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C1q/Tumor Necrosis Factor-related Protein 11 (CTRP11), a Novel Adipose Stroma-derived Regulator of Adipogenesis*

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Background: CTRP11 is a novel member of the C1q family with poorly defined function.
Results: CTRP11 inhibits 3T3-L1 adipocyte differentiation by inhibiting mitotic clonal expansion and adipogenic gene expression.
Conclusion: Adipose stroma-derived CTRP11 is a regulator of adipogenesis.
Significance: CTRP11 mediates potential paracrine cross-talk between adipocytes and cells of the stromal vascular compartment.

C1q/TNF-related proteins (CTRPs) are a family of secreted regulators of glucose and lipid metabolism. Here, we describe CTRP11, a novel and phylogenetically conserved member of the C1q family. Our studies revealed that white and brown adipose are major tissues that express CTRP11, and its expression is acutely regulated by changes in metabolic state. Within white adipose tissue, CTRP11 is primarily expressed by stromal vascular cells. As a secreted multimeric protein, CTRP11 forms disulfide-linked oligomers. Although the conserved N-terminal Cys-28 and Cys-32 are dispensable for the assembly of higher-order oligomeric structures, they are unexpectedly involved in modulating protein secretion. When co-expressed, CTRP11 forms heteromeric complexes with closely related CTRP10, CTRP13, and CRF (CTRP14) via the C-terminal globular domains, combinatorial associations that potentially generate functionally distinct complexes. Functional studies revealed a role for CTRP11 in regulating adipogenesis. Ectopic expression of CTRP11 or exposure to recombinant protein inhibited differentiation of 3T3-L1 adipocytes. The expression of peroxisome proliferator-activated receptor-γ and CAAT/enhancer binding protein-α, which drive the adipogenic gene program, was markedly suppressed by CTRP11. Impaired adipogenesis was caused by a CTRP11-mediated decrease in p42/44-MAPK signaling and inhibition of mitotic clonal expansion, a process essential for adipocyte differentiation in culture. These results implicate CTRP11 as a novel secreted regulator of adipogenesis and highlight the potential paracrine cross-talk between adipocytes and cells of the stromal vascular compartment in maintaining adipose tissue homeostasis.

The C1q protein family comprises over 30 secreted multimeric proteins, all of which share a signature C-terminal domain homologous to the globular domain of immune complement C1q (1). These include complement C1q (2), adiponectin (3), cerebellins (4), multimerins (5), emilins (6), C1q/TNF-related proteins (CTRP)[4] (7–12), CRF (13), C1qDC1 (also known as EEG1 or caprin-2) (14), otolin (15), and the nonfibrillar collagen VIII (16, 17) and collagen X (18, 19). These proteins play diverse roles in various tissue and organ systems (1).

Of the C1q family members, adiponectin and the CTRPs are considered important regulators of glucose and lipid metabolism. Adiponectin has been widely studied (20, 21); however, the metabolic function and regulation of CTRPs are only beginning to be elucidated (7–12, 22–27).

Although each of the CTRPs (CTRP1–15) has its unique tissue expression profile, they share common biochemical features (8, 9, 11, 12). They form trimers as their basic structural unit, driven by the globular C1q domain. Most CTRPs have one or more conserved Cys in their N termini that participate in intra- and inter-molecular disulfide bonding to enable the assembly of higher-order structures greater than a trimer. With the exception of CTRP4, these proteins have a variable length collagen domain, with its characteristic Gly-X-Y repeat, in the N terminus. Most secreted CTRPs circulate in plasma as potential hormones, with levels affected by metabolic states, sex, and genetic background of the animals.

Here we describe CTRP11 as a new and highly conserved member of the C1q family. We provide the first functional characterization of CTRP11 in the peripheral tissue and implicate it as a novel adipose stroma-derived regulator of adipogenesis.

MATERIALS AND METHODS

Antibodies and Reagents—Mouse anti-FLAG M2 monoclonal antibody was obtained from Sigma. Rat anti-HA (high affin-

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[4] The abbreviations used are: CTRP, C1q/tumor necrosis factor-related protein; Adpn, adiponectin; C/EBP-α, CCAAT/enhancer binding protein-α; MAPK, mitogen-activated protein kinase; PPAR-γ, peroxisome proliferator-activated receptor-γ; SVF, stromal vascular fraction; CRF, C1q-related factor; BAI3, brain-specific angiogenesis inhibitor 3; C1ql4, C1q-like protein 4; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

ity clone 3F10) antibody was obtained from Roche Applied Science. Horseradish peroxidase (HRP)-conjugated horse anti-mouse, goat anti-rabbit, and goat anti-rat IgG secondary antibodies were obtained from Cell Signaling Technology. Phospho-(Thr-202/Tyr-204) and total p42/44-MAPK antibodies, as well as C/EBPα and PPAR-γ antibodies, were obtained from Cell Signaling Technology. β-Tubulin antibody was obtained from Abcam, lipin-1 from Novus Biologicals, and adiponectin from Chemicon International. Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma, and peptide:*N*-glycosidase F from New England Biolabs.

Animals—C57BL/6 male and female mice (10–12 weeks old), and 12-week-old leptin-deficient obese (*ob*/*ob*) male mice and wild-type controls (Jackson Laboratory) were housed in polycarbonate cages on a 12-h light-dark photocycle and had access to water *ad libitum* throughout the study period. Mice were fed *ad libitum*, unless otherwise noted, standard chow diet (chow; number 5001, Lab Diet, St. Louis, MO). A separate cohort of male mice was fed a high-fat diet (60% kcal derived from fat; D12492) or an isocaloric matched low-fat diet (10% kcal derived from fat; D12450B) purchased from Research Diets Inc. (New Brunswick, NJ) for a period of 12 weeks. The high-fat fed animals are referred to as diet-induced obese mice. Epididymal fat pads were collected from these mice, frozen in liquid nitrogen, and kept at −80 °C. All animal protocols were approved by the institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Identification and Cloning of CTRP11—A search for adiponectin- and CTRP-like proteins in the NCBI GenBank™ databases identified a novel cDNA, which we designated *CTRP11* or *C1qTNF11*. Based on genomic DNA sequence and overlapping EST clones, a PCR approach was used to clone the coding region of the *Ctrp11* from a mouse testis cDNA library (Clontech). The primer pairs used were 5′-CCGTCGGGGTGGCCATGGTGCTG-3′ and 5′-GGGCGAACGAACATGAGAGTAAGGTG-3′. A 35-cycle PCR was carried out using Platinum Pfx polymerase (Invitrogen) in the presence of 8% DMSO. The PCR product was agarose gel-purified and cloned into the pCR2.1 TOPO vector (Invitrogen). The entire cDNA insert was sequenced. The nucleotide sequence of mouse CTRP11 reported in this study has been deposited in the GenBank database under accession number DQ002403.

cDNA Constructs—C-terminal FLAG (DYKDDDDK) and HA (YPYDVPDYA) epitope-tagged CTRP11 were generated by PCR and cloned into pCR2.1 TOPO vector (Invitrogen). The entire insert was excised from the pCR2.1 TOPO vector and cloned into the EcoRI restriction site of mammalian expression vector pCDNA3.1 (Invitrogen). cDNAs that encode C-terminal epitope-tagged truncated gCTRP10 (residue 1–12 and 145–287), gCTRP11 (residue 1–17 and 102–238), gCTRP13 (residue 1–22 and 124–255), and gCTRP14/CRF (residue 1–20 and 125–258) were chemically synthesized (Invitrogen) and subcloned into the EcoRI restriction site of pCDNA3.1. The overlapping PCR method was used to generate N-terminal Myc-tagged ERp44 in which the Myc epitope (EQKLISEEDL) was inserted immediately downstream of the signal peptide (residue 1–29). All constructs were verified by DNA sequencing. Mouse C-terminal HA-tagged adiponectin, CTRP1, CTRP2, CTRP3, CTRP5, CTRP6, CTRP9, CTRP10, CTRP13, CTRP14/CRF, CTRP10ΔCys, and CTRP13ΔCys constructs used in this study were described previously (8, 9, 11, 28).

Site-directed Mutagenesis—A PCR-based site-directed mutagenesis approach was used to mutate Cys-28 and Cys-32 to Ala using the high fidelity *Pfu* polymerase (Agilent Technologies). Successful mutagenesis was confirmed by DNA sequencing. The CTRP11 (C28A/C32A) mutant is designated CTRP11-HAΔCys and the CTRP14 (CRF; C28A/C32A) mutant is designated CTRP14-HAΔCys.

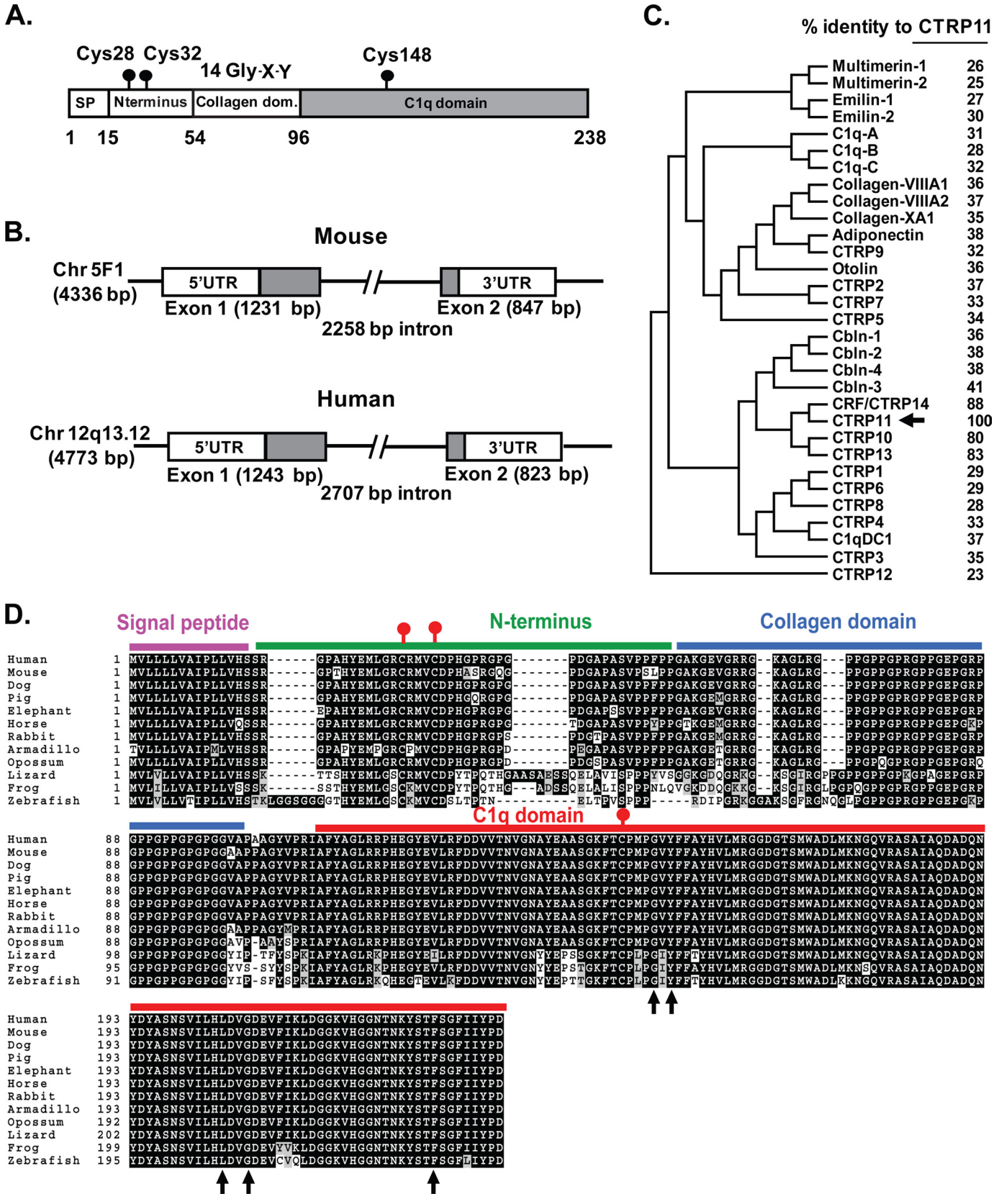
Isolation of Primary Adipocytes and Stromal Cells from Adipose Tissue—Primary adipocytes and stromal vascular cells (referred to as SVF or stromal vascular fraction) were isolated as previously described (9). Total RNA from stromal/vascular cells were extracted with TRI Reagent. Potential DNA contaminants were removed by RNase-free DNase I (Ambion).

