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The Transcription Factor Paired-Related Homeobox 1 (Prrx1) Inhibits Adipogenesis by Activating Transforming Growth Factor-β (TGFβ) Signaling*

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Background: Paired-related homeobox 1 (Prrx1) regulates mesenchymal cell fate, but whether Prrx1 impacts adipogenesis remains unknown.
Results: Prrx1 knockdown decreases transforming growth factor-β (TGFβ) ligand expression and enhances adipogenesis, whereas *Prrx1* increases in adipose tissue during obesity.
Conclusion: Prrx1 knockdown enhances adipogenesis by suppressing TGFβ signaling.
Significance: This report identifies Prrx1 as an inhibitor of adipogenesis that may impact adipose tissue function in obesity.

Differentiation of adipocytes from preadipocytes contributes to adipose tissue expansion in obesity. Impaired adipogenesis may underlie the development of metabolic diseases such as insulin resistance and type 2 diabetes. Mechanistically, a well defined transcriptional network coordinates adipocyte differentiation. The family of paired-related homeobox transcription factors, which includes Prrx1a, Prrx1b, and Prrx2, is implicated with regulation of mesenchymal cell fate, including myogenesis and skeletogenesis; however, whether these proteins impact adipogenesis remains to be addressed. In this study, we identify Prrx1a and Prrx1b as negative regulators of adipogenesis. We show that Prrx1a and Prrx1b are down-regulated during adipogenesis *in vitro* and *in vivo*. Stable knockdown of Prrx1a/b enhances adipogenesis, with increased expression of peroxisome proliferator-activated receptor-γ, CCAAT/enhancer-binding protein-α and FABP4 and increased secretion of the adipokines adiponectin and chemerin. Although stable low-level expression of Prrx1a, Prrx1b, or Prrx2 does not affect 3T3-L1 adipogenesis, transient overexpression of Prrx1a or Prrx1b inhibits peroxisome proliferator-activated receptor-γ activity. Prrx1 knockdown decreases expression of *Tgfb2* and *Tgfb3*, and inhibition of TGFβ signaling during adipogenesis mimics the effects of Prrx1 knockdown. These data support the hypothesis that endogenous Prrx1 restrains adipogenesis by regulating expression of TGFβ ligands and thereby activating TGFβ signaling. Finally, we find that expression of *Prrx1a* or *Prrx1b* in adipose tissue increases during obesity and strongly correlates with *Tgfb3* expression in BL6 mice. These observations suggest that increased Prrx1 expression may promote TGFβ activity in adipose tissue and thereby contribute to aberrant adipocyte function during obesity.

Obesity markedly increases the risk of developing type 2 diabetes, cardiovascular diseases, cancer, and many other illnesses. Therefore, the current obesity pandemic is placing a huge burden on public health worldwide. Obesity is defined by increased adiposity, which, on a cellular level, is driven by both hypertrophy and hyperplasia of adipocytes. The latter results from differentiation of new adipocytes from preadipocytes. This process, called adipogenesis, is important for adipose tissue turnover and expansion in normal and obese individuals (1). Moreover, impaired adipogenesis in obesity is implicated in the development of metabolic complications (2, 3); hence, understanding the molecular mechanisms regulating adipogenesis may provide important insights into normal adipose tissue biology and the links between obesity and associated metabolic diseases.

Extensive study of adipogenesis has led to the identification of myriad secreted ligands, intracellular signaling pathways and transcription factors that regulate this process (4–8). Transcriptional regulation is perhaps the most well understood. Adipogenesis is characterized by dynamic changes in gene expression, whereby anti-adipogenic regulators are down-regulated while gene products required for adipogenesis are transiently or terminally induced (5, 6). Such positive and negative regulators ultimately stimulate or inhibit, respectively, the expression and/or activity of peroxisome proliferator-activated

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receptor-γ (PPARγ)[4] and CCAAT/enhancer-binding protein-α (C/EBPα). These so-called "master" adipogenic transcription factors activate expression of fatty acid-binding protein 4 (*Fabp4*), adiponectin (*Adipoq*) and other genes encoding proteins responsible for mature adipocyte functions. Indeed, the transcriptional networks underlying adipogenesis have been extensively characterized, with many other pro- or anti-adipogenic transcription factors coordinating different stages of this process (5–8). Nevertheless, additional transcription factors continue to be identified, and it is clear that the full complement of adipogenic transcriptional regulators remains to be fully established.

One family of transcription factors implicated with regulation of mesenchymal cell fate is the paired-related homeobox protein family, which includes Prrx1 and Prrx2. The *Prrx1* gene is alternatively spliced to yield two proteins, Prrx1a and Prrx1b. These proteins differ at their C termini, where Prrx1a and Prrx2 contain a regulatory otp, aristaless and rax (OAR) domain, whereas Prrx1b contains a repressor domain. Functional studies suggest that Prrx1a and Prrx2 promote transcriptional activation, whereas Prrx1b acts as a transcriptional repressor (9). Prrx1 is restricted to the mesoderm during embryonic development, and both Prrx1 and Prrx2 are expressed in mesenchymal tissues in adult mice (10–14). Mice lacking *Prrx2* are viable and fertile (14, 15). In contrast, mice lacking *Prrx1* die postnatally and have impaired formation of osteogenic and chondrogenic progenitors, resulting in defective skeletogenesis (16). Mice that lack both *Prrx1* and *Prrx2* have profound defects in mesenchymal cell differentiation in the craniofacial region (14, 15). These observations suggest an important functional role for Prrx1 in regulating mesenchymal cell fate.

Like osteoblasts and chondrocytes, adipocytes are derived from mesenchymal progenitors; however, whether Prrx1a or Prrx1b impact adipogenesis has not been reported previously. In this study we investigated this possibility. Our findings indicate that Prrx1a and Prrx1b inhibit adipogenesis through regulated expression of transforming growth factor-β (TGFβ) ligands, thereby activating TGFβ signaling. Moreover, using mouse models of obesity, we find that white adipose tissue (WAT) expression of *Prrx1a* and *Prrx1b* increases with obesity and correlates with *Tgfb3* expression in a strain-specific manner. Thus, increased Prrx1 expression in WAT may contribute to dysregulated adipocyte function during obesity.

EXPERIMENTAL PROCEDURES

Animals—C57BL/6J and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For WAT fractionation, epididymal WAT was isolated from 16-week-old C57BL/6J mice and separated into stromovascular and adipocyte fractions as described previously (17). Procedures for this work were approved by the Committee on the Use and Care of Animals at the University of Michigan, with daily care of mice overseen by the unit for laboratory animal medicine. For high fat diet (HFD) studies, C57BL/6J and C3H/HeJ mice were fed a standard chow diet (Research Diets D12450b) or a HFD (Research Diets D12492) *ad libitum* from 3 to 15 weeks of age, at which point inguinal WAT was isolated for RNA purification. The Institutional Animal Care and Use Committee of Maine Medical Center Research Institute approved all procedures relevant to these HFD studies.

Cell Culture—3T3-L1 cells were maintained in culture, differentiated into adipocytes and stained with Oil Red-O as described previously (18, 19). Adipocytes were stained with BODIPY-conjugated dodecanoic acid (BODIPY® FL C_{12}) or Hoechst 33258 (Invitrogen) according to the manufacturer's instructions. 293T cells were maintained in DMEM containing 8% bovine calf serum (Denville Scientific, South Plainfield, NJ). To assess the effect of inhibition of TGFβ signaling on adipogenesis, 3T3-L1 cells were treated at the day of induction and every 2 days thereafter with vehicle control (DMSO, 0.03% v/v) or with 3 μM SB431542 (Cayman Chemical, Ann Arbor, MI), an inhibitor of the TGFβ receptor ALK5. To assess adiponectin and chemerin secretion, 3T3-L1 adipocytes at day 8 post-induction were washed and starved in DMEM (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate) from Invitrogen. After 4 or 24 h, medium was sampled for analysis by immunoblotting, as described below. To assess the effects of tumor necrosis factor-α (TNFα) on transcript expression, 3T3-L1 preadipocytes at 2 days post-confluence were fed with fresh DMEM containing 10% fetal bovine serum (Gemini Bio-products, Sacramento, CA) supplemented with recombinant murine TNFα (PeproTech, Rocky Hill, NJ) or sterile PBS as a vehicle control. After 4 or 24 h, total RNA was isolated and transcript expression analyzed as described below.

