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ORIGINAL ARTICLE

HOXC10 suppresses browning of white adipose tissues

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Given that increased thermogenesis in white adipose tissue, also known as browning, promotes energy expenditure, significant efforts have been invested to determine the molecular factors involved in this process. Here we show that HOXC10, a homeobox domain-containing transcription factor expressed in subcutaneous white adipose tissue, is a suppressor of genes involved in browning white adipose tissue. Ectopic expression of HOXC10 in adipocytes suppresses brown fat genes, whereas the depletion of HOXC10 in adipocytes and myoblasts increases the expression of brown fat genes. The protein level of HOXC10 inversely correlates with brown fat genes in subcutaneous white adipose tissue of cold-exposed mice. Expression of HOXC10 in mice suppresses cold-induced browning in subcutaneous white adipose tissue and abolishes the beneficial effect of cold exposure on glucose clearance. HOXC10 exerts its effect, at least in part, by suppressing PRDM16 expression. The results support that HOXC10 is a key negative regulator of the process of browning in white adipose tissue.
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INTRODUCTION

Increased browning of white adipose tissue (WAT) in humans can increase energy expenditure[1,2] and is proposed to be a potential therapeutic approach to prevent or treat metabolic diseases such as diabetes and obesity. In mice, both classical brown adipose tissue (BAT) and beige adipose tissue confer beneficial effects metabolically. There is evidence to indicate that the brown-like adipose depots at the supraclavicular region of humans have characteristics of beige adipocytes found in mice.[3] Therefore, there lies the important question of whether the molecular mechanisms that regulate the process of browning of WAT are different from those of classical brown fat regulators. Several positive regulators involved in brown fat differentiation/function have been identified. For example, FoxC2, PRDM16, PGC1α, EHMT1, IRF4 and Zfp516 are all involved in the development and function of classical BAT.[4–9] Many of these positive regulators of BAT are also involved in the browning of WAT. It is worth noting that most of these positive regulators were first identified using approaches that generally focus on classical BAT function, rather than the process of browning WAT itself. Therefore, there is a need to identify key regulators that are solely involved in the process of browning WAT.

The homeobox-containing transcription factor, HOXC10, is thought to mainly regulate the development of forelimbs in mammals.[10] It has been shown to be involved in breast cancer, thyroid cancer and cervical squamous cell growth.[11–14] A genome-wide association study indicates that HOXC10 could also be associated with increased waist-to-hip ratio in humans.[15] Here we identify that HOXC10 is enriched in inguinal (SubQ) WAT and is a key negative regulator in the process of browning SubQ WAT alone, with no discerning effect on classical BAT function.

MATERIALS AND METHODS

Cell culture

3T3-L1 adipocytes were differentiated with Dulbecco's modified Eagle's medium (DMEM)–high glucose containing 10% fetal bovine serum (FBS), 350 nM insulin (Gibco, Carlsbad, CA, USA), 250 nM dexamethasone and 500 nM isobutylmethylxanthine for 48 h, followed by 10% FBS–DMEM and 350 nM insulin for 48 h. C2C12 myoblasts were differentiated into myotubes with DMEM–high glucose containing 2% horse serum and 1 μM insulin. Primary cells were

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isolated from white subcutaneous or brown interscapular fat tissue from 8–10-week-old C57BL6 mice as described previously.[16] Adipocyte differentiation was induced by treating cells for 48 h in 10% FBS–DMEM containing 500 nM isobutylmethylxanthine, 125 nM indomethacin, 1 μM dexamethasone, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone (Cayman Chemical, Ann Arbor, MI, USA), followed by 48 h of 10% FBS–DMEM with 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone. HEK293 cells (for lentivirus) or Platinum-E cells (retrovirus) were used for viral production. Cells were infected overnight with viral supernatant supplemented with 4 μg ml^{-1} polybrene. All chemicals for cell culture were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

Gene expression analysis

Total RNA was isolated from cultured cells and tissues using Nucleospin RNA columns (Macherey-Nagel, Düren, Germany). Complementary DNA was prepared from total RNA using the first strand cDNA synthesis kit from Thermo Scientific (Waltham, MA, USA) and used in quantitative PCR reactions containing SYBR-Green fluorescent dye (Invitrogen, Carlsbad, CA, USA). Relative messenger RNA (mRNA) expression was determined by $\Delta\Delta^{-Ct}$ method using tata-binding protein levels as the endogenous control. Quantitative PCR was performed using the LightCycler 480 PCR machine (Roche, Basel, Switzerland). Real-time PCR oligo sequences are provided in Supplementary Table S2. For microarray analysis, an Affymetrix GeneChip Mouse Genome 430 2.0 array (Santa Clara, CA, USA) was used. The array data were analyzed using the Partek Genomics Suite 6.6 software (Partek Inc., St Louis, MO, USA). Gene enrichment was analyzed using the Enrichr program.[17]

Chromatin immunoprecipitation assays

3T3-L1 adipocytes expressing FLAG-tagged HOXC10 were used for chromatin immunoprecipitation assays. For more information, see Supplemental Experimental Procedures.

Reporter gene assays

Reporter gene assays were performed in HEK293 cells. In brief, the ~2 kb PRDM16 reporter gene was co-transfected with respective expression plasmids using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, cells were collected, and reporter gene activity was measured using the Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase reporter gene measurements were normalized using Renilla luciferase activity.

