Transcriptional Regulation of ZNF638 in Thermogenic Cells by the cAMP Response Element Binding Protein in Male Mice

Luce Perie,[1]* Narendra Verma,[1]* Lingyan Xu,[2] Xinran Ma,[2] and Elisabetta Mueller[1]

[1]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, New York University, New York, New York 10016; and [2]Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China

ORCiD numbers: 0000-0003-0292-8753 (L. Perie).

*L.P. and N.V. contributed equally to this study.

Zinc finger factors are implicated in a variety of cellular processes, including adipose tissue differentiation and thermogenesis. We have previously demonstrated that zinc finger protein 638 (ZNF638) is a transcriptional coactivator acting as an early regulator of adipogenesis *in vitro*. In this study, we show, to our knowledge for the first time, that, *in vivo*, ZNF638 abounds selectively in mature brown and subcutaneous fat tissues and in fully differentiated thermogenic adipocytes. Furthermore, gene expression studies revealed that ZNF638 is upregulated by cAMP modulators *in vitro* and by cold exposure and by pharmacological stimulation of β-adrenergic signaling *in vivo*. *In silico* analysis of the upstream regulatory region of the ZNF638 gene identified two putative cAMP response elements within 500 bp of the ZNF638 transcription start site. Detailed molecular analysis involving EMSA and chromatin immunoprecipitation assays demonstrated that cAMP response element binding protein (CREB) binds to these cAMP response element regions of the ZNF638 promoter, and functional studies revealed that CREB is necessary and sufficient to regulate the levels of ZNF638 transcripts. Taken together, these results demonstrate that ZNF638 is selectively expressed in mature thermogenic adipocytes and tissues and that its induction in response to classic stimuli that promote heat generation is mediated via CREB signaling, pointing to a possible novel role of ZNF638 in brown and beige fat tissues.

Freeform/Key Words: β-adrenergic, brown adipocytes, CREB, thermogenesis, UCP1, ZNF638

Obesity, which is caused by excessive accumulation and expansion of adipose tissue, represents a major health problem and constitutes a central contributor to the development of insulin resistance, type 2 diabetes, and cardiovascular disease [1, 2]. According to data published by the Centers for Disease Control and Prevention, 39.8% of the population in the United States is considered obese [2], and the combined percentage of overweight and obese adults is projected to rise to 85% by 2030 [3].

Abbreviations: BAT, brown adipose tissue; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, cAMP response element binding protein; DN-CREB, dominant-negative cAMP response element binding protein; eWAT, epididymal WAT; FBS, fetal bovine serum; IBMX, isobutylmethylxanthine; phospho-, phosphorylated; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor γ; qPCR, quantitative PCR; RT, room temperature; scWAT, subcutaneous white adipose tissue; siRNA, small interfering RNA; SVF, stromal vascular fraction; UCP1, uncoupling protein 1; WAT, white adipose tissue; WT, wild-type; ZNF638, zinc finger protein 638.

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Obesity results from an imbalance between the amount of calories stored in fat tissues and those consumed [4–7]. In mammals, three main types of adipocytes have been described to date—white, brown, and beige—each of them specifically localized in defined anatomical areas and with distinct gene expression signatures [8]. Whereas classic white adipose tissue (WAT) present in the visceral/perigonadal region plays key roles in the regulation of energy storage, brown adipose tissue (BAT) located in the intrascapular area in rodents mainly regulates energy dissipation via thermogenesis, the process of heat generation [9]. In addition to BAT, beige adipocytes present within the subcutaneous white adipose depots have been shown to also be capable of thermogenic processes and to have the ability to respond to external stimuli and contribute to energy expenditure [4–6, 8, 9]. Because both beige and brown fat adipocytes are able to dissipate energy [10–12], they have been proposed to be candidate target cells in the fight against metabolic disorders.

Activation of β-adrenergic receptors in response to thermogenic cues is critically involved in the induction of WAT browning and heat generation via cAMP signaling [13]. Higher intracellular levels of cAMP are known to activate protein kinase A (PKA) that in turn phosphorylates the transcription factor cAMP response element (CRE) binding protein (CREB) at a serine residue in position 133 [14–16]. This signaling culminates in the binding of phosphorylated (phospho-)CREB to promoters of genes such as uncoupling protein 1 (UCP1), ZFP516, and PGC1α, among others, involved in thermogenic responses in brown and beige fat cells [17–19].

Our laboratory recently selected zinc finger protein 638 (ZNF638) from the database based on its homology with the transcriptional cofactor PGC1α. ZNF638 is a 220-kDa protein cloned in the early 1990s, the function of which had remained unknown for more than a decade until our laboratory provided the first evidence that ZNF638 is induced during the early phases of *in vitro* adipogenesis and demonstrated via gain-of-function and loss-of-function studies that ZNF638 regulates adipocyte differentiation [20]. Furthermore, mechanistic studies demonstrated that ZNF638 acts as a transcriptional cofactor that controls the expression of the peroxisome proliferator-activated receptor γ (PPARγ) in cooperation with the CCAAT enhancer protein β or δ during adipocyte differentiation [20]. These results obtained *in vitro* suggested to us a possible novel role of ZNF638 in the regulation of adipose tissue biology [20]. In this study, we investigated, to our knowledge for the first time, the *in vivo* pattern of ZNF638 expression and the mechanisms of its transcriptional regulation in adipose tissues. Our study provides novel evidence that ZNF638 is highly expressed in mature thermogenic tissues, that it is induced by agents that elevate cAMP intracellular levels, and that it is regulated in response to exposure to low temperatures. Detailed analysis of the molecular mechanism controlling ZNF638 expression in thermogenic adipocytes revealed that ZNF638 mRNA levels are regulated by the transcription factor CREB. Overall, our studies provide new insights into ZNF638 as a novel factor regulated in response to thermogenic cues that may play a physiological role in mature beige and brown fat cells.

1. Materials and Methods

A. Cell Culture

Murine 10T1/2 and HEK-293 cells, obtained from American Type Culture Collection, and stromal vascular fraction (SVF) cells isolated from mouse subcutaneous WAT (scWAT) were cultured in DMEM (Corning, catalog no. 10-013-CV) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, catalog no. NC0959573) and 1% penicillin/streptomycin (Thermo Fisher Scientific, catalog no. 15070063) in a humidified atmosphere of 5% CO_2 at 37°C. Isolation of mouse SVF cells was performed according to previously described procedures [21]. Briefly, mouse scWAT pads were dissected, washed in PBS, minced into small pieces, and digested with 1 mg/mL collagenase type IV (Roche, catalog no. 10269638001) for 1 hour at 37°C. The resulting cell suspension was filtered through a 70-μm nylon mesh cell strainer (BD Falcon, catalog no. 352350) to remove cell clumps, and debris

and was centrifuged at 200 × *g* for 10 minutes. The cell pellet containing the stromal vascular fraction was resuspended in DMEM supplemented with 10% FBS and penicillin/streptomycin and plated in 6- or 12-well plates.