Plasmid Constructs—The J3-TK-luciferase construct contains three tandem copies of PPARγ-response elements from the J sites of the *APOA2* promoter and was generated as described previously (20). phRL-TK was from Promega (Madison, WI). pSG5-PPARγ, containing cDNA of human PPARγ, has been described previously (21). pcDNA3 was from Invitrogen. We generated pMSCVneo-Prrx1a and pMSCVneo-Prrx1b as follows. First, coding sequences of Prrx1a (−101 to +820 bp, relative to start codon) or Prrx1b (−101 to +676 bp) were amplified from 3T3-L1 preadipocyte cDNA using the following PCR primers: *Prrx1a/Prrx1b* sense, 5′-CCTCTTTCTTC-CCCACTCG-3′; *Prrx1a* antisense, 5′-GGCGGATGAA-GATATGACAGA-3′; *Prrx1b* antisense, 5′-ATGGCGC-TTTTCAGTGTCTT-3′. The *Prrx1a* or *Prrx1b* PCR products were then cloned into pCRII-TOPO (Invitrogen) according to the manufacturer's instructions, yielding pCRII-Prrx1a and pCRII-Prrx1b. Second, we subcloned inserts of Prrx1a (−112 to +827 bp) or Prrx1b (−112 to +683 bp) from pCRII-Prrx1a or pCRII-Prrx1b into the EcoRI restriction site of pMSCVneo (Clontech), yielding pMSCVneo-Prrx1a and pMSCVneo-Prrx1b. Correct orientation of inserts was confirmed by diagnostic SmaI restriction digests. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). pMSCVneo-Prrx2 (Addgene plasmid 21010) was

[4] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor-γ; HFD, high fat diet; Prrx, paired-related homeobox; SVF, stromal-vascular fraction; WAT, white adipose tissue; C/EBPα, CCAAT/enhancer-binding protein-α; EV, empty vector; qPCR, quantitative PCR; TZD, rosiglitazone; Shh, sonic hedgehog; pAb, polyclonal antibody; mAb, monoclonal antibody; MDI, methylisobutylxanthine, dexamethasone, and insulin.

purchased from Addgene (Cambridge, MA) and was generated as described previously (22). Prrx1a and Prrx1b were stably knocked down by expression of an shRNA from the pSuperior.retro.neo vector (OligoEngine, Seattle, WA). The shRNA was designed according to the manufacturer's instructions to target the following sequence of *Prrx1*: 5′-CAGAGAGCTTCTT-GATCCAAC-3′. Sequences for shRNAs used to generate the two other Prrx1 knockdown cell lines (data not shown) are available upon request.

Transient Transfection and Reporter Assays—293T cells were seeded in 24-well plates and were fed fresh medium at 24 h post-seeding (cells at 70–80% confluence). One hour later, cells were transfected with LipoD293 DNA In Vitro Transfection Reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instructions. Each well of cells was transfected with J3-TK-luciferase (0.2 μg) and phRL-TK (0.01 μg), with or without pSG5-PPARγ (0.1 μg), pMSCVneo-Prrx1a (0.05, 0.1, or 0.5 μg), and/or pMSCVneo-Prrx1b (0.05, 0.1, or 0.5 μg), as indicated. For each well, the total amount of transfected DNA was brought up to 0.81 μg using pcDNA3 empty vector. At 18–20 h post-transfection, cells were fed fresh medium supplemented with 5 μM rosiglitazone (Cayman Chemical, Ann Arbor, MI) or vehicle control (DMSO, 0.01% v/v). At 48 h post-transfection, luciferase activity was assayed using a Dual-Luciferase reporter assay system (Promega, Madison, WI). Values were normalized to *Renilla* luciferase activity derived from the co-transfected phRL-TK-Luc constitutive *Renilla* luciferase reporter.

Retroviral Infection and Constructs—Genes and shRNAs were stably introduced into 3T3-L1 cells by retroviral infection as described previously (23).

Cell Lysates and Immunoblotting—For cell lysates, cells were washed once with PBS and scraped into lysis buffer (1% SDS, 12.7 mM EDTA, 60 mM Tris-HCl, pH 6.8) heated to 95 °C. Lysates were then centrifuged at 20,000 relative centrifugal force for 15 min at 4 °C, and supernatants were transferred to fresh tubes and stored at −80 °C. Protein concentration in cell lysates was estimated using the BCA protein assay (Thermo Scientific, Waltham, MA). For analysis of adiponectin secretion, adipocyte-conditioned media were centrifuged at 300 relative centrifugal force for 1 min at 4 °C to pellet cell debris. Equal volumes of media were then transferred to fresh tubes, mixed with 4× SDS loading buffer (4% SDS, 240 mM Tris-HCl, 40% glycerol, 0.05% bromphenol blue), and supplemented with 2.5% 2-mercaptoethanol. SDS-PAGE and immunoblotting of cell lysates or conditioned media were performed as described previously (19). The following primary antibodies were used: mouse monoclonal anti-PPARγ1/2 was from Millipore (Temecula, CA); rabbit polyclonal anti-C/EBPα (catalog no. 2295) and rabbit monoclonal anti-ERK1/2 (catalog no. 4695) were from Cell Signaling Technology (Danvers, MA); mouse monoclonal anti-adiponectin (catalog no. MA1-054) was from Thermo Scientific (Rockford, IL); rabbit polyclonal anti-adiponectin (catalog no. A6354) was from Sigma; rat monoclonal anti-FABP4 (catalog no. MAB1143) and goat polyclonal anti-chemerin were from R&D Systems (Minneapolis, MN); and goat polyclonal anti-Prrx1 was from Abnova (catalog no. PAB7402), although by using this antibody we could not detect Prrx1a/b in 3T3-L1 cells.

TABLE 1
Sequences of primers for qPCR
F indicates forward; R indicates reverse.

Transcript	Primer sequence (5′ to 3′)
18 S rRNA (24)	F, CGATGCTCTTAGCTGAGTGT
	R, GGTCCAAGAATTTCACCTCT
Tbp	F, ACCTTATGCTCAGGGCTTGG
	R, GCCATAAGGCATCATTGGAC
Prrx1a	F, TTACCCGGATGCTTTTGTTC
	R, AAGTAGCCATGGCGCTGTA
Prrx1b	F, TTACCCGGATGCTTTTGTTC
	R, GCCCCTCGTGTAAACAACAT
Prrx2	F, ACGTGTCCAAGTCTGGTTCC
	R, ATCTGGGCTCATCGTGGTAG
Pparg (25)	F, GGAAAGACAACGGACAAATCAC
	R, TACGGATCGAAACTGGCAC
Fabp4 (25)	F, TGGAAGCTTGTCTCCAGTGA
	R, AATCCCCATTTACGCTGATG
Cebpa (25)	F, TGGACAAGAACAGCAACGAG
	R, TCACTGGTCAACTCCAGCAC
Adipoq (26)	F, AAGAAGGACAAGGCCGTTCTCTT
	R, GCTATGGGTAGTTGCAGTCAGTT
Tgfb1	F, TTGCTTCAGCTCCACAGAGA
	R, TGGTTGTAGAGGGCAAGGAC
Tgfb2	F, CCGGAGGTGATTTCCATCTA
	R, GCGGACGATTCTGAAGTAGG
Tgfb3	F, ATTCGACATGATCCAGGGAC
	R, TCTCCACTGAGGACACATTGA
Hspa5 (also known as Grp78)	F, GCGTGTGTGTGAGACCAGAACCG
	R, CCGACCACCGTGCCCACATC
Smo	F, AATTGGCCTGGTGCTTATTG
	R, TGGTCTCGTTGATCTTGCTG
Ptch1	F, TACGTGGAGGTGGTTCATCA
	R, AACAGGCATAGGCAAGCATC

Real Time qPCR—Total RNA was isolated from cells or tissues using RNA STAT60 reagent (Tel-Test Inc, Friendswood, TX) according to the manufacturer's instructions. Reverse transcription, primer design and qPCR were performed as described previously (19). Primer sequences are shown in Table 1.