Gel shift assays

Nucleus extract from HEK293 cells transfected with FLAG-HOXC10 expression plasmid was isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) and used in gel shift assays. Also see Supplemental Experimental Procedures.

Mice and metabolic studies

All animal experiments were performed according to the procedures approved by the Agency for Science, Technology and Research's Institutional Animal Care and Use Committee. For cold exposure, 8–10–week-old C57BL6 male mice were singly housed and exposed to 4 °C for 7 days. Body temperature was measured using a rectal probe. For the glucose tolerance test, animals were fasted for 6 h. Glucose levels in tail blood were measured with a standard glucometer before and at timed intervals following an intraperitoneal injection of 2 g D-glucose per kg body weight.

Administration of AAV vectors

The adeno-associated virus (AAV) vectors of serotype 8 were produced by Vector BioLabs (Malvern, PA, USA). To systemically administer AAV vectors into mice, AAV vectors were diluted in 200 μl of saline and injected into the lateral tail vein. For intrainterscapular BAT and intrainguinal (SubQ) WAT administrations, a longitudinal incision was performed in the skin, and 25 μl of AAV solution was injected into the fat depot.

Statistical analysis

All data are reported as mean ± s.e.m. Student's *t*-test was used to calculate significance using the Excel software package (Microsoft Corp., Redmond, WA, USA). A *P*-value <0.05 was considered significant.

RESULTS

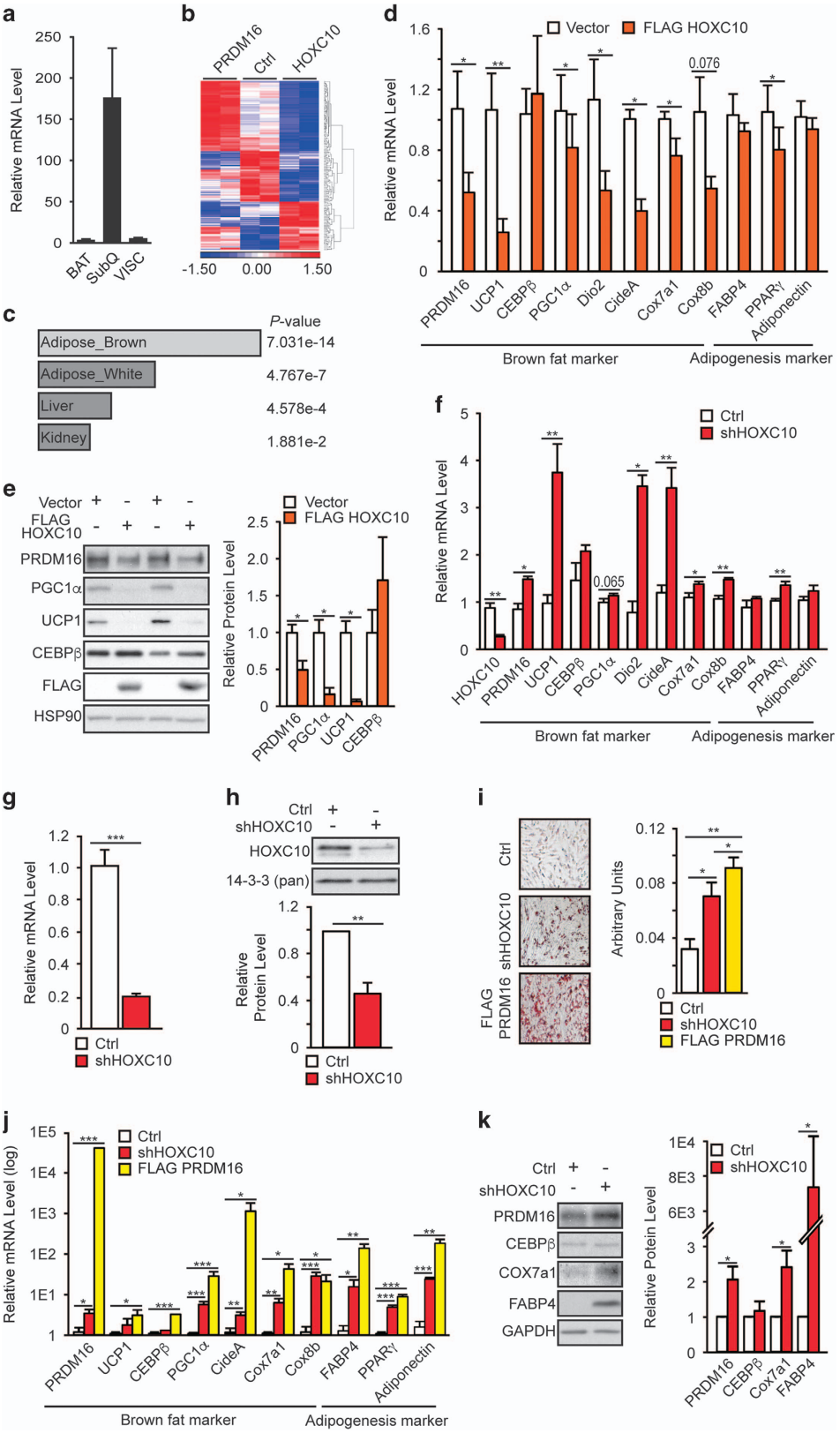
HOXC10 suppresses genes associated with BAT

