Dact1, a Nutritionally Regulated Preadipocyte Gene, Controls Adipogenesis by Coordinating the Wnt/β-Catenin Signaling Network

Claire Lagathu,[1] Constantinos Christodoulides,[1] Sam Virtue,[1] William P. Cawthorn,[1] Chiara Franzin,[1] Wendy A. Kimber,[1] Edoardo Dalla Nora,[1] Mark Campbell,[1] Gema Medina-Gomez,[1] Benjamin N.R. Cheyette,[2] Antonio J. Vidal-Puig,[1] and Jaswinder K. Sethi[1]

OBJECTIVE—Wnt signaling inhibits adipogenesis, but its regulation, physiological relevance, and molecular effectors are poorly understood. Here, we identify the Wnt modulator Dapper1/Frodo1 (*Dact1*) as a new preadipocyte gene involved in the regulation of murine and human adipogenesis.

RESEARCH DESIGN AND METHODS—Changes in *Dact1* expression were investigated in three in vitro models of adipogenesis. In vitro gain- and loss-of-function studies were used to investigate the mechanism of Dact1 action during adipogenesis. The in vivo regulation of *Dact1* and Wnt/β-catenin signaling were investigated in murine models of altered nutritional status, of pharmacological stimulation of in vivo adipogenesis, and during the development of dietary and genetic obesity.

RESULTS—*Dact1* is a preadipocyte gene that decreases during adipogenesis. However, Dact1 knockdown impairs adipogenesis through activation of the Wnt/β-catenin signaling pathway, and this is reversed by treatment with the secreted Wnt antagonist, secreted Frizzled-related protein 1 (Sfrp1). In contrast, constitutive Dact1 overexpression promotes adipogenesis and confers resistance to Wnt ligand-induced antiadipogenesis through increased expression of endogenous Sfrps and reduced expression of Wnts. In vivo, in white adipose tissue, *Dact1* and Wnt/β-catenin signaling also exhibit coordinated expression profiles in response to altered nutritional status, in response to pharmacological stimulation of in vivo adipogenesis, and during the development of dietary and genetic obesity.

CONCLUSIONS—Dact1 regulates adipogenesis through coordinated effects on gene expression that selectively alter intracellular and paracrine/autocrine components of the Wnt/β-catenin signaling pathway. These novel insights into the molecular mechanisms controlling adipose tissue plasticity provide a functional network with therapeutic potential against diseases, such as obesity and associated metabolic disorders. *Diabetes* **58: 609–619, 2009**

Dysregulated adipose function, as is observed in obesity, is associated with increased risk of developing diabetes, cardiovascular diseases, and some cancers. Recently, impaired adipose tissue expandability/plasticity has been shown to be an important factor linking obesity to its metabolic complications (1–4). This has led to the hypothesis that specific regulatory mechanisms must exist to ensure that the balance between preadipocyte recruitment and differentiation tightly matches the storage demands imposed by nutritional loads. Understanding the mechanisms that control when, how, and which preadipocytes enter the program of differentiation may aid the development of rational therapeutic strategies to improve adipose tissue functionality and lipid buffering capacity and thereby prevent and/or treat obesity-associated metabolic disorders.

Adipocyte differentiation involves the sequential activation of a cascade of transcription factors that coordinate the expression of genes responsible for the adipogenic phenotype (5,6). Briefly, CCAAT/enhancer-binding protein (C/EBP) β and δ are rapidly and transiently induced in response to adipogenic stimuli. This precedes the expression of the two adipogenic transcription factors, C/EBPα and peroxisome proliferator–activated receptor (PPAR)γ. These factors act synergistically to induce expression of adipocyte-specific genes. Although in vitro adipogenesis can be synchronously induced by exposure to a defined adipogenic cocktail, in vivo adipose tissue expansion is regulated by a combination of local and endocrine factors that act to either stimulate or inhibit adipogenesis (7) in response to demands for nutritional storage. In addition, adipocyte growth is likely to be balanced with (and may precede) recruitment of new adipocytes from the progenitor pool. In healthy individuals, tight regulation of these processes is required to ensure that the appropriate number of adipocytes form and is sufficient (but not excessive) to store nutritional surplus. As a highly conserved and widely distributed intercellular signaling pathway, the Wnt signaling network and its molecular components are good candidates to contribute to this important regulated homeostatic process.

The Wnt family of secreted glycoproteins function in a paracrine and/or autocrine manner to influence cell fate and development. Binding of specific Wnt proteins to receptor/coreceptor complexes transduces intracellular signals through either β-catenin–dependent or –independent pathways. Although both pathways may be active in preadipocytes (8), it is the former that has

been shown to potently inhibit adipogenesis both in vitro and in vivo (9). The Wnt/β-catenin signaling cascade comprises specific cell surface Frizzled receptors and LDL receptor–related protein 5 or 6 (LRP5/6) coreceptors, which on ligand binding transduce Wnt signals via intracellular Dishevelled (Dvl) proteins, ultimately leading to disassembly and inactivation of the β-catenin degradation complex (10,11). The resulting cytosolic accumulation and nuclear translocation of β-catenin leads to transcriptional coactivation of the T-cell factor (TCF)/lymphoid enhancer factor transcription factors. Wnt/β-catenin/TCF target genes include PPARδ, inhibitor of DNA binding 2 (Id2), CyclinD1, and c-Myc (myelocytomatosis oncogene). Some of these have been shown to exhibit antiadipogenic actions (9,12,13).