Statistical Analyses—Statistical significance was determined using a two-tailed Student's *t* test assuming equal variances, and is indicated as follows: * or # = $p < 0.05$; ** or ## = $p < 0.01$; *** or ### = $p < 0.001$.

RESULTS

Prrx1a, Prrx1b, and Prrx2 Are Down-regulated during Adipogenesis—Adipocyte differentiation is characterized by dynamic changes in gene expression. In general, genes that suppress adipogenesis are down-regulated, whereas genes required for adipogenesis or adipocyte function are often transiently or terminally induced (5, 6). The expression profile of a given gene during adipocyte differentiation may thereby provide insights into the potential function of this gene in regulation of adipogenesis or adipocyte biology. Based on this concept, we screened our previous microarray data (27) and publicly available microarray databases of transcript expression in 3T3-L1 adipogenesis (GEO Datasets 2659 and 2660 (28)) and in adipocyte and stromovascular fractions (SVF) of adipose tissue (GEO Dataset 2818 (29)) to identify potential transcriptional regulators of adipocyte differentiation and/or function. This approach revealed that *Prrx1* transcripts are down-regulated during 3T3-L1 adipogenesis and are reduced in adipocytes rel-

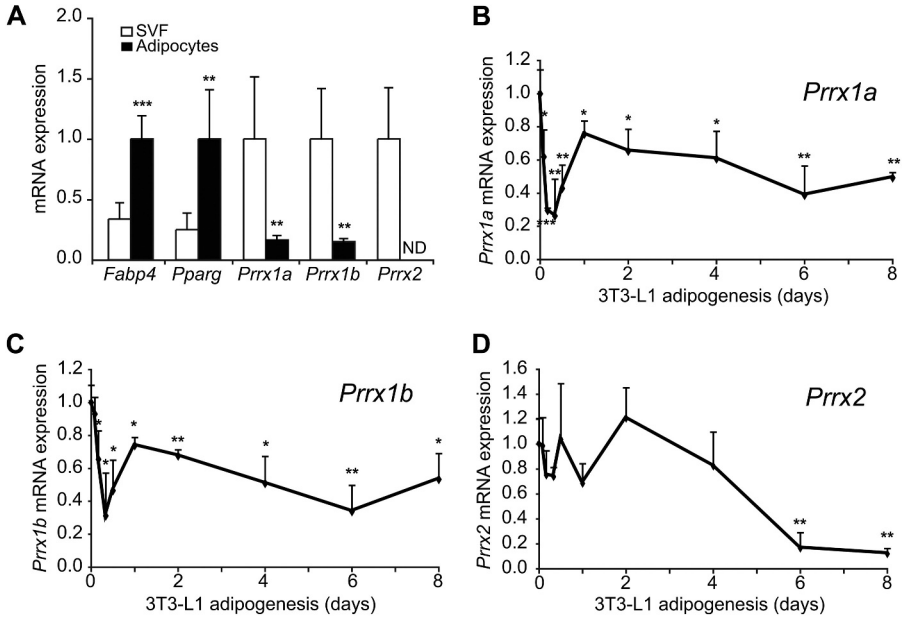

FIGURE 1. ***Prrx1a* and Prrx1b mRNA expression decreases during adipogenesis *in vivo* and *in vitro*.** *A,* gonadal WAT from WT C57BL/6 mice was separated into SVF and adipocyte fractions, as described under "Experimental Procedures." Total RNA was isolated from each fraction, and the expression of *Fabp4, Pparg, Prrx1a, Prrx1b* and *Prrx2* was analyzed by real time qPCR and normalized to 18 S rRNA. Data are presented relative to the maximum expression of each transcript as mean ± S.D. ($n = 6$); *ND,* not detectable. *B–D,* 3T3-L1 preadipocytes were induced to differentiate into adipocytes with MDI. Total RNA was isolated at the indicated time points and transcript expression of *Prrx1a, Prrx1b,* and *Prrx2* was analyzed by real time qPCR and normalized to 18 S rRNA. Data are presented relative to expression at day 0 as mean ± S.D. of three independent experiments. Significant differences between SVF and adipocyte fraction (*A*) or compared with transcript levels at day 0 (*B–D*) are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ative to SVF. To confirm and extend these observations, we used qPCR to analyze expression of *Prrx1a* and *Prrx1b*, as well as their homologue *Prrx2*, in fractionated adipose tissue and during 3T3-L1 adipogenesis. As expected, the adipocyte genes *Pparg* and *Fabp4* were enriched in adipocytes relative to SVF (Fig. 1*A*) and were up-regulated during 3T3-L1 adipogenesis (19). In contrast, *Prrx1a* and *Prrx1b* were 80% lower in adipocytes relative to SVF, whereas *Prrx2* transcripts were undetectable in the adipocyte fraction (Fig. 1*A*). During 3T3-L1 adipogenesis, *Prrx1a* and *Prrx1b* transcripts were rapidly suppressed in response to adipogenic stimuli, with ~70% down-regulation between 0 and 8 h post-induction followed by sustained 2-fold down-regulation during terminal differentiation (Fig. 1, *A* and *B*). In contrast, *Prrx2* was decreased almost 10-fold by days 6–8 post-induction but was not altered during early adipogenesis (Fig. 1*C*). Thus, in agreement with the microarray data, *Prrx1a*, *Prrx1b* and *Prrx2* are down-regulated during adipogenesis *in vitro* and in adipocytes relative to SVF *in vivo*.

Stable Knockdown of Prrx1 Enhances 3T3-L1 Adipocyte Differentiation and Gene Expression—The rapid suppression of *Prrx1a* and *Prrx1b* during adipogenesis suggests that Prrx1 might negatively regulate this process. We began to investigate this possibility through loss-of-function studies. To do so, we generated 3T3-L1 preadipocyte lines with stable expression of shRNAs against Prrx1 (shPrrx1) or a scrambled control (shControl). As shown in Fig. 2*A*, expression of both *Prrx1a* and *Prrx1b* was significantly reduced in shPrrx1 preadipocytes, whereas *Prrx2* transcripts were unaffected. Many inhibitors of adipogenesis also suppress the low levels of adipocyte gene expression detectable in preadipocytes (19); however, we found that knockdown of Prrx1 did not affect expression of *Pparg*, *Cebpa* or *Fabp4* in preadipocytes (Fig. 2*A*). In response to full adipogenic induction with MDI, Prrx1 knockdown did not enhance the transient up-regulation of *Cebpb* or *Cebpd* during early adipogenesis (data not shown), and neutral lipid accumulation appeared grossly similar between shControl and shPrrx1 adipocytes (Fig. 2*B*). However, shPrrx1 adipocytes were consistently smaller and had a higher cell density than shControl adipocytes (Fig. 2, *C* and *D*). Quantitative PCR revealed increased expression of *Pparg*, *Cebpa,* and *Fabp4* in shPrrx1 adipocytes (Fig. 2*E*), and this was also apparent at the protein level (Fig. 2*F*). In addition to *Prrx1a* and *Prrx1b*, expression of *Prrx2* was also significantly lower in shPrrx1 adipocytes (Fig. 2*E*). Given the marked suppression of *Prrx2* during terminal adipogenesis (Fig. 1*D*), decreased *Prrx2* in shPrrx1 adipocytes further suggests enhanced adipogenesis in these cells. Finally, knockdown of Prrx1 was associated with increased neutral lipid accumulation following submaximal adipogenic induction (Fig. 2*G*); importantly, this effect was also observed in two other independent Prrx1 knockdown cell lines (data not shown).

