⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 30, pp. 26516–26523, July 29, 2011
Printed in the U.S.A.

Regulation of Adipocyte Differentiation by the Zinc Finger Protein ZNF638*[S]

Received for publication, December 15, 2010, and in revised form, May 9, 2011 Published, JBC Papers in Press, May 20, 2011, DOI 10.1074/jbc.M110.212506

Sunitha Meruvu, Lynne Hugendubler, and Elisabetta Mueller[1]

From the Genetics of Development and Disease Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

Zinc finger proteins constitute the largest family of transcription regulators in eukaryotes. These factors are involved in diverse processes in many tissues, including development and differentiation. We report here the characterization of the zinc finger protein ZNF638 as a novel regulator of adipogenesis. ZNF638 is induced early during adipocyte differentiation. Ectopic expression of ZNF638 increases adipogenesis *in vitro*, whereas its knockdown inhibits differentiation and decreases the expression of adipocyte-specific genes. ZNF638 physically interacts and transcriptionally cooperates with CCAAT/enhancer-binding protein (C/EBP) *β* and C/EBP*δ*. This interaction leads to the expression of peroxisome proliferator-activated receptor *γ*, which is the key regulator of adipocyte differentiation. In summary, ZNF638 is a novel and early regulator of adipogenesis that works as a transcription cofactor of C/EBPs.

Adipogenesis is a process in which an undifferentiated mesenchymal cell becomes fully competent in storing lipids and secreting adipokines. This transformation is controlled by a tightly regulated and sequential transcriptional cascade. Multiple transcription factors have been shown to govern the induction and maintenance of the adipocyte phenotype. Gain- and loss-of-function experiments have established the sequence of events that regulate the adipocyte phenotype. Whereas CCAAT/enhancer-binding protein (C/EBP)[2] β and C/EBPδ control early events, peroxisome proliferator-activated receptor (PPAR) γ and C/EBPα regulate and maintain the later phases of adipocyte differentiation (1).

In an attempt to identify novel transcription regulators involved in determination of the preadipocyte fate, there has been an emphasis on understanding the processes that precede the expression and activation of PPARγ. The transcription factor Krox20 was shown to promote adipogenesis by regulating the expression of C/EBPβ (2), and four members of the Krüppel-like factor family (KLF15, KLF5, KLF4, and KLF9) have now been linked to induction of adipogenesis through activation of PPARγ (3). More recently, the zinc finger factor Zfp423, involved in development of the cerebellum (4), has been implicated in regulating preadipocyte commitment (5). In addition to the direct function played by transcription factors, the cofactors are also essential in regulating the early phases of adipogenesis. The coactivator SRC-3 potentiates adipocyte differentiation by cooperating with C/EBPs on the PPARγ promoter (6).

In this study, we used a candidate gene approach to identify novel factors involved in the regulation of adipogenesis. We identified uncharacterized proteins based on homology screening with the metabolic transcription coactivator PGC-1α (PPARγ coactivator 1α) to select potentially interesting molecules based on both their domain composition and their expression levels during the early phases of fat differentiation. Using this approach, we identified the zinc finger molecule ZNF638 as a factor expressed during early adipogenesis and show that its modulation via gain- or loss-of-function experiments regulates adipogenesis *in vitro*. Furthermore, we show that ZNF638 induces PPARγ expression through physical interaction and functional cooperation with C/EBPs on the PPARγ promoter.

EXPERIMENTAL PROCEDURES

Plasmids—ZNF638 cDNA was generated from a mouse cDNA library obtained from 3T3-L1 cells at day 2 of differentiation utilizing primers 5′-ACAGCCACCATGTCGAGACCCAGGTTTAATCC-3′ (forward) and 5′-CCCGGGTCACCTAGAGCTTCTTTCTTCAGTC-3′ (reverse) and cloned into the pCR2.1-TOPO TA vector (Invitrogen). Mouse ZNF638 cDNA was subcloned into the pCR3.1 vector (Invitrogen) at the BamHI and NotI sites and subsequently sequenced (GENEWIZ) to confirm the absence of mutations. FLAG-ZNF638, FLAG-ΔDBD-ZNF638 (mutant lacking the putative DNA-binding domain (DBD)), GFP-ZNF638, and GFP-ΔRS-ZNF638 (mutant lacking the arginine/serine-rich (RS) domain) were generated as indicated below. FLAG-ZNF638 was constructed utilizing primers 5′-CCATGGACTACAAGGACGACGACGACGAGACCCAGGTTTAATC-3′ (forward) and 5′-CGCCTTGTCGTCGTCGTCCTTGTAGTCTGGTGGCTGTAAGGC-3′ (reverse) and cloned into pCR3.1 at the AflII and AgeI sites. For GFP-tagged mouse ZNF638, a single fragment containing the sequences for enhanced GFP and ZNF638 was cloned into pCR3.1 at the AflII and AgeI sites. The GFP-ΔRS-ZNF638 mutant was generated using deletion primers 5′-GGAATCCTGAGATCCTCCCATCCGATGGAAAAAG-

* This work was supported in whole by the National Institutes of Health NIDDK Intramural Research Program.

⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

[1] To whom correspondence should be addressed: Genetics of Development and Disease Branch, NIDDK, NIH, Bldg. 10, Rm. 8D12B, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-594-6797; Fax: 301-402-4136; E-mail: elisabettam@niddk.nih.gov.

