences between our studies could reflect the use of different reporters or possibly strain background differences (the mice in Calo et al., 2010 are on a mixed background compared to the C57Bl/6 background used here and in Krueger et al., 2014). Labeling mature adipocytes with cytoplasmic reporters such as LacZ can also be less reliable than using membrane-targeted reporters because adipocytes have little cytoplasm, making it difficult to detect which cells express β-galactosidase (Berry et al., 2014; Berry and Rodeheffer, 2013; Sanchez-Gurmaches and Guertin, 2014; Sanchez-Gurmaches et al., 2012).

There are noteworthy comparisons between Prx1-Cre and the Myf5-Cre and Pax3-Cre-labeling patterns in adipose tissue (Sanchez-Gurmaches and Guertin, 2014). First, in the asWAT, Prx1-Cre marks mature adipocytes in a ventral to dorsal pattern. This labeling pattern is opposite to the *Myf5-Cre;R26R-mTmG* and *Pax3-Cre;R26R-mTmG* patterns in asWAT, in which cells label in a dorsal to ventral gradient. Thus, one possibility is that Myf5-Cre/Pax3-Cre and Prx1-Cre may mark two different adipocyte progenitor pools that converge in asWAT. Second, Prx1-Cre labels a significant number of periaortic brown adipocytes whereas Myf5-Cre and Pax3-Cre do not (Sanchez-Gurmaches and Guertin, 2014). paBAT may have an important role in diabetes and cardiovascular disease (Fitzgibbons and Czech, 2014; Fitzgibbons et al., 2011; Guilherme et al., 2008).

These results are consistent with a model in which brown adipocytes may arise from multiple origins. Finally, the observation that Myf5-Cre, Pax3-Cre, and Prx1-Cre label populations of both brown and white adipocytes suggests some brown and white adipocytes may share a common precursor, although further experiments are required to verify this.

Interestingly, Prx1-Cre does not distinguish between white (unilocular) and brite/beige (multilocular) adipocytes induced by CL316,243 in the psWAT. Currently, it is unclear as to whether brite/beige adipocytes arise de novo from precursors of a different lineage or form from preexisting unilocular white adipocytes (Barbatelli et al., 2010; Lee et al., 2015; Rosenwald et al., 2013; Wang et al., 2013). Our results suggest that, at least after CL316,243 treatment, any brite/beige adipocytes that might form de novo originate from either the same Prx1-Cre precursor pool as the resident subcutaneous white adipocytes or they express Prx1-Cre during differentiation. Recent studies suggest that it is likely the case that the newly observed brown-like adipocytes appearing in psWAT are being derived largely from the preexisting Prx1-Cre-marked unilocular adipocytes (Lee et al., 2015).

One interesting possibility is that Prx1-Cre marks a progenitor pool in development that gives rise to a subcutaneous white adipocyte lineage. However, there are important caveats that need to be considered when interpreting data generated from Cre-mediated cell marking or lineage tracing. For example, whereas Cre-Lox-based recombination is currently one of the best methods for lineage tracing in mice (Kretzschmar and Watt, 2012), this approach is strictly a measure of promoter activity and does not provide any information as to whether the endogenous gene products actually express in the early developmental precursors of adipocytes and/or functionally contribute to adipose tissue development. In addition, in reporter-based cell-marking/lineage-tracing studies, in which multiple tissue types label positive with a particular Cre driver, it cannot be determined whether the recombination occurred in a single lineage or alternatively in multiple independent lineages that give rise to the different tissues. Nor can it be determined when a constitutively active Cre driver first expresses. This last caveat may be addressable with the use of inducible Cre drivers, such as tamoxifen-inducible CreER drivers. However, CreER drivers are limited in many cases by their efficiency and because tamoxifen reportedly has negative effects on fat (Liu et al., 2015). The use of doxycycline-driven Cre drivers might offer a better alternative (Hudak et al., 2014; Jiang et al., 2014; Wang et al., 2013). For example, a recent study inducing β-galactosidase in adipocytes with doxycycline reports that brite/beige adipocytes in psWAT are generated by de novo adipogenesis from an undefined APC pool (Wang et al., 2013); however, other studies using inducible drivers but different reporters find that psWAT brite/beige adipocytes arise from existing white adipocytes, suggesting more work is needed to optimize methods of cell marking and lineage tracing in adipose tissue (Lee et al., 2015; Rosenwald et al., 2013). Lastly, whereas Myf5-Cre and Pax3-Cre are knockin alleles (i.e., the Cre is driven from the endogenous *Myf5* and *Pax3* promoter) and therefore more ideal for cell-labeling experiments, the Prx1-Cre used here is a transgene, and thus positional elements might also influence promoter activity for this driver.

In conclusion, we find that Prx1-Cre recombinase activity is highly selective to subcutaneous white adipocyte precursors, which may be useful for biologists aiming to manipulate the activity of genes with fat-specific functions selectively in subcutaneous WAT.

EXPERIMENTAL PROCEDURES

Mice

R26R-mTmG (stock 007676) and prx1-Cre (stock 005584) were from Jackson Laboratories. Mice were housed in the Animal Medicine facilities of the UMMS in a room set at 22°C and 45% humidity under a daily 12 hr light/dark cycle. All animal experiments were approved by the University of Massachusetts Medical School animal care and use committee.

Tissue Harvest

Adipose tissues used were carefully dissected to avoid surrounding tissue contamination. Adipose tissue notation used here was described previously (Sanchez-Gurmaches and Guertin, 2014).

FACS Analysis and Confocal Microscopy

APC analysis by FACS and whole-mount confocal and muscle histology were done as previously reported (Sanchez-Gurmaches and Guertin, 2014).

Data Presentation

Data are presented as mean + SEM, unless stated otherwise. Graphs were done using GraphPad Prism v6.0c. Per each experiment and sex, three mice were analyzed.

See also Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.02.008.

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