For brown-like fat differentiation assays, confluent 10T1/2 cells and mouse SVF cells were treated with induction medium containing DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 20 nM insulin (Sigma-Aldrich, catalog no. I1507), 1 nM T3 (Sigma-Aldrich, catalog no. T2877), 125 μM indomethacin (Sigma-Aldrich, catalog no. I7378), 1 μM dexamethasone (Sigma-Aldrich, catalog no. D4902), 1 μM rosiglitazone (Sigma-Aldrich, catalog no. 557366-M), and 0.5 μM isobutylmethylxanthine (IBMX; Sigma-Aldrich, catalog no. I5879). After 2 (for 10T1/2 cells) or 4 days (for scWAT SVF cells) of induction, the culture medium was replaced with maintenance medium containing DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 nM T3, and 20 nM insulin. Thereafter, maintenance medium was changed every 2 days until cells were fully differentiated. To test the role of cAMP modulators in the regulation of ZNF638 levels, differentiated brown-like adipocytes were treated with either forskolin (Sigma-Aldrich, catalog no. F6886), isoproterenol (Sigma-Aldrich, catalog no. I6504), or IBMX at the concentrations and time indicated before cells were collected for RNA and protein analysis.

B. Mice

Eight-week-old C57BL/6J male mice (The Jackson Laboratory, catalog no. 000664) were housed in standard cages in the animal facility at 24°C for 1 week to acclimate before they were used for subsequent studies. Mice were exposed to a 12-hour light/12-hour dark cycle and had free access to food and water. For cold exposure experiments, male mice at 9 weeks of age were either kept at room temperature (RT) or at 4°C with full access to fresh water for up to 6 hours. At the end of this time period, animals were euthanized and BAT and scWAT were harvested for subsequent analysis. For experiments involving pharmacological β-adrenergic stimulation, male mice at 9 weeks of age mice were injected IP with 100 μL of either saline alone or CL316,243 (Sigma-Aldrich, catalog no. C5976) at the concentration of 1 mg/kg body weight dissolved in saline. Three hours after the injection, mice were euthanized and BAT and scWAT were harvested for analyses. The Institutional Animal Care and Use Committee at New York University Langone Medical Center approved the mouse experimental protocol for this study.

C. Plasmids and Site-Directed Mutagenesis

The CREB and dominant-negative CREB (DN-CREB) expression plasmids were obtained from AddGene (made available by Dr. Charles Vinson, National Institutes of Health, Bethesda MA); the pGL3 luciferase reporter vector (catalog no. E1751) and the pRL *Renilla* luciferase control reporter vector (catalog no. E2231) were purchased from Promega. The 2-kb sequence upstream of the transcription start site of the ZNF638 gene was generated based on the reference sequence no. NM008717.3 and cloned into the 5′ *Kpn*I and 3′ *Xho*I sites of pGL3vector (Genewiz). Point mutations were introduced in the wild-type (WT) ZNF638 promoter luciferase reporter (ZNF638-WT-promoter) upstream sequence spanning from −463 to −409 by site-directed mutagenesis (ZNF638-mut-promoter) according to the manufacturer's protocol (New England BioLabs, catalog no. E0554S) using the following oligonucleotides: ZNF638, forward, 5′-AATCCCAGCAACTACATGGCTAATAATACACACTTATAATTCCAACAGTTG-3′; ZNF638, reverse, 5′-TGAACTCGTGAGCTTCAGAATGGTGATTAGTGCTCCTAACCACT GA-3′.

D. Transient Transfections

For gene expression studies, differentiated 10T1/2 cells were transfected using DharmaFECT transfection reagent (Dharmacon, catalog no. T-2002-03), according to the manufacturer's instructions. For experiments involving the effects of DN-CREB, 24 hours after transfection,

the culture medium was changed and cells were treated with 10 μM forskolin or vehicle for 1 hour, or as otherwise noted, before they were harvested. For CREB gain-of-function experiments, brown-like differentiated 10T1/2 cells were transfected with 5 μg of vector or CREB expression plasmid and cells were harvested 2 days later. For CREB knockdown experiments, brown-like differentiated 10T1/2 cells were transfected with 35 nM of small interfering RNA (siRNA) luciferase (Dharmacon, catalog no. D002050-01-20) or siRNA CREB (Dharmacon, catalog no. J-040959-002) and harvested 3 days later. One hour prior to collection, cells expressing siRNA luciferase or siRNA CREB were stimulated with 10 μM forskolin.

E. RNA Isolation and RT-PCR Analysis

Total RNA was extracted from cultured cells and fat tissues using an RNeasy Midi kit (Qiagen, catalog no. 75144) and TRIzol (Thermo Fisher, catalog no. 15596018), respectively. Reverse transcription was performed from 1 μg of total RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, catalog no. 1708890). For gene expression analysis, real-time quantitative PCR (qPCR) was performed in triplicate using 25 ng of cDNA and 300 nM primers with the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, catalog no. 1708880), according to the manufacturer's protocol. Relative mRNA quantification was calculated via the ΔΔCt method with normalization of each sample to the average change in cycle threshold value of the control 36B4 gene. The following primers were used for qPCR analysis: ZNF638, forward, 5′-ATTGAGAGCTGTCGGCAGTTA-3′; ZNF638, reverse, 5′-GGAATGAGAACGTCTTCTTGGAG-3′; 36B4, forward, 5′-GCTTCATTGTGGGAGCAGAC-3′; 36B4, reverse, 5′-ATGGTGTTCTTGCCCATCAG-3′; PGC1α, forward, 5′-ACCATGACTACTGTCAGTCACTC-3′; PGC1α, reverse, 5′-GTCACAGGAGGCATCTTTGAAG-3′; Ucp1, forward, 5′-GGCCCTTGTAAACAACAAAATAC-3′; Ucp1, reverse, 5′-GGCAACAAGAGCTGACAGTAAAT-3′; ZFP516, forward, 5′-AGCGCTTGGATATCCTCAGTA-3′, ZFP516, reverse, 5′-GAGGGGCCCTGCTGGCACAGT-3′: p300, forward, 5′-TACAGCAGGGCTCTCCTCAGAT-3′; p300, reverse, 5′-CAAGGCCACTTGCTCCAATCTG-3′; BRG1, forward, 5′-AGCGATGACGTCTCTGAGGT-3′; BRG1, reverse, 5′-GTACAGGGACACCAGCCACT-3′; SRC1, forward, 5′-GACCCTGCAAACCCAGAC TC-3′; SRC1, reverse, 5′-CGTGGATTTCTCTTGCTCCATT-3′; MED1, forward, 5′-TGCTTGGAAAATTCC TCAAAA-3′; MED1, reverse, 5′-GATGTCAAAGTGGCTCACCA-3′; EHTM1, forward, 5′-TAAAACAGA GGACGGTGATTGAG-3′; EHTM1, reverse, 5′-AGGGCACTATCATCTAAGGCTT-3′; CARM1, forward, 5′-TTGATGTTGGCTGTGGCTCTGG-3′; CARM1, reverse, 5′-ATGGGCTCCGAGATGATGA TGTCC-3′; PRDM16, forward, 5′-CCACCAGCGAGGACTTCAC-3′; PRDM16, reverse, 5′-GGAGGA CTCTCGTAGCTCGAA-3′.