In preadipocytes, constitutive activation of Wnt/β-catenin signaling leads to maintenance of the undifferentiated state and prevents induction of C/EBPα and PPARγ (5,6). In contrast, disruption of Wnt/β-catenin signaling promotes adipogenesis (9,12,14,15). These observations have led to suggestions that Wnt proteins may act as a functional "brake" during preadipocyte recruitment into the differentiation program. Of the 19 possible Wnt ligands, Wnt10b has been most clearly implicated as the endogenous Wnt involved in regulating adipogenesis. Wnt10b is expressed in both dividing and confluent preadipocytes, but its expression decreases during adipogenesis preceding the downregulation of intracellular β-catenin. Furthermore, ectopic expression of Wnt10b activates Wnt/β-catenin signaling and potently inhibits adipogenesis (16–18). Conversely, extracellular antagonists of Wnt/β-catenin signaling such as dickkopf homolog-1 and some of the secreted Frizzled-related proteins (sFRP) have also been shown to exert proadipogenic effects (14,15).

Although this evidence demonstrates Wnt/β-catenin–induced modulation of the adipogenic program, less is known about the physiological regulation of Wnt/β-catenin signaling network in vivo. In this report, we identify Dapper1/Frodo1 (*Dact1*) as an important preadipocyte gene that controls adipocyte differentiation by regulating the Wnt/β-catenin signaling network. Previous reports identified Dact1 as an evolutionarily conserved, Dvl-interacting protein that modulates Wnt signals (19,20); however, its mechanism of action remains ambiguous (19–25). In immortalized human embryonic kidney cells (HEK293) and in some embryonic contexts, Dact1 appears to act as an antagonist of Wnt/β-catenin signaling (19,20,26). However, a positive function for Dact1 homologs in Wnt/β-catenin signaling has also been suggested by several studies in other systems, particularly knockdown and low overexpression studies in developing embryos (22,23). Hence, it has been suggested that the function of Dact family members is cell- and tissue-context–dependent (23,26–28). Because adaptor or scaffold proteins are likely to function by bringing together other signaling components to form complexes favoring one biochemical signaling cascade over another in a particular cellular, physiological, or signaling context, it may not be meaningful to categorize Dact proteins either as general activators or inhibitors. Given this consideration, it is important to note that before this study, the role of Dact1 in adipogenesis was completely unexplored.

Here, we show that Dact1 acts to alter the Wnt/β-catenin signaling tone of preadipocytes through coordinated effects on both the efficiency of intracellular signal transduction pathway and by altering the expression profiles of

extracellular Wnt ligands and antagonists. The regulatory network that we define here incorporates cell-specific paracrine signals and is itself physiologically regulated, because expression levels of key components *Dact1*, *Wnt10b*, and *Sfrp5* are all coordinately regulated in vivo in adipose tissue by nutritional status, by proadipogenic pharmacological stimulation, and during the development of dietary and genetic obesity.

RESEARCH DESIGN AND METHODS

Tissue culture media, insulin, isobutylmethylxanthine, dexamethasone, Oil-red-O, and puromycin were purchased from Sigma (St. Louis, MO). Tissue culture sera were from HyClone (Logan, UT). Anti-Dvl1, anti-Dvl2, anti-Dvl3, and anti–extracellular signal–regulated kinase-1/2 (-ERK1/2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Dact1 antibody was from Orbigen (San Diego, CA). All antibodies were used according to the manufacturer's instructions. All horseradish peroxidase–conjugated secondary antibodies were from Cell Signaling Technology (Danvers, MA). Recombinant murine Wnt3a and human SFRP1 were from R&D Systems (Minneapolis, MN).

Retroviral expression constructs and TCF4 reporter assay. Murine *Dact1* cDNA was excised from mouse-*Dact1*-pCMV (20) with BamH1-SnaB, Klenow filled-in, and blunt-end–ligated into the SnaB1 site of the retroviral expression vector pBabe-puro. Correct orientation was confirmed by at least three different diagnostic digests. Stable knockdown of *Dact1* was achieved by retroviral expression of shRNA from the pSiren-RetroQ vector (Clontech, Mountain View, CA). Custom oligonucleotides were identified using the Dharmacon siDesign center (sequences available on request) and cloned into pSiren-RetroQ according to the manufacturer's instructions.

For TCF4 reporter assay, Dact1SH, CtrlSH, Dact1, and control 3T3-L1 cells were grown to confluence, and Topflash promoter-reporter assays were performed as previously described (15).

