Supplementary Methods

Generation of Prx 3 knockout mice

The mouse genomic DNA obtained from 129/SvJ mouse J1 embryonic stem cells was screened by PCR using two-sets of primers to isolate a 2.7kb *NotI-XhoI* fragment derived from *Prx3* gene as the 5′ short arm: the forward primer linked to *NotI* (5′ gcggccgcctgcatataagtatcgtaggt 3′); the reverse primer linked to *XhoI* (5′ ctcgagtttatgcaaagaggc 3′) and a 4.6kb *XbaI-XbaI* fragment derived from *Prx3* gene as the 3′ long arm: the forward primer (5′ accttctagaacctggcttat 3′); the reverse primer (5′gcctcttcatctagataaaag 3′). We constructed a targeting vector for deleting a segment contained sequence of region of *Prx3* gene to exon 1 from exon 4 (~5.1 kb), using 5′_2.7 kb short arm fragment and 3′_4.6 kb long arm fragment ligated into the pPNT vector. A targeting vector was designed to replace a ~5.1 kb genomic fragment containing a segment of region of *Prx3* gene to exon1 from exon 4 and positive selection marker: PGK promoter and neomycin resistance gene. Negative selection marker: HSV-1 promoter driven thymidine kinase gene was appended to the construct to select against nonhomologous recombination. The targeting vector was linearized with *Not*I and electroporated into 129/SvJ mouse J1 embryonic stem cells. Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blotting. The *Prx3* gene was modified in 2 of 466 clones screened. Two clones containing the targeted mutation were injected into C57BL/6 blastocysts, and these were subsequently transferred into pseudopregnant foster mothers. The resulting male chimeric mice were bred with C57BL/6 females to obtain heterozygous *Prx3* mice. Germ-line transmission of the mutant allele was verified by Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Interbreeding of the heterozygous mice was performed to generate homozygous *Prx3*-deficient mice.

3T3-L1 adipocytes

3T3-L1 preadipocytes obtained from the ATCC (Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (complete medium) at 37°C in humidified 5% CO_2 atmosphere. Two-day post-confluence cells (day 0) were incubated in the complete medium supplemented with 1 μM dexamethasone, 5 μg/mL insulin, and 10 μM rosiglitazone. At the end of day 2, the medium was replaced with complete medium supplemented with 5 μg/mL insulin. Starting on day 4, the medium was replaced with the complete medium every 2 days until further analysis. Preadipocytes and mature adipocytes were photographed with EVOS^{TM} microscope (AMG, Mill Creek, WA) and lipid droplets in mature adipocytes were visualized using Oil Red O (Supplementary Fig. S9). For transfection of 3T3-L1 cells, siRNA oligonucleotides specific for Prx3 mRNA were synthesized at ST Pharm (Seoul, Korea). The sequences are as follows: sense, 5′- UGACAAAGCCAAUGAAUUUdTdT-3′; antisense, 5′-AAAUUCAUUGGCUUUGUCAdTdT-3′. Scrambled siRNA (Bioneer, Daejeon, Korea) was used as a negative control. To induce gene silencing before differentiation, cells were grown until 60%–70% confluent and 50 nM siRNA was transfected into 3T3-L1 cells using RNAiMAX (Invitrogen) according to the manufacturer's protocol. 24 h later, the medium was replaced with DMEM supplemented with 10% calf serum, and the cells were incubated for additional 24 h until they were confluent. The medium was then replaced with that containing the conventional hormonal stimuli (with 0.5 mM 3-isobutylmethylxanthine and without rosiglitazone). To induce gene silencing in mature 3T3-L1 adipocytes, differentiated 3T3-L1 adipocytes were trypsinized and seeded in 6-well plates with medium containing 100 nM siRNA and RNAiMAX and used 48 h after transfection. For Prx3 overexpression, 5 μg pcDNA 3.1-Prx3 was transiently transfected using Lipofectamine LTX Plus (Invitrogen) and used 48 h after transfection.

2D-PAGE

Cultured cells and isolated adipocytes were harvested and solubilized in buffer containing 8.4 M urea, 2.4 M thiourea, 5% CHAPS, 1.6% pharmalyte 5-7, 0.4% pharmalyte 3-10, 50 mM DTT, protease inhibitor cocktail, and 5 mM Na_3VO_4. Equal amounts of proteins (200 μg for cultured cells and 125 μg for isolated adipocytes) were separated, detected, and quantitatively analyzed as described previously (1, 3). The proteins, whose expression levels were altered by at least 1.5-fold in triplicate experiments, were chosen for further analysis.

Protein identification using tandem mass spectrometry analysis

The protein spots separated on 2D-PAGE were excised, destained in solution containing 30 mM potassium ferricyanide/100 mM sodium thiosulfate, washed to remove the destaining reagent and digested with trypsin (Promega, Madison, WI) as described previously (3). Peptide sequencing was performed by nanoAcquity™ UPLC™/ESI-q-TOF tandem MS (SYNAPT™ HDMS™, Waters, London, UK) as described previously (1).

Gene expression analysis

Total RNA was extracted from 3T3-L1 preadipocytes, mature adipocytes, and isolated adipocytes using Trizol (Invitrogen) according to the standard protocol. mRNA expressions were measured by real-time PCR using ABI7300 (Applied Biosystems, Carlsbad, CA) with 20 μL reaction volume consisting of cDNA transcripts, primer pairs, and SYBR Green PCR Master Mix (Applied Biosystems). Quantifications were normalized to 18S in each reaction. The sequences for the primers are listed in Supplementary Table S2.