F. Western Blot Analysis

Whole-cell extracts from cultured cells and tissues were obtained using RIPA buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100), supplemented with a protease inhibitor cocktail (Thermo Fisher, catalog no. PIA32953). Twenty micrograms of each protein lysate was run on 8% SDS–polyacrylamide gels and transferred onto 0.45-μm polyvinylidene difluoride membranes (Millipore, catalog no. IPVH00010). Membranes were blocked using 5% nonfat dry milk (w/v) in TBST 0.1% buffer [50 mM Tris-HCl, 150 mM NaCl (pH 7.4), and 0.1% Tween 20] for 1 hour at room temperature and subsequently incubated with the primary antibody overnight at 4°C in TBST 0.1% buffer with 1% BSA (Thermo Fisher, catalog no. BP9703-100). The following antibodies were used at the indicated dilutions: anti-ZNF638 (Bethyl Laboratories, catalog no. A301-548A) at 1:2000 [22], anti-UCP1 (Abcam, catalog no. ab10983) at 1:1000 [23], anti–phospho-CREB Ser133 (Cell Signaling Technology, catalog no. 9198S) at 1:1000 [24], anti-CREB (Cell Signaling Technology, catalog no. 4820S) at 1:1000 [25], anti–β-actin (Cell Signaling, catalog no. 3700) at 1:1000 [26], and anti-vinculin (Proteintech, catalog no. 66305-1-Ig) at 1:2000 [27]. Membranes were washed in TBST 0.1% (v/v) and incubated for 1 hour at room temperature with a 1:20,000 dilution of anti-rabbit (Bio-Rad Laboratories,

catalog no. 1706515) [28] or anti-mouse (Bio-Rad Laboratories, catalog no. 1706516) [29] IgG horseradish peroxidase conjugated in TBST 0.1% with 2% nonfat dry milk (w/v). After four additional washes in TBST 0.1% (v/v), immunoblots were developed using enhanced chemiluminescence (GE Healthcare, catalog no. RPN2108) on HyBlot CL autoradiography films (Thomas Scientific, catalog no. E3012) using an X-ray film developer (Konica Minolta, catalog no. SRX-101A). All experiments were repeated at least three times. Densitometry of Western blots was performed using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD). Normalization to the signal intensity of either β-actin or vinculin used as protein loading control was performed for each sample in each experiment. Control values were set to 1.0. For densitometry analysis, the quantitation was performed within the linear range of the standard curve defined by the standard sample, β-actin, or vinculin.

G. In Silico *Analysis of the ZNF638 Promoter*

To identify possible responsive elements present in the ZNF638 promoter region, we scanned 2 kb of the genomic region upstream of the ZNF638 transcriptional start site using the Genomatrix software that predicted putative transcription factor binding motifs using MatInspector and TRANSFAC databases. This analysis identified two putative half CRE binding motifs [30], one located between −416 and −409 (TGACTCAC) and another between −463 and −456 (GACTGCT).

H. EMSAs

For EMSAs, 10T1/2 cells differentiated into brown-like adipocytes were treated for 4 hours with 10 μM forskolin and nuclear proteins were extracted using the NE-PER kit (Thermo Fisher, catalog no. 78833). Nuclear extracts were incubated with biotin-labeled WT and mutant probes spanning the sequence of the CREB putative binding elements in positions −463 to −456 and −416 to −409 from the ZNF638 transcription start site. The −463 to −456 sequence TGACTGCT was mutated into TAACGTCT and the −416 to −409 sequence TGACTCAC was mutated into CTAATAAT. A competition assay was performed using 100-fold excess of unlabeled WT probe. The binding complexes were resolved on a 4% TBE precast gel (Bio-Rad Laboratories, catalog no. 4565013) at 4°C, transferred onto nitrocellulose membrane (GE Healthcare, catalog no. RPN3038), and UV cross-linked (Stratalinker, catalog no. 2400). Detection was performed according to the manufacturer's instructions (Thermo Fisher Scientific, catalog no. 20148). The following oligonucleotides were used for EMSAs: WT-promoter-oligo, 5′-GCACTGACTGCTATTCTGAAGCTCACGAGTTCAAAT CCCAGCAACTACATG GTGACTCACAAC-3′; WT-RC-oligo, 5′-GTTGTGAGTCACCATGTAGTTGCTGGG ATTTGAACTCGTGAGCTTCAGAATAGCAGTCAGTGC-3′; Mut-promoter-oligo, 5′-GCACTA ACGTCTATTCTGAAGCTCACGAGTTCAAATC CCAGCAACT ACATGG TAA CGCACAAC-3′; Mut-RC-oligo, 5′-GTTGTGCGTTACCA TGTAGTTGCTG GGATTTGAACTCGTGAGCTTC-AGA ATAGACGTTAGTGC-3′.

I. Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) analysis of 10T1/2 cells differentiated into brown-like adipocytes was performed according to the manufacturer's protocol (Millipore, catalog no. 17-295), as previously described [21]. Briefly, differentiated cells were treated with 1% formaldehyde to cross-link protein to DNA. Cells were then washed with ice-cold PBS buffer containing protease inhibitors (Millipore, catalog no. 20-283), harvested, and sonicated for 30 minutes using Diagenode Bioruptor®. Protein/DNA complexes were immunoprecipitated overnight at 4°C using anti–phospho-CREB Ser133 antibody (Cell Signaling Technology, catalog no. 9198S) [24] or with IgG control antibody (Millipore, catalog no. 17-295). After incubation with protein A agarose beads (Millipore, catalog no. 16-201C), protein/DNA complexes were eluted for 15 minutes at room temperature using Elution Reagent C (Millipore, catalog no. 20-294). DNA fragments were purified and amplified by qPCR using the

following primers designed specifically to recognize the area spanning the putative CRE elements identified in the proximal ZNF638 promoter in position −463 to −456 and −416 to −409: PromZNF638, forward, 5′-CTCAGTGGTTAGGAGCACT-3′; PromoZNF638, reverse, 5′-GGCACCCCA GATTAGGAAT-3′.

J. Luciferase Assays

For transient transfection studies, HEK-293 cells were plated at a density of 20,000 cells per well in 12-well tissue culture plates and 24 hours later transfected with 0.5 μg of WT or mutant promoter with altered CRE motifs (ZNF638-WT-promoter and ZNF638-mut-promoter, respectively), 0.5 μg of control or 0.5 μg of CREB expression plasmid, and 50 ng of *Renilla* reporter plasmid, using Lipofectamine 2000 (Thermo Fisher, catalog no. 11668019). Forty-eight hours after transfection, cells were treated with 10 μM forskolin for 4 hours before cells were harvested for luciferase assays. The luciferase reporter activity was measured using the Dual-Luciferase reporter assay system kit (Promega, catalog no. E1910). Luciferase activity was detected using the SpectraMax 96-well plate reader (Molecular Devices), and the values were normalized to *Renilla* activity.

K. Statistical Analysis

The results were expressed as mean ± SE, unless otherwise noted in the figure legends. The groups were compared by a Student *t* test or one-way ANOVA. *P* values <0.05 were considered statistically significant. All statistical analyses were performed using Prism 7 software (GraphPad Software).

2. Results

A. ZNF638 Is Selectively Expressed in Thermogenic Fat Tissues

We have previously shown that ZNF638 is expressed during early stages of white fat differentiation and mechanistically demonstrated that it acts as a transcriptional coactivator that regulates early events of adipogenesis *in vitro* [20]. To determine whether ZNF638 is expressed in adipose tissues *in vivo* and assess its levels in different fat depots, we analyzed ZNF638 mRNA levels in BAT, scWAT, and epididymal WAT (eWAT) of 9-week-old mice. As shown in Fig. 1A, ZNF638 mRNA levels were higher in BAT and scWAT than in eWAT. Analysis of protein levels confirmed that ZNF638 is present at higher levels in BAT and scWAT compared with eWAT (Fig. 1B). These results suggest that ZNF638 is selectively enriched in thermogenic fat tissues in adult mice.