Animals. Animals were housed at a density of four animals per cage in a temperature-controlled room (20–22°C) with 12-h light/dark cycles. The effects of fasting and refeeding were studied using 8-week-old male SV129 mice (whole adipose tissue) and C57/B6 mice (tissue fractionation study). For these studies, mice were deprived of chow for a 24-h period and either killed or refed for a further period of 24 h. Mice were allowed ad libitum access to water.

The effects of genetic obesity (*ob/ob*) were studied in tissues collected as previously reported (29). The effects of long-term high-fat diet (16 weeks) were studied in 7-month-old C57/B6 mice fed either standard laboratory chow or high-fat diet (45% calories from fat; D12451; Research Diets, New Brunswick, NJ) ad libitum from weaning. The short-term high-fat diet study was performed on 16-week-old C57/B6 mice that had been fed either chow or high-fat diet for either 3 or 28 days before they were killed. Adipose tissues were collected from all mice at the same age. The effects of thiazolidinedione (TZD) treatment were studied using male C57/B6 mice fed standard chow diet supplemented with or without 10 ppm rosiglitazone (Avandia) for 3 weeks. All animal protocols used in this study were approved by the UK Home Office and the University of Cambridge.

Preadipocyte isolation, culture differentiation, and infection. Preadipocyte isolation from human adipose tissue biopsies was performed as previously described (15). Cambridge Research Ethics Committee approval was obtained, and all patients gave their informed consent. Murine primary preadipocytes were isolated from epididymal white adipose tissue (WAT) of 6-week-old male C57/B6 mice as described previously (29).

3T3-L1 cells were cultured, differentiated into adipocytes, and stained for Oil-red-O as described previously (12). Dact1 and control 3T3-L1 cell lines were generated with the pBabe-Puro retroviral vector system, and Dact1SH and CtrlSH 3T3-L1 cells were generated with the pSiren-RetroQ retroviral vector system as described previously (12,30).

Protein extraction and Western blotting. Proteins were extracted from cell monolayers as previously described (12). Proteins were electroblotted nitrocellulose membrane (Amersham Biosciences). Specific proteins were detected by incubation with the appropriate primary and horseradish peroxidase–conjugated secondary antibodies. Immune complexes were detected by enhanced chemiluminescence (Amersham Biosciences).

RNA isolation and quantitative RT-PCR. Total RNA was isolated from cultured cells using an RNeasy kit (Qiagen, Crawley, U.K.), and mRNA expression was analyzed by RT-PCR as described previously (12). The sequences of the oligonucleotides used for primers and probes are available on request.