Quantitative Real-time PCR—Quantitative real-time PCR was used to quantify the relative expression levels of *CTRP11* mRNA in human and mouse multiple tissue cDNA panels (Clontech). Every mouse tissue was pooled from 200 to 1000 mice and every human tissue was pooled from 3 to 15 individuals. Thus, the expression of *CTRP11* in each tissue represents an average value from the pooled cDNA. Other tissues profiled for *CTRP11* expression include epididymal fat pads of 12-week-old leptin-deficient (*ob*/*ob*) mice and their wild-type lean controls, primary adipocytes, and stromal vascular cells, epididymal or ovarian fat pads from C57BL/6 male and female mice (8–10 weeks old), interscapular brown adipose tissue from C57BL/6 male mice, epididymal (visceral) and inguinal (subcutaneous) fat pads from C57BL/6 male mice fed *ad libitum*, overnight (16 h) fasted, and fasted/re-fed for 2 h. Primers used were: *Ctrp11* forward, 5′-AAGGCCAAGATGAAGACAGCC-3′ and reverse, 5′-GGTTTCATTGGATCCCAAGGT-3′; *18 S rRNA* forward, 5′-GCAATTATTCCCCATGAACG-3′ and reverse, 5′-GGCCTCACTAAACCATCCAA-3′. The default PCR protocol was used on an Applied Biosystems Prism 7500 Sequence Detection System. cDNAs were synthesized from 2 μg of total RNA and 200 ng of random hexamers using SuperScript II RNase H-Reverse Transcriptase protocol (Invitrogen). For quantitative real-time PCR, samples were analyzed according to the protocol provided in SYBR® Green PCR Master Mix protocol (Applied Biosystems).

HEK 293 Cell Transfection—HEK 293 cells (GripTite™ 293 cell line from Invitrogen) were cultured in DMEM containing 10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen). Sixteen h after transfection, medium was replaced by serum-free Opti-MEM I (Invitrogen), and cells were cultured for another 24–48 h before the conditioned medium was collected for Western blot analysis.

Recombinant CTRP11 Protein Production—Full-length recombinant CTRP11 and CTRP11ΔCys (C28A/C32A) mutant were produced and purified from transfected HEK 293 cells as previously described for other related CTRPs (22). The mammalian expression system ensures proper post-translational modifications (*e.g.* glycosylation and higher-order structure assembly) of the recombinant proteins. Purified proteins were dialyzed against

20 mM Hepes buffer (pH 8.0) containing 135 mM NaCl in a 10-kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce). Protein concentration was determined using a Coomassie Plus protein assay reagent (Thermo Scientific), and samples were aliquoted and stored at −80 °C. The purity of recombinant proteins was judged to be >95% by Coomassie-stained gel.



Co-immunoprecipitation and Western Blotting—The FLAG-tagged CTRP11 was co-transfected in different combinations with HA-tagged adiponectin or CTRPs into HEK 293 cells. Aliquots of the collected supernantants (250 μl) combined with 500 μl of IP buffer (150 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-100) were subjected to co-immunoprecipitation using the EZview™ anti-FLAG affinity gel (Sigma). Samples were rotated for 4 h at 4 °C, washed 3 times with IP buffer, resuspended in 60 μl of NuPAGE sample buffer (Invitrogen) containing reducing agent (DTT), heated at 90 °C for 10 min, and separated on 10% NuPAGE BisTris gel (Invitrogen). Proteins from gels were transferred to 0.2-μm Protran BA83 nitrocellulose membranes (Whatman), blocked in 2% nonfat milk for 1 h, and probed with the anti-HA antibody in the presence of 2% nonfat milk overnight. Immunoblots were washed 3 times (10 min each) in PBS containing 0.1% Tween 20 and incubated with the anti-mouse HRP or anti-rat HRP (1:5000) for 1 h. Blots were washed 3 times (10 min each) in PBS containing 0.1% Tween 20, developed in ECL reagent (Millipore) for 2–5 min, and visualized with MultiImage III FluorChem® Q (Alpha Innotech). Quantifications of signal intensity were performed using Alphaview Software (Alpha Innotech). For input controls, aliquots of the conditioned media from transfected cells were analyzed by Western blotting using the anti-FLAG M2 or anti-HA antibody.

Protein Mixing Experiment—Conditioned media from transfected cells expressing epitope-tagged CTRP11, CTRP10, CTRP13, and CTRP14 (CRF) alone or in combination were harvested. Prior to immunoprecipitation, supernatant containing epitope-tagged CTRP10, CTRP13, or CTRP14 (CRF) was mixed with supernatant containing CTRP11. The mixtures were allowed to rotate at 4 °C for 4 h. Immunoprecipitation was performed on medium containing a single epitope-tagged protein, a mixture of two proteins expressed separately, or medium containing co-expressed proteins using 10 μl of the EZview anti-FLAG affinity gel (Sigma). Samples were washed 4 times with IP buffer and subjected to Western blot analysis using the rat anti-HA (1:2000) antibody.

Glycoprotein Detection—Approximately 50 ng of purified recombinant HA-tagged CTRP11 was separated on an SDS-polyacrylamide gel, transferred to PVDF membrane (Bio-Rad), and subjected to the ECL glycoprotein detection protocol according to the manufacturer's instructions (GE Healthcare). Briefly, any carbohydrate moiety on recombinant CTRP11 was oxidized with sodium metaperiodate, and the oxidized sugar aldehyde group was labeled with biotin using biotin hydrazide (29). The presence of a carbohydrate moiety was then detected using streptavidin conjugated to HRP and chemiluminescence substrate (Millipore).

Gel Filtration Chromatographic Analysis—Supernatant (500 μl) from HEK 293 cells containing FLAG-tagged CTRP11 was loaded onto an AKTA FPLC and fractionated through a Superdex 200 HR 10/30 column (GE Healthcare) in PBS and collected in 0.5-ml fractions. The collected fractions were subjected to immunoblot analysis.

3T3-L1 Adipocyte Differentiation—Differentiation of 3T3-L1 preadipocytes was carried out as previously described using a mixture containing 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (designated as MDI) (11). Two days before the induction of differentiation, 3T3-L1 cells were transfected with pCDNA3 control vector or vector encoding CTRP11, adiponectin, CTRP1, and CTRP10 using Lipofectamine 2000 (Invitrogen). Cells were differentiated for 8 days, and the amount of neutral lipid accumulation was quantified by Oil Red O staining. Total RNAs were isolated on a separate group of differentiated adipocytes, reverse transcribed, and subjected to real-time PCR analysis for adipogenic genes. Primers used in the real-time PCR analysis were: *PPAR-γ* forward, 5′-CCAGAGTCTGCTGATCTGCG-3′ and reverse, 5′-GCCACCTCTTTGCTCTGCTC-3′; *perilipin-1* forward, 5′-GGGACCTGTGAGTGCTTCC-3′ and reverse, 5′-GTATTGAAGAGCCGGGATCT-3′; *perilipin-2* forward, 5′-GACCTTGTGTCCTCCGCTTAT-3′ and reverse, 5′-CAACCGCAATTTGTGGCTC-3′; *perilipin-4* forward, 5′-GTGTCCACCAACTCACAGATG-3′ and reverse, 5′-GGACCATTCCTTTTGCAGCAT-3′; *adiponectin* (*Adpn*) forward, 5′-ACTGCAACTACCCATAGCCCAT-3′ and reverse, 5′-TGTCGACTGTTCCATGATTCTCC-3′; *FABP4* forward, 5′-ATCAGCGTAAATGGGGATTTGG-3′ and reverse, 5′-GTCTGCGGTGATTTCATCGAA-3′.

Oil Red O Staining and Quantification—Oil Red O staining was carried out as previously described (30). Conditioned media were aspirated from the 3T3-L1 adipocytes, and cell monolayers were washed three times with PBS then fixed for 2 min with 3.7% formaldehyde. 0.5% Stock Oil Red O prepared in isopropyl alcohol was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 1 h at room temperature. Cell monolayers were then washed with water and photographed. The pictures of the wells were taken by an EPSON scanner. The high magnification micrograph was taken by an Olympus IX50 microscope. Oil Red O stain that bound to neutral lipids within adipocytes was extracted in isopropyl alcohol and the absorbance was measured on a plate reader (BioTek, model SynergyMx) at 490–510 nm. To quantify glycerol content in the conditioned medium of adipocytes, medium (2 days old) was harvested from adipocytes on day 8 of differentiation under basal conditions (without β3-adrenergic stimulation). Glycerol levels were measured using a TG/glycerol kit from WAKO according to the manufacturer's instructions.