Given that ZNF638 mRNA is elevated in scWAT and BAT tissues **(**Fig. 1A and 1B), we assessed whether ZNF638 is expressed specifically in thermogenic adipocytes. We therefore measured the levels of ZNF638 at different time points during brown fat differentiation of SVF cells derived from scWAT and in the mesenchymal cell line 10T1/2. RNA and protein analysis revealed that ZNF638 is present at low levels in undifferentiated SVF cells obtained from scWAT but induced in fully differentiated cells (Fig. 1C and 1D). Analysis of ZNF638 during a time course of differentiation of 10T1/2 cells revealed that ZNF638 mRNA is upregulated after 72 hours from the induction of differentiation. UCP1 levels were measured in parallel to confirm that SVF cells derived from scWAT and 10T1/2 cells underwent brown-like differentiation (Fig. 1E and 1F). These results demonstrate that ZNF368 mRNA is elevated in mature thermogenic tissues *in vivo* and *in vitro* in fully differentiated brown-like adipocytes, compared with preadipocytes.

B. ZNF638 Is Induced in Response to Modulators of cAMP Levels

Given the novel evidence that ZNF638 is present in differentiated thermogenic adipocytes (Fig. 1), we assessed whether ZNF638 expression may be regulated by classic stimuli that

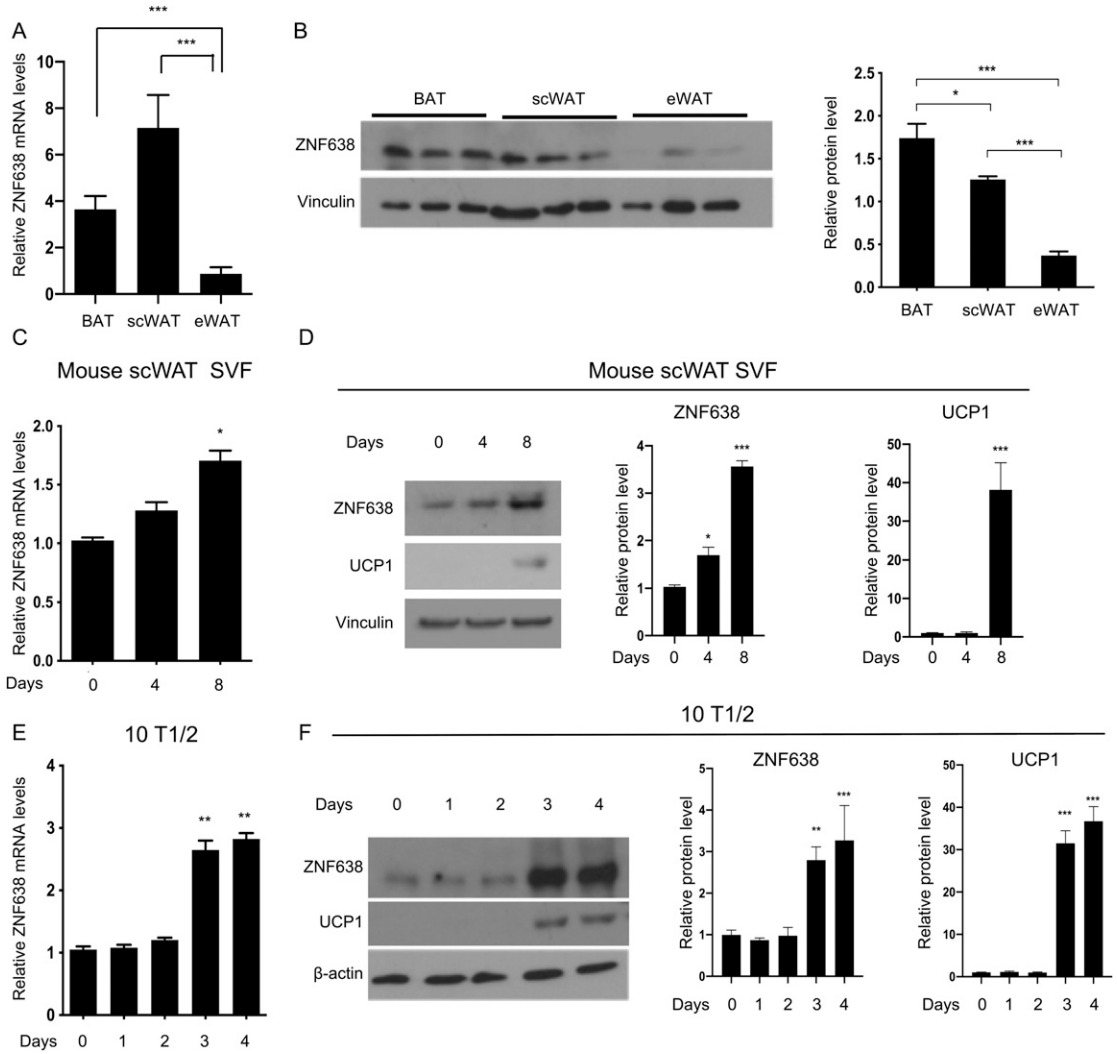

Figure 1. ZNF638 is highly expressed in thermogenic tissues and adipocytes. (A and B) ZNF638 mRNA (A) and protein levels presented as Western blot and quantified via densitometric analysis (B) in BAT, scWAT, and eWAT of mice fed a normal diet (n = 3). Vinculin was used as a loading control. (C and D) Relative ZNF638 mRNA levels (C) and ZNF638 and UCP1 (D) protein levels during a time course of brown differentiation of mouse SVF cells isolated from scWAT. Vinculin was used as a loading control. (E and F) Relative ZNF638 RNA levels (E) and ZNF638 and UCP1 (F) protein levels in a time course of differentiation of 10T1/2 cells. β-Actin was used as loading control. Results are expressed as a mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.

control the functionality of brown and beige cells [31, 32]. To determine whether modulators of cAMP levels can regulate ZNF638, we treated brown differentiated scWAT SVF cells with forskolin and observed an increase in ZNF638 levels compared with those observed in vehicle-treated cells (Fig. 2A and 2B). In parallel, we analyzed phospho-CREB levels to confirm the effectiveness of forskolin treatment in eliciting cAMP-dependent downstream events. To further investigate the dynamic of the induction of ZNF638 in response to a variety of cAMP modulators, we performed a detailed time course in differentiated 10T1/2 cells and assessed the induction of ZNF638 in response to forskolin, isoproterenol, and IBMX. As shown in Fig. 2C–2E, ZNF638 mRNA levels were rapidly induced in differentiated cells by all of the treatments tested. ZNF638 protein levels were also induced in differentiated 10T1/2 cells treated with these three cAMP modulators (Fig. 2F). In parallel to the elevation in ZNF638 mRNA, we observed an increase in the levels of ZFP516 and PGC1α, two cold-inducible