Statistical analysis. Data from densitometrical analysis, luciferase assays, and quantitative real-time PCR are presented as means ± SE of at least three
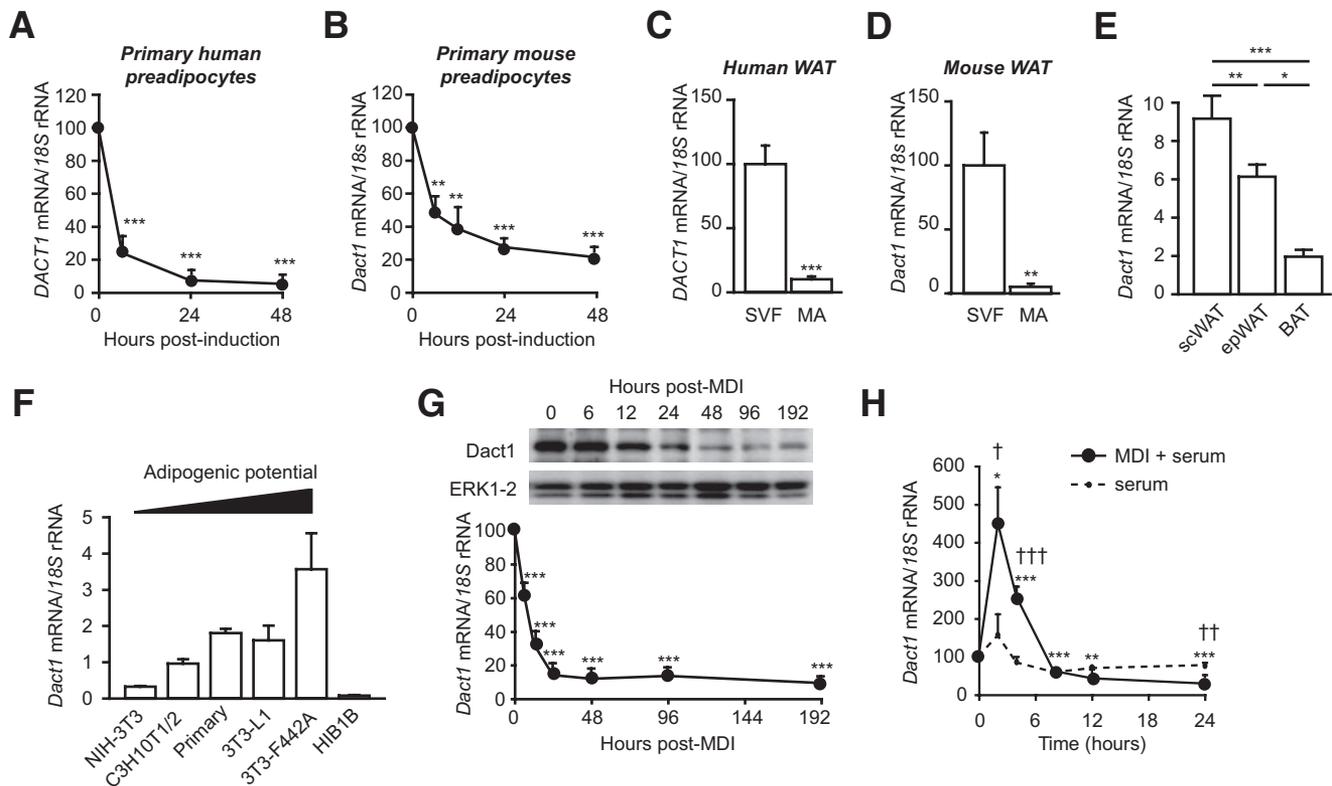
FIG. 1. DACT1 gene expression is downregulated in early adipogenesis and is found primarily the SVF of human and mouse adipose tissue. Human *DACT1* (*A*) and mouse *Dact1* (*B*) mRNA levels were measured using real-time RT-PCR at the indicated time points after induction of differentiation. **$P < 0.01$, ***$P < 0.001$ vs. time 0, $n = 7$ experiments performed in duplicate. *DACT1* and *Dact1* mRNA levels were measured in stroma-vascular cells (SVF) and mature adipocytes (MA) from human subcutaneous WAT (*C*) and mouse epididymal WAT (*D*). ***$P < 0.001$ vs. SVF, $n = 3$ replicate experiments performed in duplicate. $n = 3$ experiments performed in duplicate. *E*: Normalized *Dact1* mRNA levels were measured in epididydimal (epWAT), subcutaneous WAT (scWAT), and interscapular BAT of 10-week-old C57BL/6J. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 3$ experiments performed in duplicate. *F*: *Dact1* mRNA levels, were measured in different cell types: fibroblast (NIH3T3), mesenchymal stem cells (C3H10T1/2), white primary preadipocytes, white preadipocyte cell lines (3T3–L1 and 3T3–F442A), and brown preadipocyte cell line (HIB1B), $n = 3–4$ experiments performed in duplicate. *G*: *Dact1* mRNA levels were measured at the indicated hours after induction (with MDI) of 3T3–L1 preadipocyte differentiation. ***$P < 0.001$ vs. time 0, $n = 4$ experiments performed in duplicate. Whole-cell protein lysates were extracted at the indicated days of differentiation from 3T3 L1 cells and analyzed by immunoblotting. Representative immunoblots of Dact1 and extracellular signal–related kinase (ERK)1/2 (loading control) from three separate experiments are shown. *H*: *Dact1* mRNA levels were measured at the indicated hours after induction of 3T3–L1 preadipocytes with either control medium (serum) or MDI. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. time 0 for MDI; †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ vs. serum, $n = 3–4$ experiments performed in duplicate.

independent experiments. Statistical significance was determined with parametric (Student) or nonparametric (Mann–Whitney) tests, as appropriate (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

RESULTS

***Dact1* is decreased during adipogenesis and is restricted to the preadipocyte-enriched fractions of adipose tissue.** Microarray expression profiling of human preadipocyte differentiation revealed that *Dact1* (*DACT1*) is significantly downregulated at 6 and 48 h after induction of adipogenesis. This downregulation was confirmed and also observed during differentiation of primary preadipocytes from both human and murine sources (Fig. 1*A* and *B*) and was consistent with the enrichment of *Dact1* in the stroma-vascular fraction (SVF) when compared with mature adipocytes from WAT in both species (Fig. 1*C* and *D*). In adult mice, *Dact1* expression was also preferentially expressed in WAT depots compared with brown adipose tissue (BAT) (Fig. 1*E*). *Dact1* expression in different cellular models of fibroblasts and preadipocytes also correlated well with adipogenic potential and was barely detectable in the BAT cells HIB1B (Fig. 1*F*).

As with primary preadipocyte cultures, *Dact1* mRNA was also found to decrease by 90% after 24 h following adipogenic induction of the well-established 3T3-L1 preadipocyte cell line. This profile preceded the decrease in

Dact1 protein in the same cells (Fig. 1*G*). However, profiling the early immediate response to adipogenic stimuli revealed a rapid but transient upregulation of *Dact1* limited to the first 6 h after adipogenic induction (Fig. 1*H*).

***Dact1* is required for adipocyte differentiation of 3T3-L1 preadipocytes.** The mRNA and protein profiles of *Dact1* suggest that it might be a preadipocyte marker gene whose product contributes to the maintenance of the preadipocyte phenotype. However, given that Dact1 can act in a context-dependent manner to activate or inhibit Wnt/β-catenin signaling (19,20,22,23,26), it was possible that changes in Dact1 levels reported here were functionally either pro- or antiadipogenic. Thus, we performed gain- and loss-of-function experiments using four newly established 3T3-L1 preadipocytes cell lines, engineered to constitutively express *Dact1* ShRNA (Dact1SH), control ShRNA (CtrlSH), *Dact1* cDNA (Dact1), or empty vector control (EV).