DNA Content Measurement in Adipocytes—Cellular DNA content was extracted using the TRI Reagent kit according to

FIGURE 1. **Identification of CTRP11.** *A*, the deduced protein sequence of CTRP11 consists of four domains: a signal peptide (SP) for secretion, an N-terminal domain with two conserved Cys residues, a collagen domain with 14 Gly-X-Y repeats, and a C-terminal globular domain homologous to the immune complement C1q. The positions of Cys residues found in the mature protein are indicated with a ball-and-stick. *B*, the size, chromosomal location, and exon/intron structure of mouse and human *CTRP11* genes. *UTR*, untranslated region. *C*, cladogram of CTRP11 and related C1q family members. The percent amino acid identity (C1q domain) of each mouse protein to CTRP11 is indicated on the *right*. *D*, sequence alignment of CTRP11 derived from various vertebrate species. Identical amino acids are *shaded* and gaps are indicated by a *dash line*. The conserved Cys residue is indicated by a ball-and-stick. The signal peptide (*purple line*), N-terminal domain (*green line*), collagen domain (*blue line*), and C-terminal globular domain (*red line*) are indicated. *Arrows* indicate the conserved residues found in all C1q/TNF superfamily members (33). Amino acid numberings are indicated on the *left*.

the manufacturer's instruction. Adipocytes on day 3 of differentiation were used for DNA extraction. The DNA concentration was then measured by $OD_{260/280}$ on a Nanodrop 1000 spectrophotometer (Thermo Scientific).

Sequence Alignment and Phylogenetic Tree Analysis of CTRP11—The orthologous genes that encode the CTRP11 protein from multiple vertebrate species were extracted from the National Center for Biotechnology Information (NCBI) database or the Ensembl database. In some cases, we retrieved CTRP11 orthologs by querying a particular vertebrate genome using the CTRP11 protein sequence. Multiple sequence alignments were performed using the ClustalW program (31). Sequences used in alignment included: human (*Homo sapiens*; GenBank accession number NP_001008224), mouse (*Mus musculus*; AAY21935), dog (*Canine familiaris*; XP_851062), pig (*Sus scrofa*; XP_003126157), elephant (*Loxodonta africana*; Ensembl accession number ENSLAFP00000014689), horse (*Equus caballus*; XP_001492004), rabbit (*Oryctolagus cuniculus*; XP_002711149), armadillo (*Dasypus novemcinctus*; ENSDNOP00000013845), opossum (*Monodelphis domestica*; XP_001364371), anole lizard (*Anolis carolinensis*; XP_003223236), frog (*Xenopus laevis*; NP_001090380), and zebrafish (*Danio rerio*; XP_695928). The phylogenetic tree was generated using neighbor-joining (NJ) method and MEGA program version 4 (32) based on ClustalW alignments of the C1q domain of input protein sequences.

TABLE 1
Percent amino acid identities of CTRP11 from different vertebrate species

	Amino acid identity to mouse CTRP11	
	Full-length	C1q domain
	%	
Mouse (*M. musculus*)	100	100
Human (*H. sapiens*)	96	100
Dog (*C. familiaris*)	97	100
Elephant (*L. africana*)	96	100
Mouse lemur (*M. murinus*)	95	100
Armadillo (*D. novemcinctus*)	94	100
Cattle (*Bos taurus*)	?	100
Opossum (*M. domestica*)	93	100
Horse (*E. caballus*)	94	100
Cat (*Felis catus*)	?	100
European rabbit (*O. cuniculus*)	96	100
Tree squirrel (*Spermophilus tridecemlineatus*)	?	100
Anole lizard (*A. carolinensis*)	77	94
Frog (*X. laevis*)	73	91
Zebrafish (*D. rerio*)	72	87

RESULTS

Identification of CTRP11 cDNA and Analysis of Its Gene—In a search for proteins homologous to adiponectin and CTRPs, we identified a novel and highly conserved paralog that we designated as C1q/TNF-related protein-11 (CTRP11) (Fig. 1*A*). A PCR approach was used to clone the coding region of the *Ctrp11* from mouse testis. The deduced amino acid sequence of CTRP11 consists of four distinct domains: a signal peptide, a short N-terminal region with two conserved cysteine residues, a collagen domain with 14 Gly-X-Y repeats, and a C-terminal globular domain homologous to the C1q domain of immune complement protein C1q (Fig. 1*A*). Mouse *Ctrp11* is ~4.3 kb in size, consists of two exons, and is located on chromosome 5F1 (Fig. 1*B*). Human *CTRP11* is ~4.8 kb in size, consists of two exons, and is located on chromosome 12q13.12 (Fig. 1*B*).

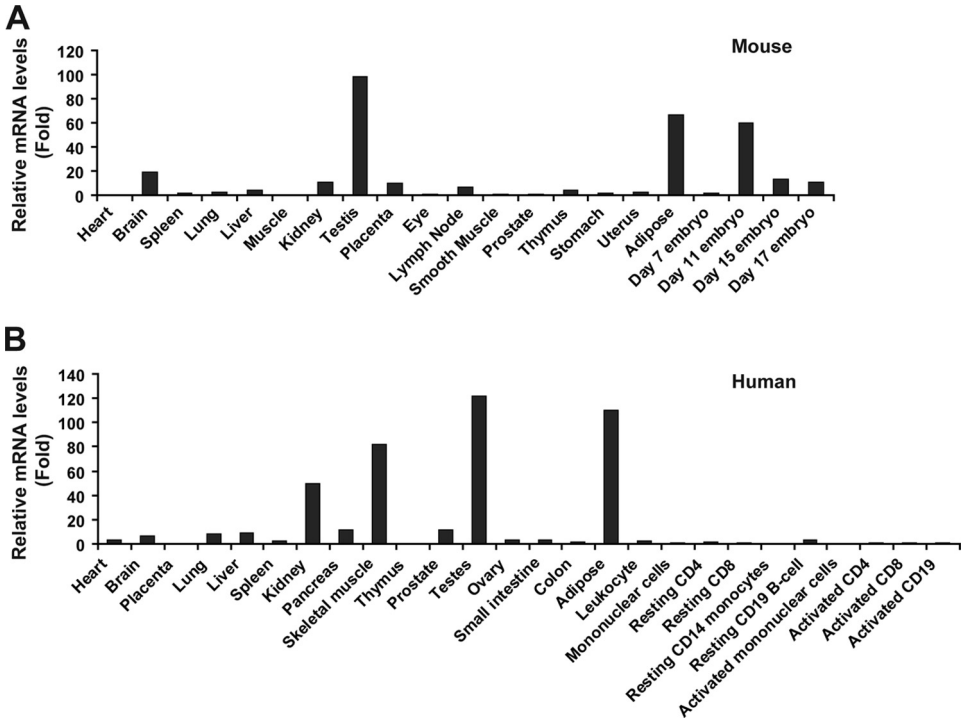


FIGURE 2. **Expression of *CTRP11* in mouse and human tissues.** Quantitative real-time PCR analysis of *CTRP11* mRNA expression in a mouse (*A*) and human (*B*) multiple tissue cDNA panel (Clontech). Expression in each tissue was first normalized to β-actin (human) and *18S rRNA* (mouse), then further normalized to tissue with the lowest expression of *CTRP11* (placenta for human and heart for mouse). Every mouse tissue was pooled from 200 to 1000 mice and every human tissue was pooled from 3 to 15 individuals (Clontech). Thus, the expression of *CTRP11* in each tissue represents an average value from the pooled cDNA.

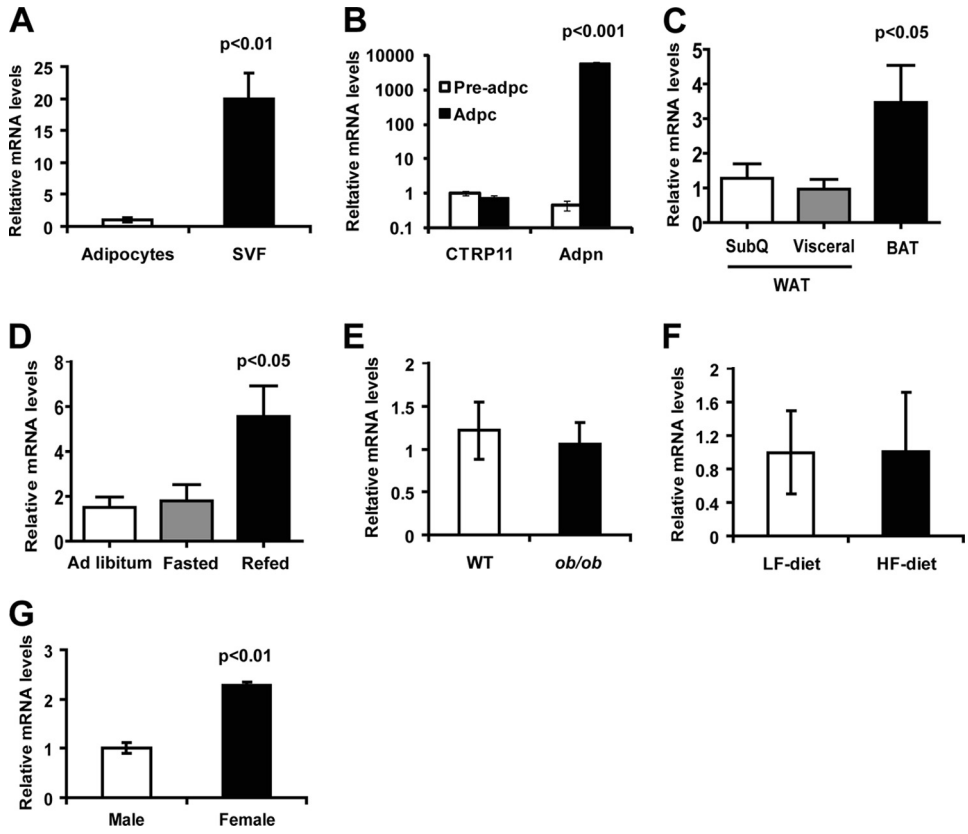


FIGURE 3. **Gender- and metabolic state-dependent expression of *Ctrp11* transcript in adipose tissue.** *A*, quantitative real-time PCR analysis of *Ctrp11* mRNA expression in primary adipocytes and SVF cells isolated from epididymal fat pads ($n = 3$). *B*, the relative expression levels of *Ctrp11* and *adiponectin* (*Adpn*) in 3T3-L1 preadipocytes (*Pre-adpc*) and adipocytes (*adpc*) ($n = 5–6$). *C*, the relative expression levels of *Ctrp11* in subcutaneous (*SubQ*), visceral adipose, and brown adipose tissue (*BAT*) ($n = 8–10$). *D*, the relative expression levels of *Ctrp11* in epididymal fat pads isolated from *ad libitum*, fasted, or fasted and re-fed mice ($n = 10$ per group). *E*, the relative expression levels of *Ctrp11* in epididymal fat pads isolated from leptin-deficient obese (*ob/ob*) mice and wild-type (*WT*) lean controls ($n = 8–10$ per group). *F*, the relative expression levels of *Ctrp11* in epididymal fat pads isolated from low-fat (*LF*) and high-fat (*HF*) diet-fed mice ($n = 8–10$ per group). *G*, the relative expression levels of *Ctrp11* in SVF cells between male and female mice ($n = 3$). All expression levels were normalized to the *18 S rRNA* levels. The *p* values (Student's *t* test) are indicated.

