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A novel role for the Wnt inhibitor APCDD1 in adipocyte differentiation: Implications for diet-induced obesity

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Impaired adipogenic differentiation during diet-induced obesity (DIO) promotes adipocyte hypertrophy and inflammation, thereby contributing to metabolic disease. Adenomatosis polyposis coli down-regulated 1 (APCDD1) has recently been identified as an inhibitor of Wnt signaling, a key regulator of adipogenic differentiation. Here we report a novel role for APCDD1 in adipogenic differentiation via repression of Wnt signaling and an epigenetic linkage between miR-130 and APCDD1 in DIO. APCDD1 expression was significantly up-regulated in mature adipocytes compared with undifferentiated preadipocytes in both human and mouse subcutaneous adipose tissues. siRNA-based silencing of APCDD1 in 3T3-L1 preadipocytes markedly increased the expression of Wnt signaling proteins (Wnt3a, Wnt5a, Wnt10b, LRP5, and β-catenin) and inhibited the expression of adipocyte differentiation markers (CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ)) and lipid droplet accumulation, whereas adenovirus-mediated overexpression of APCDD1 enhanced adipogenic differentiation. Notably, DIO mice exhibited reduced APCDD1 expression and increased Wnt expression in both subcutaneous and visceral adipose tissues and impaired adipogenic differentiation *in vitro*. Mechanistically, we found that miR-130, whose expression is up-regulated in adipose tissues of DIO mice, could directly target the 3′-untranslated region of the APCDD1 gene. Furthermore, transfection of an miR-130 inhibitor in preadipocytes enhanced, whereas an miR-130 mimic blunted, adipogenic differentiation, suggesting that miR-130 contributes to impaired adipogenic differentiation during DIO by repressing APCDD1 expression. Finally, human subcutaneous adipose tissues isolated from obese individuals exhibited reduced expression of APCDD1, C/EBPα, and PPARγ compared with those from non-obese subjects. Taken together, these novel findings suggest that APCDD1 positively regulates adipogenic differentiation and that its down-regulation by miR-130 during DIO may contribute to impaired adipogenic differentiation and obesity-related metabolic disease.

Adipose tissue is the major energy reserve in higher eukaryotes and is remarkably flexible at storing and releasing triacylglycerols during periods of caloric excess and deprivation. Adipose tissue can expand by increasing the volume of pre-existing adipocytes (hypertrophy) and/or by increasing the number of new adipocytes (hyperplasia) via adipogenic differentiation of partially committed stem cells, termed preadipocytes (1). Adipogenic differentiation of preadipocytes is a normal physiological function required for adipose tissue development and remodeling. However, in obesity, adipogenic differentiation is typically insufficient to meet metabolic demand, and the excess calories are primarily stored in pre-existing adipocytes, which become overloaded with lipid. The resulting mechanical stress leads to adipose tissue inflammation, glucose intolerance, insulin resistance, and ectopic lipid accumulation (2, 3). Studies in humans suggest that adipocyte cell size is an independent predictor of the development of obesity-related metabolic disease (4). Thus, understanding the mechanisms that regulate adipogenic differentiation during obesity may provide important insights into the pathogenesis and treatment of metabolic disease.

The Wnt signaling pathway is a fundamental regulator of cell proliferation, polarization, and differentiation (5, 6). Wnt-family proteins exert their effects on cellular processes through canonical and non-canonical pathways (7, 8). The canonical

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This work is dedicated to the late Dr. Tapan K. Chatterjee at the Vascular Biology Center, Medical College of Georgia at Augusta University.

This article contains supplemental Figs. S1–S5.

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Wnt/β-catenin pathway is activated by binding of Wnt ligands to Frizzled (Fz)[4] receptors and co-receptors, low-density lipoprotein-related protein (LRP)5/6, leading to β-catenin stabilization and subsequent translocation into the nucleus, where it affects the transcription of Wnt target genes (9–11). Wnt signaling has been reported to negatively regulate adipogenic differentiation through several mechanisms; for example, Wnt6, Wnt10a, and Wnt10b inhibit adipogenic differentiation through a β-catenin dependent mechanism (12), and Wnt3a suppresses C/EBPβ/δ-induced adipogenesis of 3T3-L1 cells by inhibiting PPARγ induction (13). Thus, regulation of Wnt signaling in obesity may impact the development and/or progression of metabolic disease.

Recently, adenomatosis polyposis coli down-regulated 1 (APCDD1), a membrane-bound protein expressed during tissue development, has been identified as a novel Wnt inhibitor (14). Mechanistically, APCDD1 was reported to physically interact with the canonical Wnt3a ligand and LRP5 receptor at the cell surface, thereby repressing the biological effects of Wnt signaling and contributing to neural development in chicks and in *Xenopus* embryos. APCDD1 is widely expressed in adult human tissues, including the heart, pancreas, prostate, hair follicles, liver, kidney, and adipose tissues (14–16), but the biological functions of APCDD1 are poorly understood. Previously, using an unbiased genome-wide microarray approach, we observed that APCDD1 expression was significantly higher in well differentiated subcutaneous adipocytes compared with poorly differentiated visceral (perivascular) adipocytes isolated from the same human subjects (17), suggesting the possibility that APCDD1 could play a role in adipogenic differentiation, perhaps through its ability to inhibit Wnt signaling.

In this study, we investigated the role of APCDD1 in regulating the differentiation of human and mouse preadipocytes. We provide evidence that down-regulation of endogenous APCDD1 expression increases the Wnt/β-catenin signaling pathway, leading to the inhibition of key adipogenic transcription factors (C/EBPα and PPARγ) and impaired adipogenic differentiation. Conversely, overexpression of APCDD1 promotes adipogenic differentiation. We also report that APCDD1 expression in adipose tissue is decreased under obese conditions in both mouse and human subjects compared with non-obese controls. Furthermore, miR-130 was identified as a posttranscriptional regulator of APCDD1 gene expression during DIO. These findings may have important implications for the role of APCDD1 in the pathogenesis of obesity-related metabolic disease.

Results

Increased APCDD1 expression in conjunction with decreased Wnt signaling during adipogenic differentiation

Wnt expression was reported to be down-regulated during differentiation of murine preadipocytes (18). To confirm that Wnt expression is down-regulated during *in vitro* adipogenic differentiation of human preadipocytes, we assayed the mRNA expression of Wnt1, Wnt3a, and Wnt10b. All three genes were significantly down-regulated 6 days after induction of differentiation compared with undifferentiated cells (Fig. 1*A*). Next, to examine whether APCDD1 is differentially expressed in mature adipocytes *versus* undifferentiated preadipocytes (PA), we fractionated human subcutaneous adipose tissues to obtain floating mature adipocytes and the stromal vascular fraction, which is enriched in preadipocytes. Interestingly, APCDD1 mRNA levels were markedly higher in mature human adipocytes compared with the stromal vascular fraction (Fig. 1*B*). In addition, APCDD1 mRNA expression was significantly increased during *in vitro* differentiation of human subcutaneous preadipocytes (Fig. 1*C*). Time course experiments in mouse 3T3-L1 preadipocytes demonstrated that APCDD1 mRNA expression is rapidly induced following the onset of adipogenic differentiation, with levels peaking by day 3 and sustained for up to 12 days (Fig. 1*D*). Furthermore, APCDD1 protein expression was markedly up-regulated during adipogenic differentiation in isolated murine adipocytes (Fig. 1*E*) and in 3T3-L1 preadipocytes (Fig. 1*F* and supplemental Fig. S1) compared with undifferentiated cells. Thus, Wnt expression is down-regulated during adipogenic differentiation in conjunction with increased APCDD1 expression in both humans and mice.

APCDD1 gene silencing attenuates adipogenic differentiation in conjunction with increased Wnt signaling

To investigate whether APCDD1 expression participates in adipogenic differentiation, we silenced *APCDD1* in 3T3-L1 preadipocytes by transfection with siRNA specific for *APCDD1* or a scrambled control. Knockdown of *APCDD1* blunted adipogenic differentiation, as demonstrated by reduced lipid droplet accumulation (Fig. 2*A*) and adipogenic marker (C/EBPα and PPARγ) expression (Fig. 2, *B* and *C*). Furthermore, APCDD1 gene silencing increased the expression of proteins associated with the Wnt/β-catenin signaling pathway, including Wnt3a, Wnt5a, Wnt10b, LRP5, and β-catenin, in 3T3-L1 preadipocytes (Fig. 2, *B* and *C*). These data indicate that knockdown of APCDD1 blocks adipogenic differentiation while up-regulating Wnt signaling.