In *Dact1*SH 3T3-L1 cells, endogenous expression of *Dact1* mRNA was decreased by ~90% and remained at this level throughout the differentiation process (Fig. 2*A*). In contrast *Dact1* 3T3-L1 cells displayed a 10-fold increase of *Dact1* mRNA compared with EV 3T3-L1 cells (Fig. 2*A*). These transcript levels were also reflected at the amount of Dact1 protein (Fig. 2*B*). To evaluate the impact of altered Dact1 levels on adipogenic potential, each cell line
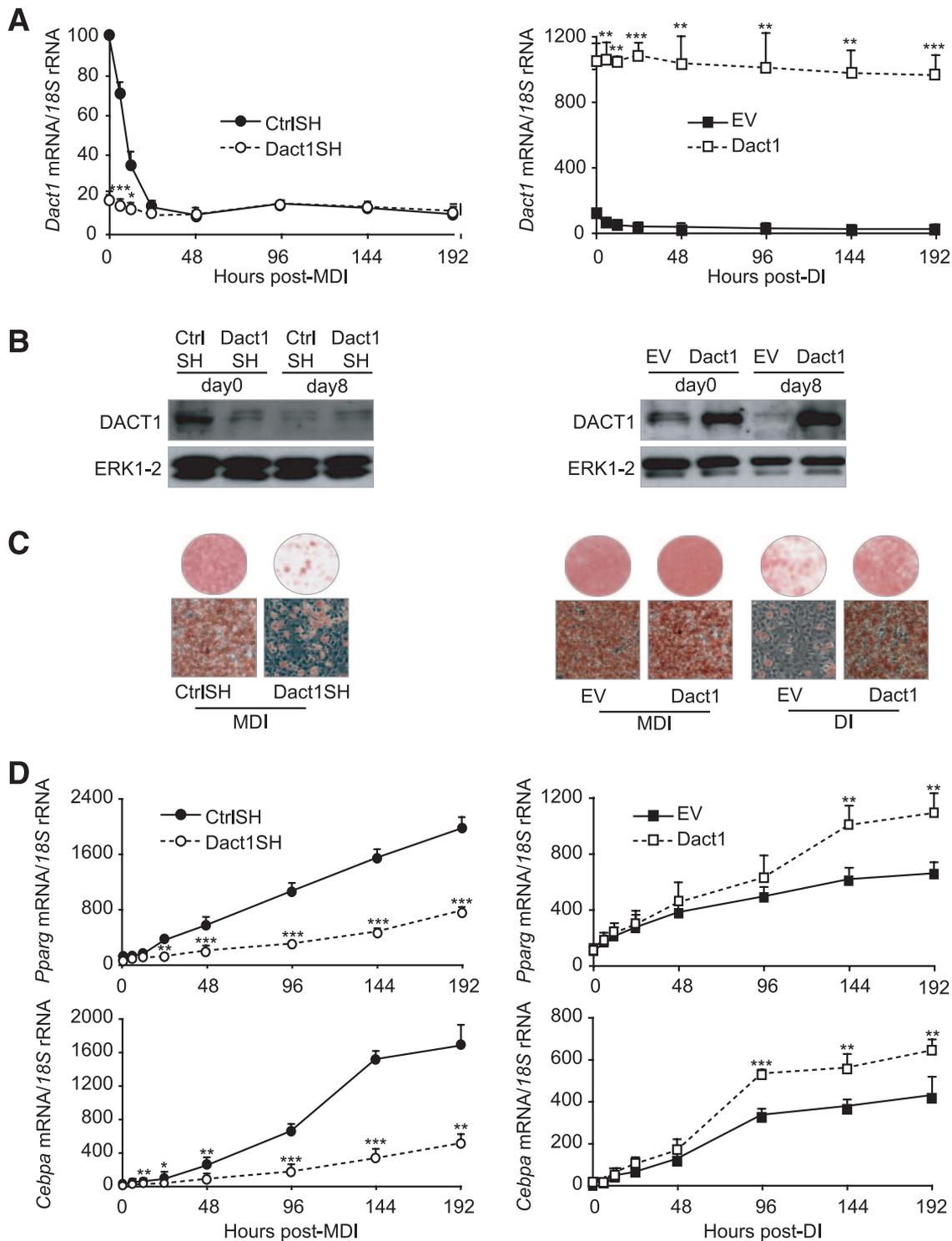
FIG. 2. Dact1 is required for adipocyte differentiation of 3T3–L1 preadipocytes. Preadipocytes were infected with a retrovirus carrying Dact1 ShRNA (Dact1SH), control ShRNA (CtrlSH), Dact1 (Dact1), or vector alone (EV). *A*: Dact1 mRNA levels, normalized to 18S rRNA levels, were measured using real-time RT-PCR at the indicated hours of differentiation in Dact1SH- and CtrlSH-differentiated MDI and in Dact1 and EV cells differentiated with DI. $*P < 0.05$ and $***P < 0.001$ vs. CtrlSH or EV, $n = 3$ experiments performed in duplicate. *B*: Whole-cell lysates were extracted at 0 and day 8 days of differentiation from CtrlSH, Dact1SH, EV, and Dact1 cells and analyzed by immunoblotting. Representative immunoblots of Dact1 and extracellular signal–related kinase (ERK)1/2 (loading control) from three separate experiments are shown. *C*: Dact1SH and CtrlSH 3T3–L1 cells were differentiated with MDI, Dact1, and EV 3T3–L1 cells were differentiated with MDI or DI. Cells were stained with Oil Red-O to visualize lipid droplets 8 days after induction, $n = 5$. *D*: *Ppar γ* and *Cebp α* mRNA levels, normalized to 18S rRNA levels, were measured using real-time RT-PCR at the indicated hours of differentiation in Dact1SH and CtrlSH cells differentiated with MDI and in Dact1 and EV cells differentiated with DI. At day 0, only PPARγ mRNA was significantly altered in Dact1-SH preadipocytes. Neither PPARγ not CEBPα were significantly altered in Dact vs. EV cells at time 0 h. $*P < 0.05$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. CtrlSH or EV, $n = 3$ experiments performed in duplicate. (Please see http://dx.doi.org/10.2337/db08-1180 for a high-quality digital representation of this figure.)