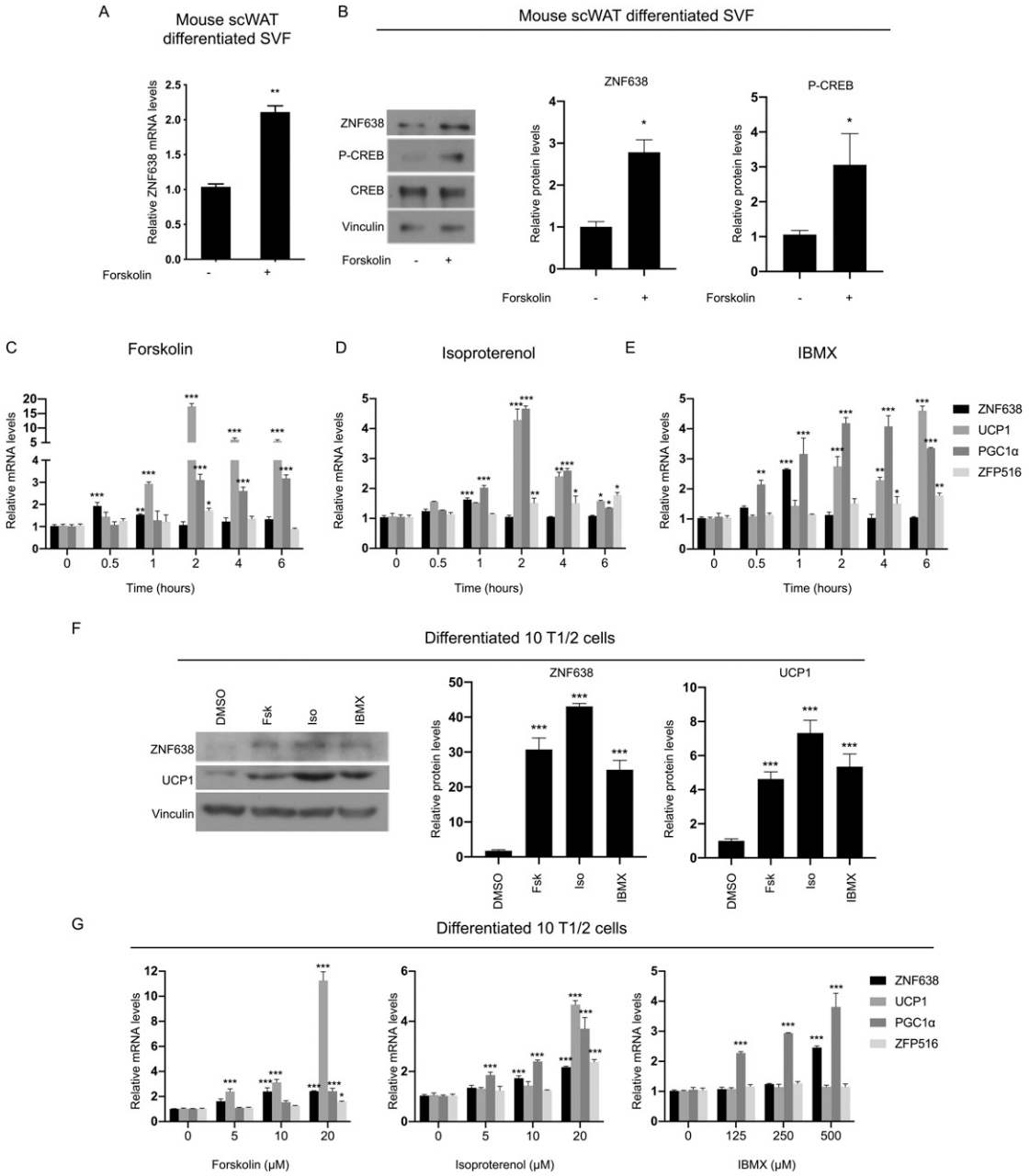

Figure 2. Activators of cAMP upregulate ZNF638 levels in thermogenic adipocytes. (A and B) Relative ZNF638 mRNA (A) and protein (B) levels in differentiated mouse SVF cells isolated from scWAT treated for 4 h with 10 μM forskolin. (C–E) Relative mRNA levels of ZNF638, UCP1, PGC1α, and ZFP516 in differentiated 10T1/2 cells treated with 10 μM forskolin (C), 10 μM isoproterenol (D), or 500 μM IBMX (E) for the indicated times. (F) ZNF638 protein levels in differentiated 10T1/2 cells treated with 10 μM forskolin, 10 μM isoproterenol, or 500 μM IBMX for 6 h. (G) Dose response of ZNF638 induction in response to forskolin, isoproterenol, and IBMX treatments at the doses indicated for 1 h. Relative ZNF638 levels were calculated by densitometric analysis via ImageJ. Results are expressed as a mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.

transcriptional (co)factors known to be induced in response to cAMP modulators [17, 18]. Elevation of UCP1 levels further confirmed the effectiveness of the different compounds used (Fig. 2C–2E). Additionally, a dose response analysis of the induction of ZNF638 confirmed the ability of forskolin, isoproterenol, and IBMX (Fig. 2E) to rapidly induce ZNF638 levels. Taken

together, these results suggest that ZNF638 is regulated by modulators of cAMP in thermogenic adipocytes and that the induction of ZNF638 mRNA occurs rapidly and precedes that of UCP1.

C. ZNF638 Is Induced by Cold Temperatures and by Pharmacological Activation of β-Adrenergic Signaling in Thermogenic Fat Tissues

Given that ZNF638 is expressed in thermogenic cells and tissues and that it is upregulated by modulators of cAMP levels *in vitro,* we assessed whether ZNF638 is controlled also *in vivo* in response to thermogenic stimuli. As shown in Fig. 3A and 3B, BAT and scWAT obtained from mice exposed for 6 hours to 4°C showed increased ZNF638 RNA and protein in comparison with the levels detected in control mice kept at RT. The effectiveness of the cold exposure in eliciting thermogenic gene expression was confirmed through analysis of the levels of classic markers of brown fat functionality such as UCP1, ZFP516, and PGC1α, as shown in Fig. 3A and 3B. To establish the relative magnitude of ZNF638 induction in comparison with that of other cofactors known to be relevant in thermogenic fat tissues, we measured the extent of induction of a number of cofactors in response to thermogenic stimuli. As shown in Fig. 3E, ZNF638 is among the most highly induced cofactors in scWAT by cold stimuli. To further assess the responsiveness of ZNF638 to pharmacological activation of the β-adrenergic signaling pathway *in vivo*, we analyzed the levels of ZNF638 in mice treated with the β-adrenergic agonist CL316,243 and observed an increase in ZNF638 in BAT and scWAT in CL316,243-treated mice, compared with controls (Fig. 3C and 3D). As shown in Fig. 3C and 3D, ZNF638 induction occurred in parallel to that of the classic thermogenic markers UCP1, PGC1α, and ZFP516. Taken together, these results suggest that ZNF638 mRNA expression is regulated in response to thermogenic cues both *in vitro* and *in vivo* in thermogenic adipocytes and tissues and that the elevation in ZNF638 levels is concomitant to that of classic transcriptional regulators involved in thermogenic responses.

D. CREB Regulates ZNF638 Expression

Given that CREB is downstream of PKA signaling and critically involved in the regulation of the expression of a number of genes involved in thermogenesis [15], we asked whether CREB may also control ZNF638 levels in response to cAMP elevation. *In silico* analysis of the ZNF638 promoter revealed the presence of two putative CREB binding motifs (CRE), one in position −463 to −456 and another at −409 to −416 from the ZNF638 transcription start site (Fig. 4A). EMSAs demonstrated that proteins present in nuclear extracts obtained from differentiated 10T1/2 cells treated with forskolin were able to generate a shift when incubated with a labeled probe corresponding to the sequence spanning the ZNF638 promoter region with the putative CRE binding elements. The binding to these sites appeared to be specific given that it was competed off when nuclear extracts were pretreated with excess unlabeled oligonucleotides and because no shift was observed when nuclear extracts were incubated with oligonucleotides containing mutated CRE sequences (Fig. 4B). These data suggest that the putative CRE elements in position -463 to -456 and -409 to -416 are bound by proteins present in nuclear extracts of cells stimulated with forskolin. To more definitively establish whether CREB binds to the putative CRE motifs present in the ZNF638 promoter, we performed ChIP assays. Our results demonstrated that endogenous phospho-CREB present in differentiated 10T1/2 cells treated with forskolin can occupy the putative CRE sites we identified in the ZNF638 promoter (Fig. 4C). These results indicate that CRE motifs present in the promoter region of ZNF638 can recruit phospho-CREB in cells stimulated with forskolin.