To examine whether the HOX10 paralogs (HOXA10, HOXC10 and HOXD10) have any role in adipose tissue function, transcript levels of these three genes were examined in inguinal (SubQ) WAT, BAT and visceral WAT (Supplementary Figure S1a). HOXC10 had the highest gene expression level in all three adipose depots examined (Supplementary Figure S1a). The *HOXC10* mRNA expression level was highest in the SubQ WAT and low in BAT and visceral (Figure 1a), indicating that HOXC10 may have a unique role in the SubQ WAT. To obtain a snapshot of the function of HOXC10 in SubQ WAT, global transcript was examined in forskolin-treated adipocytes that were differentiated from stromal-vascular fraction (SVF) isolated from SubQ WAT ectopically expressing HOXC10 (Figure 1b). HOXC10 overexpression was confirmed by an increase in HOXC10 transcript level (Supplementary Figure S1b). A total of 173 genes were differentially expressed (1.5-fold) in adipocytes expressing HOXC10 (Supplementary Table S1). Enrichment analysis on genes that were suppressed by HOXC10 revealed an over-representation of BAT-enriched genes ($P<7.031e-14$; Figure 1c). The majority of the genes suppressed by HOXC10 were highly induced in adipocytes expressing PRDM16 (Figure 1b). PRDM16 overexpression was confirmed by an increase in PRDM16 transcript (Supplementary Figure S1b). These data indicate that HOXC10 may have a role in suppressing the expression of genes involved in browning of adipocytes.

To confirm the role of HOXC10 as a suppressor of brown fat genes, an N-terminal FLAG-tagged HOXC10 was expressed in 3T3-L1 cells. Expression of HOXC10 did not affect lipid accumulation (Supplementary Figure S1c) or adipogenic markers, such as *FABP4* and *adiponectin* (Figure 1d). With the exception of *C/EBPβ*, the expression of HOXC10 suppressed most brown fat genes (Figure 1d). PRDM16, UCP1 and PGC1α protein levels were markedly reduced in HOXC10-expressing adipocytes (Figure 1e). PGC1α and UCP1 were not detectable in 3T3-L1 adipocytes in the untreated condition. Upon forskolin treatment, low levels of PGC1α and UCP1 could be detected in these cell lysates compared with lysates from BAT (Supplementary Figure S2d). In agreement with the data obtained from 3T3-L1 adipocytes, HOXC10

expression in adipocytes derived from SVF of BAT reduced expression of brown fat genes, with minimal effect on adipogenesis genes (Supplementary Figure S1d).

To determine whether the suppressive effect of HOXC10 could be attributed to its DNA-binding domain, a point mutation was introduced to the three alpha helices within



the DNA-binding motif (Supplementary Figure S1d). Mutations in the first (DBM1) and third (DBM3) alpha helices completely abolished the suppressive effect of HOXC10 on brown fat genes (Supplementary Figure S1e). These data indicate that HOXC10 is a key regulator that suppresses browning of adipocytes, and that this effect is dependent on its DNA-binding capability.

To determine whether HOXC10 is necessary for suppressing the browning process, short hairpin RNA targeting HOXC10 was expressed in adipocytes differentiated from SVF isolated from SubQ WAT (Figure 1f). *HOXC10* mRNA level was reduced by ~70% (Figure 1f). Reduction of *HOXC10* expression led to the increased expression of brown fat genes, with minimal effect on lipid accumulation (Supplementary Figure S1f) or adipogenesis genes (Figure 1f).

In the myogenic Myf5+ cell line, C2C12, the expression of key regulators such as PRDM16[18] or Zfp516[5] reduced its myogenic properties and induced adipogenesis and expression of brown fat genes. Knockdown of HOXC10 in C2C12 cells resulted in a significant reduction in both *HOXC10* at the transcript (Figure 1g) and protein levels (Figure 1h). Similar to the expression of PRDM16, knockdown of HOXC10 in C2C12 cells undergoing myogenic differentiation led to a reduction in myotube-specific genes, including *Myf5*, *Myf6*, *MEF2A*, *Myg* and *MCK*, and reduced protein levels of Myf5 and MEF2A (Supplemtentary Figure S1f and S1g). An adipogenic cocktail treatment in control cells failed to induce adipogenesis, as evidenced by the lack of lipid staining and low expression of adipogenic markers (Figure 1i–k). In contrast, HOXC10-deficient C2C12 cells accumulated lipid droplets (Figure 1i) and had increased expression of adipogenic markers, such as *adiponectin*, *FABP4* and *PPARγ* (Figure 1j). Importantly, expression of the brown fat genes *PRDM16*, *PGC1α*, *CideA*, *Cox7a1* and *Cox8b* was significantly upregulated in shHOXC10- and PRDM16-expressing C2C12 cells (Figure 1j). Protein levels of the adipogenic marker FABP4 and brown fat genes PRDM16 and Cox7a1 were also increased in shHOXC10-expressing cells (Figure 1k). Collectively, these data indicate that HOXC10 can affect the adipogenic properties of C2C12 myogenic cells and are both necessary and sufficient to suppress brown fat genes in cultured adipocytes.