was induced to differentiate and lipid accumulation visualized with Oil Red-O staining. Knockdown of Dact1 (Dact1SH) almost completely blocked lipid accumulation

(Fig. 2*C*). This was consistent with the reduced expression levels of the adipogenic markers C/EBPα and PPARγ in these cells (Fig. 2*D*). Conversely, Dact1 3T3-L1 cells and
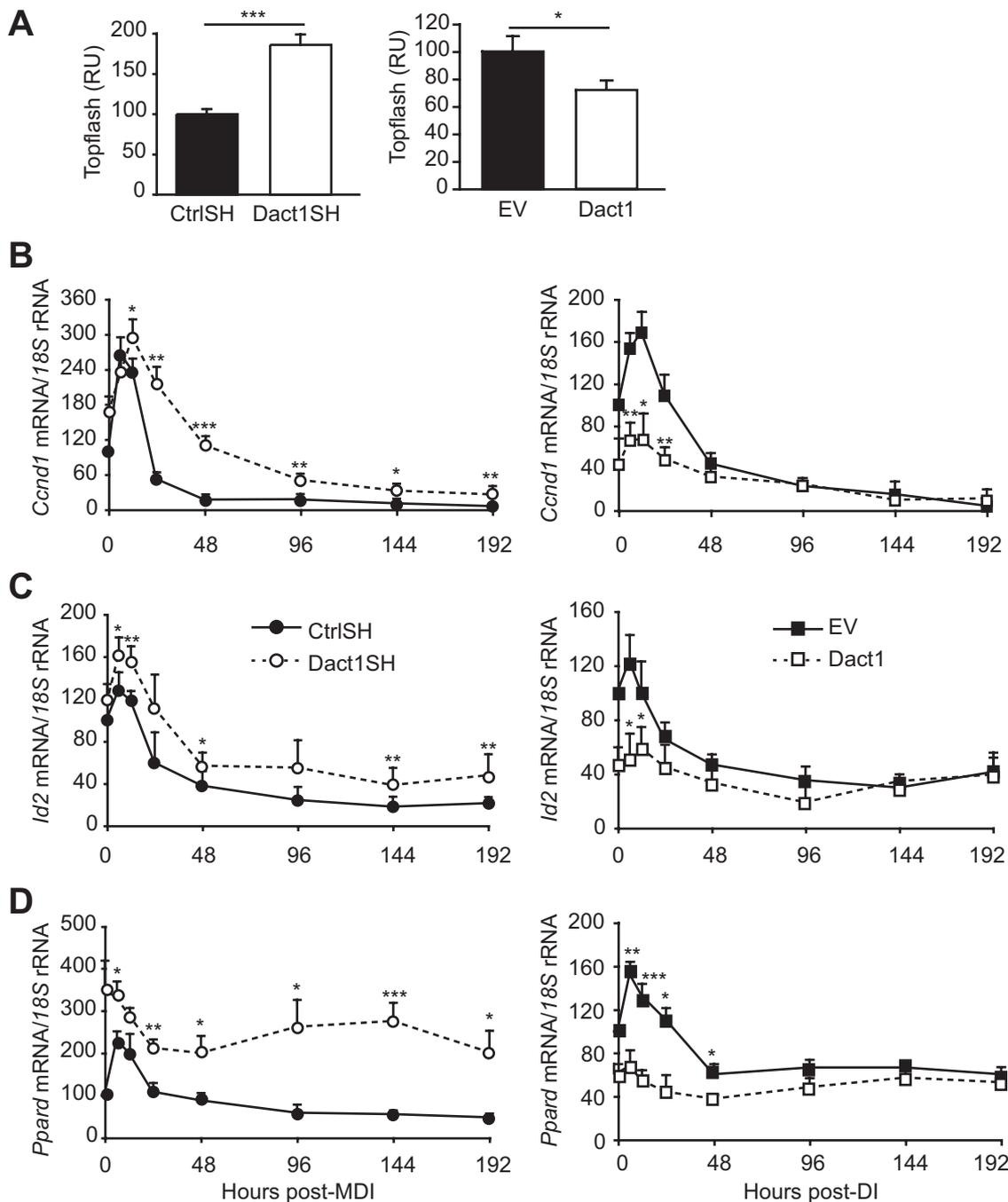
FIG. 3. Dact1 antagonizes Wnt/β-catenin signaling in 3T3–L1 cells. *A*: Topflash reporter activity in 3T3–L1 cells expressing control ShRNA (CtrlSH), Dact1 ShRNA (Dact1SH), empty vector (EV), or Dact1, *n* = 3–5 experiments performed in triplicate. Results are expressed as fold difference relative to CtrlSH. Results are the mean ± SE of at least four independent experiments. *Ccnd1* (CyclinD1) (*B*), *Id2* (*C*), and *Ppard* (*D*) mRNA levels, normalized to *18S* rRNA levels, were measured at the indicated hours of differentiation in Dact1SH and CtrlSH cells differentiated with MDI and in EV and Dact1 cells differentiated with DI. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. CrtlSH or EV, *n* = 3 experiments performed in duplicate. RU, relative luciferase unit.