Unlike most CTRPs (7), the globular C1q domain of CTRP11 is encoded by two exons instead of a single exon.

Phylogenetic analysis revealed that CTRP11 is modestly related to most members of the C1q family (Fig. 1*C*), sharing 23–38% amino acid identity at the C1q domain. However, three of the family members, CTRP10, CTRP13, and CTRP14 (also known as CRF), are closely related to CTRP11 and form a separate clade on the cladogram. They share 80–88% amino acid identity with CTRP11 at the C1q domain.

Conservation of CTRP11 throughout Vertebrate Evolution—Sequence alignment revealed striking conservation of CTRP11 throughout vertebrate evolution (Fig. 1*D* and Table 1). Mouse CTRP11 and its corresponding human, dog, elephant, lemur, armadillo, cattle, opossum, horse, cat, rabbit, and squirrel orthologs share >93% amino acid identity over the full-length protein and a striking 100% amino acid identity in their presumed functional C-terminal globular domain. Distantly related vertebrate species such the anole lizard (a reptile), frog (an amphibian), and zebrafish (a teleost) share an impressive 94, 91, and 87% amino acid identity, respectively, to the mouse CTRP11 in their globular C1q domain (Table 1). Such a remarkable degree of conservation over 250 million years of evolution (since the emergence of teleost) implies an essential and highly conserved function for CTRP11. Structure-based alignment of adiponectin, complement C1q, and TNF family members (TNF-α, TNF-β, and CD40L) indicates multiple highly conserved residues important for the packing of the hydrophobic core of the protomer (33). These conserved residues shared by the C1q and TNF-α superfamily of proteins are also conserved in CTRP11 (Fig. 1*D*, *arrows*).

Adipose as a Major Tissue That Expresses Ctrp11 mRNA—A survey of 17 adult mouse tissues and four developmental stages of the mouse embryo using quantitative real-time PCR showed that testis and adipose tissue expressed the highest levels of *Ctrp11* transcript (Fig. 2*A*). Other tissues, such as the brain, kidney, and placenta, expressed lower levels of the transcript. Interestingly, the *Ctrp11* transcript is also expressed highly during the early stage (embryonic day 11) of mouse development, indicating a potential role in development. In humans, *CTRP11* is also expressed at the highest levels in testis and adipose tissue (Fig. 2*B*). In contrast to mouse, human skeletal muscle and kidney also expressed *CTRP11* (Fig. 2*B*). None of the immune cells express appreciable amounts of *CTRP11* transcript.

Stromal Vascular Cells and Brown Adipose Tissue Express Ctrp11—In addition to adipocytes, adipose tissue contains endothelial, fibroblastic, and immune cells. Collectively, these nonadipocytic cells are referred to as the SVF. We sought to examine whether *Ctrp11* is expressed by adipocytes or SVF

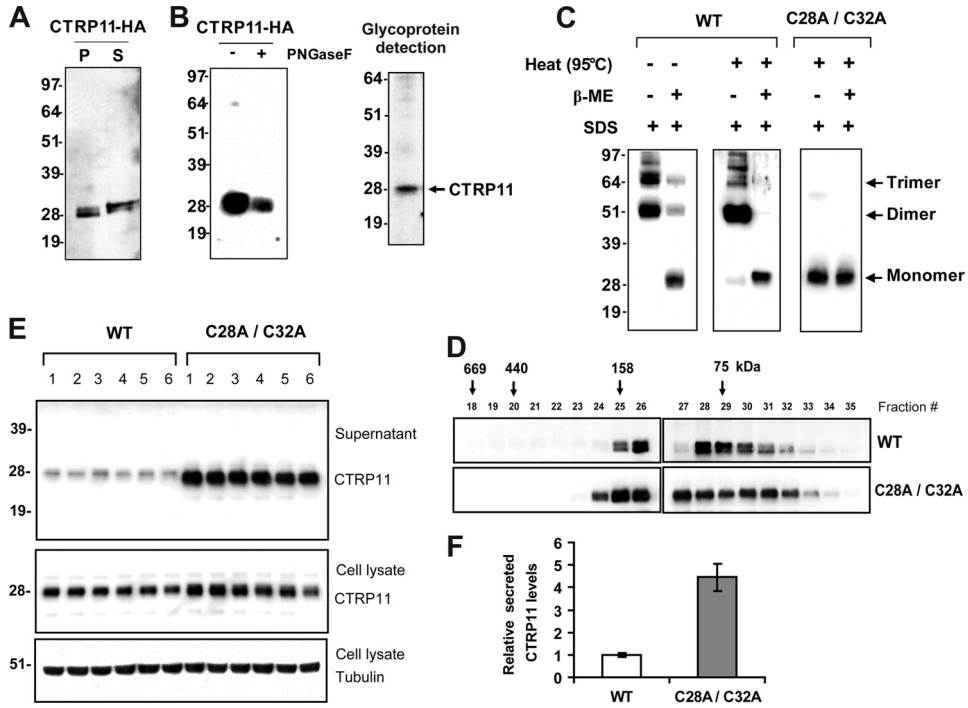


FIGURE 4. **CTRP11 is a secreted disulfide-linked multimeric glycoprotein.** *A*, Western blot analysis of cell lysate/pellet (*P*) and supernatant (*S*) from HEK 293 cells expressing epitope-tagged CTRP11 using the anti-HA antibody. *B*, Western blot analysis of recombinant CTRP11-HA digested with endoglycosidase (*PNGase F*) (*left*) and detection of sugar moiety on recombinant CTRP11 using a glycoprotein detection kit (see "Materials and Methods") (*right*). *C*, nonreducing and reducing immunoblot analysis of wild-type (*WT*) CTRP11 and the cysteine mutant (C28A/C32A) using the anti-HA antibody. Molecular weight markers are indicated on the *left* of each blot. *D*, size exclusion chromatographic analysis was carried out on FLAG-tagged wild-type CTRP11 and CTRP11ΔCys mutant (C28A/C32A). Aliquots from FPLC fractions 18–35 were subjected to Western blot analysis using the anti-FLAG antibody. *Arrows* indicate the molecular weight markers that correspond to the peak elution fraction of protein standards for thyroglobulin, ferritin, aldolase, and conalbumin, respectively. *E*, immunoblot analysis of WT CTRP11 and the Cys mutant in transfected HEK 293 cell lysates and supernatants (representing 6 independent transfections). *F*, quantification of results as shown in *E*.

within the adipose tissue. Quantitative real-time PCR revealed that *Ctrp11* is expressed predominantly by SVF rather than adipocytes (Fig. 3*A*). Unlike adiponectin, *Ctrp11* is not induced by adipogenesis (Fig. 3*B*), seen by comparing pre-adipocytes with mature adipocytes. Of the different adipose depots, brown adipose tissue (BAT) expresses a higher level of *Ctrp11* transcript than subcutaneous (inguinal) and visceral (epididymal) white adipose tissue (Fig. 3*C*).

Expression of Ctrp11 mRNA Is Regulated by Metabolic State—Expression of several CTRPs is responsive to acute changes in metabolic state induced by fasting and re-feeding, indicative of their involvement in energy homeostasis (12, 22). Similarly, expression of *Ctrp11* was significantly up-regulated in re-fed compared with fasted mice (Fig. 3*D*), suggestive of its role in energy metabolism. However, the expression of *Ctrp11* was unchanged in two mouse models of obesity (leptin-deficient *ob*/*ob* and diet-induced obese) representing chronic excess caloric intake (Fig. 3, *E* and *F*).

Sexually Dimorphic Expression Patterns of Ctrp11—Several of the CTRPs (*e.g. Ctrp5* and *Ctrp13*) exhibit sexually dimorphic expression patterns, with female mice expressing higher levels of the transcript compared with male mice (9, 11). Likewise, we observed that females express ~2.5-fold higher levels of *Ctrp11* mRNA in their adipose SVF relative to male mice (Fig. 3*G*).

CTRP11 Is a Secreted Glycoprotein—When expressed in heterologous HEK 293 cells, secreted CTRP11 protein was readily detected in the conditioned medium (Fig. 4*A*). The predicted molecular mass of mature CTRP11 is 23 kDa. However, the apparent molecular mass of the secreted CTRP11 on immunoblot is ~29 kDa, suggesting the presence of post-translational modification. Peptide:*N*-glycanase treatment of CTRP11 did not result in mobility shift of the protein on immunoblot, indicating the absence of *N*-linked glycans (Fig. 4*B*, *left panel*), consistent with the lack of a consensus Asn residue needed for the attachment of an *N*-linked carbohydrate. However, using a more general method to detect the presence of the sugar moiety, we showed that mammalian cell-produced CTRP11 indeed contains carbohydrate moiety (Fig. 4*B*, *right panel*). The nature of the glycans is unknown; however, CTRP11 contains a potential lysine residue (Lys-57) in its collagen domain that conforms to the pentapeptide motif G*X*KGE, known to be glycosylated in other CTRPs (8, 9).