HOXC10 suppresses browning of SubQ WAT *in vivo*

To determine whether HOXC10 has a regulatory role in the browning process of WAT *in vivo*, mice were kept at 4 °C for 7 days. *PRDM16*, *PGC1α* and *UCP1* mRNA (Supplementary Figure S2a) and protein levels (Figure 2a) were highly upregulated in cold-exposed mice. In contrast, HOXC10 protein expression (Figure 2a) was markedly reduced despite an increase in the mRNA level (Supplementary Figure S2a). These data indicate that during browning, reduction of HOXC10 protein may facilitate the induction of brown fat genes, and that HOXC10 may act as a negative regulator in the process of browning.

To examine whether an increased level of HOXC10 can prevent browning *in vivo*, HOXC10 was placed under the control of a mini-aP2 promoter and expressed using AAV in mice. AAV8-aP2-FLAG-HOXC10 or AAV8-aP2-Emerald vectors were administered systemically through intravascular injection. Immunoblotting confirmed that FLAG-HOXC10 was expressed in SubQ WAT, visceral WAT and BAT (Figure 2b). At room temperature, no difference was detected in the body temperature of these mice (Figure 2c). After cold exposure for 7 days, mice expressing HOXC10 had lower body temperature (Figure 2c), indicating that HOXC10 inhibits the thermogenic response during exposure to cold conditions. In agreement with the cell culture data (Figure 1), the expression of HOXC10 suppressed the mRNA levels of *PRDM16*, *PGC1α*, *UCP1*, *Dio2*, *CideA*, *CoX7a1* and *Elvol3* in the SubQ WAT (Figure 2d). Protein levels of PRDM16, UCP1 PGC1α and Cox7a1 were also markedly reduced (Figure 2e). In cold-exposed control mice, multilocular adipocytes were observed in SubQ WAT, and immunohistochemistry confirmed that those were UCP1-positive adipocytes (Figure 2g). In contrast, the SubQ WAT of HOXC10-expressing mice remained mainly unilocular and lacked UCP1 staining (Figure 2g). It was previously

Figure 1 Ectopic expression of HOXC10 suppresses and knockdown of HOXC10 increases brown-fat-specific genes. (**a**) Quantitative PCR (qPCR) analysis of *HOXC10* messenger RNA (mRNA) in brown adipose tissue (BAT), SubQ white adipose tissue (WAT) and visceral (VISC) WAT. (**b**) Heatmap representation and cluster analysis of genes altered >1.5-fold. Ectopically expressed HOXC10 or PRDM16 in adipocytes derived from stromal-vascular fraction (SVF) of SubQ WAT treated with 10 μM forskolin for 6 h. Transcripts were analyzed using Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Data were processed using Partek Genomics Suite. A complete list of genes is provided in Supplementary Table S1. (**c**) Analysis of genes repressed by HOXC10 using ENRICHR program. (**d**) qPCR analysis of genes in 3T3-L1 adipocytes transduced with either empty vector control or FLAG-HOXC10. Cells were treated with 10 μM forskolin for 2 h before harvest. (**e**) Immunoblot and densitometry analysis for PRDM16, PGC1α, UCP1, CEBPβ and FLAG in 3T3-L1 adipocytes described in **d**. HSP90 served as a loading control. (**f**) qPCR analysis of *HOXC10* mRNA, brown-fat- and white-fat-specific (adipogenesis) genes in adipocytes derived from SVF of SubQ WAT transduced with HOXC10 short hairpin RNA (shRNA) or a control shRNA. $n=3$ (**g**) qPCR analysis of *HOXC10* mRNA in C2C12 myoblasts transduced with HOXC10 shRNA or a control shRNA. (**h**) Immunoblot and densitometry analysis for HOXC10 in C2C12 myoblasts transduced with HOXC10 shRNA or a control shRNA; 14-3-3 (pan) served as a loading control. (**i**) Oil Red O staining and absorbance reading of C2C12 myoblasts transduced with HOXC10 shRNA, a control shRNA, or expressing FLAG-PRDM16 at day 6 of adipocyte differentiation. (**j**) qPCR analysis of brown-fat- and white-fat-specific genes in C2C12 myoblasts transduced with HOXC10 shRNA, a control shRNA, and FLAG-PRDM16 at day 6 of adipocyte differentiation. (**k**) Immunoblot and densitometry analysis for PRDM16, CEBPβ, Cox7a1 and FABP4 in C2C12 myoblasts transduced with HOXC10 shRNA, a control shRNA, or expressing FLAG-PRDM16 at day 6 of adipocyte differentiation. GAPDH served as a loading control. All data are presented as means ± s.e.m.; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. $n=4$. Ctrl, control.