EV 3T3-L1 cells differentiated to a similar extent when induced with full induction cocktail (MDI). However, induction with submaximal stimulation (DI) revealed that Dact1 3T3-L1 cells have an increased propensity for lipid accumulation (Fig. 2*C*). DI-treated Dact1 3T3-L1 cells also showed increased expression of the adipogenic markers PPARγ and C/EBPα when compared with similarly treated EV 3T3-L1 cells (Fig. 2*D*). Taken together, these data suggest that *Dact1* is proadipogenic and is required for adipogenesis.

***Dact1* antagonizes Wnt/β-catenin signaling in 3T3-L1 cells.** To assess the function of Dact1 in Wnt/β-catenin signaling in 3T3-L1 preadipocytes, we first investigated alterations in β-catenin/TCF promoter activity (TOPflash) in each cell line after adipogenic induction. Figure 3*A* shows that β-catenin/TCF promoter activity is reciprocally related to the level of *Dact1* expression in each cell line, such that Dact1SH 3T3-L1 cells show significantly increased reporter activity, whereas Dact1 3T3-L1 cells show significantly reduced reporter activity. These results

are further supported by parallel changes in the expression profiles of the endogenous Wnt target genes Cyclin D1, Id2, and PPARδ (Fig. 3*B*–*D*) in these cell lines. In control cells, after maximal adipogenic induction by MDI (CtrlSH cells) or submaximal induction by DI (EV cells), Wnt target gene expression was transiently upregulated between 0 and 24 h after induction (Fig. 3*B*–*D*). This response was significantly blunted in Dact1 cells, whereas Dact1SH cells maintained elevated target gene expression levels throughout the time course of adipogenesis (Fig. 3*B*–*D*). These data suggest that Dact1 inhibits Wnt/β-catenin signaling in preadipocytes and also affects the magnitude of their transcriptional response to adipogenic treatment.

Dvl protein levels change dynamically during adipogenesis and are regulated by Dact1 in 3T3-L1 cells. Dact1 was originally identified as a Dvl-interacting protein (19,22) and has been reported in some contexts to antagonize Wnt signaling by inducing Dvl degradation (20). The expression levels of *Dvl* family members have not previously been reported during adipogenesis nor has any study systematically assessed the effect of *Dact1* levels on all three members of the Dvl family concurrently. Figure 4*A* shows the protein expression profiles of all three members of the Dvl family: *Dvl1*, *Dvl2*, and *Dvl3* during adipogenesis in our in vitro models. Only Dvl2 and Dvl3 proteins are detected in confluent preadipocytes. However, during the adipogenic program each Dvl family member exhibits a distinct expression profile. The protein expression profiles closely parallel the corresponding mRNA profiles for each *Dvl* gene (Fig. 4*B*–*D*), suggesting that changes in Dvl protein levels are substantially regulated at the transcript level.