Overexpression of APCDD1 enhances adipogenic differentiation and inhibits Wnt signaling

To establish APCDD1 as a positive regulator of adipogenic differentiation, 3T3-L1 preadipocytes were transduced with adenovirus overexpressing APCDD1 or GFP control. At 7 days of adipogenic differentiation, APCDD1-overexpressing cells exhibited increased lipid droplet accumulation (Fig. 3*A*) and adipogenic marker (adiponectin, FABP4, C/EBPα, and PPARγ) expression (Fig. 3, *B* and *C*) compared with GFP control-transduced cells. Moreover, the expression of Wnt signaling proteins, including Wnt3a, Wnt5a, LRP5, and β-catenin, was attenuated in APCDD1-overexpressing cells compared with the control. These findings suggest that APCDD1 positively regulates adipogenic differentiation while suppressing the Wnt signaling pathway.

Reduced APCDD1 expression in parallel with impaired adipocyte differentiation in obese mice

To investigate the potential role of APCDD1 in DIO *in vivo*, we quantified APCDD1 expression in the subcutaneous adi-

[4] The abbreviations used are: Fz, Frizzled; PA, preadipocyte; CD, chow diet; HFD, high-fat diet; SQ, subcutaneous; SV, stromal vascular; qPCR, quantitative PCR; C/EBPα, CCAAT/enhancer-binding protein α; PPARγ, peroxisome proliferator-activated receptor γ; BMI, body mass index.

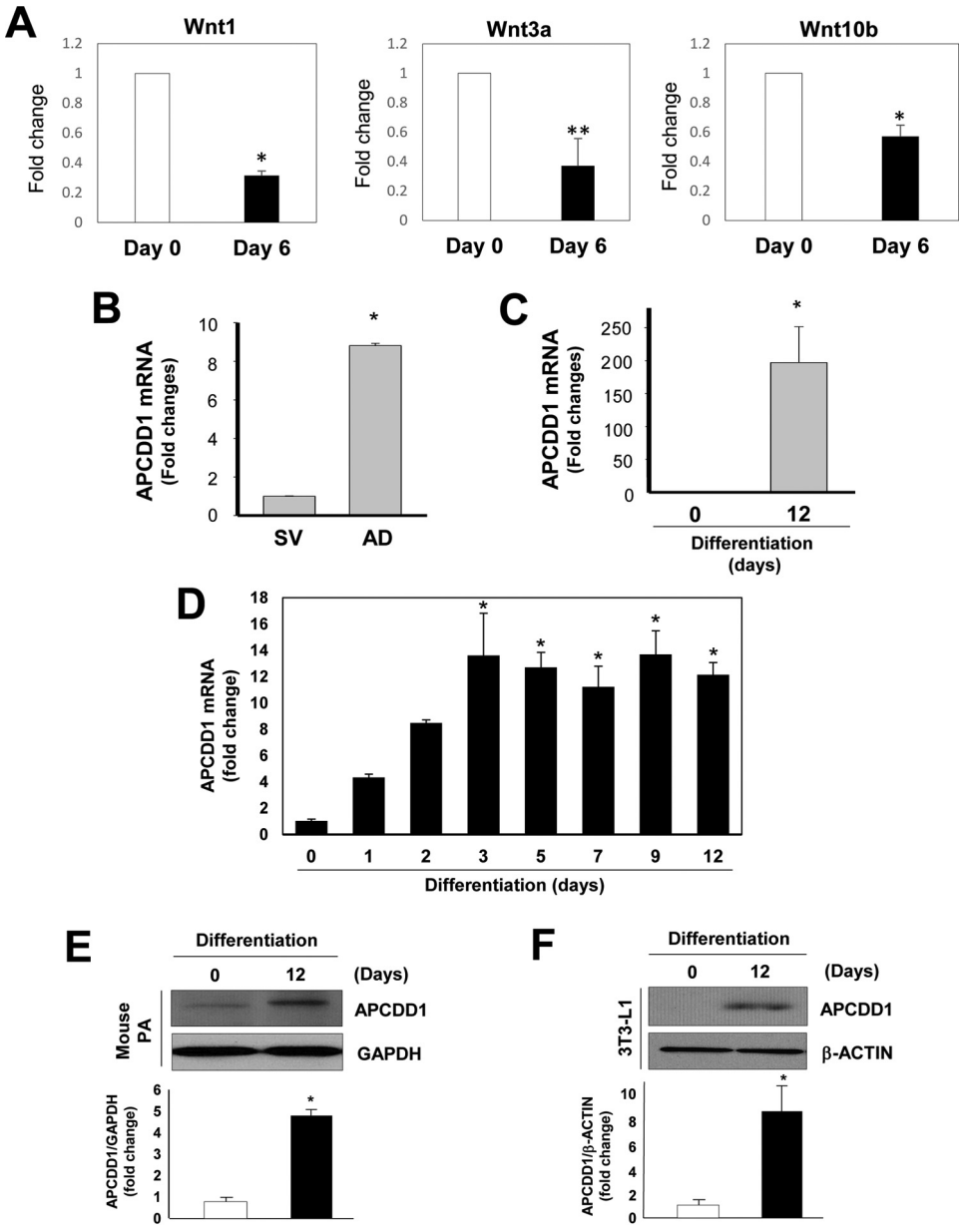


Figure 1. Wnt and APCDD1 expression in relation to adipogenic differentiation. *A*, expression of Wnt1, Wnt3a, and Wnt10b mRNAs was down-regulated during adipogenic differentiation (6 days) of human subcutaneous preadipocytes ($n = 3$). *, $p < 0.01$; **, $p < 0.05$ *versus* control (*Day 0*). *B*, the APCDD1 mRNA level was up-regulated in mature human adipocytes (*AD*) compared with PAs within the SV fraction ($n = 3$). *, $p < 0.01$ *versus* control (SV). *C*, APCDD1 mRNA expression was increased during *in vitro* adipogenic differentiation (12 days) of primary cultured human preadipocytes ($n = 3$). *, $p < 0.01$ *versus* control (*Day 0*). *D*, time course expression of APCDD1 mRNA during adipogenic differentiation of 3T3-L1 preadipocytes ($n = 3$). *, $p < 0.05$ *versus* control (*Day 0*). *E* and *F*, APCDD1 protein expression was increased during *in vitro* adipogenic differentiation (12 days) of 3T3-L1 preadipocytes and primary cultured mouse preadipocytes ($n = 3$). *, $p < 0.05$ *versus* control (*Day 0*). Levels of mRNA and protein expressions were determined by qPCR and Western blotting, respectively.

pose tissues of lean chow diet (CD)-fed mice and obese high-fat diet (HFD) mice. Body weight was significantly increased by HFD feeding (60% calories from lard fat) in WT (C57Bl/6) mice (Fig. 4*A*). Intriguingly, after 18 weeks of HFD feeding, APCDD1 mRNA and protein expression were reduced in subcutaneous (SQ) adipose tissues compared with CD mice (Fig. 4, *B* and *C*). Similarly, APCDD1 protein expression was also significantly decreased in HFD mouse-derived visceral adipose tissues (Fig. 4*D*). On the other hand, Wnt3a protein expression was increased in adipose tissues isolated from HFD mice. Importantly, as we reported previously (19), preadipocytes isolated from subcutaneous adipose tissue of HFD male mice demonstrated impaired *in vitro* differentiation compared with that from CD mice, as evidenced by diminished lipid droplet accumulation (Fig. 4*E*). These results indicate that impaired adipogenic differentiation during HFD is associated with reduced APCDD1 expression.

miR-130 overexpression induced by HFD directly targets APCDD1 gene expression

Next, we investigated the potential mechanisms whereby APCDD1 expression is down-regulated during DIO. We