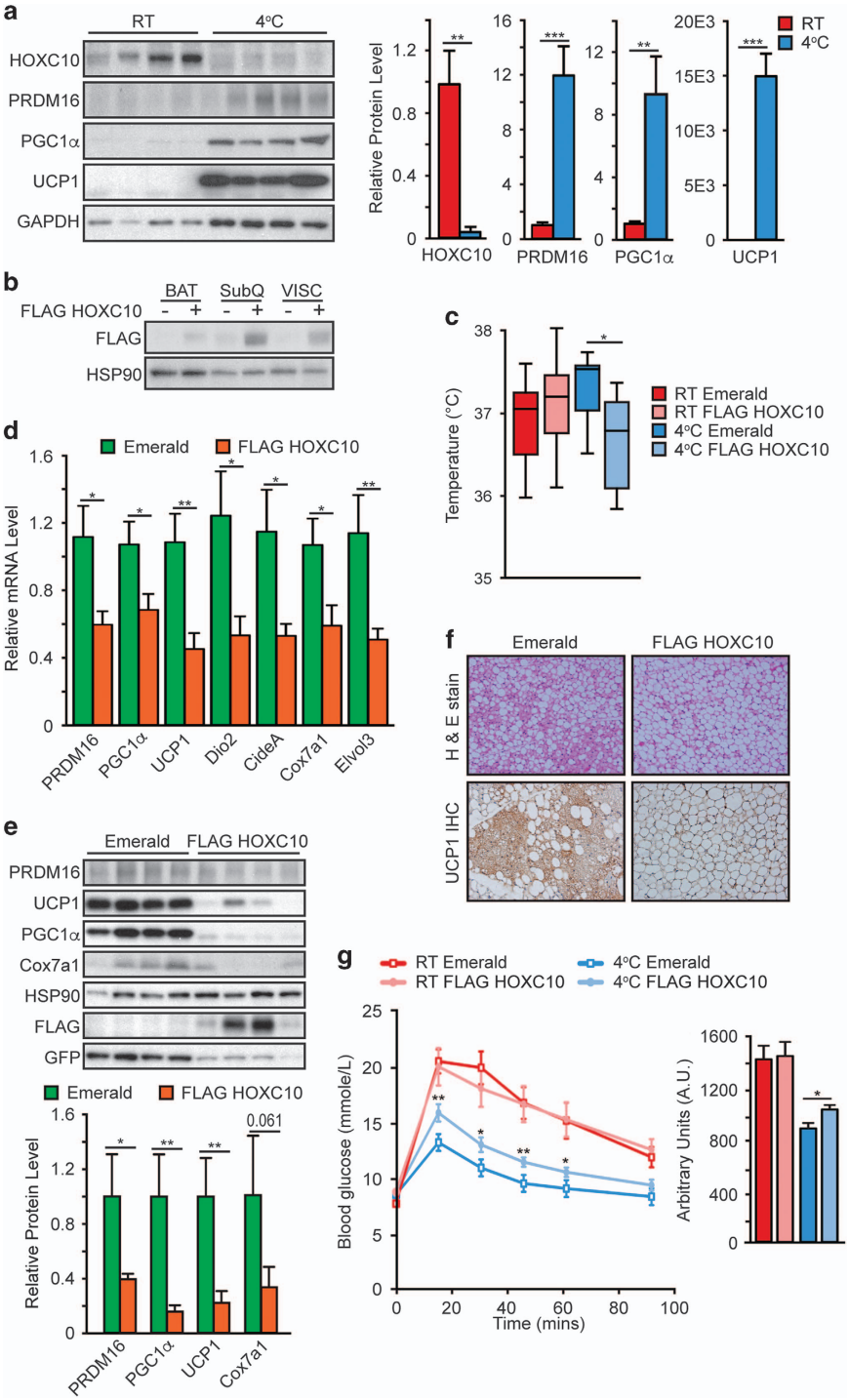


Figure 2 Intravenous delivery of adeno-associated virus (AAV)-FLAG-HOXC10 suppresses browning of SubQ WAT. (**a**) Immunoblot and densitometry analysis for HOXC10, PRDM16, PGC1α and UCP1 in SubQ white adipose tissue (WAT) of mice at room temperature (RT) and mice exposed to 4 °C for 7 days. GAPDH served as a loading control. Cold exposure was conducted with male 8-week-old mice. $n=5$. (**b**) Immunoblot analysis of FLAG and HSP90 in brown adipose tissue (BAT), SubQ and visceral (VISC) 4 weeks after the intravascular administration of 4×10^{12} GC of AAV-FLAG-HOXC10 vectors. (**c**) Rectal temperature of male mice at RT and after 7 days of exposure at 4 °C. Readings were taken after 4 weeks of intravascular administration of 4×10^{12} GC of AAV-FLAG-HOXC10 or AAV-Emerald vectors. $n=8$–10 per group. (**d**) Quantitative PCR analysis of brown-fat-specific genes in SubQ WAT of AAV-transduced mice after exposure to 4 °C for 7 days. $n=9$–11. (**e**) Immunoblot and densitometry analysis for PRDM16, UCP1, PGC1α, Cox7a1, FLAG and GFP in SubQ WAT of AAV-transduced mice after exposure to 4 °C for 7 days. HSP90 served as a loading control. $n=5$. (**f**) Hematoxylin and eosin staining, and immunostaining of UCP1 in SubQ WAT of AAV-transduced mice after 7 days of exposure at 4 °C. Data are presented as means ± s.e.m.; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ (**g**) Intraperitoneal glucose tolerance test in AAV-transduced mice at RT and after 7 days of exposure at 4 °C. $n=11$–13 per group

demonstrated that cold exposure improved the glucose clearance of rodents during a glucose tolerance test.[19] In agreement with the previous findings, control mice showed markedly improved glucose tolerance after cold exposure (Figure 2f). In mice expressing HOXC10, glucose disposal was less efficient compared with control mice (Figure 2f). HOXC10 expression had minimal effect on BAT function, as determined by mRNA and protein levels (Supplementary Figure S2b and S2c). These data indicate that HOXC10 suppressed cold-induced browning of the SubQ WAT *in vivo*.