Role of the Conserved N-terminal Cys-28 and Cys-32 in Protein Secretion—On the nonreducing immunoblot, CTRP11 existed predominantly as dimers and trimers (Fig. 4*C*, *lanes 1* and *3*). Two of the N-terminal Cys residues (Cys-28 and Cys-32) are highly conserved from zebrafish to humans (Fig. 1*D*). Mutations of the two conserved Cys residues abrogated the formation of intermolecular disulfide linkage (Fig. 4*C*, *lane 5*). The assembly of trimeric and hexameric complexes of wild-type CTRP11 was further confirmed by gel filtration chromatographic analysis (Fig. 4*D*). Surprisingly, the assembly of higher-order multimeric complexes was largely unaffected in the

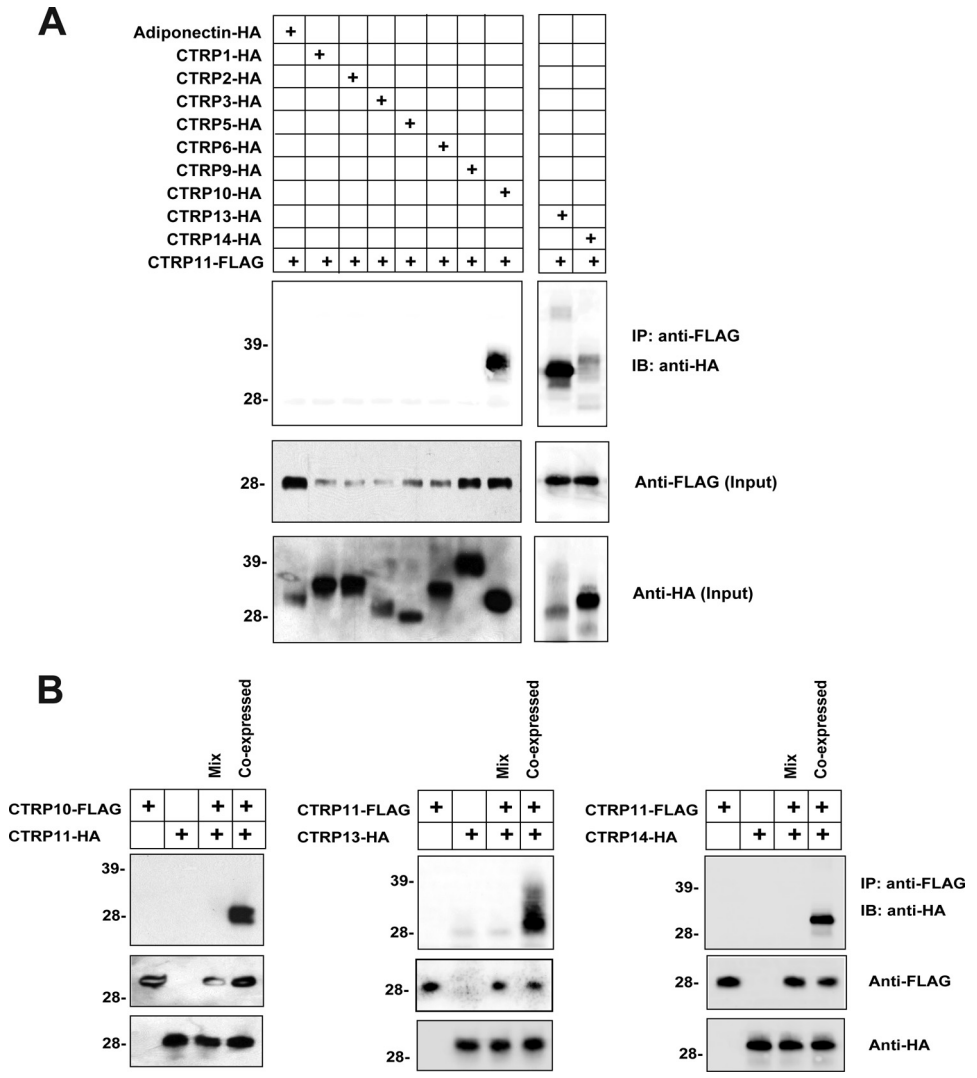
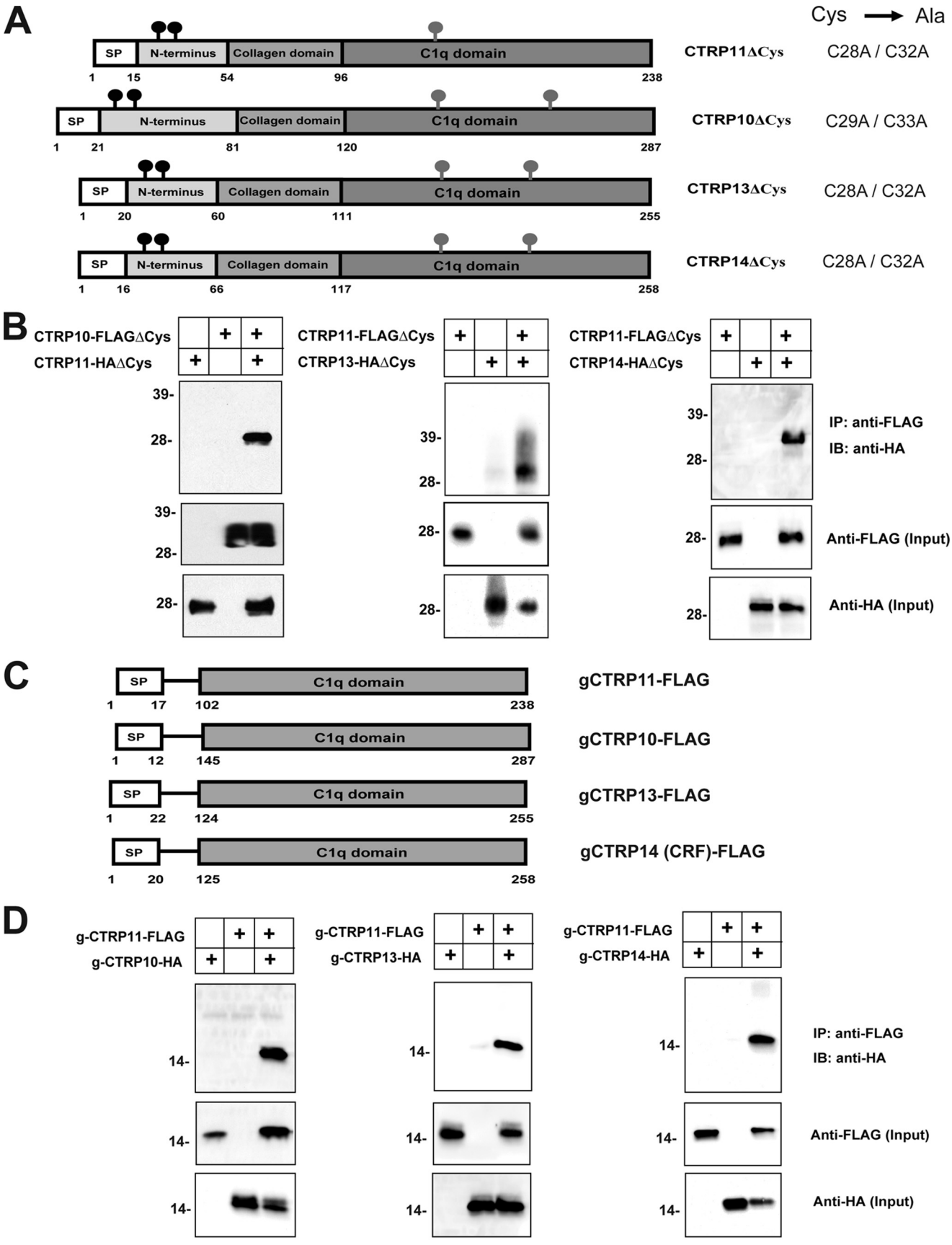


FIGURE 5. **CTRP11 forms heteromeric complexes with CTRP10, CTRP13, and CTRP14.** *A*, conditioned media from cells co-expressing FLAG-tagged CTRP11 with HA-tagged adiponectin, CTRP1, CTRP2, CTRP3, CTRP5, CTRP6, CTRP9, CTRP10, CTRP13, or CTRP14 were subjected to immunoprecipitation (*IP*) using the anti-FLAG affinity gel and immunoblotted (*IB*) with the anti-HA antibody (*top panel*). The *second* and *bottom panels* indicate the presence of FLAG and HA-tagged input proteins in the supernatants. *B,* conditioned media containing individually expressed, mixed, and co-expressed proteins were subjected to IP followed by IB with the anti-HA antibody (*top panel*). The *middle* and *bottom panels* indicate the presence of input proteins in the supernatants.

C28A/C32A double mutant (Fig. 4*D*) despite involvement of the cysteine residues in intermolecular disulfide bridges (Fig. 4*C*, compare *lanes 3* and *5*). Unexpectedly, Cys-28 and Cys-32 appeared to be involved in modulating protein secretion; mutations led to a striking enhancement in the amount of CTRP11 secreted from transfected HEK 293 cells compared with wild-type protein (Fig. 4, *E* and *F*). This is analogous to adiponectin in which the conserved N-terminal Cys-39 regulates protein secretion by forming a disulfide linkage with ERp44, an endoplasmic reticulum chaperone (34). However, unlike adiponectin, both FLAG-tagged wild-type CTRP11 and the mutant (C28A/C32A) physically interacted with Myc-tagged ERp44 equally well when co-expressed in HEK 293 cells (data not shown), suggesting that increased protein secretion of the Cys mutant is independent of ERp44.

CTRP11 and CTRP10 Form Heteromeric Complexes—C1q family members are known to form heteromeric complexes (8, 9, 11, 12). When co-expressed in mammalian cells, epitope-tagged CTRP11 co-immunoprecipitated with the closely related CTRP10, CTRP13, and CTRP14 (CRF) from the conditioned medium of transfected cells (Fig. 5*A*). The heteromeric association is specific; CTRP11 did not co-immunoprecipitate with other related C1q family members when co-expressed (Fig. 5*A*). Heteromeric complexes formed during biosynthesis; mixing of supernatant containing individually expressed protein did not result in co-immunoprecipitation (Fig. 5*B*). The conserved N-terminal Cys residues are not required for heteromeric complex assembly; mutations of these Cys residues (Cys-28 and Cys-32 in CTRP11, Cys-29 and Cys-33 in CTRP10, Cys-28 and Cys-32 in CTRP13, Cys-28 and Cys-32 in CTRP14) did not abrogate their physical association when co-expressed (Fig. 6, *A* and *B*), consistent with our previous study implicating the role of the C-terminal globular domain in homo- and heteromeric complex formation (8, 9). Indeed, co-expressing just the globular C1q domains of CTRP10, CTRP11, and CTRP13 was sufficient for the formation of heteromeric complexes (Fig. 6, *C* and *D*).