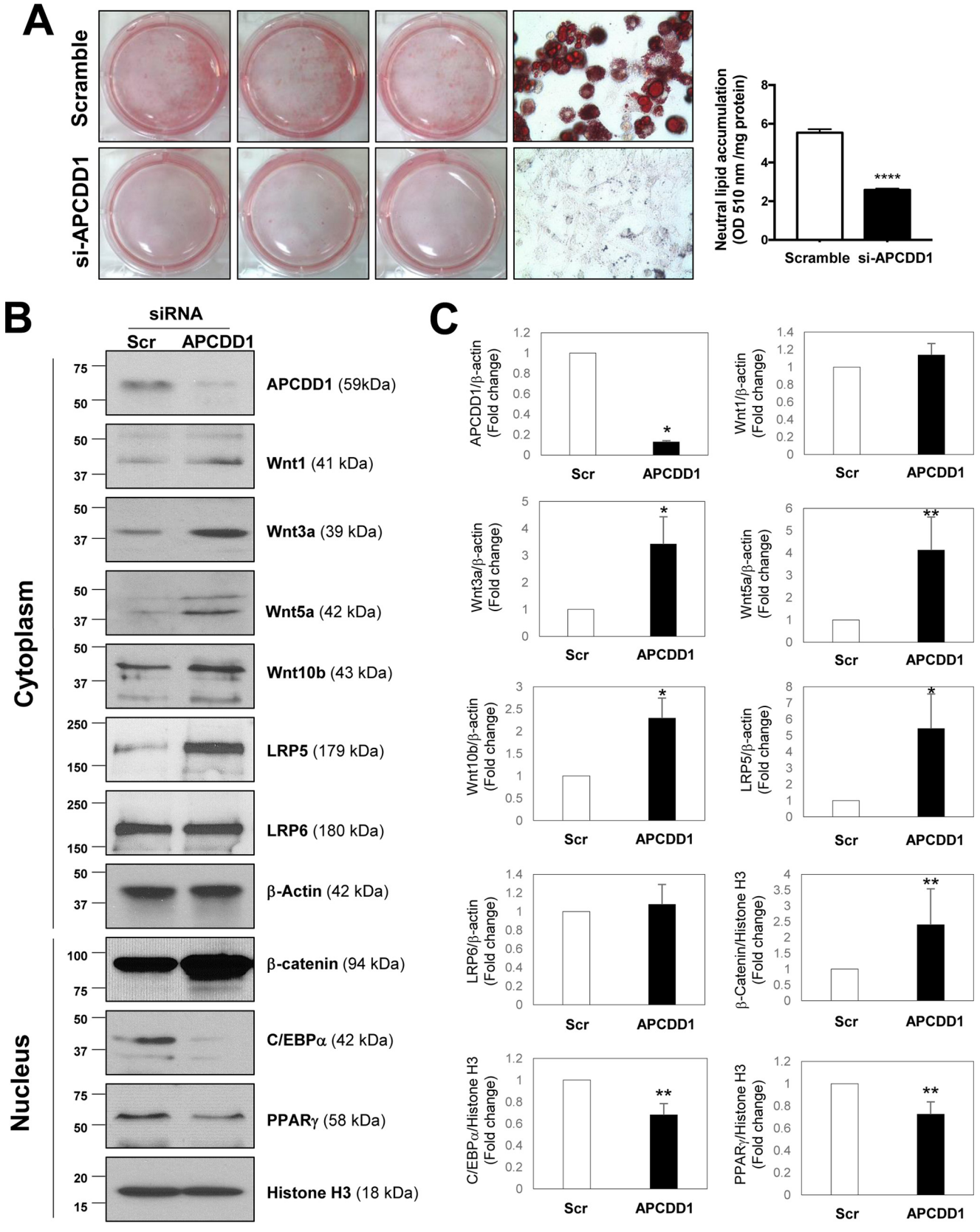
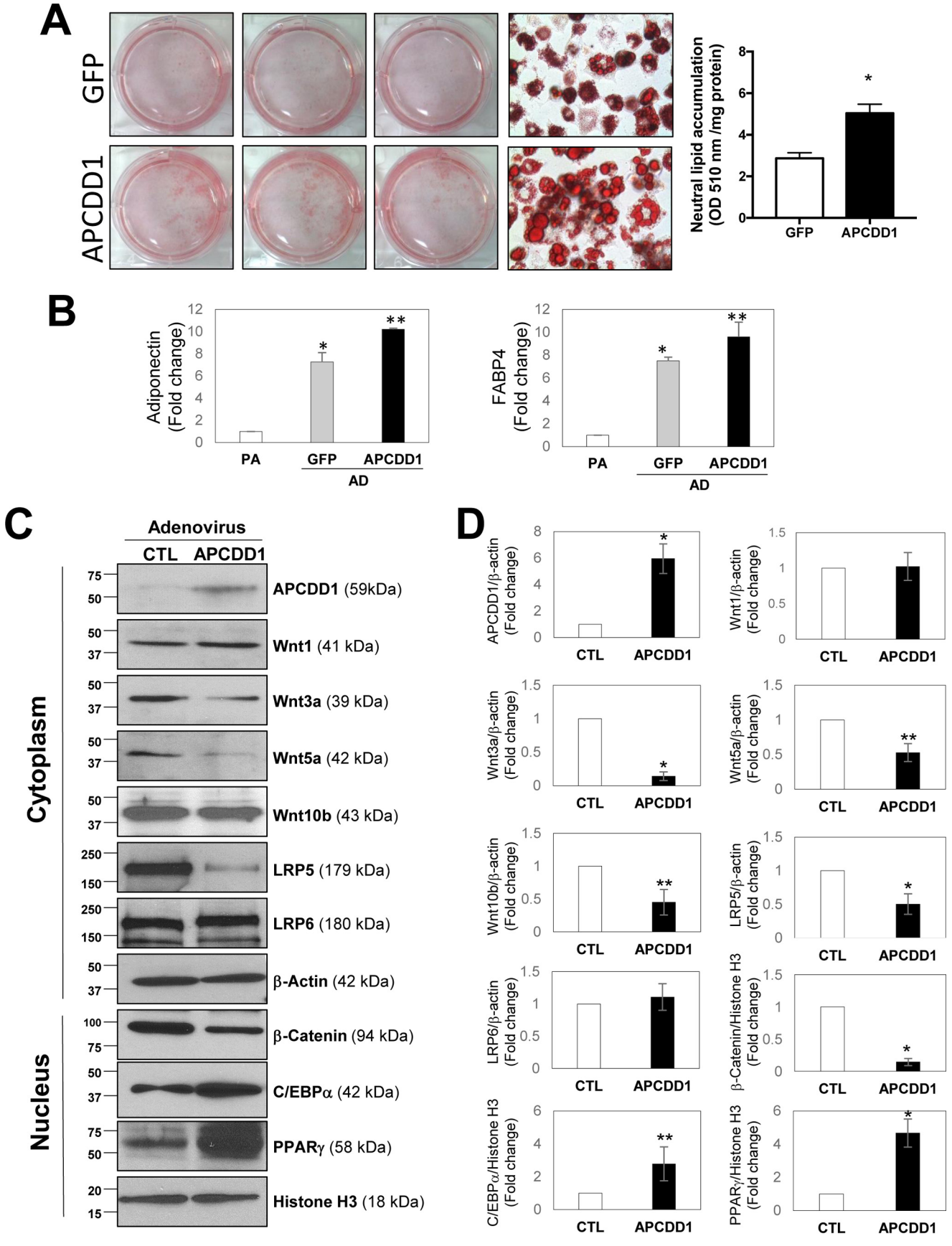


Figure 2. APCDD1 gene silencing suppresses adipogenic differentiation in conjunction with up-regulated expression of Wnt signaling proteins in 3T3-L1 preadipocytes. *A*, adipogenic differentiation was impaired in 3T3-L1 preadipocytes transfected with APCDD1 siRNA compared with the scrambled control, as indicated by reduced cytoplasmic lipid droplet accumulation (Oil red O). ****, $p < 0.0001$ *versus* control (*Scramble*). *B* and *C*, transfection of 3T3-L1 preadipocytes with APCDD1 siRNA increased expression of Wnt signaling proteins (Wnt3a, Wnt5a, Wnt10b, LRP5, and β-catenin) and decreased expression of adipogenic markers (C/EBPα and PPARγ) ($n = 3$). β-catenin, C/EBPα, and PPARγ expression levels were examined in the nuclear fraction. *, $p < 0.01$; **, $p < 0.05$ *versus* control. Protein expressions were determined by Western blotting and densitometry analysis. *Scr*, scrambled.