These observations show that knockdown of Prrx1 during adipogenesis enhances adipocyte gene and protein expression. Therefore, we were surprised to find that, despite similar expression of *Adipoq* transcripts between shPrrx1 and shControl adipocytes (Fig. 2*E*), shPrrx1 adipocytes had markedly decreased intracellular adiponectin protein and modest reductions in adiponectin secretion (Fig. 2*F*, *mouse mAb*). In contrast, expression and secretion of another adipokine, chemerin (30, 31), was higher in shPrrx1 adipocytes (Fig. 2*F*). This suggested that the decreases in adiponectin were not a general phenomenon for all secreted proteins. Therefore, we postulated that shPrrx1 adipocytes might have increased degradation of adiponectin protein. Because endoplasmic reticulum stress

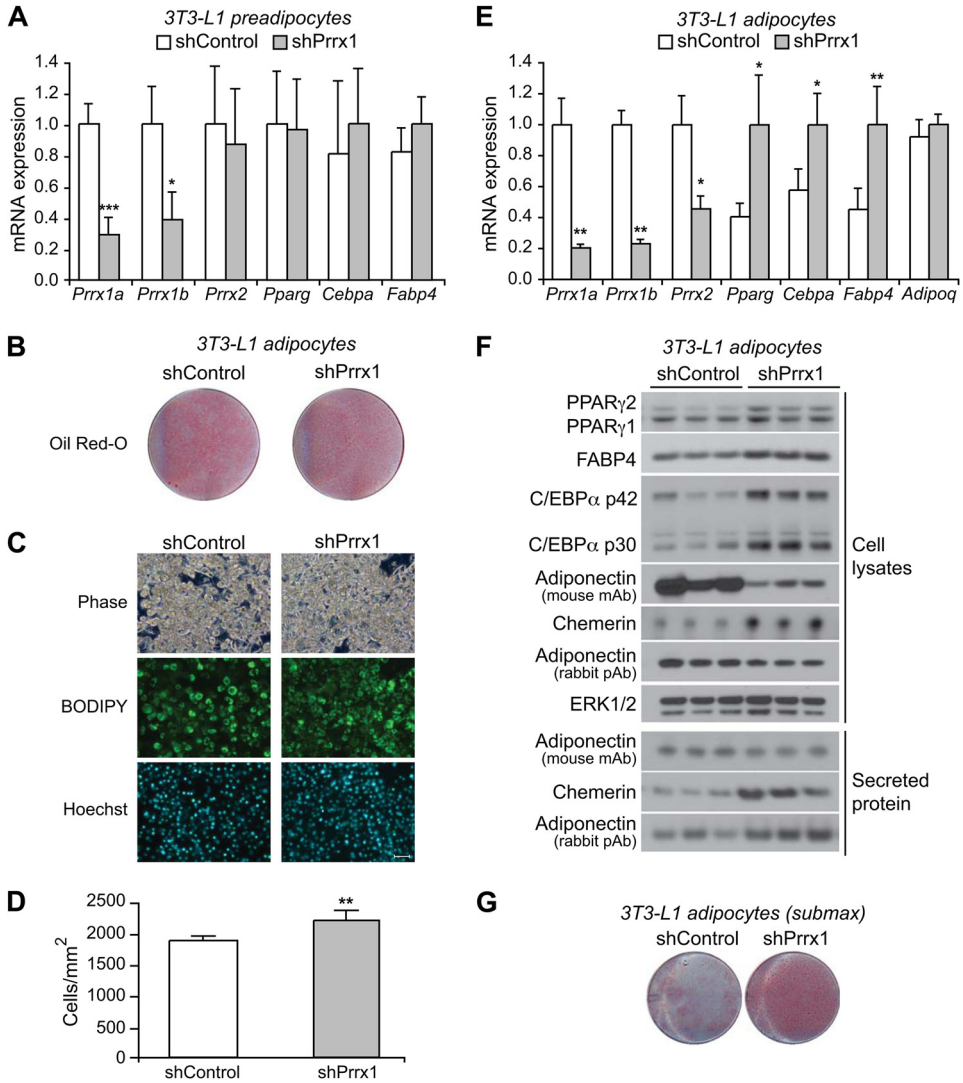

FIGURE 2. **Stable knockdown of Prrx1 enhances 3T3-L1 adipocyte differentiation and gene expression.** 3T3-L1 preadipocytes were infected with retroviruses for stable expression of shRNAs against Prrx1 or a scrambled control (*shControl*). *A,* cells were grown to 2 days post-confluence; total RNA was isolated, and expression of the indicated transcripts was analyzed by qPCR. *B–G,* shControl and shPrrx1 3T3-L1 preadipocytes were induced to differentiate into adipocytes with MDI (*B–F*) or dexamethasone and insulin only (*G*). At day 8 post-induction, the extent of adipogenesis was assessed by staining with Oil Red-O (*B* and *G*) or BODIPY (*C*), by qPCR analysis of adipocyte gene expression (*E*), or by immunoblotting (*F*). *C*, phase-contrast and fluorescent micrographs show live adipocytes through a ×20 objective; Hoechst-stained cells are shown with hue adjusted to aid visualization. *Scale bar,* 50 μm. *D,* cell density was quantified from micrographs of Hoechst-stained cells using ImageJ software and is presented as mean ± S.D. of 15 micrographs. To assess adiponectin and chemerin secretion in *F*, adipocytes at day 8 post-induction were cultured in serum-free medium. After 24 h, medium was sampled and adiponectin or chemerin was detected by immunoblotting. Immunoblots and images of live or stained adipocytes are representative of at least three independent experiments. Transcript expression in *A* and *E* was normalized to *Tbp* mRNA and is presented relative to the maximum expression of each transcript as mean ± S.D. of at least three independent experiments. Significant differences in cell density or transcript expression between shPrrx1 and shControl cells are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

promotes adiponectin degradation (32), we analyzed endoplasmic reticulum stress markers in shControl and shPrrx1 adipocytes. However, we found that endoplasmic reticulum stress did not differ between these cell types, as determined by expression of Grp78 mRNA and protein and analysis of *Xbp1* splicing (data not shown). Further analysis based on time courses of cycloheximide treatment revealed no difference in rates of adiponectin degradation between shControl and shPrrx1 adipocytes (data not shown). Eventually, we tested another anti-adiponectin antibody (rabbit pAb) to see whether this affected detection of adiponectin. Surprisingly, immunoblotting with this rabbit pAb revealed only minor reductions in intracellular adiponectin in shPrrx1 adipocytes and increased adiponectin secretion from these cells (Fig. 2*F*, *rabbit pAb*); the latter observation is consistent with enhanced function of shPrrx1 adipocytes. As detailed further under the "Discussion," we believe that shPrrx1 adipocytes may have enhanced post-translational modification of adiponectin that impairs detection by the mouse mAb but not by the rabbit pAb. These findings highlight the importance of antibody selection when detecting adiponectin protein.

Stable Expression of Prrx1a, Prrx1b, or Prrx2 Does Not Affect 3T3-L1 Adipogenesis—The above data show that knockdown of Prrx1 enhances adipogenesis. Based on these findings, we postulated that enforced expression of Prrx1a or Prrx1b would inhibit 3T3-L1 adipogenesis. To investigate this possibility, we