To test whether CREB signaling is overall necessary for the regulation of the expression of endogenous ZNF638 in response to forskolin, we expressed a DN-CREB in differentiated 10T1/2 cells. As shown in Fig. 5A, forskolin-dependent induction of ZNF638 was suppressed in the presence of a DN-CREB. These data further suggest that CREB may be involved in the

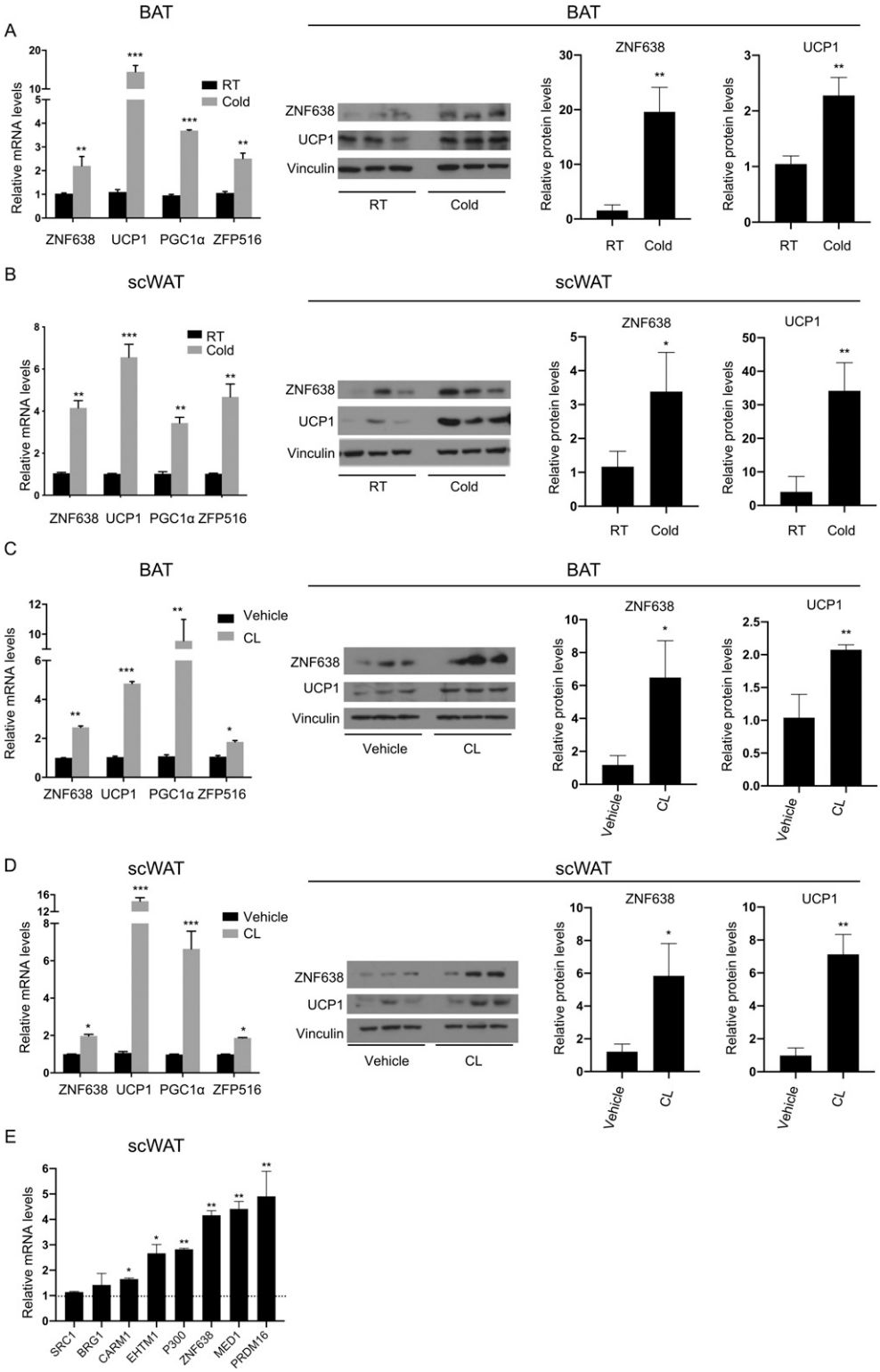

Figure 3. ZNF638 is induced by cold exposure and pharmacological activation of β-adrenergic signaling in thermogenic tissues. (A) Relative ZNF638, UCP1, PGC1α, ZFP516, and UCP1 mRNA levels and ZNF638 and UCP1 protein levels in BAT of mice exposed to cold temperature for 6 h of mice (n = 3). (B) Relative ZNF638, UCP1, PGC1α, ZFP516, and UCP1 mRNA levels in scWAT and ZNF638 and UCP1 protein levels in scWAT of mice exposed to cold temperature for 6 h (n = 3). (C) Relative ZNF638, UCP1, PGC1α, ZFP516, and UCP1 mRNA levels and ZNF638 and UCP1 protein levels in BAT of mice treated with CL316,243 (CL) for 3 h (n = 3). (D). Relative ZNF638, UCP1, PGC1α, ZFP516, and UCP1 mRNA levels and ZNF638 and UCP1 protein levels in scWAT of mice treated with CL316,243 (CL) for 3 h (n = 3). (E) Relative mRNA levels of cofactors expressed in scWAT at RT and after cold exposure. Results are expressed as a mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.