A
GFP
APCDD1
Neutral lipid accumulation (OD 510 nm /mg protein)
GFP
APCDD1
B
Adiponectin (Fold change)
PA
GFP
APCDD1
AD
FABP4 (Fold change)
C
Adenovirus
CTL
APCDD1
Cytoplasm
Nucleus
APCDD1 (59kDa)
Wnt1 (41 kDa)
Wnt3a (39 kDa)
Wnt5a (42 kDa)
Wnt10b (43 kDa)
LRP5 (179 kDa)
LRP6 (180 kDa)
β-Actin (42 kDa)
β-Catenin (94 kDa)
C/EBPα (42 kDa)
PPARγ (58 kDa)
Histone H3 (18 kDa)
D
APCDD1/β-actin (Fold change)
Wnt1/β-actin (Fold change)
Wnt3a/β-actin (Fold change)
Wnt5a/β-actin (Fold change)
Wnt10b/β-actin (Fold change)
LRP5/β-actin (Fold change)
LRP6/β-actin (Fold change)
β-Catenin/Histone H3 (Fold change)
C/EBPα/Histone H3 (Fold change)
PPARγ/Histone H3 (Fold change)
CTL
APCDD1

hypothesized that a specific set of miRNAs may be induced by HFD to regulate APCDD1 expression in preadipocytes. To test this hypothesis, we first examined databases (Targetscan, miRWalk, and PicTar) to search for miRNAs potentially targeting *APCDD1*, which predicted that miRNA 130a-3p and miRNA 130b-3p directly bind to the 3′ UTR of APCDD1 mRNA (Fig. 5*A*). We quantified the expression of miR-130a-3p and miR-130b-3p in 3T3-L1 cells and observed that both of these miRNAs were down-regulated within 1 day after induction of differentiation and remained repressed for up to 12 days (Fig. 5*B* and supplemental Fig. S2). To experimentally validate that APCDD1 is a target gene of miR-130, expression of miR-130a-3p and miR-130b-3p was knocked down by specific antisense inhibitors. Transfection of 3T3-L1 preadipocytes with anti-miR-130a-3p or anti-miR-130b-3p significantly augmented APCDD1 protein expression (Fig. 5*C*), indicating that APCDD1 is a direct target of miR-130. To investigate whether miR-130 is induced by HFD, we examined the expression levels of miR-130 in subcutaneous and visceral adipose tissues. Interestingly, expression of miR-130a-3p and miR-130b-3p was markedly increased in both subcutaneous and visceral adipose tissues from HFD mice compared with control mice (Fig. 5*D*). Furthermore, transfection of 3T3-L1 preadipocytes with miR-130a-3p inhibitor enhanced, whereas overexpression of miR-130a-3p blunted, adipogenic differentiation, as evaluated by cytoplasmic lipid droplet measurement (Fig. 5, *D–F*).

MiR-130 could potentially inhibit adipogenic differentiation by targeting genes other than APCDD1. Thus, we tested the effects of forced overexpression of APCDD1 in 3T3-L1 preadipocytes transfected with an miR-130a-3p mimic. As shown in supplemental Fig. S3, the miR-130a-3p mimic failed to inhibit adipogenic differentiation in APCDD-1-overexpressing cells. These findings support a mechanistic link between miR-130 and APCDD1 in the inhibition of adipogenic differentiation. Moreover, our results are consistent with the notion that HFD-induced miR-130 targets APCDD1 in DIO mice to impair adipogenic differentiation.

APCDD1 expression is down-regulated in obese individuals in conjunction with reduced adipogenic gene expression

To investigate the functional relevance of APCDD1 in human obesity, we examined APCDD1 expression in subcutaneous adipose tissues isolated from obese (BMI $>$ 30) and non-obese (BMI $<$ 30) human subjects. Interestingly, APCDD1 protein expression was significantly down-regulated in obese compared with non-obese subjects. Furthermore, adipogenic markers (C/EBPα and PPARγ) protein expression was markedly reduced in obese subjects (Fig. 6, *A* and *B*). There was also a strong trend toward decreased adiponectin gene expression with increasing BMI in our patient population (supplemental Fig. S4). Notably, expression of Wnt3a, one of the targets of APCDD1, tended to be up-regulated in adipose tissues isolated from obese subjects ($p = 0.06$ *versus* non-obese control). miR-130 expression in human subcutaneous adipose tissues exhibited considerable variability but showed a trend toward an increase in obese individuals (supplemental Fig. S5). These findings in human adipose tissues support our data obtained from mice as well as *in vitro* cell culture experiments in that impaired adipogenic differentiation during DIO could potentially be linked to reduced APCDD1 expression.

Discussion

The Wnt signaling pathway negatively regulates adipogenic differentiation and may play an important role in promoting metabolic dysfunction in obesity. Here we report that APCDD1 is a positive regulator of adipogenic differentiation through its repression of Wnt signaling. APCDD1 is weakly expressed in subcutaneous adipose tissues isolated from obese mice and humans, consistent with reports of impaired adipocyte differentiation in DIO. Moreover, overexpression of APCDD1 in 3T3-LI cells enhances adipogenic differentiation and inhibits expression of proteins in the Wnt signaling pathway, whereas siRNA-mediated APCDD1 gene silencing blunts adipogenic differentiation and up-regulates Wnt protein expression. Mechanistically, we demonstrated that APCDD1 is directly targeted by miR-130, which is overexpressed in adipose tissues of obese mice. Taken together, these findings uncover a novel role for APCDD1 in adipogenic differentiation and raise the possibility that suppression of APCDD1 expression by miR-130 in adipose tissues contributes to obesity-related metabolic disease.

Wnt signaling has been reported to repress adipogenic differentiation by blocking the induction of key adipogenic transcription factors, PPARγ and C/EBPα (18, 20). Wnts are secreted, cysteine-rich glycoproteins that act as autocrine or paracrine factors, regulating a variety of developmental processes, including cell proliferation and differentiation (5, 21). Wnt signaling encompasses both canonical and non-canonical pathways, with the former being better characterized in adipogenic differentiation. Pioneering studies demonstrated that overexpression of Wnt1 or a GSK3β phosphorylation-defective β-catenin mutant inhibited adipogenic differentiation in 3T3-L1 preadipocytes (18). Overexpression of canonical Wnt10b ligand stabilized β-catenin and blocked adipogenic differentiation in 3T3-L1 preadipocytes (18). Also, transgenic mice overexpressing Wnt10b under the control of the adipose-specific FABP4 promoter exhibited a 50% reduction in total body adiposity and resistance to HFD-induced white adipose tissue expansion (22). Although the canonical Wnt pathway has been consistently implicated in

Figure 3. APCDD1 overexpression accelerates adipogenic differentiation and down-regulates the expression of Wnt signaling proteins in 3T3-L1 preadipocytes. *A*, adipogenic differentiation, as indicated by lipid droplet accumulation (Oil red O), was increased in 3T3-L1 preadipocytes transfected with adenoviral APCDD1 compared with the control. *, $p < 0.01$ *versus* control (Adeno-GFP). *B*, mRNA expression of adipogenic markers (adiponectin and FABP4) was up-regulated in adenoviral APCDD1-transduced 3T3-L1 preadipocytes during differentiation (7 days) ($n = 4$). *, $p < 0.01$ *versus* control (PA); **, $p < 0.05$ *versus* GFP. *AD*, adipocyte. *C* and *D*, transduction of 3T3-L1 preadipocytes with adenoviral APCDD1 down-regulated Wnt-associated protein expression (Wnt3a, Wnt5a, Wnt10b, LRP5, and β-catenin) and up-regulated adipogenic marker (C/EBPα and PPARγ) protein expression during differentiation ($n = 3$). *, $p < 0.01$; **, $p < 0.05$ *versus* control (*CTL*). Levels of mRNA and protein expressions were determined by qPCR and Western blotting, respectively. Representative blots are shown in *C* and densitometry in *D*.