[2] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; DBD, DNA-binding domain; RS, arginine/serine-rich; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

TABLE 1
Cloning primers for GFP- and GST-ZNF638 constructs

Construct	Primers
ZNF638-(1–610)	
GFP-tagged	
Forward	GAA TTC TGC AGT CGA CAT GGC GAG ACC CAG GTT TAA TCC
Reverse	CCG CGG TAC CGT CGA CTC AGG CAA GTT TTG GCT TTT GTG C
GST-tagged	
Forward	GAA GGT CGT GGG ATC CAT GGC GAG ACC CAG GTT TAA TCC
Reverse	AGT CAC GAT GCG GCC GCT CAG GCA AGT TTT GGC TTT TGT GC
ZNF638-(607–1118)	
GFP-tagged	
Forward	GAA TTC TGC AGT CGA CAT GGC ACA AAA GCC AAA ACT TGC C
Reverse	CCG CGG TAC CGT CGA CTC ACT ATC AGC TGC TGT TTG TAC CTC
GST-tagged	
Forward	GAA GGT CGT GGG ATC CAT GGC ACA AAA GCC AAA ACT TGC C
Reverse	AGT CAC GAT GCG GCC GCT CAC TAT CAG CTG CTG TTT GTA CCT C
ZNF638-(1110–1780)	
GFP-tagged	
Forward	GAA TTC TGC AGT CGA CAT GGA GGT ACA AAC AGC AGC TGA TAG
Reverse	CCG CGG TAC CGT CGA CTC ATC GTG CTA ACT CAA CTT TTG AAT C
GST-tagged	
Forward	GAA GGT CGT GGG ATC CAT GGA GGT ACA AAC AGC AGC TGA TAG
Reverse	AGT CAC GAT GCG GCC GCT CAT CGT GCT AAC TCA ACT TTT GAA TC
ZNF638-(1773–1927)	
GFP-tagged	
Forward	GAA TTC TGC AGT CGA CAT GGA TTC AAA AGT TGA GTT AGC ACG A
Reverse	CCG CGG TAC CGT CGA CTC ACC TAG AGC TTC TTT CTT CAG TCT C
GST-tagged	
Forward	GAA GGT CGT GGG ATC CAT GGA TTC AAA AGT TGA GTT AGC ACG A
Reverse	AGT CAC GAT GCG GCC GCT CAC CTA GAG CTT CTT TCT TCA GTC TC

GCATTAG (forward) and 5′-GTTGTAAATTTCTTCTGT-AGAATATCCTTTGTTAG-3′ (reverse). FLAG-ΔDBD-ZNF638 was prepared using primers 5′-GTTACAAACCCTGAAACT-GAATTAGCAGTATCTGAC-3′ (forward) and 5′-CTGTTT-CCTGAGACTGTTTTGTTATTGGCTTTTCTCTTATTG-CTGC (reverse). Four ZNF638 regions encompassing amino acids 1–610, 607–1118, 1110–1780, and 1773–1927 were cloned into the pGEX4T (GE Healthcare) and pAcGFP1-C1 (Clontech) vectors via the In-Fusion™ Advantage PCR cloning kit (Clontech) to generate GST- and GFP-tagged proteins using the primers given in Table 1. For knockdown studies, control and ZNF638 shRNA lentiviruses (sc-150040, Santa Cruz Biotechnology) and siRNAs (Thermo Scientific) were used. C/EBPβ and C/EBPδ were obtained from Addgene, and C/EBPα was a gift from Kai Ge (NIDDK, National Institutes of Health).

Cell Culture—U2OS, HEK-293, 3T3-L1, and 10T1/2 cells (American Type Culture Collection) were cultured in DMEM (Mediatech) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Mediatech). For differentiation assays, confluent 3T3-L1 cells were treated with a differentiation medium containing 0.5 μM isobutylmethylxanthine (Sigma), 1 μM dexamethasone (Fluka), and 5 μg/ml insulin (Sigma) (MDI medium). For differentiation of 10T1/2 cells, MDI medium and 100 nM rosiglitazone (ChemPacific Corp.) were used. 2 days after induction, the differentiation medium was replaced with maintenance medium containing 10% FBS, 1% penicillin/streptomycin, and 5 μg/ml insulin. The maintenance medium was changed every 2 days until cells were harvested at the indicated days. For ZNF638 knockdown studies, 10T1/2 cells were transduced with control or ZNF638 shRNA lentiviral particles, and stable cell lines resistant to puromycin were selected. These cells were transfected with either control or ZNF638 siRNAs, respectively, to obtain robust knockdown.

Protein Analysis—Cytosolic and nuclear extracts were prepared from 3T3-L1 or 10T1/2 cells according to a previously described protocol with some modifications (8). Briefly, cells were washed twice with ice-cold PBS, harvested into 1 ml of PBS, and centrifuged at 700 rpm for 10 min at 4 °C. Cell pellets were dissolved in hypotonic solution (10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% Nonidet P-40, and protease inhibitors (Roche Applied Science)), incubated for 5 min on ice, mixed, and spun at 1000 × *g* for 5 min to pellet the nuclei. The nuclei were lysed in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors) and shaken at 4 °C for 20 min. Samples were spun at 16,000 × *g* for 10 min. Protein amounts were measured using the protein concentration assay (Bio-Rad). For immunoprecipitation assays, anti-FLAG M2 affinity gel (Sigma) or GFP-Trap® beads (Chromotek-GFP-Trap, Allele Biotechnology) were used. Briefly, for FLAG immunoprecipitation assays, nuclear extracts were obtained from transfected HEK-293 cells. 40 μl of anti-FLAG M2 beads were added to 200 μl of nuclear lysates and incubated overnight at 4 °C. For GFP immunoprecipitation assays, transfected HEK-293 cells were lysed, and 30 μl of GFP-Trap beads were added to the lysate and incubated for 2 h at 4 °C. The beads were subsequently pelleted, washed twice and resuspended in 100 μl of 2× SDS sample buffer, and run on a NuPAGE 4–12% BisTris gradient gel (Invitrogen). Separated proteins were transferred onto Immobilon-P PVDF membranes (Millipore), and blots were incubated overnight with primary antibody and for 1 h at room temperature with HRP-linked secondary antibody (GE Healthcare). Blots were developed (ECL, Pierce) and exposed to film (Kodak).

RNA Analysis—RNA was extracted from cultured cells using TRIzol solution (Invitrogen). Briefly, cells were harvested in 1 ml of TRIzol, and 0.2 ml of chloroform was added. RNA was precipitated with 0.5 volume of isopropyl alcohol, washed with

TABLE 2
Mouse primers for real-time PCR
LPL, lipoprotein lipase.