A
Cys → Ala
SP
N-terminus
Collagen domain
C1q domain
1 15 54 96 238
CTRP11ΔCys C28A / C32A
1 21 81 120 287
CTRP10ΔCys C29A / C33A
1 20 60 111 255
CTRP13ΔCys C28A / C32A
1 16 66 117 258
CTRP14ΔCys C28A / C32A
B
CTRP10-FLAGΔCys
CTRP11-HAΔCys
CTRP11-FLAGΔCys
CTRP13-HAΔCys
CTRP11-FLAGΔCys
CTRP14-HAΔCys
39-
28-
IP: anti-FLAG
IB: anti-HA
Anti-FLAG (Input)
Anti-HA (Input)
C
gCTRP11-FLAG
1 17 102 238
gCTRP10-FLAG
1 12 145 287
gCTRP13-FLAG
1 22 124 255
gCTRP14 (CRF)-FLAG
1 20 125 258
D
g-CTRP11-FLAG
g-CTRP10-HA
g-CTRP11-FLAG
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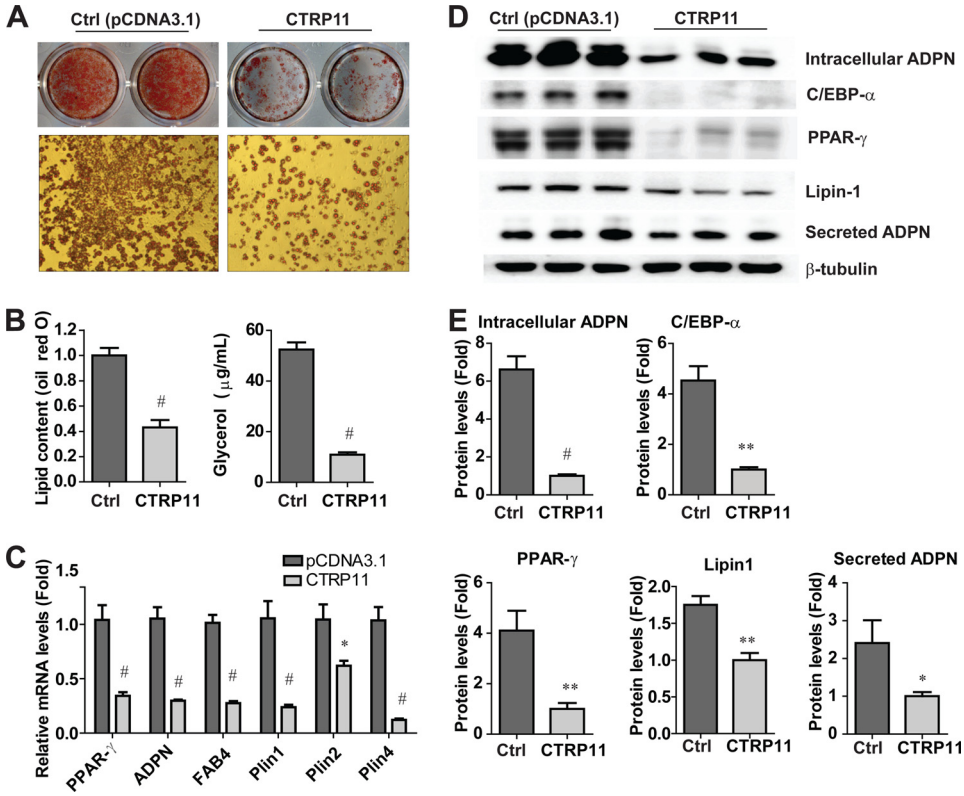

FIGURE 7. **Ectopic expression of CTRP11 suppresses 3T3-L1 adipocyte differentiation.** *A*, Oil Red O staining of adipocytes transfected with control vector (pCNDA3.1) or vector expressing CTRP11 2 days before (day −2) the induction of differentiation. Images were taken on day 8 of adipocyte differentiation. *Top panels* are photographs of adipocyte staining, and *bottom panels* are micrographs of adipocyte staining at ×10 magnification. *Ctrl*, control. *B*, quantification of Oil Red O staining of adipocytes, and glycerol released from the adipocytes expressing control vector or CTRP11 ($n = 3$). *C*, quantitative real-time PCR analyses of markers in adipocytes expressing control vector or *CTRP11* ($n = 6$). The relative expression levels were normalized to *18 S rRNA* levels. *Adpn, Adiponectin*; *FABP4, fatty acid-binding protein 4; Plin1, perilipin-1; Plin2, perilipin-2; Plin4, perilipin-4*. *D*, Western blot analyses of markers in adipocytes expressing control vector or CTRP11. Intracellular β-tubulin serves as the loading control. *E*, quantification of adipocyte markers as shown in *panel D* ($n = 3$). The relative expression levels were normalized to β-tubulin levels. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$.

Ectopic Expression of CTRP11 Suppresses 3T3-L1 Adipocyte Differentiation—Because CTRP11 is expressed by stromal vascular cells within the adipose compartment, we explored whether this secreted protein plays a role in regulating adipocyte differentiation. When ectopically expressed, CTRP11 significantly inhibited 3T3-L1 preadipocyte differentiation compared with cells expressing vector control, as revealed by a marked reduction in Oil Red O staining of neutral lipids within the lipid droplets of adipocytes (Fig. 7*A*). Quantification revealed a 60% decrease in neutral lipid accumulation in CTRP11-expressing adipocytes relative to cells expressing vector control (Fig. 7*B*, *left panel*). The reduced accumulation of intracellular neutral lipids also correlated with a 5-fold reduction in glycerol levels found in the supernatant of CTRP11-expressing adipocytes compared with control cells (Fig. 7*B*, *right panel*). Glycerol release reflects nonstimulated basal lipolysis of 3T3-L1 adipocytes and the extent of which is comparable to a previous study (10–30 nmol/ml/h; ~44–132 μg/ml in 48 h) (35). The greater the number of lipid droplets in adipocytes, the greater the amount of glycerol released from nonstimulated lipolysis. Suppression of adipogenesis by CTRP11 is specific; ectopic expression of adiponectin, CTRP1, and CTRP10, three related members of the C1q protein family, has little or no effect on neutral lipid (triglyceride) accumulation in differentiated adipocytes (data not shown). Quantitative real-time PCR analysis indicated that the expression of PPAR-γ, a major transcription factor controlling adipogenesis (36), was greatly reduced in CTRP11-expressing 3T3-L1 cells (Fig. 7*C*). Accordingly, a number of adipocyte-specific genes, such as *adiponectin* and *FABP4*, and genes involved in lipid droplet metabolism and formation, including perilipin-1 (*Plin1*), perilipin-2 (*Plin2*), and perilipin-4 (*Plin4*), were also significantly reduced in CTRP11-expressing adipocytes (Fig. 7*C*). At the protein level, both C/EBP-α and PPAR-γ expression, two major transcription factors driving the expression of adipogenic genes (36–38), were greatly reduced (Fig. 7, *D* and *E*). Protein markers for mature adipocytes, including adiponectin (intracellular and secreted) and lipin-1, were substantially down-regulated in

FIGURE 6. **Globular C1q domain mediates heteromeric complex formation.** *A*, domain structure of CTRP10, CTRP11, and CTRP13. Only the Cys residues located in the N-terminal region (*black* ball-and-stick) were mutated, whereas Cys residues located in the globular C1q domain (*gray* ball-and-stick) were spared. *B*, conditioned media containing individually expressed or co-expressed proteins were subjected to immunoprecipitation (*IP*) followed by immunoblot (*IB*) analysis with the anti-HA antibody (*top panel*). The *middle* and *bottom panels* indicate the presence of input proteins in the supernatants. *C*, truncation constructs encoding only the signal peptide (*SP*) and globular C1q domain of CTRP10, CTRP11, and CTRP13. *D*, conditioned media containing individually expressed or co-expressed proteins were subjected to immunoprecipitation followed by immunoblot analysis with the anti-HA antibody (*top panel*). The *middle* and *bottom panels* indicate the presence of input proteins in the supernatants.

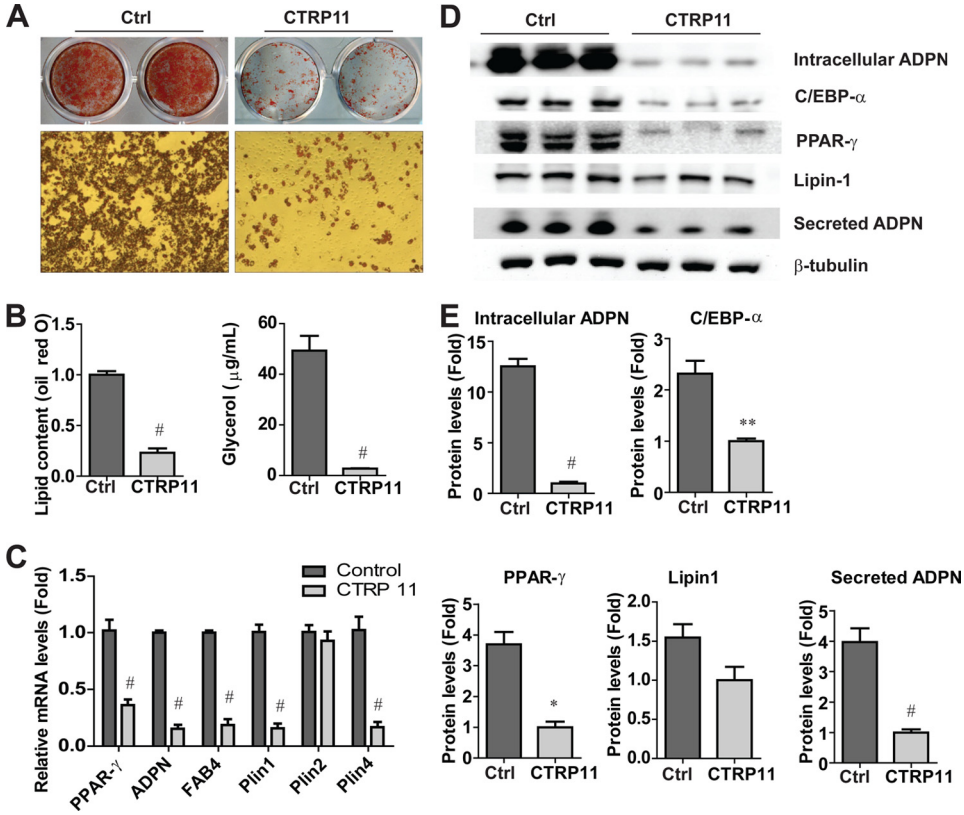

FIGURE 8. **Conditioned media containing CTRP11 suppresses 3T3-L1 adipocyte differentiation.** *A*, Oil Red O staining of adipocytes incubated with control conditioned media or conditioned media containing CTRP11 on day −2 and day 0. Images were taken on day 8 of adipocyte differentiation. *Top panels* are photographs of adipocyte staining, and *bottom panels* are micrographs of adipocyte staining at ×10 magnification. *Ctrl*, control. *B*, quantification of Oil Red O staining of adipocytes, and glycerol released from the adipocytes incubated with control or conditioned media containing CTRP11 ($n = 3$). *C*, quantitative real-time PCR analyses of markers in adipocytes incubated with control or media containing CTRP11 ($n = 6$). *Adpn, adiponectin; FABP4, fatty acid-binding protein 4; Plin1, perilipin-1; Plin2, perilipin-2; Plin4, perilipin-4*. The relative expression levels were normalized to *18 S rRNA* levels. *D,* Western blot analyses of adipocyte markers incubated with control or media containing CTRP11. Intracellular β-tubulin serves as the loading control. *E*, quantification of adipocyte markers as shown in *panel D* ($n = 3$). The relative expression levels were normalized to β-tubulin levels. *, $p < 0.05$; *, *$p < 0.01$; #, $p < 0.001$.

CTRP11-expressing adipocytes compared with control cells (Fig. 7, *D* and *E*). These data indicate that CTRP11-expressing 3T3-L1 cells have reduced adipogenesis, leading to reduced intracellular neutral lipid accumulation, glycerol release (basal lipolysis), and expression of adipogenic genes and mature adipocyte markers.