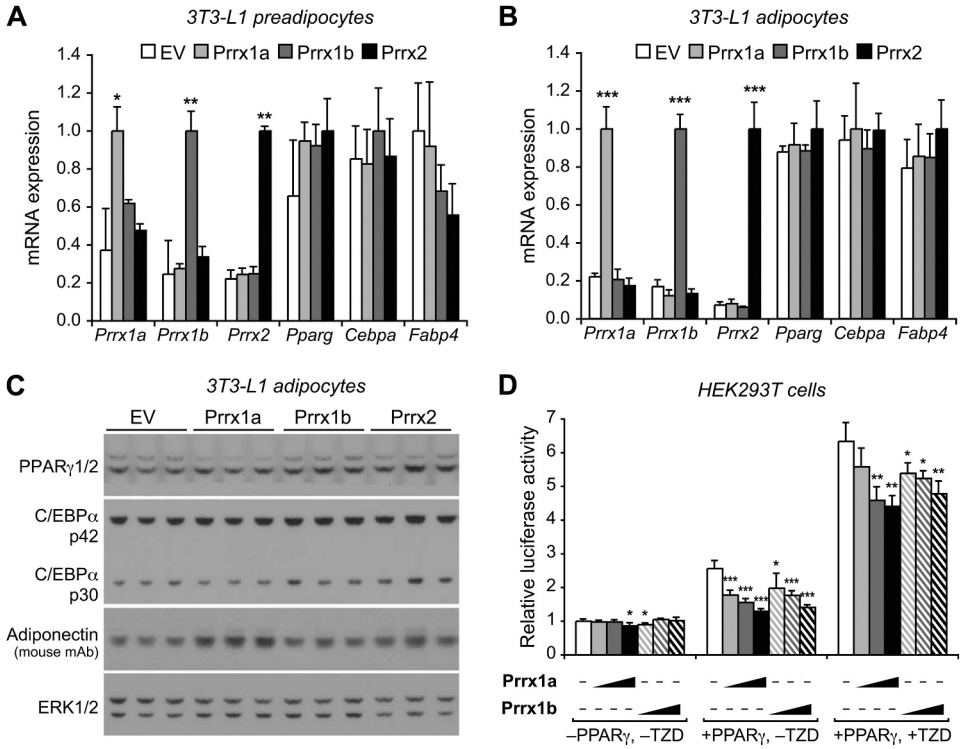

FIGURE 3. **Stable exogenous expression of Prrx1a, Prrx1b, or Prrx2 does not affect 3T3-L1 adipogenesis but overexpression suppresses PPARγ activity.** *A–C,* 3T3-L1 preadipocytes were infected with retroviruses for stable expression of Prrx1a, Prrx1b, Prrx2 or an EV control. *A,* cells were grown to 2 days post-confluence; total RNA was isolated, and expression of the indicated transcripts was analyzed by qPCR. *B* and *C,* EV- and Prrx-expressing 3T3-L1 preadipocytes were induced to differentiate into adipocytes. At day 8 post-induction, the extent of adipogenesis was assessed by analysis of adipocyte gene expression by qPCR (*B*) and immunoblotting (*C*). Immunoblots are representative of at least three independent experiments. Transcript expression was normalized to 18 S rRNA (*A*) or *Tbp* mRNA (*B*) and is presented relative to maximum expression of each transcript as mean ± S.D. of at least three independent experiments. *D,* HEK293T cells were transiently transfected with a PPARγ-response element-luciferase reporter construct along with an empty control vector (pcDNA3) and/or expression vectors for PPARγ, Prrx1a, or Prrx1b, as indicated. At 18–20 h post-transfection, cells were fed with fresh medium supplemented with 5 μM TZD or vehicle control (DMSO). Luciferase activity was assayed at 48 h post-transfection and is reported relative to control −TZD as mean ± S.D. of 3–4 biological replicates, representative of 2–7 independent experiments. Significant differences in transcript expression (*A* and *B*) or reporter activity (*D*) between control and Prrx-expressing cells are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

generated 3T3-L1 preadipocyte lines with stable exogenous expression of Prrx1a, Prrx1b, or an empty vector control (EV); we also generated a cell line with stable expression of Prrx2 to assess whether this family member might also regulate adipogenesis. Quantitative PCR confirmed increased expression of *Prrx1a*, *Prrx1b,* or *Prrx2* in each cell line, relative to EV cells (Fig. 3*A*). Consistent with our above findings (Fig. 2*A*), expression of *Pparg, Cebpa,* and *Fabp4* did not differ between EV- and Prrx-expressing 3T3-L1 preadipocytes (Fig. 3*A*). Therefore, we next investigated effects of stable Prrx expression on 3T3-L1 adipogenesis. Quantitative PCR confirmed maintenance of exogenous Prrx expression after differentiation into adipocytes (Fig. 3*B*). Nevertheless, each of the Prrx-expressing cells accumulated lipid to a similar extent as EV cells following maximal or submaximal adipogenic induction (data not shown). Similarly, expression of adipocyte genes or proteins did not differ between EV- and Prrx-expressing adipocytes (Fig. 3, *B* and *C*). In some cases, PPARγ2 protein appeared decreased and adiponectin increased in Prrx1a-expressing adipocytes (Fig. 3*C*); however, this was not observed across all experiments. The extent of adipogenesis in EV- and Prrx-expressing cells also did not differ in response to submaximal adipogenic induction (data not shown). Thus, these data show that enforced stable expression of Prrx1a, Prrx1b, or Prrx2 is not sufficient to inhibit 3T3-L1 adipogenesis.

Transient Exogenous Expression of Prrx1a or Prrx1b Inhibits PPARγ Activity—One drawback to these stable expression studies is that, using the relatively poor anti-Prrx1 antibody that is currently available, we were unable to detect endogenous or exogenous Prrx1a or Prrx1b in any of our 3T3-L1 cell lines (data not shown). Thus, it is possible that the modest increases in *Prrx1a*, *Prrx1b,* or *Prrx2* expression in these cell lines (Fig. 3*A*) were insufficient to exert functional effects. To overcome potential deficits in the extent of exogenous expression, we turned to transient overexpression studies to further investigate anti-adipogenic effects of increased Prrx1 expression. Following transient transfection of HEK293T cells, we isolated nuclear protein lysates and confirmed exogenous expression of Prrx1a and Prrx1b protein from these vectors by immunoblotting (data not shown). Because many inhibitors of adipogenesis block PPARγ activity (33–36), we next co-transfected a luciferase reporter driven by a PPARγ-response element to determine whether exogenous Prrx1a or Prrx1b could affect PPARγ activity. In the absence of exogenous PPARγ, expression of Prrx1a or Prrx1b was associated with weak suppression of luciferase activity under some conditions tested (Fig. 3*D*). Exogenous PPARγ increased reporter activity by almost 3-fold, with further enhancement following treatment with rosiglitazone (TZD), a synthetic PPARγ agonist (Fig. 3*D*). With or without TZD treatment, Prrx1a or Prrx1b dose-dependently sup-

pressed reporter activation (Fig. 3*D*) without affecting nuclear localization of exogenous PPARγ (data not shown). These data demonstrate that, at increased expression levels, Prrx1a or Prrx1b can suppress basal and ligand-induced PPARγ activity.

Prrx1 Regulates Expression of TGFβ Ligands in Preadipocytes—We next sought to determine how Prrx1 modulates adipogenesis. A previous study suggested that Prrx1 stimulates sonic hedgehog (Shh) activity in mesenchymal tissues (37). Given that Shh signaling blocks adipogenesis (38), we postulated that Prrx1 knockdown might enhance adipogenesis by suppressing Shh signaling. A major transcriptional target of Shh signaling is *Ptch1* (39), which encodes the transmembrane receptor for Shh (40). Thus, to determine whether Prrx1 knockdown impacts Shh signaling, we analyzed expression of *Ptch1* by qPCR as a readout of Shh activity. We found that Prrx1 knockdown did not affect expression of *Ptch1* in preadipocytes (data not shown); expression of *Smo,* a positive effector of Shh signaling, was also unaffected (data not shown). These data suggest that suppression of Shh signaling does not account for enhanced adipogenesis in shPrrx1 cells.

Another established inhibitor of adipogenesis is TGFβ signaling (41). A previous study demonstrates that Prrx1a directly transactivates the *Tgfb3* promoter and thereby induces *Tgfb3* expression in hepatic stellate cells (42). Therefore, we investigated whether Prrx1 knockdown affects expression of *Tgfb1*, *Tgfb2*, or *Tgfb3* in 3T3-L1 preadipocytes. *Tgfb1* showed a trend for decreased expression, whereas *Tgfb2* and *Tgfb3* were significantly lower in shPrrx1 preadipocytes (Fig. 4*A*). This suggests that Prrx1 stimulates expression of TGFβ ligands in preadipocytes.