	Primers
ZNF638	
Forward	ATT GAG AGC TGT CGG CAG TTA
Reverse	GGA ATG AGA ACG TCT TCT TGG AG
PPARγ	
Forward	AGT CTG CTG ATC TGC GAG CC
Reverse	CTT TCC TGT CAA GAT CGC CC
aP2	
Forward	TCG ATG AAA TCA CCG CAG AC
Reverse	TGT GGT CGA CTT TCC ATC CC
C/EBPα	
Forward	GAA CAG CAA CGA GTA CCG GGT A
Reverse	GCC ATG GCC TTG ACC AAG GAG
Adiponectin	
Forward	TGT TCC TCT TAA TCC TGC CCA
Reverse	CCA ACC TGC ACA AGT TCC CTT
Leptin	
Forward	GAG ACC CCT GTG TCG GTT C
Reverse	CTG CGT GTG TGA AAT GTC ATT G
Glut4	
Forward	ACC GGA TTC CAT CCC ACA AG
Reverse	TCC CAA CCA TTG AGA AAT GAT GC
Lipoprotein lipase (LPL)	
Forward	GGG AGT TTG GCT CCA GAG TTT
Reverse	TGT GTC TTC AGG GGT CCT TAG
Perilipin	
Forward	ACA GCA GAA TAT GCC GCC AA
Reverse	GGC TGA CTC CTT GTC TGG TG
18 S	
Forward	AGT CCC TGC CCT TTG TAC ACA
Reverse	CGA TCC GAG GGC CTC ACT A
36B4	
Forward	GCT TCA TTG TGG GAG CAG AC
Reverse	ATG GTG TTC TTG CCC ATC AG

70% ethanol, and air-dried. RNA pellets were dissolved in H_2O and treated with TURBO DNA-*free*™ (Ambion). cDNA was generated with the high capacity cDNA reverse transcription kit (Applied Biosystems), and reverse transcription-PCR was performed using the indicated primers (Integrated DNA Technologies) and FastStart Universal SYBR Green Master (ROX, reference dye, Roche Applied Science) on an ABI PRISM machine (Applied Biosystems). The real-time PCR primers are listed in Table 2.

GST Pulldown Assays—GST-ZNF638 fragments were purified from BL21 bacterial cells (Invitrogen) according to the following protocol. Cells were collected, pelleted, and lysed in 2 ml of lysis buffer (20 mM Tris (pH 8), 400 mM KCl, 1 mM EDTA, 0.1% Triton X-100, and 1 mM DTT). Following 30 min of incubation on ice, lysed cells were centrifuged at 10,000 rpm for 15 min at 4 °C. Lysates were incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 2 h at 4 °C and subsequently washed three times with lysis buffer. *In vitro* translated and radiolabeled C/EBPs were generated (Promega) with EasyTag EXPRESS^{35}S protein labeling mix (PerkinElmer Life Sciences). GST-ZNF638 fragments were incubated with *in vitro* translated proteins in 20 mM HEPES (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40, 2 mM DTT, and 10% glycerol for 1 h at room temperature. Unbound *in vitro* translated proteins were removed by washing with the above buffer. Samples were eluted and run on a 15% SDS-polyacrylamide gel along with one-fifth of *in vitro* translated inputs. Gels were stained with Coomassie Blue (Bio-Rad), dried in a gel dryer (Bio-Rad), and exposed to film (Kodak).

Transfections—24 h prior to transfections, cells were plated at 90% confluency in 10% FBS-containing DMEM. For luciferase assays, on the day of transfection, the medium was changed to 0.5% FBS-containing DMEM. 100 ng of either C/EBP-luciferase or PPARγ2-luciferase and 150 ng of ZNF638, 10 ng of C/EBP, or control DNAs were cotransfected in HEK-293 cells plated in 24-well plates using FuGENE 6 (Roche Applied Science). 48 h after transfection, cells were lysed in 400 μl/well lysis buffer and assayed using firefly luciferase substrates (Pharmingen). For differentiation assays, 6 μg of pCR3.1-ZNF638 or empty vector were transfected into 10T1/2 cells plated in 6-well plates using Lipofectamine 2000 reagent (Invitrogen). For knockdown studies, stable 10T1/2 cells expressing control or ZNF638 shRNAs were transfected with 200 nM of luciferase or ZNF638 siRNAs, respectively. 24 h after transfection, MDI medium and 100 nM rosiglitazone were added. After 2 days of induction, maintenance medium containing insulin was added and changed every 2 days. For immunostaining, 3 μg of pCR3.1-ZNF638, GFP-ZNF638, GFP-ΔRS-ZNF638, individual GFP-ZNF638 fragments, or vectors were transfected in U2OS cells plated on slides. For immunoprecipitation assays, 5 μg of FLAG-ZNF638 and 1 μg of C/EBPβ were transfected in HEK-293 cells either in combination or individually. 3 μg of GFP-ZNF638-(607–1118) or GFP-ZNF638-(1773–1927) and 3 μg of C/EBPβ were transfected in HEK-293 cells for GFP immunoprecipitation assays. Assays were performed at least three times in triplicate.

Immunohistochemistry—Cells plated on slides (after transfection or differentiation) were fixed in 4% formaldehyde solution, permeabilized, blocked, and probed with anti-ZNF638 primary antibody (Bethyl) and then stained with Alexa Fluor 488-labeled secondary antibody (Invitrogen).