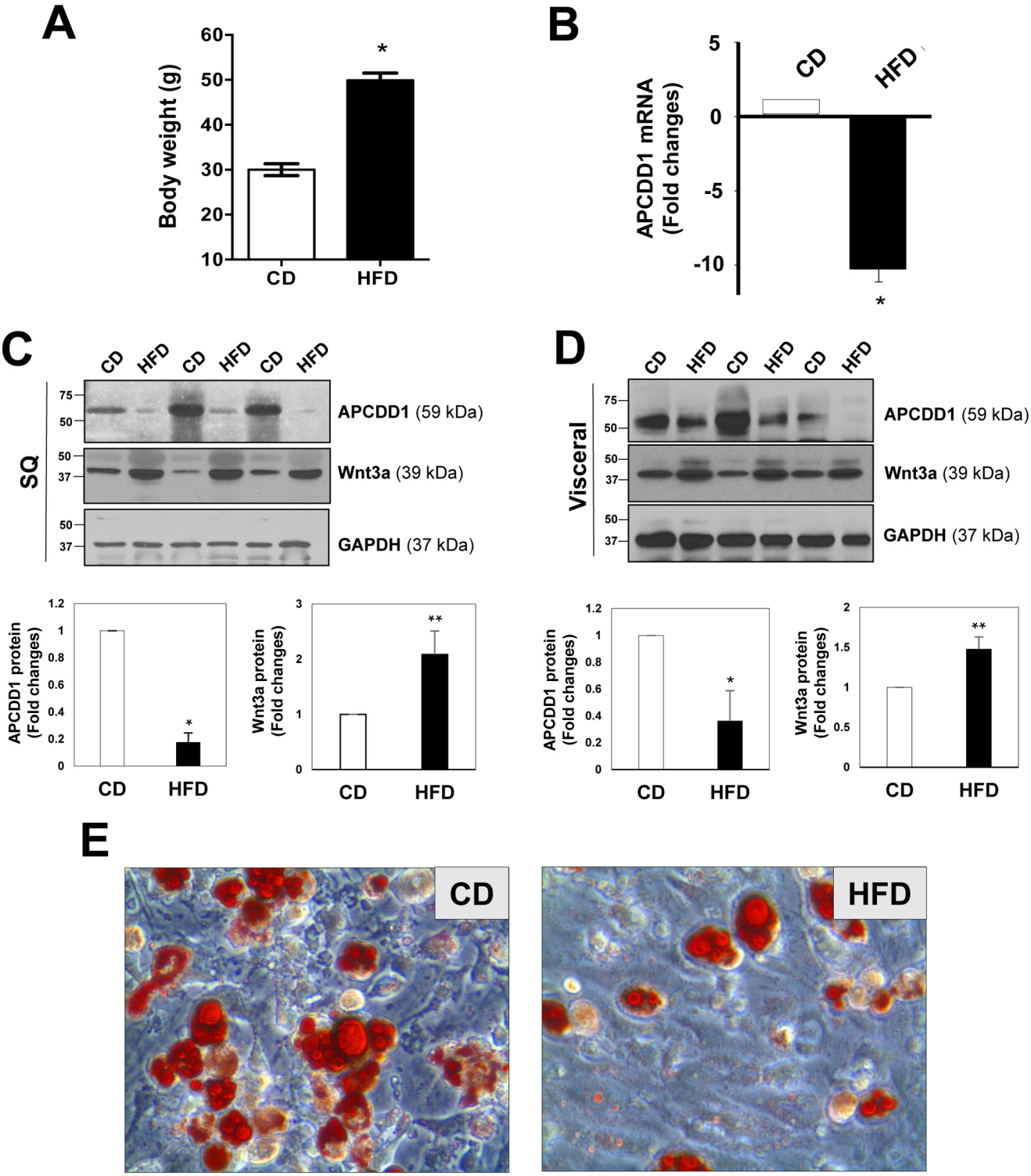


Figure 4. Reduced APCDD1 expression is associated with up-regulated Wnt3a and blunted adipogenic differentiation in DIO mice. *A*, 18 weeks of HFD feeding, compared with CD feeding, significantly increased body weight in mice ($n = 3$). *B*—*D*, APCDD1 mRNA (*B*) and protein (*C*) levels were down-regulated, whereas Wnt3a was up-regulated, in extracts of SQ (*C*) and visceral (*D*) adipose tissues isolated from HFD-fed DIO mice compared with lean controls ($n = 3$). Relative mRNA and protein expression were quantified by qPCR and Western blotting, respectively. *, $p < 0.01$; **, $p < 0.05$ *versus* control (*CD*). *E*, adipogenic differentiation (7 days) was impaired in preadipocytes isolated from subcutaneous adipose tissues of DIO mice. Light microscopy of neutral cytoplasmic lipid droplet accumulation was assessed by Oil red O staining.

inhibition of adipogenic differentiation *in vitro* and *in vivo*, non-canonical Wnt pathways (independent of β-catenin) are poorly understood and may exert opposing effects on adipogenesis. For instance, Wnt5b, a non-canonical ligand, is transiently induced during adipogenic differentiation and partially inhibits the canonical Wnt/β-catenin pathway to facilitate differentiation (23). Thus, the canonical and non-canonical Wnt pathways are interconnected and cross-talk