Inhibition of TGFβ Signaling during Adipogenesis Phenocopies Effects of Prrx1 Knockdown—These findings raise the possibility that Prrx1 knockdown enhances adipogenesis by suppressing TGFβ signaling. To address this, we investigated if inhibition of endogenous TGFβ signaling would phenocopy knockdown of Prrx1. To do so, we induced adipogenesis in both shControl and shPrrx1 preadipocytes in the absence or presence of SB431542, which blocks TGFβ signaling by inhibiting the TGFβ receptor ALK5. Following adipogenesis in the absence of SB431542, cell density, expression of PPARγ and C/EBPα, and secretion of adiponectin and chemerin were increased in shPrrx1 adipocytes compared with shControl cells, whereas intracellular adiponectin was markedly lower in shPrrx1 adipocytes when detected with the mouse mAb (Fig. 4, *B–D*). These observations are consistent with our above findings (Fig. 2). Strikingly, each of these differences also occurred in shControl adipocytes that were differentiated in the presence of SB431542 (Fig. 4, *B–D*). In contrast, no effects were observed following treatment of mature shControl adipocytes with SB431542 (data not shown). Thus, inhibition of TGFβ signaling during adipogenesis, but not in mature adipocytes, has similar effects on adipocyte biology as Prrx1 knockdown. Notably, adipogenesis with SB431542 did not significantly affect cell density, adipocyte protein expression, or adipokine secretion in shPrrx1 adipocytes (Fig. 4, *B–D*), possibly because endogenous TGFβ activity is already reduced in these cells. These data support the conclusion that Prrx1 knockdown enhances adipocyte protein expression and adipokine secretion, at least in part, by suppressing TGFβ signaling during adipogenesis.

Prrx1 Is Not Required for Inhibition of Adipogenesis by Tumor Necrosis Factor-α or Wnt3a—Like TGFβ, tumor necrosis factor-α (TNFα) and Wnt/β-catenin signaling are well-established inhibitors of adipogenesis (19, 43). A recent study shows that TNFα induces expression of *Prrx1a*, *Prrx1b,* and *Prrx2* in C3H10T1/2 cells and in MC3T3 preosteoblasts and that increased Prrx1 expression contributes to the ability of TNFα to suppress differentiation of these osteoblast progenitors (44). Similarly, Wnt7b targets *Prrx1* in vascular smooth muscle cells (45). Based on these findings, we postulated that TNFα or Wnt ligands might also induce *Prrx* expression in preadipocytes to inhibit adipogenesis. Consistent with a previous study (18), we found that TNFα markedly suppressed *Cebpa* in preadipocytes (Fig. 5, *A* and *B*). In contrast, TNFα did not affect expression of *Prrx1a*, *Prrx1b* or *Prrx2* in these cells (Fig. 5, *A* and *B*). Moreover, neither knockdown of Prrx1 nor stable expression of Prrx1a, Prrx1b, or Prrx2 affected the ability of TNFα to suppress *Cebpa* in preadipocytes or inhibit adipogenesis (data not shown). Equally, Prrx1 knockdown did not alter nuclear translocation of β-catenin in 3T3-L1 preadipocytes and exogenous Prrx expression did not affect Wnt3a-induced anti-adipogenesis (data not shown). Thus, neither Prrx1 nor Prrx2 appear to be involved in the inhibition of adipogenesis by TNFα or Wnt3a.

Adipose Tissue Expression of Prrx1a and Prrx1b Increases with Obesity and Correlates with Tgfb3 in a Strain-specific Manner—During obesity, adipose tissue or primary adipocytes have increased expression of TGFβ (46) and decreased expression of adipogenic genes (47). Given that Prrx1 knockdown is associated with decreased TGFβ ligand expression and increased adipogenic gene expression, we postulated that WAT expression of *Prrx1a* and/or *Prrx1b* might increase during obesity. To test this hypothesis, we fed C57BL/6J and C3H/HeJ mice a chow (control) or HFD from 3 to 15 weeks of age, resulting in development of obesity (Fig. 6*A*). We then used qPCR to determine the effect of obesity on gene expression in inguinal WAT. Consistent with previous studies (48, 49), transcripts for leptin and *Pparg2* mRNA were markedly up-regulated in inguinal WAT with obesity (Fig. 6*B*). Obesity in C3H mice was also associated with significant down-regulation of *Fasn* and *Scd1* (Fig. 6*B*), but these changes were not observed in BL6 mice (Fig. 6*B*). Given that both *Fasn* and *Scd1* are induced by insulin in adipocytes (50, 51), these findings suggest impaired insulin action in inguinal WAT of C3H mice, but not yet in the BL6 mice. In contrast to *Fasn* and *Scd1*, obesity was associated with increased expression of *Prrx1a, Prrx1b,* and *Tgfb3* in BL6 mice but not in C3H mice (Fig. 6*B*). Moreover, there was a significant positive correlation between expression of *Tgfb3* and *Prrx1a* or *Prrx1b* in BL6 mice but not in C3H mice (Fig. 6*C*). We also analyzed expression of *Prrx2*, *Tgfb1,* and *Tgfb2* in these samples; however, these transcripts were not detectable under our conditions (data not shown). These observations demonstrate that *Prrx1a* and *Prrx1b* are increased in adipose tissue of BL6 mice prior to the onset of insulin resistance and suggest that up-regulation of *Prrx1a* and/or *Prrx1b* may contribute to increased WAT expression of *Tgfb3* during obesity.