ChIP Assay—ChIP assays were performed using a commercial kit (EZ-ChIP™, Millipore) according to the manufacturer's instructions. Briefly, 3T3-L1 cells at day 2 of MDI medium stimulation were treated with 1% formaldehyde to cross-link proteins to DNA. Cells were subsequently collected, lysed in SDS-containing buffer, sonicated (Bioruptor®, Diagenode), and immunoprecipitated with 5 μg of anti-ZNF638 (Bethyl), anti-C/EBPβ, or IgG (Santa Cruz Biotechnology) antibodies. The protein-antibody complexes were incubated with protein A-agarose beads and washed, and DNA was eluted after reversing the protein/DNA cross-link with 5 M NaCl. 2 μl of DNA were used for PCR with primers designed to detect the C/EBP site present on the PPARγ or SREBP1c promoter. GAPDH primers were used as controls. The primers used for ChIP were as follows: C/EBP site on the PPARγ promoter, GGCCAAATACGTTTATCTGGTG (forward) and TCACTGTTCTGTGAGGGGC (reverse); GAPDH, TGTGCCAAGCACTTGTATAAC (forward) and TATGTCTGACCAGAGGAGAGCA (reverse); and C/EBP site on the SREBP1c promoter (15), CCACTGAGGGCTCTTGATCCT (forward) and AAACAGTCACGCTTGTGCTCA (reverse).

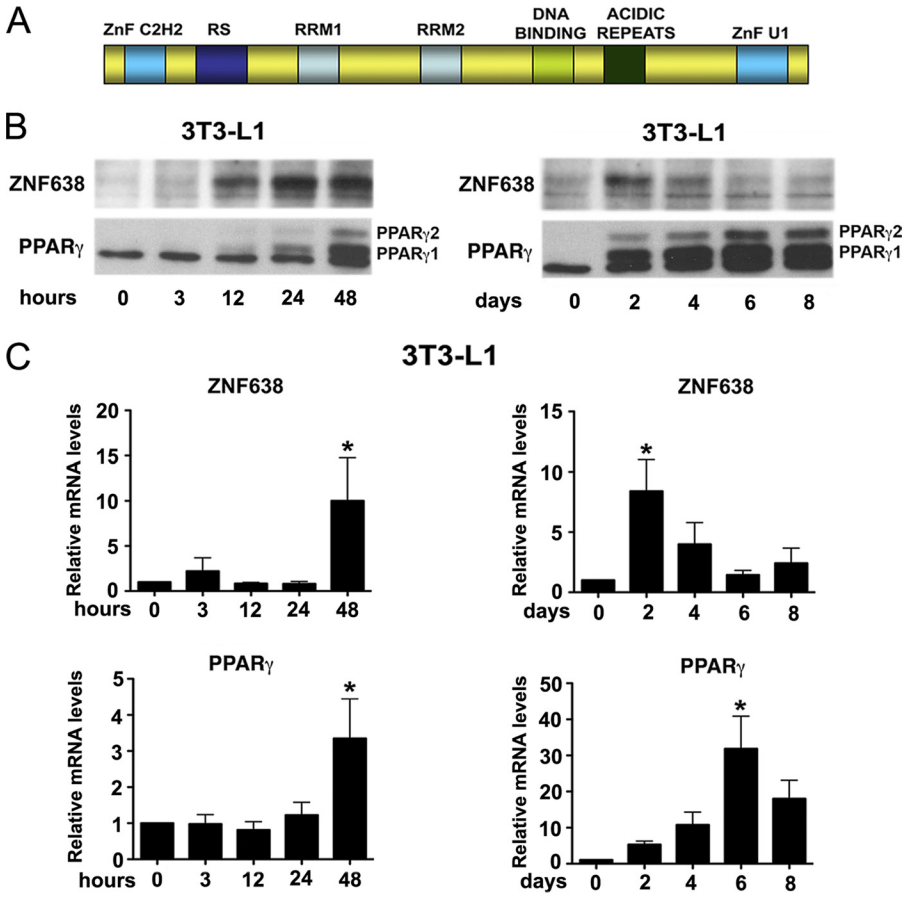

FIGURE 1. **Zinc finger factor ZNF638 is expressed during early phases of adipogenesis.** *A*, schematic representation of the zinc finger protein ZNF638. *ZnF*, zinc finger; *RRM*, RNA recognition motif. *B*, ZNF638 and PPARγ protein levels during 3T3-L1 differentiation. *C*, ZNF638 and PPARγ mRNA levels during a time course of 3T3-L1 differentiation (* $p < 0.05$).

RESULTS

Zinc Finger Factor ZNF638 Is Induced during Early Adipogenesis—To identify novel zinc finger factors involved in adipogenesis, we performed an *in silico* screen for molecules with a similar domain composition to PGC-1α. Through this candidate gene approach, we identified the zinc finger protein ZNF638, containing an RS motif and two RNA recognition motifs, as shown in Fig. 1*A*, with sequence homology to PGC-1α (supplemental Fig. 1). In addition to these domains in common with PGC-1α, ZNF638 contains two zinc finger motifs, a putative DBD, and nine acidic repeats (LVTVDEVIEEEDL) (9). On the basis of its domain composition and molecular weight, we hypothesized that this factor could potentially play a role as a scaffolding molecule and exert a function in transcription by acting as a cofactor. We first examined whether ZNF638 is expressed during adipogenesis by assessing its protein and mRNA levels (Fig. 1, *B* and *C*) during a time course of differentiation of 3T3-L1 cells. This analysis revealed that ZNF638 was induced at the protein and RNA levels shortly after exposure to the induction mixture, peaking before PPARγ was induced and rapidly decreasing during later stages of differentiation (Fig. 1, *B* and *C*). This expression pattern suggested a potential role for ZNF638 in the early stages of adipogenesis. To further study this molecule, we proceeded to clone ZNF638 from a cDNA library generated from mRNAs obtained from 3T3-L1 cells during the early stages of adipocyte differentiation, when the expression of ZNF638 appeared to be highly enriched.