Conditioned Medium Containing CTRP11 Suppresses 3T3-L1 Adipocyte Differentiation—To verify the paracrine effect of stromal vascular cell-derived CTRP11 on adipocyte differentiation, we collected serum-free conditioned media from control vector- or CTRP11-transfected HEK 293 cells. Control or CTRP11-containing media were given to 3T3-L1 preadipocytes when they reached 100% confluence (day −2), and fresh media containing CTRP11 or control were replaced 2 days after (day 0), followed by adipocyte differentiation protocol (see "Materials and Methods"). Conditioned medium containing CTRP11 robustly inhibited the adipogenesis program of 3T3-L1 cells relative to control medium, reflected by a marked reduction (80%) of intracellular neutral lipid accumulation (Fig. 8, *A* and *B*) and glycerol released into the supernatant (derived from basal lipolysis) (Fig. 8*B*, *right panel*). At the mRNA level, medium containing CTRP11 suppressed PPAR-γ, adipogenic and lipid droplet biogenesis genes (Fig. 8*C*). At the protein level, significant suppression of expression of adipogenic proteins and markers by CTRP11-containing medium was also observed (Fig. 8, *D* and *E*). Of these, adiponectin, an adipocyte-specific protein, showed the most dramatic reduction (Fig. 8, *D* and *E*). The observed differences were greater for intracellular adiponectin compared with adiponectin secreted into the conditioned medium. Intracellular fraction of adiponectin reflects synthesis and secretion, whereas the secreted protein accumulates over time (day 7–8 conditioned medium). Together, these results confirm that CTRP11 functions in a paracrine fashion to inhibit adipocyte differentiation.

CTRP11 Has No Effect on Terminal Differentiation of Adipocytes—Strong inhibition of adipocyte differentiation was observed when 3T3-L1 cells were incubated with CTRP11-containing medium on day −2 and day 0, suggesting that CTRP11 acts on the early stage of differentiation to regulate adipogenesis. To address whether CTRP11 also regulates the late stage of differentiation, CTRP11-containing medium was given to 3T3-L1 cells on day 3 of differentiation. As shown in Fig. 9*A*, CTRP11 treatment, if given after cells have already undergone 2 days of differentiation, had no effect on neutral lipid accumulation, glycerol release, or adipogenic gene and marker expression in differentiating adipocytes (Fig. 9). These results suggest that CTRP11 acts at the early stage to inhibit adipocyte differentiation.

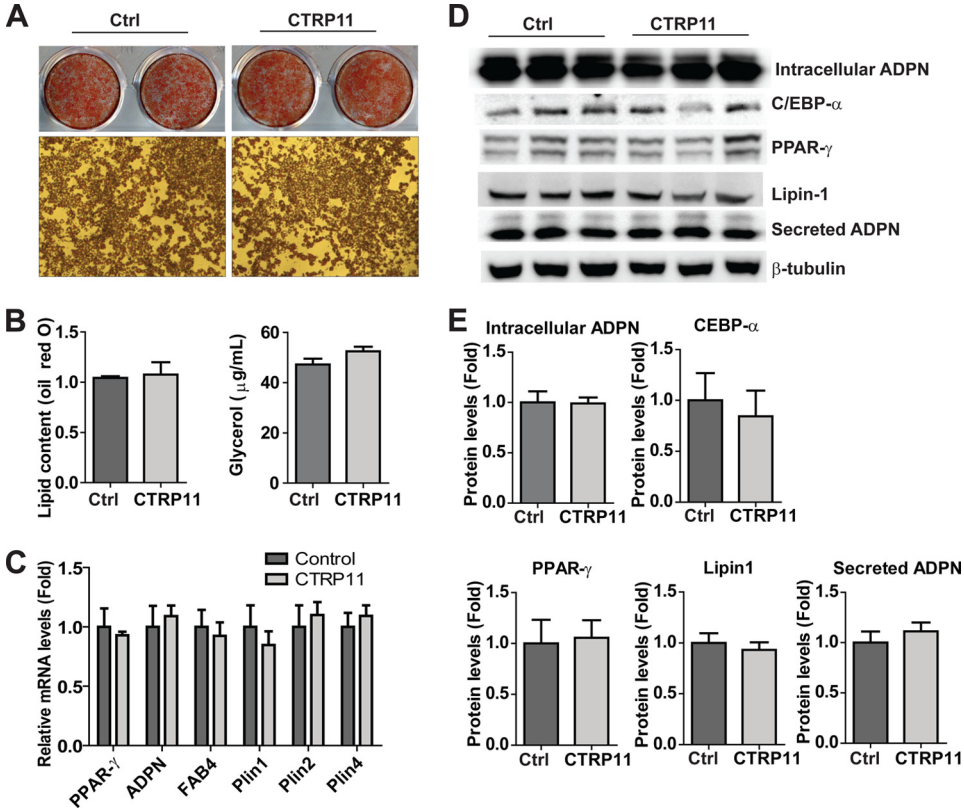

FIGURE 9. **CTRP11 has no effect on the late stage of 3T3-L1 adipocyte differentiation.** *A*, Oil Red O staining of adipocytes incubated with control conditioned media or conditioned media containing CTRP11 on day 3. Images were taken on day 8 of adipocyte differentiation. *Top panels* are photographs of adipocyte staining, and *bottom panels* are micrographs of adipocyte staining at ×10 magnification. *Ctrl*, control. *B*, quantification of Oil Red O staining of adipocytes, and glycerol released from the adipocytes incubated with control or conditioned media containing CTRP11 ($n = 3$). *C*, quantitative real-time PCR analyses of adipocyte markers in adipocytes incubated with control or media containing CTRP11 ($n = 6$). The relative expression levels were normalized to *18 S rRNA* levels. *Adpn, adiponectin; FABP4, fatty acid-binding protein 4; Plin1, perilipin-1; Plin2, perilipin-2; Plin4, perilipin-4*. *D*, Western blot analyses of markers in adipocytes treated with control or media containing CTRP11. Intracellular β-tubulin serves as the loading control. *E*, quantification of markers in adipocytes as shown in *panel D* ($n = 3$). The relative expression levels were normalized to β-tubulin levels.

Purified Recombinant CTRP11 Suppresses 3T3-L1 Adipocyte Differentiation—To further confirm and provide a quantitative evaluation of the inhibitory action of CTRP11 on adipocyte differentiation, we treated cells with purified recombinant CTRP11 protein. As shown in Fig. 10*A*, when 3T3-L1 pre-adipocytes were treated with 5 μg/ml of purified CTRP11 at day −2 and day 0 of differentiation, adipogenesis was strikingly suppressed (Fig. 10, *A* and *B*). The CTRP11ΔCys mutant (C28A/C32A) inhibited adipogenesis to the same extent as the wild-type protein (Fig. 10, *A* and *B*), consistent with the role of Cys-28 and Cys-32 in modulating protein secretion and not the assembly of higher-order oligomeric structures.

CTRP11 Inhibits p44/42 MAPK Signaling and Mitotic Expansion of 3T3-L1 Cells—We examined the specific process targeted by CTRP11 in the early stage of 3T3-L1 differentiation. It is known that growth-arrested 3T3-L1 cells undergo additional rounds of cell division upon induction of differentiation by hormonal signals during the onset of adipogenesis. This mitotic clonal expansion is essential for the induction of adipogenesis (39). Inhibition of p42/44 MAPK signaling with MAPK kinase inhibitor U-0126 suppresses mitotic clonal expansion and adipogenesis (39). We explored whether CTRP11 inhibits adipogenesis by inhibiting mitotic clonal expansion of 3T3-L1 cells. Indeed, ectopic expression of CTRP11 diminished the differentiation mixture MDI-induced p44/42 MAPK activation in 3T3-L1 cells compared with controls (Fig. 11*A*). When DNA content was quantified as a read-out for cell proliferation, MDI induced a 2-fold increase in cellular DNA content in cells expressing vector control, indicative of mitotic clonal expansion (Fig. 11*B*). However, 3T3-L1 cells expressing CTRP11 failed to undergo MDI-induced mitotic clonal expansion as judged by the lack of increase in cellular DNA content. Similarly, differentiating cells incubated with conditioned medium containing recombinant CTRP11 also showed reduced p44/42 MAPK activation and failed to undergo MDI-induced mitotic clonal expansion needed for differentiation to proceed (Fig. 11, *C* and *D*). Together, our results indicate that CTRP11 suppresses adipogenesis in culture via inhibition of mitotic clonal expansion.

DISCUSSION

We provided evidence here that CTRP11 is a novel adipose stroma-derived secretory protein of the C1q family. It shares multiple common biochemical features with the other CTRPs we have recently characterized (7–9, 11, 12). These include similar domain structure and post-translational modifications like glycosylation and the assembly of higher-order oligomeric structures.

CTRP11 stands out among proteins of the C1q family, exhibiting striking conservation throughout vertebrate evolution.

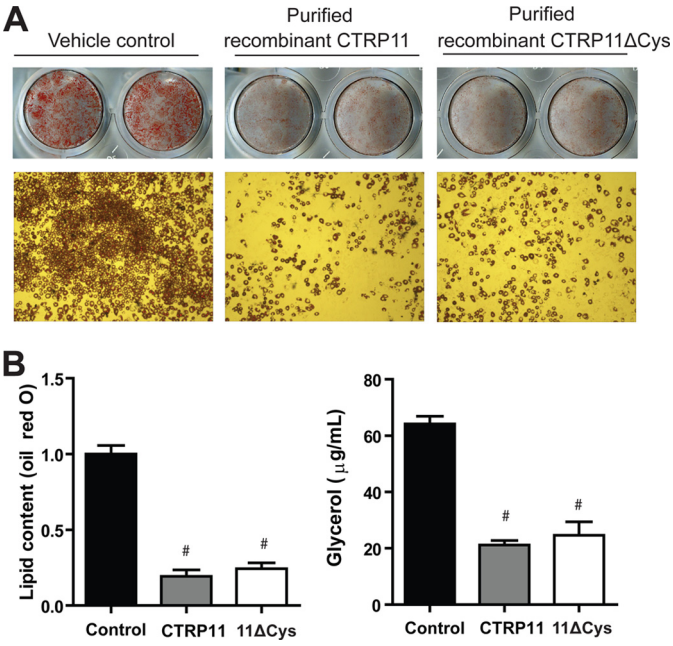

FIGURE 10. **Purified recombinant CTRP11 and the Cys mutant suppress 3T3-L1 adipocyte differentiation.** *A*, Oil Red O staining of adipocytes treated with vehicle buffer, CTRP11 (5 μg/ml), or CTRP11ΔCys mutant (C28A/C32A) (5 μg/ml) on day −2 and day 0 of differentiation. Images were taken on day 8 of adipocyte differentiation. *Top panels* are photographs of stained adipocytes and *bottom panels* are micrographs of stained adipocytes at ×10 magnification. *B*, quantification of Oil Red O staining of adipocytes (*left panel*) and glycerol released (*right panel*) from the adipocytes incubated with vehicle buffer or recombinant proteins ($n = 3$). #, $p < 0.001$.