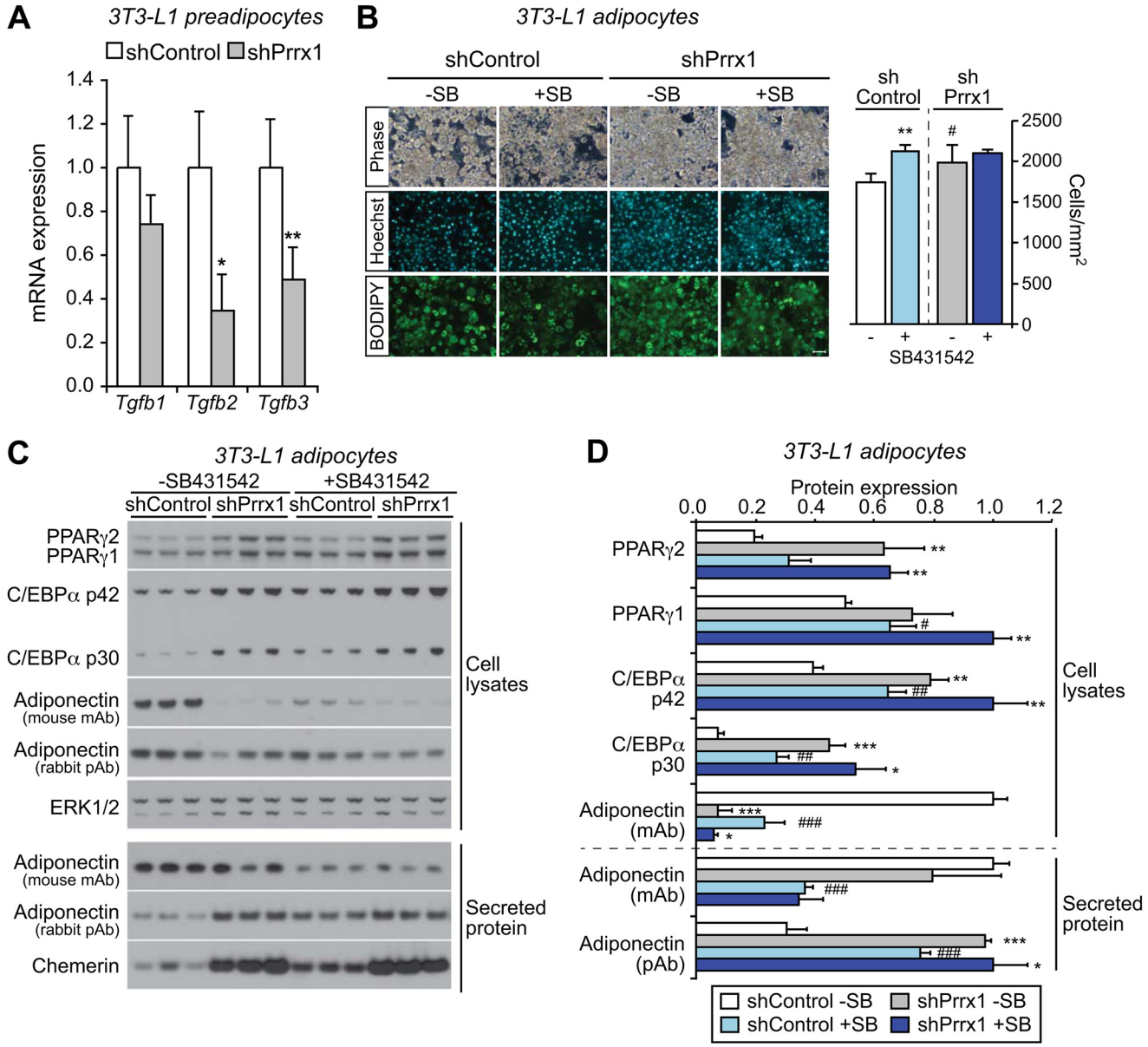

FIGURE 4. **Inhibition of TGFβ signaling phenocopies effects of Prrx1 knockdown on adipogenesis.** *A,* 3T3-L1 preadipocytes expressing shControl or shPrrx1 were grown to 2 days post-confluence. Total RNA was isolated and expression of *Tgfb1*, *Tgfb2* and *Tgfb3* was analyzed by qPCR. Transcript expression was normalized to *Tbp* mRNA and is presented as mean ± S.D. of at least three independent experiments. *B* and *D,* shControl and shPrrx1 3T3-L1 preadipocytes were induced to differentiate into adipocytes with MDI in the absence or presence of the TGFβ receptor inhibitor SB431542 (*SB*) (3 μM). Effects on adipogenesis were assessed at day 8 post-induction by fluorescent microscopy (*B*) and immunoblotting (*C*). Micrographs in *B* show live or stained adipocytes through a ×20 objective; Hoechst-stained cells are shown with hue adjusted to aid visualization. *Scale bar,* 50 μm. Cell density was quantified as described for Fig. 2*D* and is presented as mean ± S.D. of five micrographs. Immunoblots and micrographs are representative of at least three independent experiments. *D,* quantification of protein expression from *C*. Protein expression was normalized to ERK1 (p44) and is presented relative to maximum levels of each protein as mean ± S.D. of biological triplicates from *C*. Significant differences in transcript or protein expression are indicated as follows: shControl *versus* shPrrx1, *, $p < 0.05$; **, $p < 0.01$; ***, = $p < 0.001$. −SB431542 *versus* + SB431542, #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

DISCUSSION

Prrx1 as a Regulator of Adipogenesis—Previous reports have suggested that Prrx1 regulates mesenchymal cell fate, including myogenesis, osteoblastogenesis, and chondrogenesis (11, 13–16, 44, 52). However, this study is the first to demonstrate that Prrx1 regulates adipocyte differentiation and biology. Our findings suggest that Prrx1a and Prrx1b stimulate TGFβ signaling and suppress PPARγ activity, thereby restraining expression of adipocyte genes.

A previous report identifies Prrx1a as a transcriptional activator but Prrx1b as a transcriptional repressor (9). In contrast, we find similar regulation of *Prrx1a* and *Prrx1b* expression during 3T3-L1 adipogenesis and in WAT during obesity and that both Prrx1a and Prrx1b suppress PPARγ activity. Thus, our observations suggest that Prrx1a and Prrx1b have similar functions in the context of adipocyte biology. One possibility is that functional characteristics of Prrx1a and Prrx1b are cell type-specific. Indeed, our experiments were done in 3T3-L1 and HEK293T cells and WAT, whereas the previous study relied on NIH3T3 fibroblasts and C2C12 myoblasts (9). Whether Prrx1a and Prrx1b differentially impact adipogenesis or adipose tissue biology also remains unclear because both isoforms are sup-

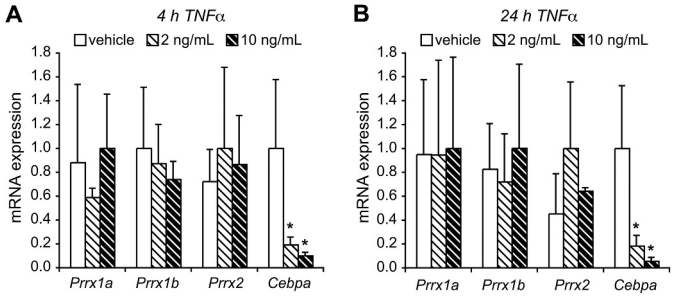

FIGURE 5. **TNFα does not inhibit adipogenesis through Prrx1.** 3T3-L1 preadipocytes at 2 days post-confluence were treated with vehicle control (PBS) or with TNFα at the indicated concentrations. After 4 h (*A*) or 24 h (*B*), total RNA was isolated and expression of *Cebpa*, *Prrx1a*, *Prrx1b* and *Prrx2* was analyzed by qPCR. Transcript expression was normalized to 18 S rRNA and is presented relative to the maximum expression of each transcript as mean ± S.D. of four biological replicates from one experiment, representative of two independent experiments. Statistical significance compared with vehicle treatment is indicated by * ($p < 0.05$).

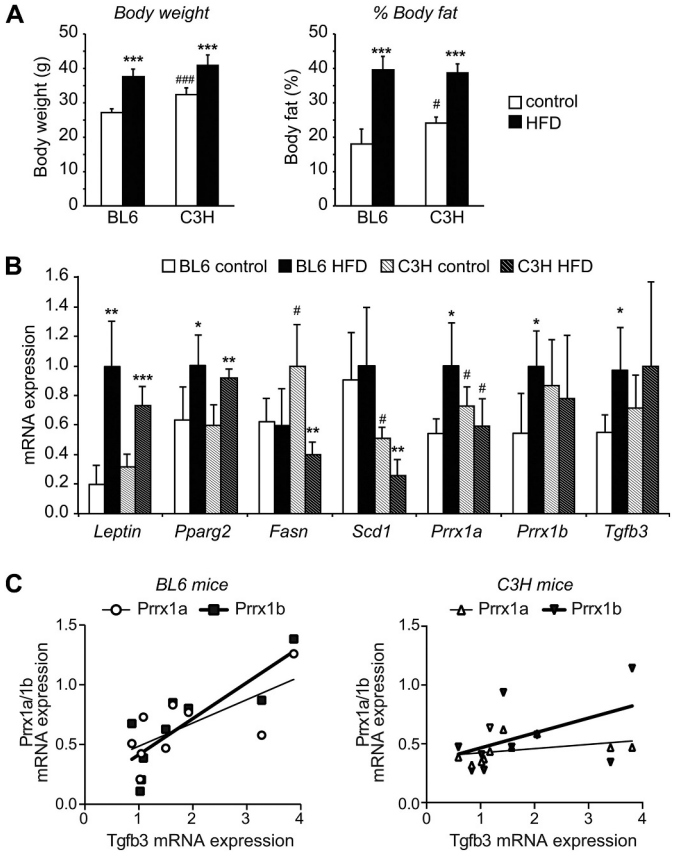

FIGURE 6. **Adipose tissue expression of *Prrx1a* and *Prrx1b* increases with obesity and correlates with *Tgfb3* in a strain-specific manner.** Male C57BL/6J (BL6) or C3H/HeJ (*C3H*) mice were fed normal chow (*control*) or HFD from 3 to 15 weeks of age. *A,* at 15 weeks mice were weighed and % body fat was determined by dual-energy x-ray absorptiometry. *B* and *C,* total RNA was isolated from inguinal WAT of 15-week-old mice. Expression of *Leptin, Pparg2, Fasn, Scd1, Prrx1a, Prrx1b* and *Tgfb3* was analyzed by qPCR and normalized to *Tbp* mRNA. Body weights and % body fat in *A* and transcript expression (relative to maximum for each transcript) in *B* and *C* are presented as mean ± S.D. of 4–5 mice. Significant differences are indicated as follows: control *versus* HFD, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. BL6 *versus* C3H/HeJ, #, $p < 0.05$; ###, $p < 0.001$. *C,* expression of *Prrx1a* or *Prrx1b* significantly correlates with *Tgfb3* expression in inguinal WAT of BL6 mice but not C3H/HeJ mice. For each correlation, R^2 and p values are as follows: BL6, *Prrx1a versus Tgfb3* ($R^2 = 0.4768$, $p = 0.0395$); BL6, *Prrx1b versus Tgfb3* ($R^2 = 0.6860$, $p = 0.0058$); C3H/HeJ, *Prrx1a versus Tgfb3* ($R^2 = 0.1568$, $p = 0.2574$); C3H/HeJ, *Prrx1b versus Tgfb3* ($R^2 = 0.2354$, $p = 0.1552$).

pressed in our shPrrx1 cells. Although the minor differences in *Prrx1a* and *Prrx1b* transcripts may prove difficult to target using shRNAs, individual knockdown of each isoform in preadipocytes and/or adipocytes would clearly be informative. Future studies should explore this approach.