ZNF638 Is a Nuclear Protein Localized in Speckles in Differentiating Adipocytes—We first assessed whether ZNF638 is a *bona fide* nuclear protein. Nuclear localization was predicted by ZNF638 sequence analysis, indicating two putative nuclear localization sequences, present at the N and C termini, and was previously shown for NP220, the human homolog of ZNF638 (10). U2OS cells ectopically expressing full-length ZNF638 were immunostained, demonstrating nuclear localization of ZNF638 (Fig. 2*A*). To determine whether ZNF638 is nuclear during adipocyte differentiation, we analyzed ZNF638 by immunostaining in 3T3-L1 cells undergoing adipocyte conversion (Fig. 2*B*). Interestingly, endogenous ZNF638 was localized in nuclear speckles also in differentiating adipocytes. To further characterize which domains of ZNF638 are involved in localization to speckles, we generated four ZNF638 mutants fused to GFP, transfected them into U2OS cells, and monitored their subcellular localization. As shown in Fig. 2*C*, only the ZNF638 fragment spanning amino acids 1–610 (containing a nuclear localization sequence and an RS domain) localized to the nuclear speckles, whereas the fragments encompassing the two RNA recognition motifs or the putative DBD with acidic repeats were mainly cytoplasmic. Interestingly, the putative nuclear localization sequence present at the C terminus also

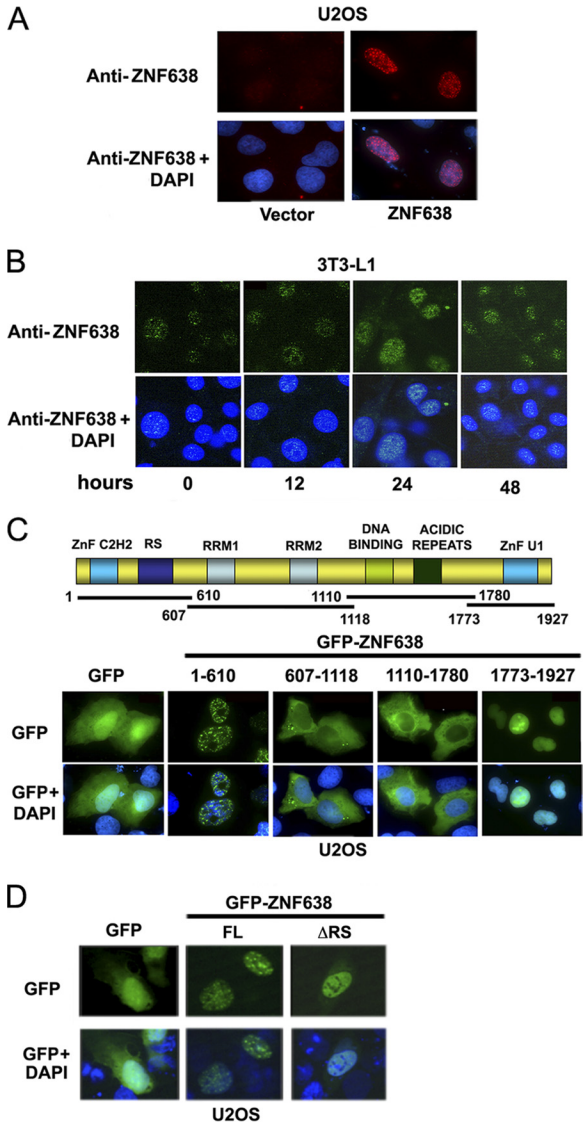

FIGURE 2. **ZNF638 is localized in the nucleus.** *A*, subcellular localization of ZNF638 in U2OS cells assessed by fluorescence microscopy (magnification ×100). *B*, detection of endogenous ZNF638 by immunohistochemistry in 3T3-L1 cells during differentiation by confocal imaging. DAPI staining was used as a nuclear staining control. *C*, subcellular distribution of discrete domains of GFP-ZNF638 assessed by fluorescence microscopy (magnification ×100). *ZnF*, zinc finger; *RRM*, RNA recognition motif. *D*, differential nuclear localization of GFP-ZNF638 and of a mutant of ZNF638 lacking the RS motif (GFP-ΔRS-ZNF638) detected by confocal imaging (63 × magnification, 1.4 NA). *FL*, full-length GFP-ZNF638.

conferred nuclear localization to ZNF638. To determine whether the RS domain is responsible for targeting ZNF638 to nuclear speckles, we generated an RS domain deletion mutant of GFP-ZNF638 (GFP-ΔRS-ZNF638) and monitored its localization. As shown in Fig. 2*D*, although GFP-ΔRS-ZNF638 was still nuclear, it was no longer localized in speckles. These data suggest that the RS domain is responsible for the speckled distribution of ZNF638 and that the two putative nuclear localization sequences present at the N and C termini are necessary for its *in vivo* nuclear localization.

ZNF638 Promotes Adipocyte Differentiation in Vitro—To determine whether ZNF638 modulates adipogenesis, we ectopically expressed ZNF638 in 10T1/2 preadipocytes (Fig. 3*A*) and compared adipocyte conversion with that in cells expressing only an empty vector. ZNF638-expressing 10T1/2 cells showed a higher degree of Oil Red O staining, a measure of lipid accumulation (Fig. 3*B*), and an increased number of lipid-accumulating cells (Fig. 3*C*) compared with cells expressing a control vector. These morphological changes were accompanied by increased expression of specific markers of fat differentiation, including aP2 and PPARγ (Fig. 3*D*), suggesting that ZNF638 promotes adipogenesis. To determine whether ZNF638 is not only sufficient but also necessary for the adipogenic process, we performed knockdown experiments with ZNF638 in 10T1/2 preadipocytes (Fig. 3*E*). Fig. 3*F* shows Oil Red O staining of control 10T1/2 cells and ZNF638 knockdown 10T1/2 cells that had been induced to undergo differentiation. ZNF638 knockdown 10T1/2 cells showed decreased overall lipid accumulation (Fig. 3*F*), a decreased number of lipid-laden cells (Fig. 3*G*), and lower expression of adipocyte markers (Fig. 3*H*) compared with control cells.