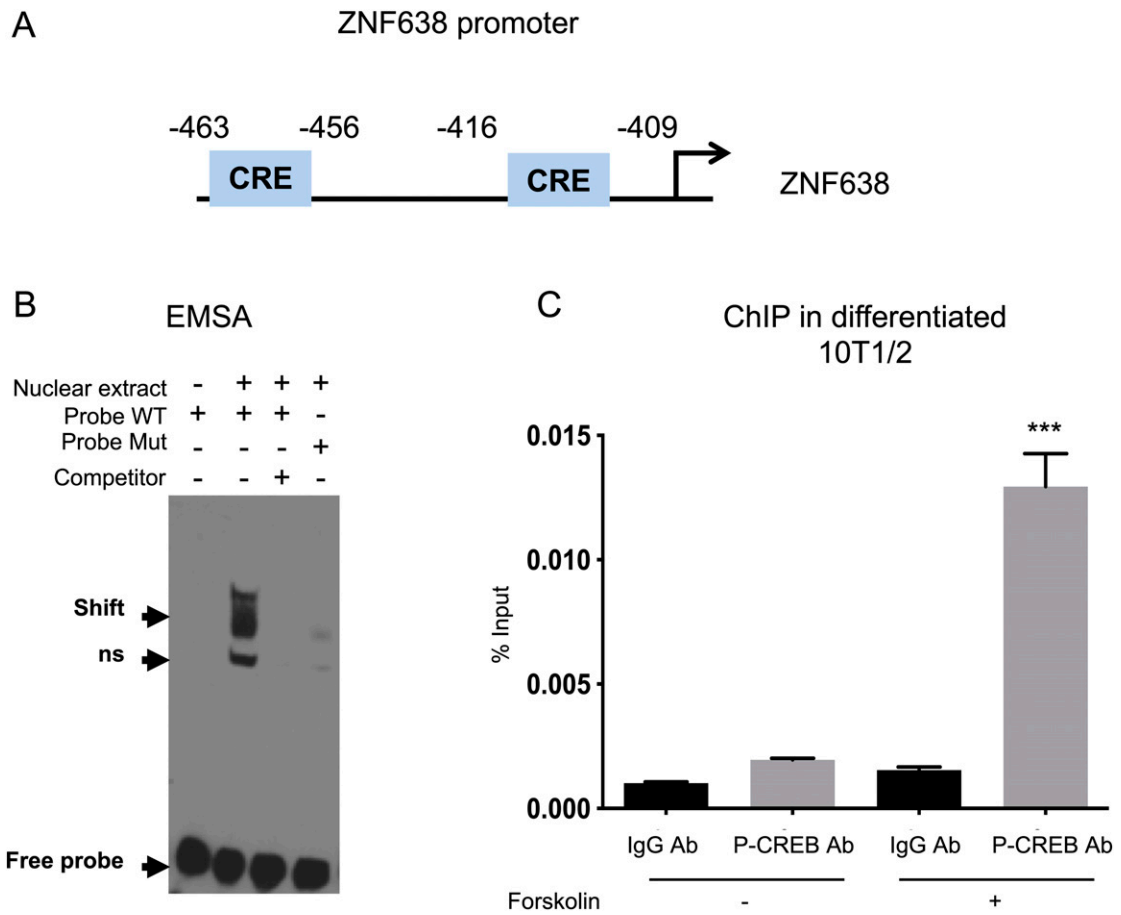

Figure 4. CREB binds to the ZNF638 promoter. (A) *In silico* analysis using the Genomatix program predicted two CREB binding sites, one at −409 to −416 and one at −456 to −463 in the promoter region of ZNF638. (B) EMSA with nuclear extracts obtained from 10T1/2 differentiated into brown-like fat cells treated with 10 μM forskolin for 1 h. Nuclear extracts incubated with biotin-labeled DNA probes corresponding to the putative CRE binding sequences generated a shift (lane 2), detected only in the presence of a probe containing the wild-type CRE (probe WT) sequence, but not with a mutated CRE (designated probe MT). For competition assays, a 100-fold excess of unlabeled WT oligonucleotide was used. Specific complexes are indicated with arrows, and nonspecific complexes are indicated by ns. (C) ChIP assay performed in 10T1/2 differentiated into brown-like fat cells. IgG and phospho-CREB antibodies were used to assess the occupancy of CREB at the putative CRE sites in the ZNF638 promoter in the presence (+) or absence (−) of forskolin at the concentration of 10 μM, for 1 h. Results are expressed as a mean ± SEM from three independent experiments. ***$P < 0.001$.

regulation of ZNF638 expression in response to forskolin. To further determine whether CREB is sufficient to modulate ZNF638 levels in response to thermogenic signals, we performed luciferase assays and gene expression studies. To measure the effects of CREB in regulating ZNF638, we first generated a ZNF638 promoter reporter gene by cloning 2 kb of the sequence upstream of the ZNF638 transcription start site containing the two CRE elements. In addition to this WT ZNF638 promoter reporter construct, we generated a reporter containing the mutated putative CRE binding motifs to more specifically assess the requirement and necessity of the CRE sites for ZNF638 regulation. As shown in Fig. 5B, luciferase assays revealed that the CRE sites are required for the regulation of the expression of the reporter gene, suggesting the involvement of CRE sites in the regulation of ZNF638 induction. Furthermore, coexpression of CREB with the ZNF638 promoter reporter constructs demonstrated that luciferase activity is induced only when CREB is coexpressed with

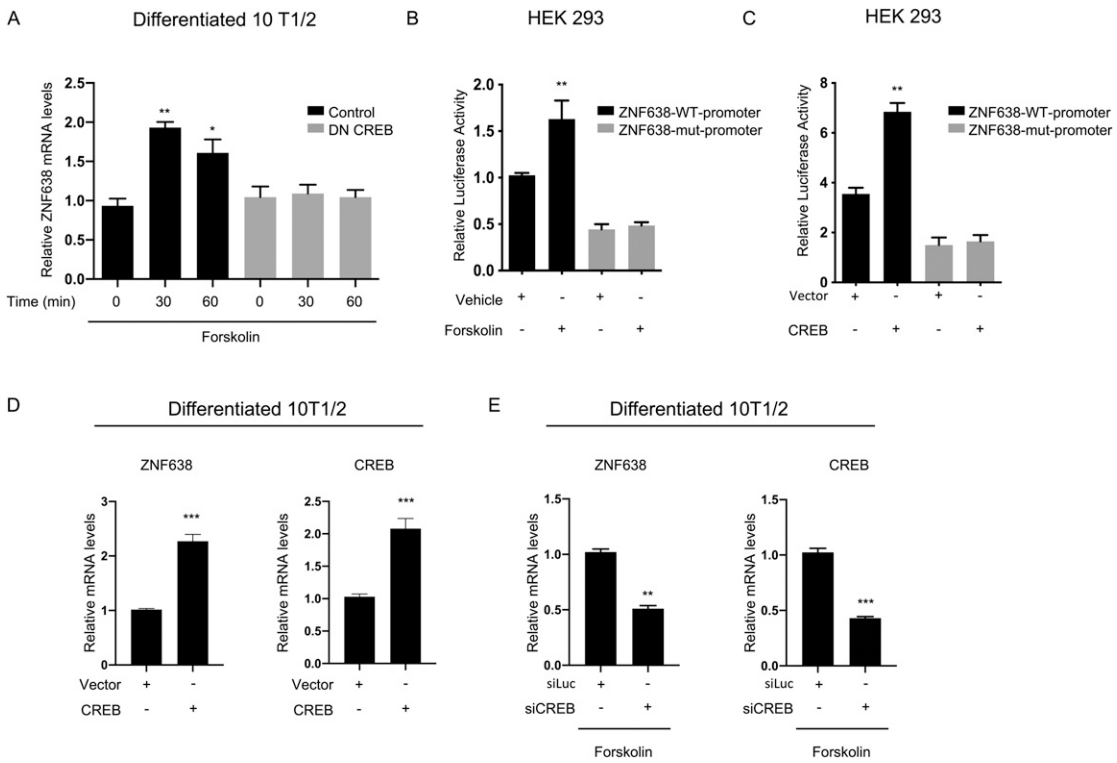

Figure 5. CREB is required for the induction of ZNF638 in response to elevation in cAMP levels. (A) Relative ZNF638 mRNA levels in response to forskolin treatment in differentiated 10T1/2 cells expressing either vector control or a DN-CREB for the indicated times. (B) Luciferase assays were performed in HEK-293 cells expressing either a luciferase reporter vector with the WT ZNF638 promoter containing two CRE putative motifs (ZNF638-WT-promoter) or a luciferase reporter vector with the mutated ZNF638 promoter with altered CRE putative motifs (ZNF638-mut-promoter) after treatment with vehicle or 10 μM forskolin for 4 h. (C) Luciferase assay in HEK-293 cells cotransfected with either vector or CREB with either the WT or the mutant ZNF638 luciferase reporter construct. Results show a representative experiment performed in triplicate. (D and E) Relative RNA levels of ZNF638 and CREB in 10T1/2 differentiated cells with either CREB overexpression (D) or knockdown (E) treated with vehicle or 10 μM forskolin. Data are represented as mean ± SD (n = 3). $*P < 0.05$, $**P < 0.005$.

the WT ZNF638 promoter, but not when the mutated reporter is used (Fig. 5C). These data suggest that CREB can bind to CRE putative elements present in the ZNF638 promoter and that CREB is required for the induction of the ZNF638 promoter luciferase reporter.