Orthologous CTRP11 found in human, mouse, dog, elephant, lemur, armadillo, cattle, opossum, horse, cat, rabbit, and squirrel share >93% amino acid identity over the full-length protein and 100% amino acid identity in their presumed functional globular C1q domain (constituting 60% of the protein). Distantly related species such as *Xenopus* and zebrafish also share an impressive 91 and 87% amino acid identity, respectively, at the globular domain with mouse CTRP11, suggesting an essential and conserved function of CTRP11.

Both white and brown adipose tissues express CTRP11. The functional relevance of CTRP11 in brown adipose tissue is unclear. Within white adipose tissue, CTRP11 is primarily produced by cells of the stromal vascular fraction and not by adipocytes within white adipose tissue. Thus, endothelial cells, fibroblasts, immune cells (*e.g.* macrophages), and preadipocytes are among the potential cell types that express CTRP11. In the obese state, large numbers of macrophages are known to infiltrate the adipose tissue (40, 41). However, expression of the *Ctrp11* transcript remains unchanged in two different obese mouse models (*ob*/*ob* and diet-induced obese), suggesting that CTRP11 is most likely not expressed by macrophages. Re-feeding following a fast acutely up-regulates CTRP11 expression, indicating that the CTRP-11-expressing cell type within white adipose tissue is responsive to acute alterations in metabolic state. Due to its high degree of conservation, attempts to generate a CTRP11-specific antibody have not been successful, precluding analysis of the endogenous CTRP11 protein and limiting the current study. It remains to be determined whether CTRP11 circulates in plasma to act as a hormone, like other CTRPs described to date (8–10, 12).

Unlike the roles played by other CTRPs in regulating glucose and fatty acid metabolism (8–12, 22, 23), CTRP11 functions as an adipose stroma-derived regulator of adipogenesis. Ectopic expression of CTRP11 in 3T3-L1 cells inhibited adipocyte differentiation by suppressing the expression of *PPAR-γ* and *C/EBP-α*, two critical transcriptional regulators of adipogenesis (36–38), and genes involved in lipid droplet metabolism and formation (*i.e. perilipins*) (42). The potential paracrine action of CTRP11 on adipocyte differentiation was confirmed by treating 3T3-L1 adipocytes with conditioned medium containing CTRP11 or purified recombinant protein.

Notably, the suppression of adipogenesis by CTRP11 occurs within the first 2–3 days of 3T3-L1 cell differentiation in culture; no inhibition of adipogenesis was observed if 3T3-L1 cells were treated with CTRP11 on day 3 of differentiation. In growth-arrested 3T3-L1 cells, hormonal signals provided by MDI differentiation mixture induces additional rounds of cell division, a mitotic clonal expansion process essential for the induction of adipogenesis in culture (39). Differentiation of preadipocytes entails sequential and ordered induction of C/EBPs and PPAR-γ, transcription factors that orchestrate the program of adipogenesis (38, 43). Failure to undergo mitotic clonal expansion prevents implementation of the adipogenic program (39, 44). Inhibition of p42/44-MAPK signaling with MAPK kinase inhibitor U-0126 also suppresses mitotic clonal expansion and adipogenesis (39). We showed that the ectopic expression of CTRP11 or treatment of cells with CTRP11 decreased MDI-induced p44/42 MAPK activation and suppressed mitotic clonal expansion of differentiating 3T3-L1 preadipocytes. Our results suggest that the inhibition of PPAR-γ and C/EBP-α expression and adipogenesis by CTRP11 is mechanistically linked to the inhibition of MDI-induced mitotic clonal expansion in cultured 3T3-L1 cells.

Unlike other tissues or organs, adipose tissue seems to have a vast ability to expand, as seen in many striking gain- and loss-of-function mouse models (45–48), as well as in morbidly obese humans (49, 50). The mechanism controlling the size of fat mass is not well understood. Among the secreted factors, insulin and bone morphogenetic proteins play a positive or instructive role in governing fat mass, either by directly regulating adipocyte function (*e.g.* promoting triglyceride storage) as in the case of insulin (51–53) or by promoting the differentiation of mesenchymal precursor cells along the adipocytic lineage (54, 55). In contrast, secreted proteins of the Wnt family negatively regulate adipocyte differentiation (56–58). The cleaved ectodomain of an epidermal growth factor-like repeat-containing transmembrane protein, Pref-1, derived from pre-adipocytes, is also a potent negative regulator of fat cell differentiation (59–61). In this context, our study suggests that CTRP11, a secreted protein of the C1q family, represents a potential negative regulator of fat mass through its action on adipocyte differentiation.

What roles CTRP11 may play in other tissues such as the brain, testis, and kidney that also express *Ctrp11* transcript are not clear and speculative at best. While current work was ongoing, Iijima *et al.* (62) described C1q-like protein 4 (C1ql4), a protein identical to CTRP11 that is expressed in the central nervous system. The authors did not examine the expression of

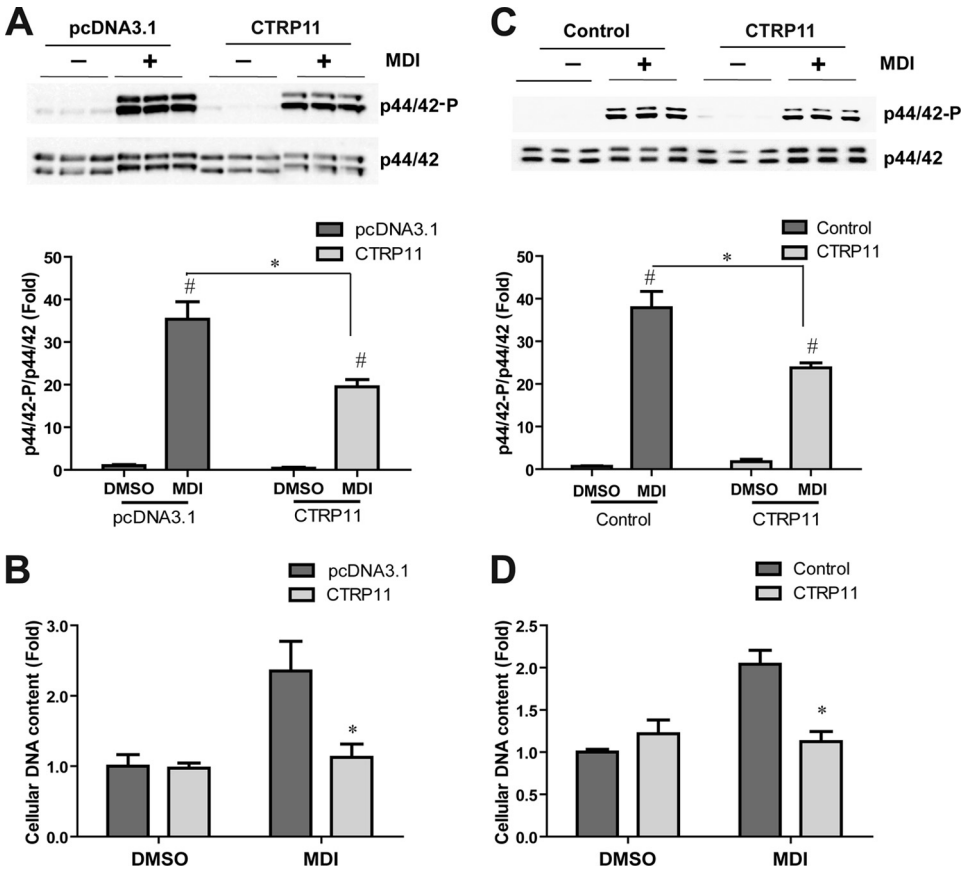

FIGURE 11. **CTRP11 decreases MDI-induced p44/42 MAPK signaling and inhibits mitotic clonal expansion in 3T3-L1 cells.** *A* and *B*, 3T3-L1 cells were transfected with control vector or vector encoding CTRP11 2 days before (day −2) being treated with differentiation mixture (MDI) containing 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. On the day of differentiation (day 0), cells were treated with control DMSO or MDI for 3 days, and cell lysates were harvested and subjected to Western blot analyses and quantification of p44/42 MAPK signaling (*A*; $n = 3$) or quantification of cellular DNA content (*B*; $n = 3$). *C* and *D*, 3T3-L1 cells were incubated with control conditioned media or conditioned media containing CTRP11 on day −2 and day 0. On the day of differentiation (day 0), cells were treated with control DMSO or MDI for 3 days, and cell lysates were harvested and subjected to Western blot analyses and quantification of p44/42 MAPK signaling (*C*; $n = 3$) or quantification of cellular DNA content (*D*; $n = 3$). *, $p < 0.05$; #, $p < 0.001$.

CTRP11/C1ql4 in adipose tissue nor demonstrate a function for CTRP11/C1ql4 in the brain. More recently, Bolliger *et al.* (63) showed that brain-specific angiogenesis inhibitor 3 (BAI3), an orphan G protein-coupled receptor, binds to multiple C1q-like proteins, including CTRP11/C1ql4. In cultured primary mouse hippocampal neurons, treatment of cells with recombinant gC1ql4 (just the globular domain fused to GST) produced in bacteria caused a reduction in synapse density. It is unclear whether the GST fusion protein made in bacteria is properly assembled into its native higher-order oligomeric structures, likely to be functionally relevant. Importantly, the expression of BAI3 is highly restricted to the brain (64, 65), in striking contrast to the expression of CTRP11 by a variety of tissues in addition to the central nervous system. Although published studies (64, 65) and the available database in NCBI indicate that BAI3 is not expressed in human and mouse adipose tissue, it still remains to be determined whether pre-adipocytes express BAI3 and whether it mediates the suppressive effect of CTRP11 on adipocyte differentiation. Alternatively, CTRP11 may bind to a different receptor in the peripheral tissue to exert its biological function. A loss-of-function mouse model will help confirm our *in vitro* study and elucidate additional *in vivo* physiological function of CTRP11.

In summary, our study establishes a potentially novel paracrine pathway by which CTRP11 secreted from stromal vascular cells negatively regulates adipocyte differentiation. Although the secreted endogenous regulators of adipogenesis within the adipose compartment *in vivo* remain poorly defined, CTRP11 derived from the stromal vascular compartment may mediate paracrine cross-talk necessary to maintain adipose tissue homeostasis.

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