ZNF638 Regulates PPARγ Expression via Functional and Physical Interaction with C/EBPs—The timing of expression of ZNF638 during adipogenesis suggested that ZNF638 may be an early effector of adipocyte differentiation. To test this idea, we assessed the ability of ZNF638 to activate transcription in combination with C/EBPs. As shown in Fig. 4*A*, coexpression of ZNF638 and select C/EBPs in HEK-293 cells increased the transcriptional activation of a luciferase reporter gene driven by a C/EBP-responsive element compared with the levels obtained by ZNF638 or C/EBPs when individually expressed. Similar results were obtained when we repeated this assay on a luciferase reporter gene driven by the PPARγ promoter containing its native C/EBP sites (Fig. 4*B*). To exclude the possibility that ZNF638 binds directly at the C/EBP-binding site through its putative DNA-binding motif, we generated a ZNF638 mutant that lacks the DBD (ΔDBD-ZNF638) and tested its ability to transactivate the PPARγ promoter. The FLAG-ΔDBD-ZNF638 mutant was able to activate the PPARγ promoter in combination with C/EBPβ to the same extent as wild-type ZNF638 (supplemental Fig. 2).

We next examined whether ZNF638 forms a complex with C/EBPs by direct physical interaction by generating four GST fusion proteins containing distinct ZNF638 domains. As shown in Fig. 4*C*, GST-ZNF638 interacted directly with C/EBPs. To confirm that this interaction also occurs in cells, we performed immunoprecipitation assays by transfecting HEK-293 cells with C/EBPβ and ZNF638 either individually or in combination. As shown in Fig. 4*D* and supplemental Fig. 3 (*A* and *B*), ZNF638 interacted with C/EBPβ also *in vivo*. Given the observation that ZNF638 activated the PPARγ promoter by cooperating with C/EBPs *in vitro*, we next performed a ChIP assay in 3T3-L1 cells undergoing differentiation to examine whether ZNF638 is present at the C/EBP site in the PPARγ promoter. As shown in Fig. 4*E*, when we performed ChIP using an antibody against endogenous ZNF638, we detected ZNF638 at the C/EBP site. As a control, we performed ChIP using an anti-C/EBPβ antibody to verify that this C/EBP-binding site is indeed occupied by C/EBPβ, as expected and previously reported at this time point. To further determine whether ZNF638 is present at C/EBP-binding sites in the promoters of other early pro-