Western blot analysis

3T3-L1 cells or isolated adipocytes were lysed in TGN buffer (2), separated by SDS-PAGE and transferred to PVDF membrane for 45 min and probed with various antibodies (cofilin 1, pyruvate carboxylase, PCNA, PAI-1, prohibitin, GAPDH, and α-tubulins: Santa Cruz Biotechnology, Santa Cruz, CA; Prx1, Prx3, Prx5, and Prx3-SO_3: AbFrontier, Seoul, Korea, Hsp70/Hsc70: Stressgen, San Diego, CA; Hsp60:

Epitomics, Burlingame, CA; adiponectin: Adipogen, Seoul, Korea; aP2, C/EBPα, and PPARγ: Cell Signaling Technology, Danvers, MA). The blots were detected using LAS-3000 (Fuji photo film, Tokyo, Japan).

Histology and immunohistochemistry

Epididymal adipose tissues from WT and Prx3 KO mice were fixed in 10% formalin, dehydrated, and embedded in paraffin. Five micron sections were used for hematoxylin and eosin (H&E) staining. For quantification, the number of adipocytes per microscope picture was divided by the area of the picture (mm^2). For each mouse, four random pictures were used. For immunohistochemistry, anti-nitrotyrosine (1:100, Santa Cruz Biotechnology) and anti-Hsp 60 (1:100, Epitomics) antibodies were used. Stained sections were counterstained with Mayer's hematoxylin solution.

Mitochondrial isolation

Subcutaneous, epididymal, and omental fat pads of WT and Prx3 KO mice were homogenized, washed in 10 m*M* Tris-HCl, and lysed with 300 m*M* sucrose, and centrifuged at 700 *g* to remove nuclei, unbroken cells, and the lipid cake. Supernatant was centrifuged at 14,000 *g* to obtain mitochondria and the pellet was lysed with 2X Laemmli buffer for protein carbonylation assay.

WAT analysis of db/db mice and human subjects

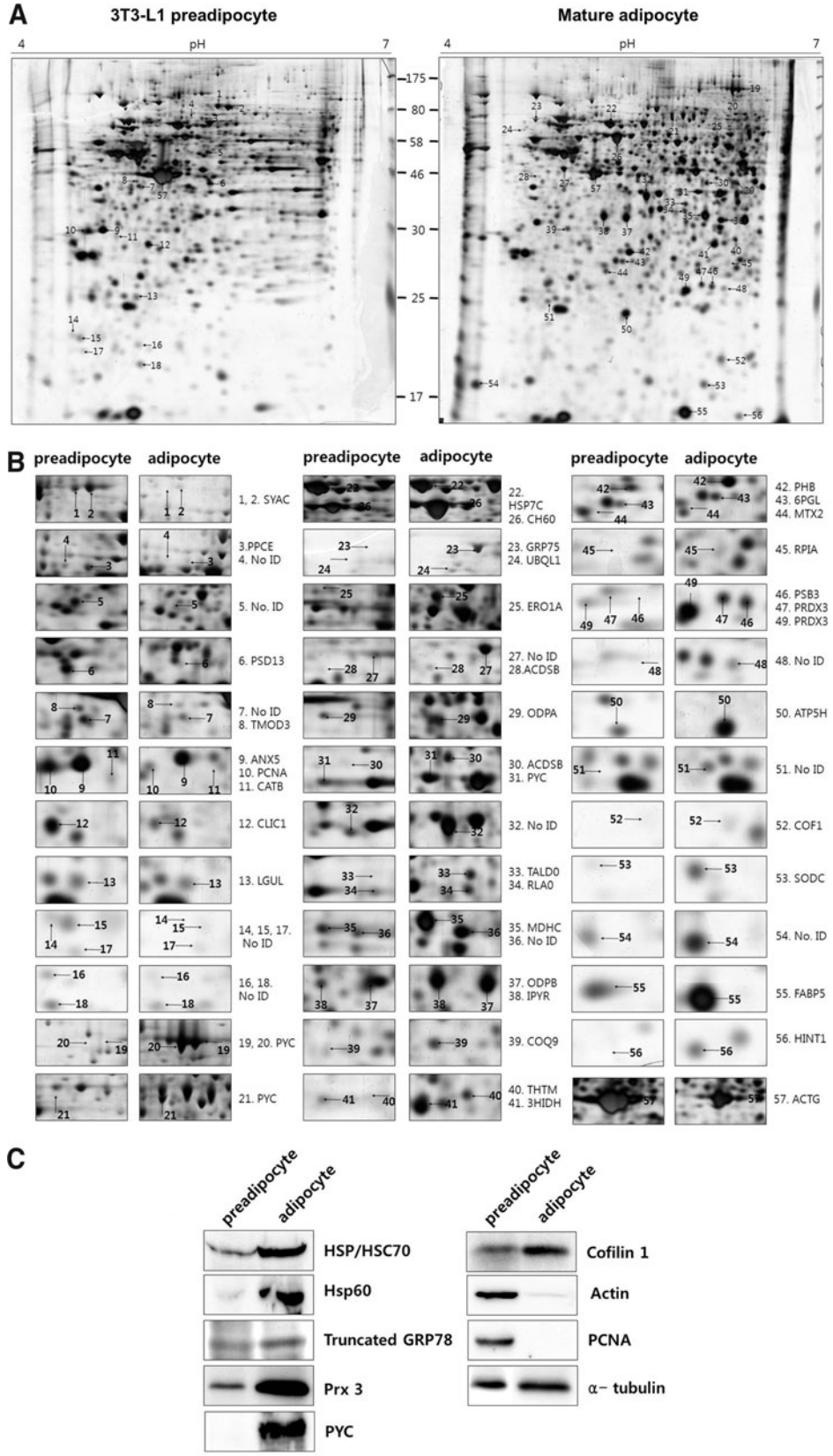
RNA was extracted from epididymal fat pads of *db/m* and *db/db* mice. Heart, liver, kidney, and WAT of *db/m* and *db/db* mice were lysed and separated by SDS-PAGE as described above. Human peri-renal fat tissues were obtained from redundant soft tissues attached to the extracted donor kidneys for 28 living donor kidney transplantation.