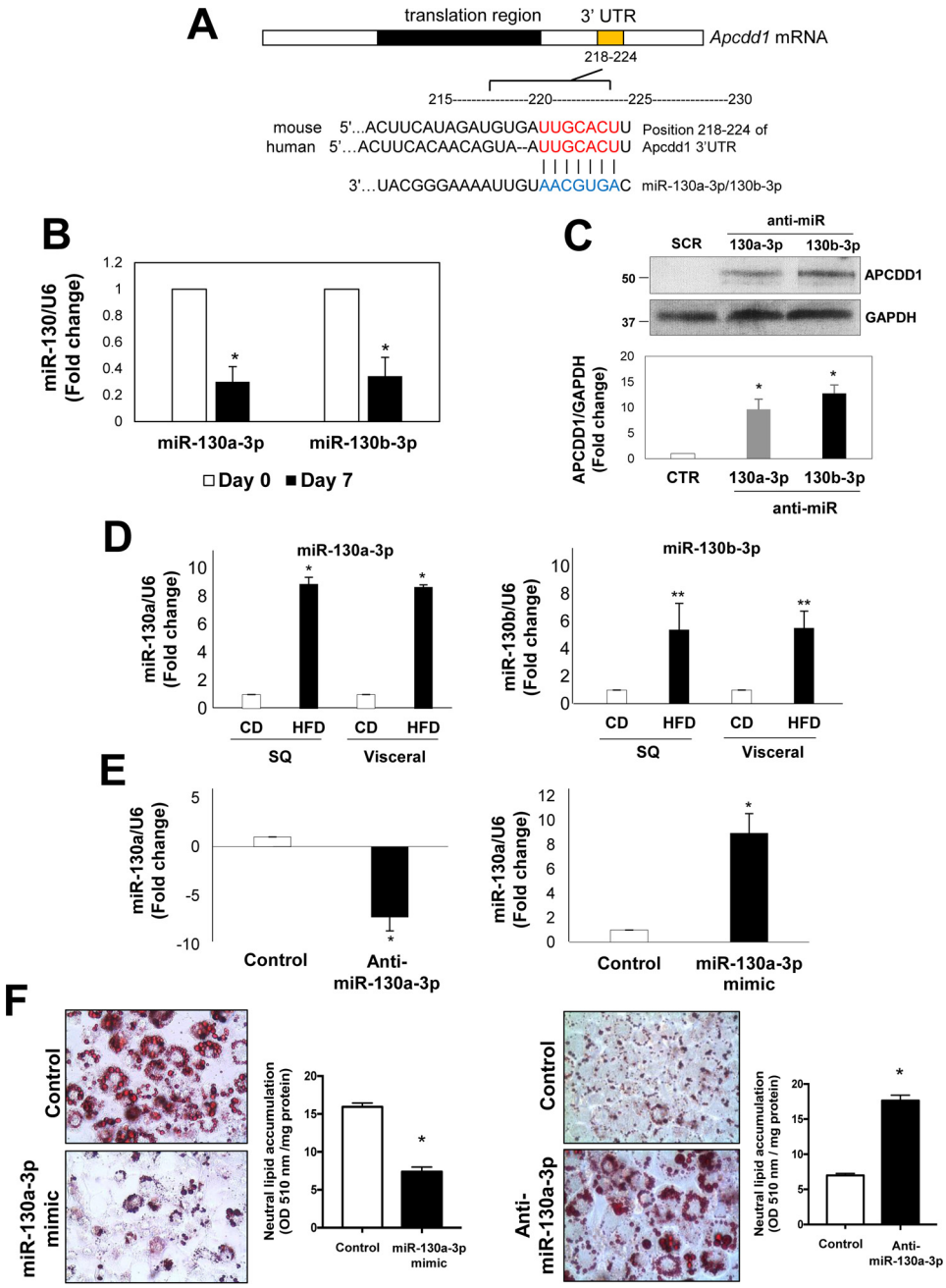


Figure 5. HFD-induced miR-130 targets *APCDD1* and inhibits adipogenic differentiation. *A*, computational analysis predicted that miR-130a-3p and miR-130b-3p can directly bind the 3′ UTR of APCDD1. *B*, miR-130a-3p and miR103b-3p levels were down-regulated during adipogenic differentiation in 3T3-L1 preadipocytes. *, $p < 0.05$ *versus* control (*Day 0*). *C*, APCDD1 protein expression was increased by transfection of miR-130 inhibitor in 3T3-L1 preadipocytes ($n = 3$). *, $p < 0.05$ *versus* control (*CTR*). *SCR*, scramble. *D*, miR-130a-3p and miR-130b-3p levels were increased in the SQ and visceral adipose depots of HFD-fed DIO mice ($n = 5$). *, $p < 0.01$; **, $p < 0.05$ *versus* control (*CD*). *E*, miR-130a-3p expression was significantly reduced or increased by transfection of miR-130a-3p inhibitor or mimic, respectively, in 3T3-L1 preadipocytes ($n = 3$). *, $p < 0.01$ *versus* control. *F*, adipogenic differentiation of 3T3-L1 preadipocytes was enhanced by transfection of miR-130a-3p inhibitor and reduced by transfection of miR-130a-3p mimic. *, $p < 0.0001$ *versus* control. Light microscopy of neutral cytoplasmic lipid droplets accumulation was evaluated by Oil red O staining and optical density measurement by spectrophotometer.

with one another to regulate adipogenic differentiation in a complex manner.

The components of Wnt signaling pathways are modulated by a number of endogenous activators and inhibitors, among them APCDD1 (24). APCDD1 is an evolutionarily conserved plasma membrane glycoprotein that has only been recently identified as a Wnt inhibitor (14). The APCDD1 gene is located on chromosome 18 in mice and humans and was first identified in colon cancer tissues (14, 16, 25). It encodes an ~58-kDa transmembrane protein whose transcription is regulated by the β-catenin·Tcf complex (14, 16). APCDD1 expression was found to be elevated in 18 of 27 primary colon cancer tissues compared with corresponding noncancerous mucosa, and its exogenous expression was shown to promote cancer cell growth (16). In humans, APCDD1 is expressed during tissue development and in numerous adult tissue types (14–16), sug-

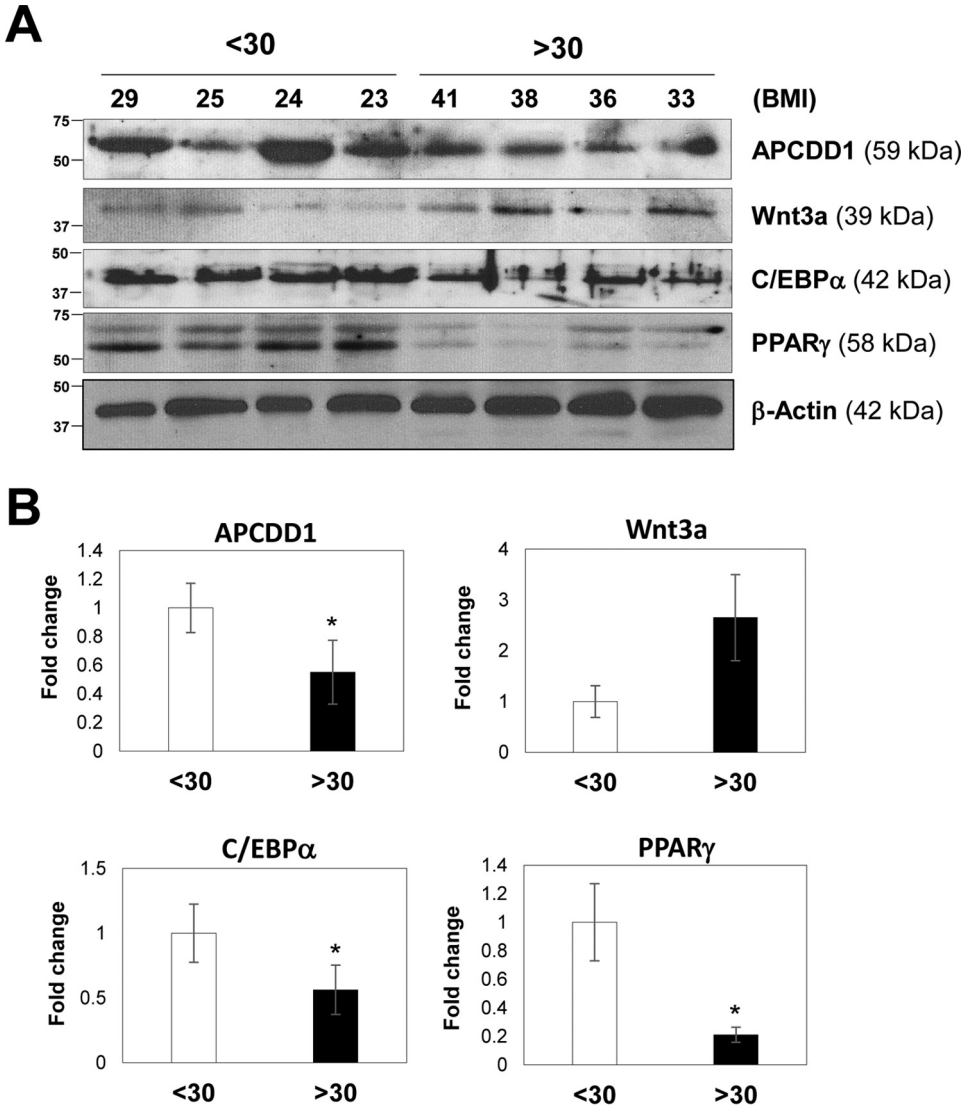


Figure 6. Down-regulated expression of APCDD1 in adipose tissues from obese humans is associated with reduced adipogenic gene expression. *A* and *B*, protein expression of APCDD1, in conjunction with C/EBPα, PPARγ, was down-regulated, whereas Wnt3a levels tended to be up-regulated ($p = 0.06$), in subcutaneous adipose tissues isolated from obese (BMI $>$ 30) compared with non-obese subjects (BMI $<$ 30). *, $p < 0.05$ *versus* non-obese control. Protein expressions were analyzed by Western blotting (*A*, representative data) and densitometry (*B*, $n = 8$).

gesting a diverse role for APCDD1 in biological processes associated with Wnt signaling. Indeed, recent studies have documented that APCDD1 is involved in gliogenesis, tooth morphogenesis, oligodendrocyte differentiation, osteogenic differentiation of human dental follicle cells, and diseases such as hereditary hypotrichosis simplex (14, 26–29). Although most studies support an inhibitory role for APCDD1 in Wnt/β-catenin signaling, Morsczeck and co-workers (28) reported that APCDD1 sustained the expression and activation of β-catenin during osteogenic differentiation of human dental follicle cells. Moreover, microarray results indicated that APCDD1 expression is >300-fold higher in dental follicle cells than in mesenchymal stem cells (30). This suggests that the level of APCDD1 expression and its impact on Wnt/β-catenin signaling may vary considerably in individual cells and tissues.