To test whether CREB is sufficient and necessary to induce the expression of endogenous ZNF638 in cells, we performed gain-of-function and loss-of-function experiments in differentiated 10T1/2 cells and assessed the effects of the modulation of CREB on ZNF638 mRNA levels. As shown in Fig. 5D, overexpression of CREB led to the induction of ZNF638 whereas its knockdown decreased ZNF638 levels in response to forskolin (Fig. 5E). Collectively, these data demonstrate that the putative CRE sites identified are *bona fide* CREB binding elements and that CREB is necessary and sufficient to regulate ZNF638 levels (Fig. 6).

3. Discussion

We have previously characterized the zinc finger protein ZNF638 as a novel coactivator involved in early events of white adipocyte differentiation *in vitro* [20], but whether ZNF638 is expressed in mature fat tissues *in vivo* and whether it is expressed in a fat depot–selective manner are currently unknown. In this study, we provide novel evidence that ZNF638 is

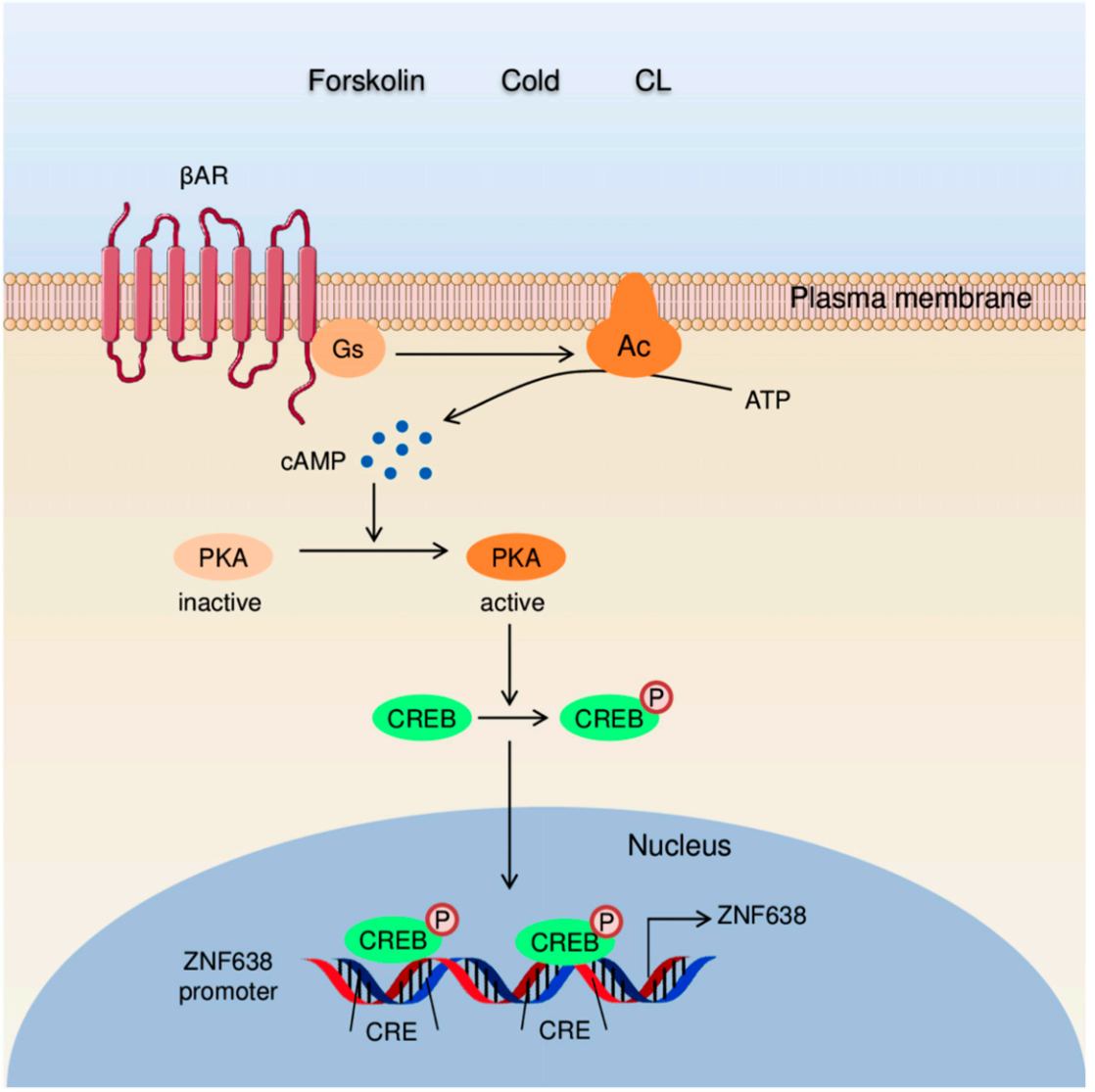

Figure 6. Schematic representation of the regulation of the expression of ZNF638 in response to thermogenic cues. Shown are the possible molecular events leading to the induction of ZNF638 levels in response to thermogenic stimuli involving the cAMP/CREB-dependent pathway. Ac, adenylyl cyclase; CL, CL316,243; βAR, β-adrenergic receptor.

expressed at higher levels in thermogenic adipocytes and tissues and demonstrate, to our knowledge for the first time, that ZNF638 levels are induced in response to thermogenic stimuli *in vivo* and controlled by the transcription factor CREB.

It has been extensively demonstrated that factors such as PGC1α, ZFP516 and UCP1 involved in brown fat thermogenesis and functionality [17, 18, 33] are induced in response to elevation of cAMP levels *in vitro* and by cold and pharmacological β-adrenergic stimulation *in vivo*. Our data showing that both pharmacological agents that elevate the levels of cAMP and cold exposure lead to a rapid increase in ZNF638 mRNA levels, provide the first evidence that ZNF638 is upregulated by thermogenic stimuli. Furthermore, our data reveal that ZNF638 elevation occurs concomitantly to that of known critical transcriptional regulators of thermogenic responses (Fig. 2) and precedes the induction of UCP1. These data suggest that ZNF638 may be involved in the concerted transcriptional response to thermogenic stimuli involved in the control of brown/beige fat tissue functionality.

ZNF638 expression levels have been studied previously only during the adipogenic process *in vitro* and shown to be transiently upregulated during early events of differentiation, right after the induction phase [20]. The data we provide herein indicate that the pattern of ZNF638 gene expression in brown-like cells and thermogenic tissues is very different from that observed during *in vitro* white fat adipogenesis, given the novel evidence we provide that ZNF638 mRNA is maintained at high levels after brown-like differentiation is achieved and it is rapidly increased after stimulation with modulators of cAMP levels. These data suggest that ZNF638 may be subjected to differential transcriptional regulation depending on the fat tissue type.

Our results reveal that ZNF638 is a novel transcriptional regulator expressed in brown fat tissues that is specifically turned on in response to thermogenic cues at levels comparable to those of other cofactors relevant for brown and beige adipocyte biology and may provide novel insights into the molecular mechanisms controlling brown and beige fat functionality. Future functional studies involving modulation of ZNF638 levels selectively in thermogenic fat tissues may permit us to assess more definitively the role of ZNF638 in mature fat and may allow the evaluation of its requirement for the functionality of thermogenic adipose tissues *in vivo*.

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Additional Information

Correspondence: Elisabetta Mueller, PhD, Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, New York University, New Science Building, Room 612, 435 East 30th Street, New York, New York 10016. E-mail: Elisabetta.Mueller@nyumc.org.

Disclosure Summary: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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