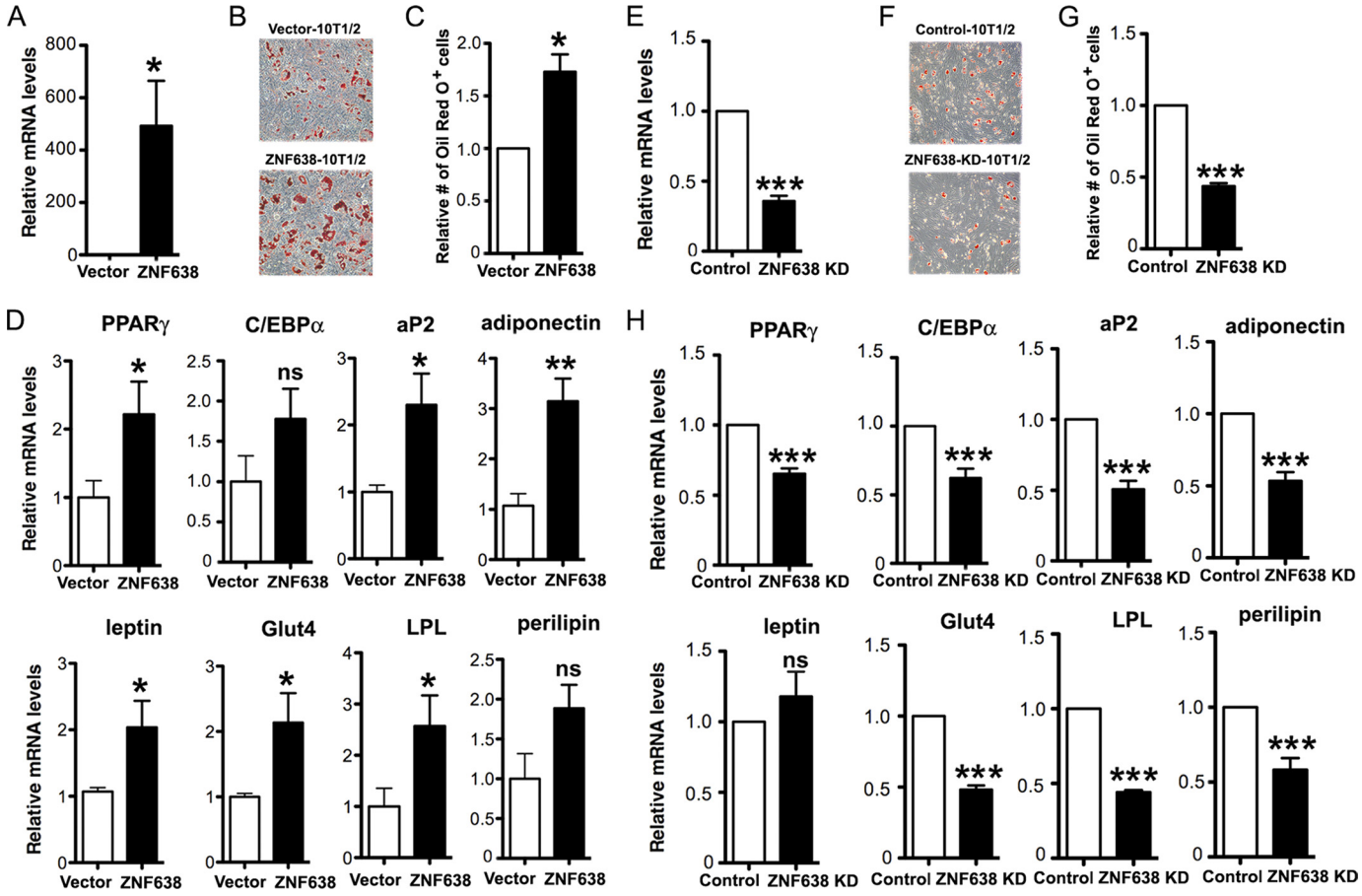

FIGURE 3. **ZNF638 regulates adipogenesis.** *A*, mRNA levels of ZNF638 in 10T1/2 cells ectopically expressing vector or ZNF638. *B*, Oil Red O staining of 10T1/2 cells ectopically expressing either vector or ZNF638. *C*, number of lipid-laden cells in 10T1/2 cells expressing vector or ZNF638. *D*, mRNA levels of adipocyte markers in 10T1/2 cells ectopically expressing vector or ZNF638. *LPL*, lipoprotein lipase. *E*, mRNA levels of ZNF638 in control and ZNF638 knockdown 10T1/2 cells. *F*, Oil Red O staining of control and ZNF638 knockdown 10T1/2 cells. *G*, number of lipid-containing cells present in control and ZNF638 knockdown 10T1/2 cells. *H*, mRNA levels of adipocyte markers in control or ZNF638 knockdown 10T1/2 cells (*** $p < 0.001$; ** $p < 0.005$; * $p < 0.05$; *ns*, not significant).

adipogenic genes, we assessed ZNF638 occupancy at the C/EBP-binding site present in the *SREBP1c* promoter. An antibody directed against ZNF638 was able to immunoprecipitate the C/EBP-binding site present in the *SREBP1c* promoter at day 2 of differentiation (supplemental Fig. 4). These data showing the presence of ZNF638 at C/EBP-binding sites suggest a functional cooperation between ZNF638 and C/EBPβ on promoters of proadipogenic genes.

DISCUSSION

Adipogenesis is governed by a robust gene regulatory network. Understanding how this network functions provides insights into pathological conditions, such as obesity and lipodystrophy, and represents an important step toward the rational design of targeted therapeutic interventions for these metabolic disorders.

This study has shown that the zinc finger factor ZNF638 is a novel regulator of the early phases of adipocyte differentiation. Several lines of evidence support this claim. First, ZNF638 is regulated specifically during the early phases of adipogenic differentiation, with its levels declining as adipocytes fully mature. Second, regulation of ZNF638 parallels the induction of C/EBPβ and C/EBPδ, known early regulators of adipogenesis, and the gain or loss of ZNF638 affects PPARγ expression and adipocyte differentiation. Finally, ZNF638 interacts with C/EBPs to control the expression of PPARγ.

ZNF638 was previously reported to be expressed in a variety of human cancer cell lines (9) and tumors (11) and in the mouse heart (9). Our finding that ZNF638 expression has a temporal specific pattern during early adipogenesis suggests a novel role for this factor in mesenchymal differentiation. Previously, based on its sequence and domain composition, the cellular function of ZNF638 was inferred to have a putative role in splicing and DNA/RNA binding (10). However to date, no *in vivo* data have shown whether ZNF638 is involved in any of these predicted functions. Although the human homolog of ZNF638, NP220, was previously reported to directly interact with the transcription cofactor FHL2 and shuttle it to the nuclear compartment (12), ZNF638 has not been implicated in a direct interaction with a transcription factor. Our results demonstrate that C/EBPs recruit ZNF638 to the PPARγ promoter, supporting a role for this zinc finger factor as a transcription cofactor.

During adipocyte differentiation, ZNF638 is driven by its RS domain to localize in nuclear speckles. Because speckles represent areas for storage of splicing factors, the presence of ZNF638 in a speckled pattern suggests that it may co-localize with proteins involved in splicing and play a potential role in cou-