To further determine whether the effect of HOXC10 on browning SubQ WAT is restricted to this depot or due to its effect on other adipose depots (Figure 2b), AAV8-aP2-FLAG-HOXC10 or AAV8-aP2-Emerald virus was injected directly into the SubQ WAT (Figure 3). Expression of HOXC10 or Emerald was restricted to the injected SubQ WAT (Figure 3a). SubQ WAT depot-specific expression of HOXC10 reduced the body temperature of mice after cold treatment (Figure 3b), suppressed brown fat genes and protein levels (Figure 3c and d) and reduced glucose clearance (Figure 3e), recapitulating the effects observed in Figure 2. Conversely, the expression of HOXC10 specifically in BAT had no discerning effects on any of the parameters examined (Supplementary Figure S3). These data indicate that the expression of HOXC10 specifically suppresses the browning of SubQ WAT *in vivo* with no effect on BAT.

HOXC10 suppresses genes involved in browning partially via suppressing PRDM16 gene expression

Given that expression of HOXC10 had a marked effect on preventing the process of browning, we surmised that HOXC10 may suppress brown fat genes via key regulators involved in this process. PRDM16 was proposed to be a master regulator for many genes involved in browning.[4] As HOXC10 suppressed a major portion of genes upregulated by PRDM16 (Figure 1b), we investigated whether HOXC10 could suppress brown fat genes via PRDM16.