Statistics

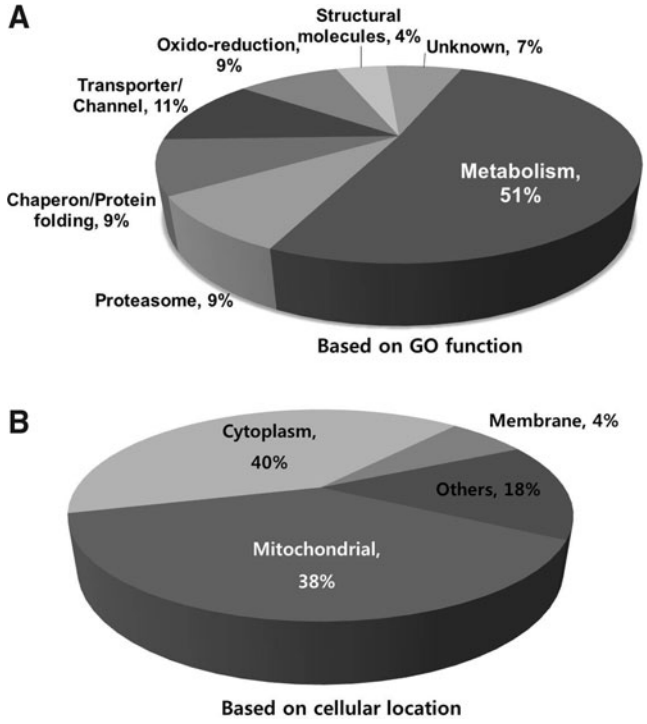
Data are presented as mean ± S.E. Mean values obtained from each group were compared by ANOVA with subsequent Fisher's significant difference method. Nonparametric analyses were also used where appropriate. P value of < 0.05 was used as the criterion for a statistically significant difference.

References

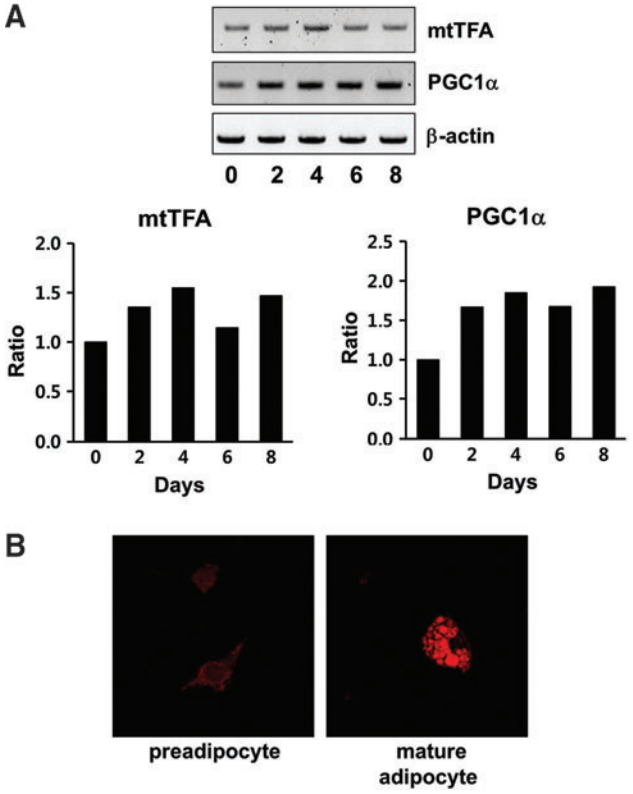
1. Seo J, Jeong J, Kim YM, Hwang N, Paek E, and Lee KJ. Strategy for comprehensive identification of post-translational modifications in cellular proteins, including low abundant modifications: application to glyceraldehyde-3-phosphate dehydrogenase. *J Proteome Res* 7: 587–602, 2008.
2. Seo JB, Moon HM, Noh MJ, Lee YS, Jeong HW, Yoo EJ, Kim WS, Park J, Youn BS, Kim JW, Park SD, and Kim JB. Adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c regulates mouse adiponectin expression. *J Biol Chem* 279: 22108–22117, 2004.
3. Song EJ, Yim SH, Kim E, Kim NS, and Lee KJ. Human Fas-associated factor 1, interacting with ubiquitinated proteins and valosin-containing protein, is involved in the ubiquitin-proteasome pathway. *Mol Cell Biol* 25: 2511–2524, 2005.