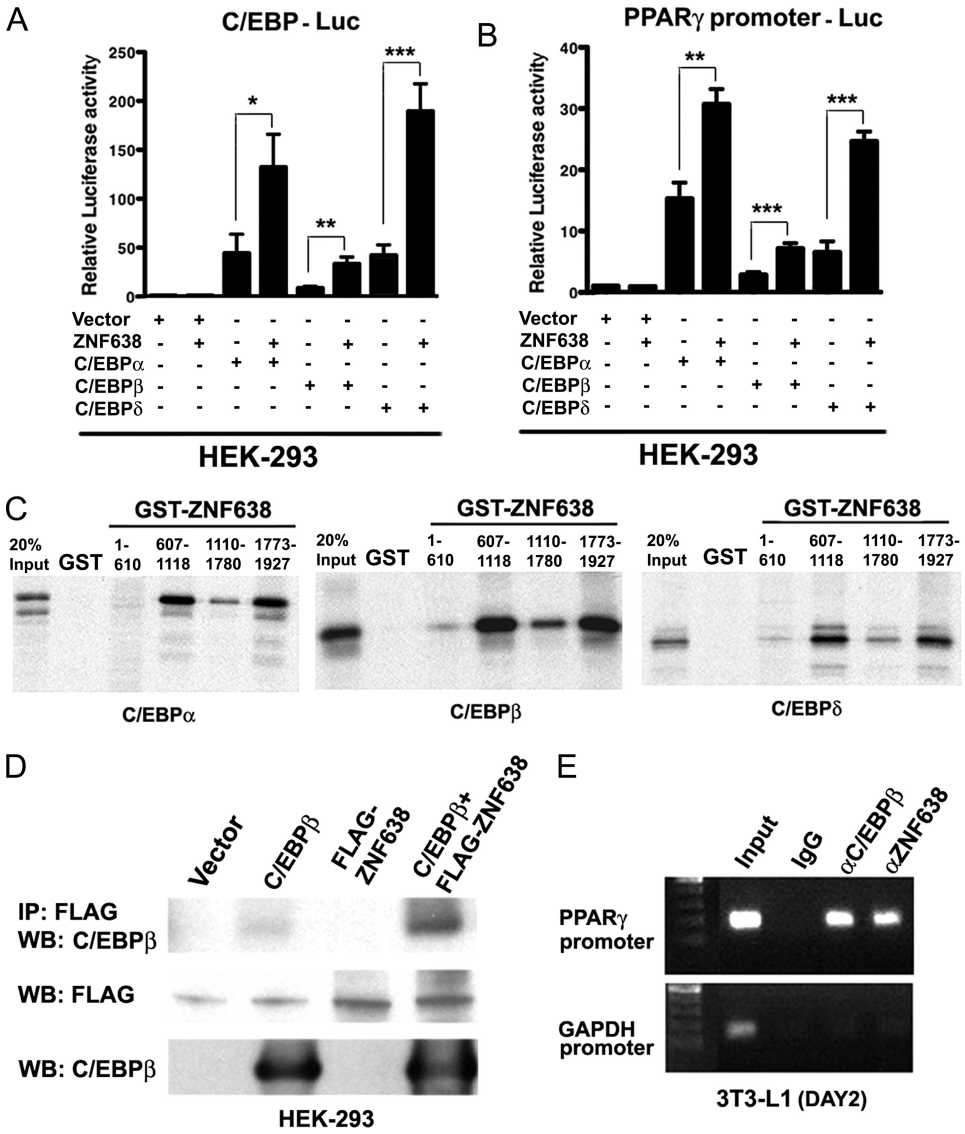

FIGURE 4. **ZNF638 interacts functionally and physically with C/EBPs.** *A*, luciferase activity of C/EBPα, C/EBPβ, and C/EBPδ in the presence or absence of ZNF638 on a luciferase (*Luc*) reporter gene driven by a C/EBP-responsive element. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$. *B*, transcriptional activity of C/EBPs on a luciferase reporter driven by the PPARγ promoter containing a C/EBP-binding site in the presence or absence of ZNF638. ***, $p < 0.001$; **, $p < 0.005$. *C*, GST pulldowns of fragments of GST-ZNF638 (amino acids 1–610, 607–1118, 1110–1780, and 1773–1927) in the presence of *in vitro* translated C/EBPα, C/EBPβ, and C/EBPδ. *D*, interaction between FLAG-ZNF638 and C/EBPβ in HEK-293 cells detected by immunoprecipitation (*IP*). *WB*, Western blot. *E*, ChIP assay at the C/EBP site present in the PPARγ promoter using either an anti-C/EBPβ antibody or an antibody directed against ZNF638 in 3T3-L1 cells at day 2 of differentiation.

pling transcription to RNA processing, a similar role to what was previously demonstrated for PGC-1α (13). Interestingly, in addition to their role in transcription, several known coactivators are also involved in pre-mRNA splicing (14). It remains to be determined whether ZNF638 is also a dual function cofactor.

Our study highlights the role of ZNF638 in regulating early fat differentiation and characterizes it as a novel cofactor. An analogous role in adipogenesis has been previously attributed to the transcription coactivator SRC-3, which is also activated early in differentiation and induces PPARγ expression by cooperating with C/EBPs (6). Despite the similarity of ZNF638 to SRC-3 with regard to its ability to physically and functionally interact with C/EBPs on the PPARγ promoter, ZNF638 differs from SRC-3 in that it lacks domains involved in histone modification. Additionally, the presence of multiple conserved motifs in this zinc finger factor may suggest that ZNF638 could function as an adaptor molecule to assemble other proteins in a functional transcription complex. Future experiments will determine whether ZNF638 functions by recruiting chromatin-remodeling factors or components of the transcriptional machinery to potentiate transcription. Finally, it will be of interest to determine whether hormonal cues modify the activity of ZNF638 to promote its functions during the early phases of adipocyte differentiation.

Acknowledgments—We thank Dr. Pasha Sarraf for critical discussions and for comments on the manuscript and Yun-Ping Wu and Rick Dreyfuss for assistance with confocal and fluorescence microscopy.

REFERENCES

1. Farmer, S. R. (2006) *Cell Metab.* **4,** 263–273
2. Chen, Z., Torrens, J. I., Anand, A., Spiegelman, B. M., and Friedman, J. M. (2005) *Cell Metab.* **1,** 93–106
3. Brey, C. W., Nelder, M. P., Hailemariam, T., Gaugler, R., and Hashmi, S. (2009) *Int. J. Biol. Sci.* **5,** 622–636
4. Warming, S., Rachel, R. A., Jenkins, N. A., and Copeland, N. G. (2006) *Mol. Cell. Biol.* **26,** 6913–6922
5. Gupta, R. K., Arany, Z., Seale, P., Mepani, R. J., Ye, L., Conroe, H. M., Roby, Y. A., Kulaga, H., Reed, R. R., and Spiegelman, B. M. (2010) *Nature* **464,** 619–623
6. Louet, J. F., Coste, A., Amazit, L., Tannour-Louet, M., Wu, R. C., Tsai, S. Y., Tsai, M. J., Auwerx, J., and O'Malley, B. W. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 17868–17873
7. Deleted in proof
8. Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983) *Methods Enzymol.* **101,** 582–598
9. Matsushima, Y., Ohshima, M., Sonoda, M., and Kitagawa, Y. (1996) *Biochem. Biophys. Res. Commun.* **223,** 427–433
10. Inagaki, H., Matsushima, Y., Nakamura, K., Ohshima, M., Kadowaki, T., and Kitagawa, Y. (1996) *J. Biol. Chem.* **271,** 12525–12531
11. Eichmuller, S., Usener, D., Dummer, R., Stein, A., Thiel, D., and Schadendorf, D. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 629–634
12. Ng, E. K., Chan, K. K., Wong, C. H., Tsui, S. K., Ngai, S. M., Lee, S. M., Kotaka, M., Lee, C. Y., Waye, M. M., and Fung, K. P. (2002) *J. Cell. Biochem.* **84,** 556–566
13. Monsalve, M., Wu, Z., Adelmant, G., Puigserver, P., Fan, M., and Spiegelman, B. M. (2000) *Mol. Cell* **6,** 307–316
14. Auboeuf, D., Dowhan, D. H., Dutertre, M., Martin, N., Berget, S. M., and O'Malley, B. W. (2005) *Mol. Cell. Biol.* **25,** 5307–5316
15. Payne, V. A., Au, W. S., Lowe, C. E., Rahman, S. M., Friedman, J. E., O'Rahilly, S., and Rochford, J. J. (2010) *Biochem. J.* **425,** 215–223