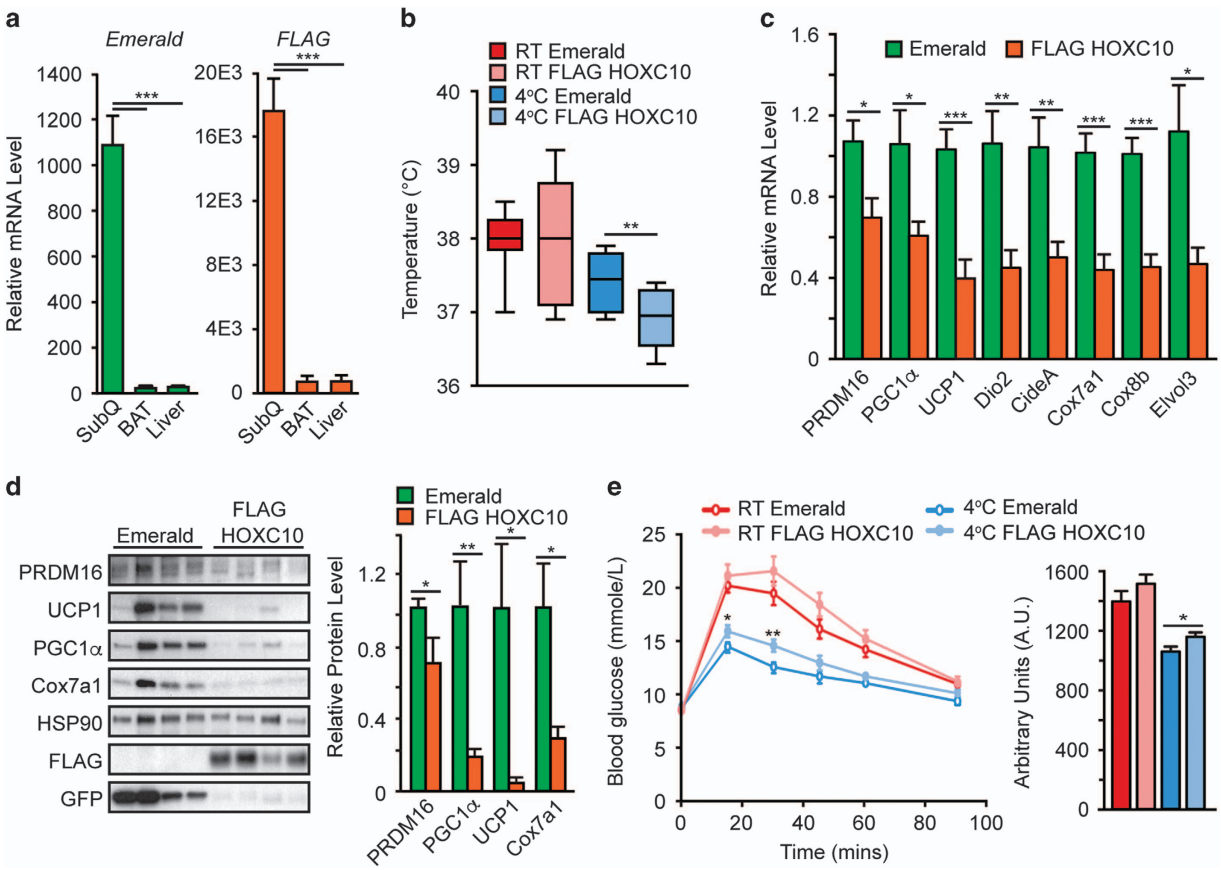


Figure 3 Expression of adeno-associated virus (AAV)-FLAG-HOXC10 in SubQ WAT suppresses browning. (**a**) Quantitative PCR (qPCR) analysis of *FLAG* or *Emerald* genes in SubQ white adipose tissue (WAT), brown adipose tissue (BAT) and liver tissues of mice transduced with either AAV-FLAG-HOXC10 or AAV-Emerald vectors, respectively. $n=7$. (**b**) Rectal temperature of male mice at room temperature (RT) and after 7 days of exposure at 4 °C. Readings were taken after 4 weeks of intrainguinal WAT administration of 1×10^{10} GC of AAV-FLAG-HOXC10 or AAV-Emerald vectors. $n=10$ per group. (**c**) qPCR analysis of brown-fat-specific genes in subcutaneous WAT of AAV-transduced mice after exposure to 4 °C for 7 days. $n=7$. (**d**) Immunoblot and densitometry analysis for PRDM16, UCP1, PGC1α, Cox7a1, FLAG and GFP in subcutaneous WAT of AAV-transduced mice after exposure to 4 °C for 7 days. HSP90 served as a loading control. $n=4$. (**e**) Intraperitoneal glucose tolerance test in AAV-transduced mice at RT and after 7 days of exposure at 4 °C. $n=9$–10 per group. Data are presented as means ± s.e.m.; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

To determine whether HOXC10 has a regulatory role in the 5′ regulatory region of PRDM16 (−1 to −2074), we generated a reporter construct by replacing the PRDM16-coding region with a luciferase reporter. In cultured cells, the PRDM16 5′ regulatory region induced luciferase activity, which was suppressed by the concomitant expression of wild-type HOXC10 but not the DNA-binding mutant DBM1 (Figure 4a). Chromatin immunoprecipitation assay revealed that HOXC10 bound to −1667 to −1412 of the 5′ regulatory region of PRDM16 (Figure 4b). Mobility shift was observed using the 256-bp fragment (−1667 to −1412) of PRDM16 5′ regulatory region, and this band shift collapsed when a corresponding cold probe was used (Figure 4c). To confirm the identity of the binding protein, an antibody against HOXC10 was used in the assay. Dose-dependent reduction of the band shift was observed in the presence of HOXC10 antibodies, whereas antibodies of another closely related HOXC protein, HOXC8, did not have this effect (Figure 4c). Taken together, these data indicate that HOXC10 interacts directly with the 5′ regulatory region of PRDM16 to regulate the expression of PRDM16.

To determine whether HOXC10 suppresses brown fat genes solely through PRDM16, we next determined whether ectopic PRDM16 expression could overcome the effect of HOXC10 expression. Both HOXC10 and PRDM16 were expressed to a similar level in adipocytes differentiated from BAT SVF (Figure 4d). Consistent with the *in vitro* data (Figure 1d; Supplementary Figure S1c), the expression of HOXC10 alone suppressed brown fat genes. Expression of PRDM16 alone increased most brown fat gene expression (Figure 4e and f), with the exception of *Dio2* and *Elvol3* (Figure 4g). Expression of PRDM16 alleviated HOXC10-dependent suppression of *PGC1α*, *Cox7a1* and *Cox8b* (Figure 4e), whereas *UCP1* and *CideA* expression was not fully restored (Figure 4g). Similar observations were made in adipocytes isolated from SubQ WAT (Supplementary Figure S4). These data indicate that HOXC10 can suppress brown genes in a unique manner with a distinct mechanism from PRDM16.

DISCUSSION

Our current study identified HOXC10 as a critical negative regulator in the process of browning SubQ WAT *in vivo*. Several lines of evidence support this role of HOXC10. Expression of HOXC10 suppressed brown fat genes with the minimal effects on adipogenesis markers in adipocytes, and knockdown of HOXC10 increased brown fat gene expression