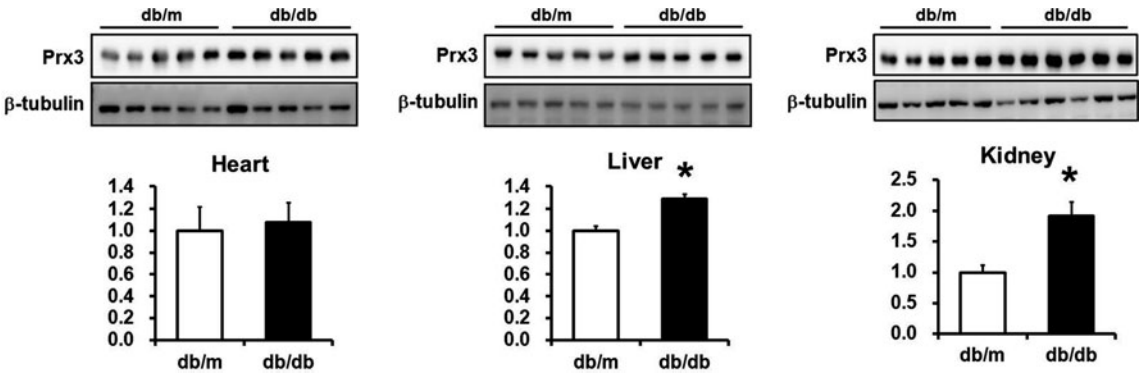
SUPPLEMENTARY FIG. S1. Protein expression profiles of 3T3-L1 preadipocytes and differentiated mature adipocytes. **(A)** Representative 2-DE gel images of 3T3-L1 preadipocytes and mature adipocytes. Cell lysates were separated on 2D-PAGE (pH 4–7), detected with silver staining, and semi-quantitatively analyzed with image analysis. Differentially expressed proteins were excised and analyzed by in-gel digestion and UPLC-ESI-q-TOF tandem MS. **(B)** Enlarged image of each spot identified by tandem MS. **(C)** Differentially expressed proteins were confirmed by Western blot analysis.



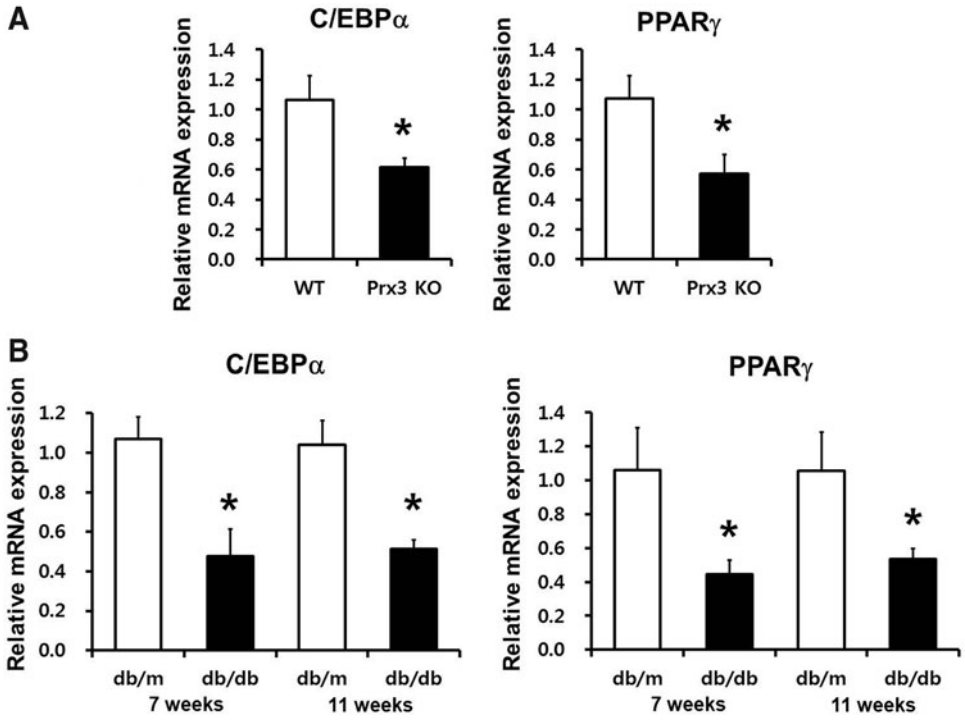
SUPPLEMENTARY FIG. S2. Identified proteins in preadipocytes and mature 3T3-L1 adipocytes were classified based on (A) biological function and (B) cellular location using gene ontology.



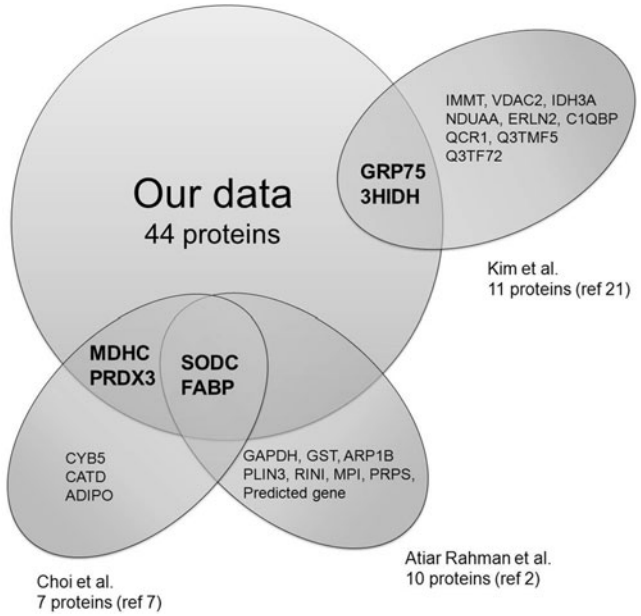
SUPPLEMENTARY FIG. S3. Mitochondrial biogenesis during 3T3-L1 adipocyte differentiation. (A) RT-PCR results of mtTFA and PGC1α during 3T3-L1 differentiation. *β*-Actin was used as an internal control. **(B)** Mitochondria staining of 3T3-L1 preadipocytes and mature adipocytes were performed with MitoTracker Red CMS Ros and examined using a confocal microscope.