Enforced expression of inhibitors of adipogenesis is often sufficient to block adipocyte differentiation (19). Thus, considering the strong effects of Prrx1 knockdown, we were surprised to observe no consistent phenotype with stable expression of *Prrx1a, Prrx1b,* or *Prrx2*. These exogenous transcripts are clearly capable of translation to functional protein, because the same vectors yield detectable Prrx1a and Prrx1b protein following transient transfection into HEK293T cells (data not shown). One possibility is that Prrx1a and Prrx1b must both be up-regulated to affect 3T3-L1 adipogenesis, such that enforced expression of *Prrx1a* or *Prrx1b* alone has no effect. Another possible explanation is that, in 3T3-L1 preadipocytes, endogenous expression of *Prrx1a, Prrx1b,* and *Prrx2* already exceeds the threshold for full activation, such that further increases are ineffective. Indeed, the fold increases in *Prrx* expression between EV- and Prrx-expressing cells are only 2.5–4.5; these are very low considering that our Prrx-expression constructs utilize high-level expression from the porcine cytomegalovirus promoter (53). Moreover, qPCR cycle thresholds for each of these endogenous transcripts in preadipocytes are typically 19–21, which under our assay conditions indicates very high expression. In this light it is surprising that endogenous or exogenous Prrx1a or Prrx1b proteins are undetectable by immunoblotting in any of our 3T3-L1 cell lines (data not shown). We believe this discrepancy relates to limited functionality of the currently available Prrx1 antibody. Indeed, even with transient overexpression in HEK293T cells, exogenous Prrx1a and Prrx1b proteins are still barely detectable, and only in nuclear lysates (data not shown). Thus, improved antibodies should be developed to aid future study of Prrx1. Despite these issues, our immunoblots do confirm greater absolute expression of Prrx1a and Prrx1b after transient transfection compared with stable expression; this may explain why effects of exogenous Prrx expression are observed in HEK293T cells but not in 3T3-L1 preadipocytes.

Differential Detection of Adiponectin Protein—A curious finding of our study is that detection of adiponectin by immunoblotting differs depending on the primary antibody used. Our initial results using the mouse mAb show strikingly decreased adiponectin expression, but not secretion, in shPrrx1 adipocytes (Figs. 2*F* and 4*C*). We spent many months investigating this phenomenon before eventually discovering very different results using the rabbit pAb. We believe that these discrepancies derive from differential sensitivity of the two antibodies to post-translational modification of adiponectin. Thus, the rabbit pAb is raised against the globular domain of adiponectin, which is not post-translationally modified (54). In contrast, the mouse mAb is raised against full-length adiponectin. This includes the collagenous domain, which is extensively modified post-translationally to facilitate adiponectin multimerization and secretion (54). Considering the enhanced adiponectin secretion from shPrrx1 adipocytes (Figs. 2*F* and 4*C*), these cells likely have increased post-translational modification

of adiponectin compared with shControl adipocytes. We believe these modifications impair binding of adiponectin by the mouse mAb but not by the rabbit pAb. Thus, the rabbit pAb can detect both modified and unmodified adiponectin, whereas the mouse mAb cannot. Although this capacity for differential detection may prove useful, our observations establish that choice of primary antibody must be carefully considered for reliable detection of total adiponectin protein.

TGFβ as a Target of Prrx1 in Adipogenesis and Adipose Tissue—Several previous studies support our conclusion that Prrx1 regulates TGFβ ligand expression in preadipocytes and thereby modulates adipogenesis. For example, TGFβ ligand expression decreases during 3T3-L1 adipogenesis (28, 55) and is lower in adipocytes relative to SVF of WAT (29). Given that knockdown of *Prrx1* suppresses *Tgfb2* and *Tgfb3* in preadipocytes, it is possible that down-regulation of *Prrx1a* and *Prrx1b* contributes to decreased TGFβ ligand expression during adipogenesis. At least one study shows that Prrx1a binds to and transactivates the *Tgfb3* promoter, thereby inducing *Tgfb3* expression in hepatic stellate cells (42). Consistent with these observations, in some experiments we detected increased *Tgfb2* and *Tgfb3* in 3T3-L1 preadipocytes stably expressing *Prrx1a, Prrx1b,* or *Prrx2* (data not shown); however, this effect was not routinely observed across all experiments. Nevertheless, the strong correlation between *Tgfb3* and *Prrx1a* or *Prrx1b* in BL6 WAT further supports the notion that Prrx1a and/or Prrx1b regulate *Tgfb3* expression in adipose tissue. Additionally, inhibition of endogenous TGFβ signaling through expression of dominant-negative TGFβ receptor II results in accelerated adipogenesis, with increased expression of *Pparg, Cebpa*, and *Fapb4* transcripts but no increases in *Cebpb* or *Cebpd* (56). These effects are similar to those of Prrx1 knockdown, further supporting the conclusion that knockdown of Prrx1 enhances adipogenesis, in part, through suppression of endogenous TGFβ signaling.

Prrx1 as an Effector of Other Adipogenic Regulators—We demonstrate that Prrx1a or Prrx1b is not required for inhibition of adipogenesis by TNFα or Wnt3a. Nevertheless, other signaling pathways might target Prrx1 to regulate adipogenesis. For example, bone morphogenetic protein-2 (BMP-2) down-regulates expression of Prrx1 in osteoblast precursors, whereas parathyroid hormone-related protein up-regulates Prrx1 in these cells (13). These effects may contribute to the ability of BMP-2 to promote preadipocyte commitment (4, 57) or to inhibition of adipogenesis by parathyroid hormone-related protein (58). Thus, future studies should investigate whether Prrx1 acts downstream of these or other adipogenic regulators.

Effects of Prrx1 on Adipose Tissue in Vivo—Our conclusions are based largely on experiments in cell models. Whether Prrx1 also impacts adipocyte formation or function *in vivo* remains to be established. Microarray analyses suggest that, in mice, *Prrx1* is expressed most highly in osteoblasts, with lower expression in WAT (59, 60). In contrast, among human tissues *Prrx1* expression is highest in WAT (59), and this increases with obesity in humans (61). The latter observation is consistent with our findings in WAT of BL6 mice (Fig. 6). *Prrx1* expression also increases in subcutaneous WAT after fat loss during bariatric surgery (62). Together, these data suggest that altered metabolic states impact WAT *Prrx1* expression in both mice and humans. Unfortunately, mice lacking *Prrx1* die postnatally (16), and whether such mice have altered WAT development has not been reported. Therefore, a better approach would be to generate mice with adipose tissue-specific knock-out of *Prrx1*, for example using targeted embryonic stem cells available through the International Knock-out Mouse Consortium (63). If such mice were viable they would allow more thorough investigation of Prrx1 in WAT formation and function *in vivo*. Considering our present findings showing the ability of Prrx1 to modulate adipogenesis and PPARγ activity, such *in vivo* studies are clearly warranted.

In conclusion, we have identified Prrx1 as an endogenous regulator of adipocyte function that is down-regulated during adipocyte differentiation but increases in WAT with obesity in BL6 mice. Prrx1 knockdown enhances adipogenesis, at least in part, by reducing *Tgfb2* and *Tgfb3* expression and thereby suppressing TGFβ signaling during adipogenesis. Moreover, up-regulation of *Prrx1a* or *Prrx1b* inhibits PPARγ activity and may contribute to increased WAT expression of *Tgfb3* during obesity, before the onset of insulin resistance. Future studies should address whether Prrx1 impacts adipose tissue formation and function *in vivo* and in the context of obesity and other metabolic diseases.

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