Although a role for APCDD1 in mammalian adipose development and adipocyte differentiation is heretofore unreported, Ullah *et al.* (15) recently identified APCDD1 as a potential human adipocyte-specific marker gene using GeneChips analysis and bioinformatics. Our study is the first to directly examine the functional significance of APCDD1 in adipogenic differentiation of human and murine preadipocytes *in vitro*. We demonstrate that APCDD1 gene expression is rapidly induced during adipogenic differentiation and propose a functional link between APCDD1 and the adipogenic program through a Wnt signaling-dependent mechanism. APCDD1 was demonstrated to physically interact with Wnt3a and Fz co-receptor LRP5 proteins, thereby preventing the formation of Wnt receptor complexes (14). Here we show that APCDD1 inhibits the expression of Wnt pathway-related proteins, including Wnt3a, LRP5, and β-catenin, in 3T3-L1 preadipocytes, revealing a mechanism by which APCDD1 could disrupt canonical Wnt signaling. In addition to canonical Wnt signaling, we found that APCDD1 may also regulate the expression of non-canonical Wnt5a. The precise mechanisms whereby APCDD1 inhibits Wnt pathways

in the context of adipogenic differentiation thus appear to be complex. It is also possible that APCDD1 regulates adipocyte differentiation via Wnt-independent mechanisms. Future studies will be required to investigate these possibilities.

Although adipose tissue contains a large number of preadipocytes that are potentially able to differentiate into fully mature, lipid-storing adipocytes, it nevertheless primarily expands by enlargement of pre-existing adipocytes in obesity (31, 32), thus promoting insulin resistance and hepatic steatosis, key aspects of metabolic syndrome (3, 33). This suggests that the level of adipogenic differentiation is insufficient to match the metabolic demand in obesity. The rate of preadipocyte replication is actually *increased* in adipose tissues of obese mice (34), suggesting that the insufficient level of adipocyte differentiation in obesity is not caused by reduced adipogenic precursor cell abundance. Interestingly, we have reported previously that HFD impairs adipogenic differentiation, promoting accumulation of inefficiently differentiated adipocytes that exhibit diminished expression of adipogenic differentiation-specific genes (35). This impairment in adipogenic differentiation in HFD-induced obesity therefore may be related to the inability of preadipocytes to efficiently undergo differentiation and/or maintain the differentiated state.

Mounting evidence indicates that Wnt signaling may be dysregulated in obesity and thus could potentially contribute to the impaired adipogenic differentiation. Two independent groups have reported increased circulating Wnt5a in obese patients compared with lean control subjects (36, 37). Expression of Wnt5a mRNA in visceral adipose tissues was also found to be increased, whereas secreted Fz-related protein 5 (SFRP5), an adipokine that represses Wnt signaling by binding and sequestering Wnt ligands, was reduced in obese patients (37). In line with these findings, we showed that obese subjects (BMI $>$ 30) tended to display increased Wnt3a expression in their subcutaneous adipose tissues compared with non-obese subjects (BMI $<$ 30). The mechanisms responsible for up-regulated Wnt ligand expression in obesity are unknown. Interestingly, we observed decreased APCDD1 protein expression in both obese human and mouse adipose tissues compared with their corresponding controls. Based on our findings, we postulate that reduced APCDD1 expression in obese adipose tissues may potentiate Wnt signaling, thereby contributing to impaired adipogenic differentiation *in vivo*. Future studies directly targeting APCDD1 in animal models of obesity will be required to test this hypothesis.

Identifying the mechanisms whereby APCDD1 expression is down-regulated in obese adipose tissues may provide insights into the pathogenesis of metabolic syndrome and new approaches for treatment. Here we focused on epigenetic mechanisms of gene regulation, which play a prominent role in the pathogenesis of obesity-related diabetes (38). In particular miRNAs, through their potent effects on posttranscriptional regulation, are functionally important in adipogenic differentiation. For instance, miR-143 stimulates adipogenic differentiation through inhibition of ERK5 (39), whereas miR-210 enhances adipogenesis by inhibiting TCF7L2, a key transcription factor in Wnt signaling (40). Moreover, miR-124 and miR-17–92 promote adipogenic differentiation by repressing Dlx5 and retinoblastoma (RB)-family Rb2/p130, respectively (41, 42). On the other hand, several miRNAs have been reported to negatively regulate adipogenesis. TNF-α-induced up-regulation of miR-155 inhibits adipogenic differentiation by down-regulating cAMP-response element-binding protein (CREB) and C/EBPβ (43), and let-7 was reported to inhibit adipogenesis by regulating the expression of high mobility group AT-hook2 (HMGA-2) (44). Notably, the miR-130 family has been shown to impair human preadipocyte differentiation by repressing PPARγ biosynthesis, and TNF-α-induced miR-130 was reported to promote adipocyte dysfunction during obesity (45, 46). Here we confirmed a previous report that miR-130 expression is up-regulated in adipose tissues of HFD-fed obese mice (46). Computational analysis of the 3′ UTR of the APCDD1 gene predicted a binding site for miR-130a and miR-130b, which share the same seed sequence but are encoded by two independent loci (miRBase Database). Knockdown of miR-130 in preadipocytes increased APCDD1 expression, suggesting that it may directly target APCDD1. Indeed, transfection of an miR-130a-3p inhibitor in preadipocytes enhanced, whereas an miR-130a-3p mimic blunted, adipogenic differentiation. Based on these findings, we hypothesize that elevated miR-130 in obese adipose tissues may contribute to impaired adipogenic differentiation via its inhibitory effect on APCDD1 expression (Fig. 7). Although this study focused on the role of miRNA, the importance of other epigenetic mechanisms, including DNA methylation and histone modifications, in regulating APCDD1 gene expression in obesity remains to be determined. Finally, what role, if any, adipocyte enlargement in obesity plays in down-regulating APCDD1 expression also remains to be determined.

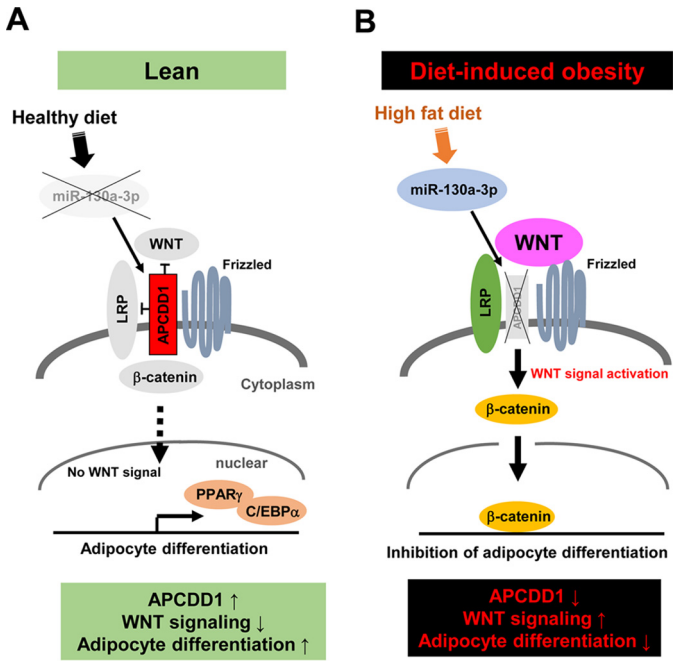


Figure 7. Schematic depicting the potential mechanisms of APCDD1-mediated adipogenic differentiation in the lean state *versus* DIO. Under normal physiological conditions, APCDD1 inhibits the expression of Wnt signaling proteins, leading to the induction of key adipogenic transcription factors (C/EBPα and PPARγ) and adipogenic differentiation. In DIO, HFD-induced miR-130 in adipose tissues blocks APCDD1 gene expression, thereby augmenting Wnt signaling and repressing adipogenic differentiation.

In conclusion, we provide novel evidence that APCDD1, an endogenous inhibitor of Wnt signaling, is an essential component of the adipogenic differentiation program. Based on our findings, we propose a working model (Fig. 7, *A* and *B*) to suggest that, under normal physiological conditions, APCDD1 inhibits the expression of Wnt signaling proteins, leading to the induction of key adipogenic transcription factors (C/EBPα and PPARγ) and induction of adipogenic differentiation. In DIO, up-regulated expression of miR-130 in adipose tissues blocks APCDD1 gene expression, thereby augmenting Wnt signaling and repressing adipogenic differentiation. These findings may have important implications for the pathogenesis of obesity-related metabolic disease.