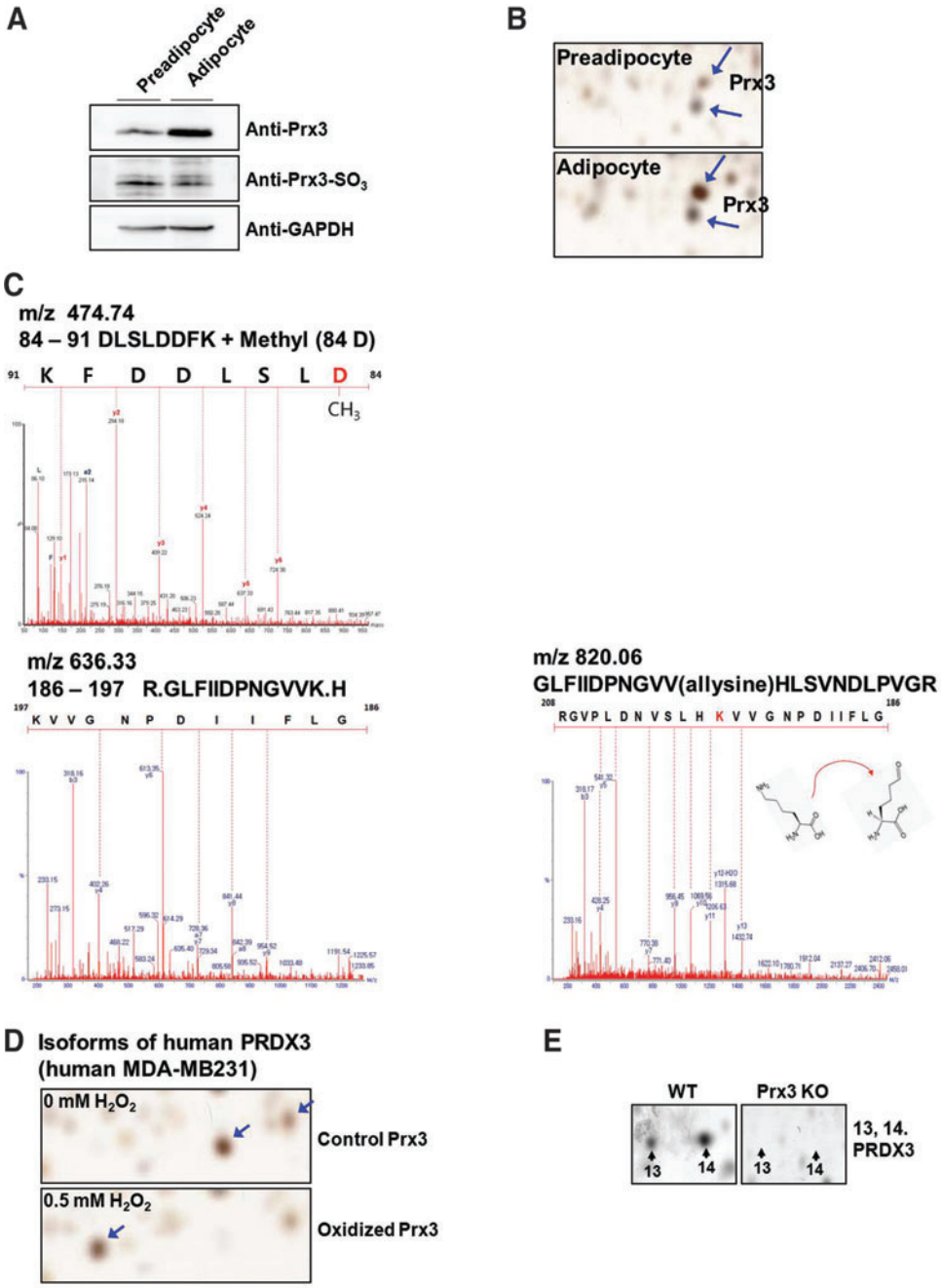
SUPPLEMENTARY FIG. S4. Prx3 is differentially expressed in various tissues. Prx3 protein expression levels in heart, liver, and kidney of *db/m* and *db/db* mice. Values were normalized to tubulin. Values are means ± SE of 5–6 mice per group. *$p < 0.05$ vs. *db/m*.