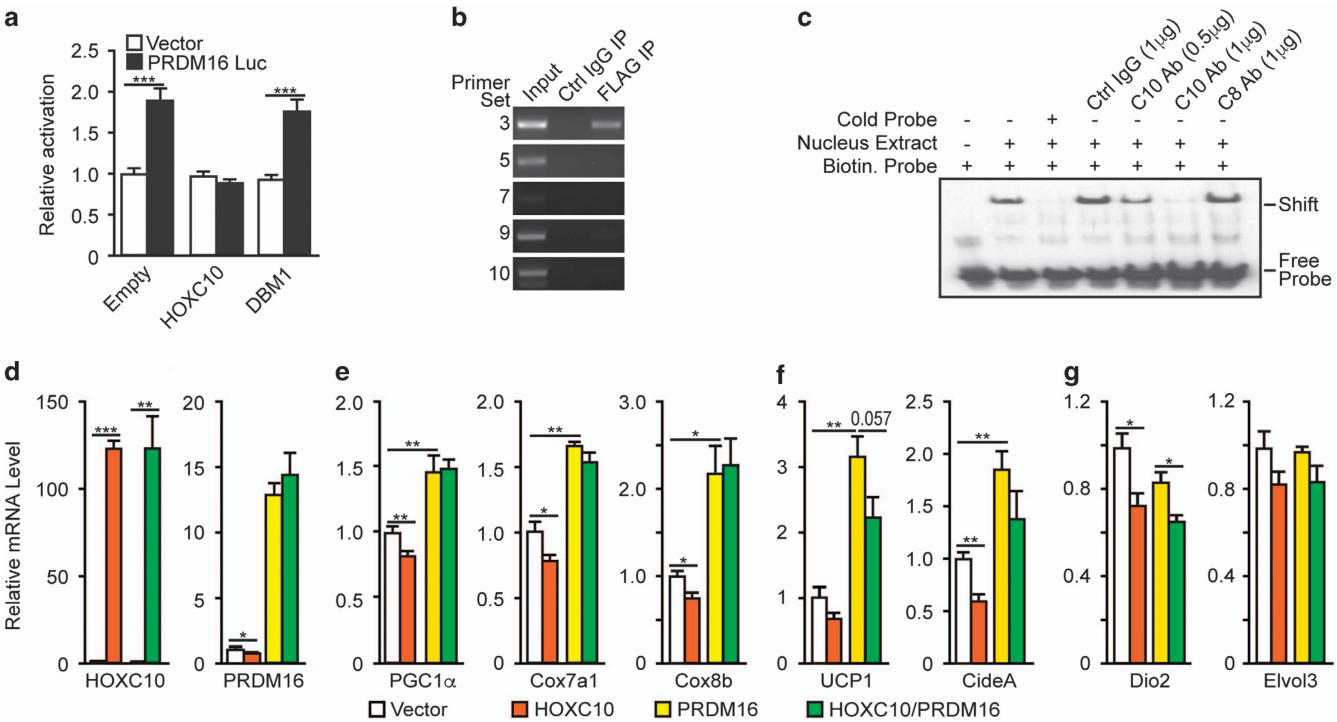


Figure 4 HOXC10 binds to PRDM16 promoter and suppresses its gene expression. (**a**) Luciferase reporter activity in HEK293 cells co-transfected with FLAG-HOXC10, homeodomain-mutated HOXC10 (DBM1), or a control vector together with either PRDM16 or a control vector. Data are presented as means ± s.e.m.; ***$P<0.001$; $n=4$. (**b**) The chromatin immunoprecipitation analysis in 3T3-L1 adipocytes expressing FLAG-HOXC10. Immunoprecipitation was performed with FLAG or control IgG antibodies and PCR with specific primers for the PRDM16 promoter sequence. (**c**) Gel shift assay using biotinylated oligo in nuclear extracts from HEK293 cells transfected with FLAG-HOXC10. Control rabbit IgG, HOXC10 and HOXC8 antibodies were used for the super shift assay. (**d**–**g**) Quantitative PCR analysis of genes in adipocytes derived from stromal-vascular fraction of brown adipose tissue co-transduced with either empty vector control or FLAG-HOXC10 with either empty vector control or FLAG-PRDM16. Data are presented as means ± s.e.m.; *$P<0.05$, **$P<0.01$ and ***$P<0.001$. $n=3$. Ctrl, control

in adipocytes and a myogenic cell line. In mice, cold exposure led to a reduction of HOXC10 protein in SubQ WAT and ectopic expression of HOXC10 in SubQ WAT suppressed browning of this depot, and reduced the ability of these mice to maintain body temperature upon cold exposure.

Given that HOXC10 can act as a negative regulator for brown fat gene expression, it is not surprising that the endogenous HOXC10 level is much lower in BAT compared with SubQ WAT of mice at ambient temperature. Although HOXC10 suppresses browning in SubQ WAT as well as the other cell lines examined, we noted that ectopic expression of HOXC10 specifically in BAT had no effect on brown fat gene expression and did not affect BAT function. This finding is in contrast to the role of TLE3, another negative regulator of browning.[20] Expression of TLE3 not only suppresses browning in SubQ WAT but also reduces BAT function. This effect is attributed to TLE3 acting reciprocally with PRDM16 to regulate the thermogenic program because TLE3 favors the expression of WAT genes in both BAT and WAT depots. However, HOXC10 acts mainly by suppressing brown fat genes and has a minimal effect on the classical WAT genes examined. Another possible explanation is that HOXC10 primarily affects the process of browning rather than matured classical BAT function. This action also highlights the presence of distinct regulatory machineries that govern classical BAT function versus the process of browning in WAT.

In addition to HOXC10 described in this study, HOXC8 and HOXC9 were also shown to be involved in adipogenesis[21] or associated with adiposity.[22] HOXC8 was reported as another negative regulator of brown fat genes in WAT.[21] Although HOXC8 and HOXC10 belong to the same HOXC cluster, their regulation of brown fat gene expression seems to differ. HOXC8 represses C/EBPβ,[21] a regulator of adipogenesis, whereas one of the targets that HOXC10 suppresses is PRDM16. Despite a substantial similarity in amino-acid sequence with HOXC8, HOXC10 had no discernible effect on C/EBPβ expression, indicating that additional regulators may determine the downstream target specificity between HOXC10 and HOXC8. There is precedence that PRDM16 can be regulated by HOX proteins. In a cell line model of human acute myeloid leukemia, it was demonstrated that HOXB4 represses PRDM16 gene expression, whereas HOXA9 and HOXA10 upregulate its expression.[23] Therefore, different HOX proteins could differentially regulate the same target in a tissue/cell line-specific manner. In summary, we have identified HOXC10 as a key negative regulator in the process of browning adipocytes *in vitro* and *in vivo*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: YN and SXT conceived and performed most of the experiments and wrote the manuscript. SYC and LS performed short hairpin RNA knockdown experiments in SubQ SVF. WH and WH provided supervision and feedback.

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