Experimental procedures

Preparation of mouse adipose tissues

Male C57BL/6J mice were maintained on chow diet after weaning. At 6 weeks of age, these mice were either maintained on CD (Harlan Teklad, LM-485) or switched to an HFD (Research Diet, D12492, 60% calories from lard fat) for 18 weeks. Mice were euthanized, blood was collected via cardiocentesis, and subcutaneous and visceral adipose tissues were collected following tissue perfusion with saline. All animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the University of Cincinnati College of Medicine and Medical College of Georgia at Augusta University.

Preparation of human adipose tissues

Human adipose tissues were collected from obese (BMI $>$ 30) and non-obese (BMI $<$ 30) patients undergoing abdominal surgeries. The study protocol was approved by the Institutional Review Boards of the University of Cincinnati and Medical College of Georgia at Augusta University.

Isolation of adipocytes, preadipocytes, and in vitro adipogenic differentiation

To isolate adipocytes and preadipocytes from adipose tissues, mouse or human adipose tissues were thoroughly minced, digested with collagenase (Worthington), filtered, and centrifuged to separate mature floating adipocytes from the pelleted stromal vascular (SV) fraction cells (preadipocyte-enriched) as described previously (47). Then, SV pellets were resuspended, plated, and grown in preadipocyte growth medium (Cell Applications) as described previously (47). The cells were expanded for two to four passages in culture and differentiated in the presence of adipocyte differentiation medium (Cell Applications), which was replaced with fresh medium every 2 days. 3T3-L1 preadipocytes were cultured in 3T3-L1 preadipocytes medium and differentiated in 3T3-L1 differentiation medium (ZenBio). These commercial media include a standard combination of adipogenic differentiation factors, including FBS (5–10%), insulin (100–1700 nM), dexamethasone (250–1000 nM), 3-isobutyl-1-methylxanthine (100–500 nM), indomethacin, biotin, pantothenate, etc. and induce adipogenic differentiation with high efficiency. For molecular studies, adipose tissues were flash-frozen in liquid nitrogen immediately after collection for subsequent analysis of RNA or protein expression. 3T3-L1 preadipocytes, obtained from the ATCC, were grown in preadipocyte medium (ZenBio) and differentiated in the presence of adipocyte differentiation medium (ZenBio) according to the instructions of the manufacturer.

Cytoplasmic lipid droplet measurement by Oil red O

0.4% of Oil red O (Sigma) in isopropanol (stock solution) was diluted at 3:2 (Oil red O:ddH_2O) ratio to form Oil red O working solution. Cells were washed with PBS and fixed with 4% paraformaldehyde for 1 h. Cells were then washed with ddH_2O followed by 60% isopropanol. Oil red O working solution was then added to the fixed cells and incubated for 10 min at room temperature. After incubation, cells were washed with ddH_2O and imaged using light microscopy. Oil red O quantification was carried out by measuring the optical density (510 nm) with a spectrophotometer.

siRNA transfection of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were transfected with APCDD1-specific siRNA (Santa Cruz Biotechnology; final concentration, 20 nM) or scramble control by using Lipofectamine 2000^{TM} (Invitrogen) according to the instructions of the manufacturer, and then the cells were differentiated in the presence of adipocyte differentiation medium. After 72 h of differentiation, the cells were retransfected with APCDD1 siRNA or scramble control in the same manner to boost the transfection efficiency. At 7 days of differentiation, cells were stained with Oil red O to evaluate lipid accumulation (an index of adipocyte differentiation) or harvested for molecular studies.

Adenovirus transduction of 3T3-L1 preadipocytes

The human APCDD1 adenovirus (Applied Biological Materials) was amplified by transducing HEK 293 cells, and the medium supernatant containing the adenovirus was collected and titrated according to the instructions of the manufacturer. 3T3-L1 preadipocytes were transduced with adenoviruses carrying APCDD1 or a control gene at a multiplicity of infection of 500 with 0.5 μg/ml poly-L-lysine in preadipocyte medium for 6 h and then differentiated in the presence of adipocyte differentiation medium. At 7 days of differentiation, cells were stained with Oil red O or harvested for molecular studies.

Quantitative PCR

Total RNA was extracted from tissues or cells with QIAzol lysis reagent and purified with the RNeasy lipid tissue mini kit (Qiagen) according to the instructions of the manufacturer. Real-time quantification of mRNA levels of the genes of interest was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) according to the instructions of the manufacturer. Normalized Ct values were subjected to statistical analysis, and the -fold difference was calculated by the $\Delta\Delta$ Ct method as described previously (48).

Western blotting

Proteins were extracted from tissues or cells by radioimmune precipitation (RIPA) assay lysis buffer, separated on SDS-PAGE gel, transferred to nitrocellulose membranes, and probed with

the appropriate antibodies, and subsequently blots were developed using ECL system (Thermo Scientific). For detection of nuclear proteins (β-catenin, C/EBPα, and PPARγ), nuclear fractionation was performed using an NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific) according to the protocol of the manufacturer. The specific antibodies used in this study were as follows: APCDD1, C/EBPα, Wnt1, Wnt3a, Wnt10b, LRP5, LRP6, and β-actin (Santa Cruz Biotechnology); Wnt5a (Abcam); GAPDH (Ambion); PPARγ (Novus Biologicals); histone H3 (Cell Signaling Technology); and β-catenin (Upstate).

miRNA target prediction

Computational miRNA target prediction analysis was performed using three databases (TargetScan, PicTar, and miRWalk algorithms) to predict potential binding between 3′ UTR of target genes and selective miRNAs.

miRNA isolation and detection

Total RNA, including miRNA, was extracted using the *mir*Vana™ miRNA isolation kit (Ambion), and miR-130a-3p and miR-130b-3p were detected using All-in-One first-strand cDNA synthesis kits and All-in-One miRNA quantitative RT-PCR reagent kits (GeneCopoeia). Specific miR-130 primers for quantitative RT-PCR were as follows: miRNA universal reverse, 5′-CCAGTGCAGGGTCCGAGGTA; miR-130a-3p RT, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGCCCTT; miR-130a-3p forward, 5′-ACACTCCAGCTGGGCAGTGCAATGTTAAAA; miR-130b-3p RT, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGCCCTT; and miR-130a-3p forward, 5′-ACACTCCAGCTGGGCAGTGCAATGATGAAA.

Transfection of 3T3-L1 preadipocytes with miRNA inhibitor or mimic

miR-130 mimic (miRIDIAN miRNA mimic) and anti-miR-130 (hairpin inhibitor) were synthesized by Dharmacon. 3T3-L1 preadipocytes were transfected with 50 nM miRNA anti-miR or mimic using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer, and then the cells were differentiated in the presence of adipocyte differentiation medium. After 48 h of differentiation, the cells were retransfected in the same manner to boost transfection efficiency. At 5 days of differentiation, cells were stained with Oil red O. In some experiments, 3T3-L1 preadipocytes were co-transfected with the APCDD1 plasmid and miR-130a-3p mimic by adenovirus and Lipofectamine 2000, respectively, in preadipocyte medium for 6 h and then differentiated in the presence of adipocyte differentiation medium.

Statistical analysis

Data are expressed as mean ± S.E. Comparison between two mean values was evaluated by an unpaired Student's two-tailed *t* test and between three or more groups by one-way analysis of variance followed by Bonferroni post hoc analysis. The statistical relationship between two continuous variables was evaluated by linear repression analysis. $p < 0.05$ was considered statistically significant.

Author contributions— N. K. H. Y., T. K. C., H. W. K., and N. L. W. were involved in study conception and design, data acquisition, analysis, interpretation, and manuscript writing. R. P. performed the experiments and analyzed the results. Z. B., V. P., V. M. K., and S. M. R. provided human tissue samples and reviewed the manuscript. Y. L. T., B. K. S., D. J. F., D. W. S., W. C., S. E. L., and D. Y. H. contributed to interpretation of data and manuscript review.

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