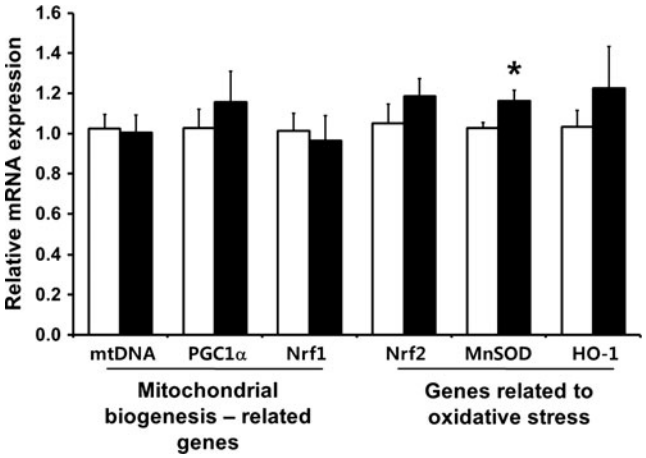
SUPPLEMENTARY FIG. S5. Adipogenic genes were downregulated in Prx3 KO mice and *db/db* mice adipocytes. C/EBPα and PPARγ levels were examined by real-time PCR in **(A)** WT and Prx3 KO mice, and **(B)** *db/m* and *db/db* mice white adipose tissues. Quantifications were normalized to 18S in each reaction. Values are means ± SE of 4–5 mice per group. *$p < 0.05$ vs. WT or *db/m*.



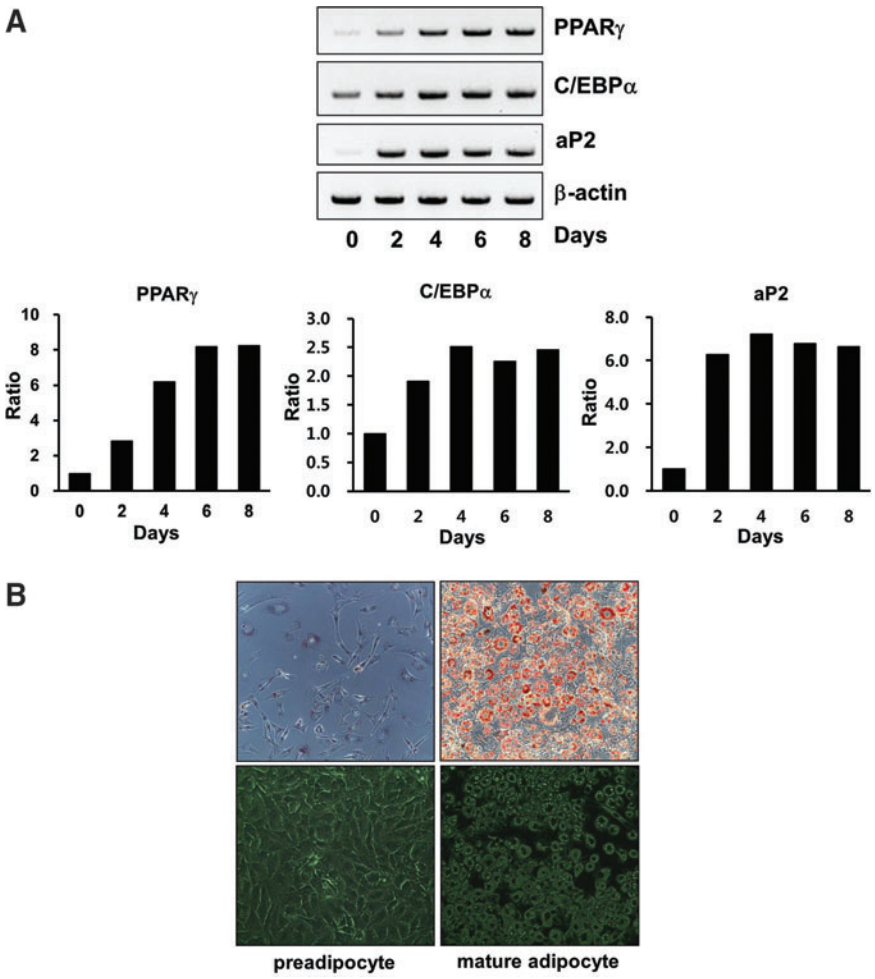
SUPPLEMENTARY FIG. S6. Venn diagram illustrating the overlap in proteins identified in adipogenic-differentiated cells in various proteomic methodologies.



SUPPLEMENTARY FIG. S7. Post-translational modification of Prx3. (A) Prx3 expression and its oxidation product Prx3-SO_3 in preadipocytes and adipocytes were detected by Western analysis using anti-Prx3 and anti-Prx3-SO_3 antibodies. **(B)** Comparison of Prx3 spots between preadipocytes and adipocytes on 2D-PAGE. **(C)** MS/MS spectra of modified peptides of Prx3. **(D)** 2D-PAGE of control and oxidized Prx3 in human MDA-MB231 cells. **(E)** 2D profiling of Prx3 in WT and Prx3 KO mouse adipocytes. Spot 13 is oxidized Prx3, and spot 14 is control Prx3.



SUPPLEMENTARY FIG. S8. Liver mRNA levels were similar between wild-type and Prx3 KO mice. Genes related to mitochondrial biogenesis and oxidative stress were examined by real-time PCR. Quantifications were normalized to 18S in each reaction. Values are means ± SE of 5–7 mice per group. $*p < 0.05$ vs. WT.



SUPPLEMENTARY FIG. S9. Differentiation of 3T3-L1 preadipocytes. (A) RT-PCR results of PPARγ, C/EBPα, and aP2 during 3T3-L1 differentiation. β-Actin was used as an internal control. **(B)** Representative picture of 3T3-L1 preadipocytes and mature adipocytes. The images were captured by EVOS™ light microscope. Lipid droplets were stained with Oil Red O.

Supplementary Table S1A. Upregulated Proteins in Mature 3T3-L1 Adipocytes

No.	Score	%Cov.	Mr	pI	Accession No.	Symbol	Protein Name	Cellular Component
Proteins that function in metabolism								
19	2.40E+17	40	129685.7	6.3	Q05920	PYC	Pyruvate carboxylase, mitochondrial precursor	Mito
20	1.01E+11	31	129685.7	6.3	Q05920	PYC	Pyruvate carboxylase, mitochondrial precursor	Mito
21	160	7	129685.7	6.3	Q05920	PYC	Pyruvate carboxylase, mitochondrial precursor	Mito
28	6.46E+03	24	47874.5	8.0	Q9DBL1	ACDSB	Acyl-CoA dehydrogenase, short/branched chain specific, mitochondrial precursor	Mito
29	6.52E+05	36	43232.1	8.5	P35486	ODPA	Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial precursor	Mito
30	4.37E+03	23	102536.1	5.8	O08528	HXK2	Hexokinase type II	Mito
31	129602	14	129685.7	6.3	Q05920	PYC	Pyruvate carboxylase, mitochondrial precursor	Mito
34	7.25E+05	39	34216.7	5.9	P14869	RLA0	60S acidic ribosomal protein P0	Ribo
35	4.89E+05	41	36346.3	6.2	P14152	MDHC	Malate dehydrogenase, cytoplasmic	Cyto
37	4.59E+10	61	38937.5	6.4	Q9D051	ODPB	Pyruvate dehydrogenase E1 component beta subunit, mitochondrial precursor	Mito
38	3.07E+04	41	32667.4	5.4	Q9D819	IPYR	Inorganic pyrophosphatase	Cyto
39	112	10	35061	4.9	Q8K1Z0	COQ9	Ubiquinone biosynthesis protein COQ9, mitochondrial precursor	Mito
42	1.22E+05	59	29820.3	5.0	P67778	PHB	Prohibitin	Memb
43	8.14E+04	61	27254.7	5.6	Q9CQ60	6PGL	6-phosphogluconolactonase	Cyto
45	9.31E+03	42	26000	6.1	P47968	RPIA	Ribose-5-phosphate isomerase	
55	3.67E+09	84	15137.6	6.1	Q05816	FABP5	Fatty acid-binding protein, epidermal	Cyto
Proteins that relate with proteasome								
24	6.93E+03	16	61977	4.9	Q8R317	UBQL1	Ubiquilin 1	Cyto, Nu, Proteasome
46	3.08E+05	65	22965.1	6.2	Q9R1P1	PSB3	Proteasome subunit beta type 3	Cyto, Nu, Proteasome
Proteins that function in chaperone/mediator of protein folding								
22	1.20E+09	40	70871.6	5.4	P63017	HSP7C	Heat shock cognate 71kDa protein	Cyto
23	54	13	73483	5.9	P38647	GRP75	Stress-70 protein, mitochondrial precursor	Mito
26	5.53E+04	34	60956	5.9	P63038	CH60	60kDa Heat shock protein, mitochondrial precursor	Mito
33	69	8	72377	5.1	P20029	GRP78	78kDa glucose-regulated protein precursor	ER
Proteins that act as transporter/channel								
25	3.25E+03	23	54038.8	6.1	Q8R180	ERO1A	ERO1-like protein alpha precursor	ER
40	1.05E+07	48	32892.3	6.1	Q99J99	THTM	3-mercaptopyruvate sulfurtransferase	Cyto, Mito
44	1.79E+04	31	29758.4	5.4	O88441	MTX2	Metaxin 2	Mito
50	5.57E+05	65	18618.4	5.5	Q9DCX2	ATP5H	ATP synthase D chain, mitochondrial	Mito
Proteins that act in oxido-reduction								
41	2.50E+08	65	35440.3	8.4	Q99L13	3HIDH	3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor	Mito

(*continued*)

Supplementary Table S1A. (Continued)

No.	Score	%Cov.	Mr	pI	Accession No.	Symbol	Protein Name	Cellular Component
47	120	24	28109	7.2	P20108	PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	Mito
49	3.35E+04	56	28127.3	7.2	P20108	PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	Mito
53	2.78E+03	43	15811.6	6.0	P08228	SODC	Superoxide dismutase [Cu-Zn]	Cyto
Proteins that function in structural molecules								
52	112	20	18417	6.4	P18760	COF1	Cofilin-1	Cyto, CK, Nu
57	490	55	41766	5.3	P63260	ACTG	Alpha actin	
Unknown								
56	3.82E+03	52	13645.8	6.4	P70349	HINT1	Histidine triad nucleotide-binding protein 1	Cyto, Nu
No ID								
27, 32, 36, 48, 51, 54								

Supplementary Table S1B. Downregulated Proteins in Mature 3T3-L1 Adipocytes

No.	Score	%Cov.	Mr	pI	Accession No.	Symbol	Protein Name	
Proteins that function in metabolism								
1	1.21E+04	20	106909.5	5.45	Q8BGQ7	SYAC	Alany-tRNA synthetase	Cyto
2	1.21E+04	20	106909.5	5.45	Q8BGQ7	SYAC	Alany-tRNA synthetase	Cyto
8	1.42E+03	26	39503	5.02	Q9JHJ0	TMOD3	Tropomodulin-3	Cyto, CK
10	649	43	28785.1	4.66	P17918	PCNA	Proliferating cell nuclear antigen;Cyclin	Nu
13	7.87E+03	40	20678.6	5.25	Q9CPU0	LGUL	Lactoylglutathione lyase	
Proteins that relate with proteasome								
3	4.21E+10	38	80752.3	5.44	Q9QUR6	PPCE	Prolyl endopeptidase	Cyto
6	1.23E+06	59	42809	5.46	Q9WVJ2	PSD13	26S Proteasome non-AT-Pase regulatory subunit 13	Cyto, Nu, Memb
Proteins that act as transporter/channel								
12	9.62E+06	57	26881.9	5.09	Q9Z1Q5	CLIC1	Chloride intracellular channel protein 1	Memb,Cyto, Nu
Unknown								
9	5.72E+09	59	35752.7	4.83	P48036	ANXA5	Annexin A5	
11	4.77E+04	30	37280.2	5.56	P10605	CATB	Cathepsin B precursor	Lys
No ID								
4, 5, 7, 14, 15, 16, 17, 18								

Supplementary Table S2. Sequences of Primers

Gene name	*Forward*	*Reverse*
Mouse primers		
aP2	5′-ACATGATCATCAGCGTTAATGGG-3′	5′-TCATAACACATTCCACCACCAGC-3′
C/EBPα	5′-GAACAGCAACGAGTACCGGGTA-3′	5′-CCATGGCCTTGACCAAGGAG-3′
PPARγ	5′-GGTGAAACTCTGGGAGATTC-3′	5′-CAACCATTGGGTCAGCTCTT-3′
PGC1α	5′-TCGATGTGTCGCCTTCTTGC-3′	5′-ACGAGAGCGCATCCTTTGG-3′
mtTFA	5′-CGATTTTCCACAGAACAGCT-3′	5′-CCATCAGCTGACTTGGAGTT-3′
mtDNA	5′-CCACTTCATCTTACCATTTA-3′	5′-ATCTGCATCTGAGTTTAATC-3′
Nrf1	5′-CAACAGGGAAGAAACGGAAA-3′	5′-GCACCACATTCTCCAAAGGT-3′
Nrf2	5′-CTCTCTGAACTCCTGGACGG-3′	5′-GGGTCTCCGTAAATGGAAG-3′
MnSOD	5′-CCGAGGAGAAGTACCACGAG-3′	5′-GCTTGATAGCCTCCAGCAAC-3′
HO-1	5′-CACGCATATACCCGCTACCT-3′	5′-CCAGAGTGTTCATTCGAGCA-3′
Hsp60	5′-GTCCCTCACTCGCCGCAGAC-3′	5′-GATGAGGAGCCAGTGCCCGG-3′
Prx1	5′-CCAAGCGCACCATTGCTCAGGAT-3′	5′-GAGCGGCCAACGGGAAGATCG-3′
Prx3	5′-GCAGCTGCGGGAAGGTTGCT-3′	5′-TGCTGGGTGACAGCAGGGGT-3′
Prx5	5′-CCAAGGGAGCGCAGGTGGTG-3′	5′-GCCTTCTGCCTGGTGGGCTC-3′
Adiponectin	5′- AGGAGCTGAAGGGCCACGGG -3′	5′- TGGGAACAGTGACGCGGGTC-3′
PAI-1	5′-AGGGCTTCATGCCCCACTTCTTCA-3′	5′-AGTAGAGGGCATTCACCAGCACCA-3′
Leptin	5′-GGGAGCACCGTGAAGGCTGC-3′	5′-CCTGGCTGACCCCCAAAGCC-3′
FAS	5′-CCTGGATAGCATTCCGAACCT-3′	5′-GCACATCTCGAAGGCTACACA-3′
Lpl	5′-GCCGCGTAGTTCCAGCAGCA-3′	5′-CCCTCCTCGGAAGGCGGTCA-3′
ATGL	5′-CTCATTCGCTGGCTGCGGCT-3′	5′-CCCCAGTGACCAGCGCTGTG-3′
HSL	5′-TGGGGAGCTCCAGTCGGAAGAGG-3′	5′-CATTAGACAGCCGCCGTGCTG-3′
MCAD	5′-CAACACTCGAAAGCGGCTCA-3′	5′-ACTTGCGGGCAGTTGCTTG-3′
CPT1a	5′-ACCACTGGCCGCATGTCAAG-3′	5′-AGCGAGTAGCGCATAGTCAT-3′
β-actin	5′-ACCCACACTGTGCCCATCTA-3′	5′-GCCACAGGATTCCATACCCA-3′
18S	5′-CGAAAGCATTTGCCAAGAAT-3′	5′-AGTCGGCATCGTTTATGGTC-3′
Human primers		
Gpx3	5′-TGAAATCCCAGCCGCCTAGCGA-3′	5′-GCCACCGTCCGTCTGAGGTGT-3′
Prx3	5′-TGCCCGACATGTGAGTGCCAT-3′	5′-GCAGGTGCATGGCATGAGGAACTG-3′
mtDNA	5′-ACGACCTCGATGTTGGATC-3′	5′-GCTCTGCCATCTTAACAAACC-3′
28S	5′-TTAAGGTAGCCAAATGCCTCG-3′	5′-CCTTGGCTGTGGTTTCGCT-3′