To investigate whether altered *Dact1* expression affects Dvl protein levels during adipogenesis, we profiled Dvl protein and mRNA expression in both Dact1SH and Dact1 3T3-L1 cells on days 0 and 8 after induction (Fig. 4*B*, *D*, *F*, and *H*). Parallel changes were observed at both the protein and RNA levels. Before adipogenic induction (day 0), knockdown of Dact1 had no effect on levels of Dvl1 but reduced levels of Dvl2 and increased levels of Dvl3. In contrast, overexpression of Dact1 at day 0 had no significant effect on Dvl1 or Dvl2 levels but reduced levels of Dvl3. At day 8 after adipogenic treatment, the level of *Dvl1* was significantly lower in Dact1SH 3T3-L1 cells but significantly higher in Dact1 3T3-L1 cells (Fig. 4*B* and *D*). In contrast, at day 8, *Dvl2* levels were minimal and unaffected by Dact1 manipulation (Fig. 4*B* and *F*), whereas day 8 *Dvl3* levels correlated reciprocally with that of Dact1, being significantly higher in Dact1SH 3T3-L1 cells and lower in Dact1 3T3-L1 cells (Fig. 4*B* and *H*). Hence, each of the Dvl family members shows a distinct expression profile during adipogenesis in 3T3-L1 cell lines and is likely to be primarily regulated at the level of mRNA transcription. Although levels of *Dvl1* appear to correlate with extent of adipocyte conversion, alterations in Dact1 levels does impact directly on basal *Dvl2* and *Dvl3* expression.

Dact1 alters extracellular Wnt signals in preadipocytes and in differentiating adipocytes. We next investigated whether extracellular components of the Wnt/β-catenin signaling cascade may be altered by changes in *Dact1*. In Dact1SH 3T3-L1 cells before adipogenic induction, we observed an increase in mRNA levels of *Wnt10b* and *Wnt3a* when compared with CtrlSH 3T3-L1 cells (Fig. 5*A*). Conversely, in Dact1-overexpressing 3T3-L1 cells, *Wnt10b* and *Wnt3a* mRNA levels are decreased when

compared with EV 3T3-L1 cells (Fig. 5*A*). As expected, after full induction of adipogenesis by MDI (CtrlSH) or partial induction by DI (EV), *Wnt10b* levels fall significantly by day 8 (Fig. 5*A*, *top*). However, in Dact1SH 3T3-L1 cells, levels of *Wnt10b* and *Wnt3a* remain higher than in CtrlSH 3T3-L1 cells during adipogenic induction. Next, we examined the mRNA expression profile of two closely related secreted Wnt antagonists, *Sfrp1* and *Sfrp5*. In contrast to the decreasing expression of *Wnt10b* and *Wnt3a*, *Sfrp1* and *Sfrp5* mRNA expression increased as adipocytes differentiate (Fig. 5*B*). The fold increase was proportional to the strength of adipogenic stimulation and conversion.

To assess whether the effect of Dact1 on adipogenesis is primarily due to altered production of extracellular Wnt ligands and antagonists, we investigated the ability of recombinant SFRP1 to reverse the antiadipogenic phenotype of Dact1SH 3T3-L1 cells. As shown in Fig. 5*C*, Dact1SH 3T3-L1 cells treated with SFRP1 accumulate more lipids than untreated Dact1SH 3T3-L1. SFRP1 acts extracellularly by sequestering Wnt ligands, suggesting that increased Wnt and decreased SFRP production are in part responsible for the reduced adipogenic response of Dact1SH cells.

Conversely, by treating Dact1 3T3-L1 cells with recombinant Wnt ligands, we assessed whether these cells were more resistant to the antiadipogenic actions of extracellular Wnts. Treatment with recombinant Wnt3a practically eliminated the ability of EV 3T3-L1 cells to accumulate lipids, whereas *Dact1* 3T3-L1 cells accumulate lipid even in the presence of Wnt3a. Together with our prior assays of Wnt signaling, these data show that ectopically expressed Dact1 in 3T3-L1 cells inhibits the efficiency of Wnt/β-catenin signal transduction, thereby allowing these cells to differentiate even in the presence of exogenous Wnts. Taken together, our results suggest that changes in Dact1 protein levels alter Wnt/β-catenin signaling tone in 3T3-L1 cells through coordinated effects on both the efficiency of intracellular signal transduction pathway and by altering the expression profiles of extracellular Wnt ligands and antagonists.

***Dact1*, *Wnt10b*, and *Sfrp5* are all regulated by nutritional status in vivo.** We sought to determine whether *Dact1*, *Wnt10b*, and *Sfrp5* levels are regulated in vivo by nutritional status. Fasting and/or refeeding experimental conditions were selected as opposing physiological paradigms with respect to acute fuel availability. In response to fasting, the expression of *Dact1*, *Wnt10b*, and *Sfrp5* were all decreased in WAT. However, in response to refeeding, the mRNA levels of all three genes were markedly increased significantly beyond the levels found in the fed state (Fig. 6). Interestingly, the expression of the Wnt/β-catenin target gene *Ccnd1* was also markedly decreased after fasting but remained low following refeeding (Fig. 6*G*). Figure 6*E*–*H* confirmed that these changes predominantly reflected alterations in SVFs (for *Dact1* and *Wnt10b*) and also in mature adipocytes (for *Sfrp5*). However, appreciable levels of *Ccnd1* was found in both fractions. Taken together, these data suggest that in response to increased nutritional availability (refeeding), adipose tissue undergoes dynamic changes in the expression of key components of the Wnt/β-catenin signaling pathway, including *Dact1* in preadipocytes and *Sfrp5* in mature adipocytes. Furthermore, there is not only coordinated upregulation of proadipogenic signals but also a simultaneous upregulation of antiadipogenic Wnt-related
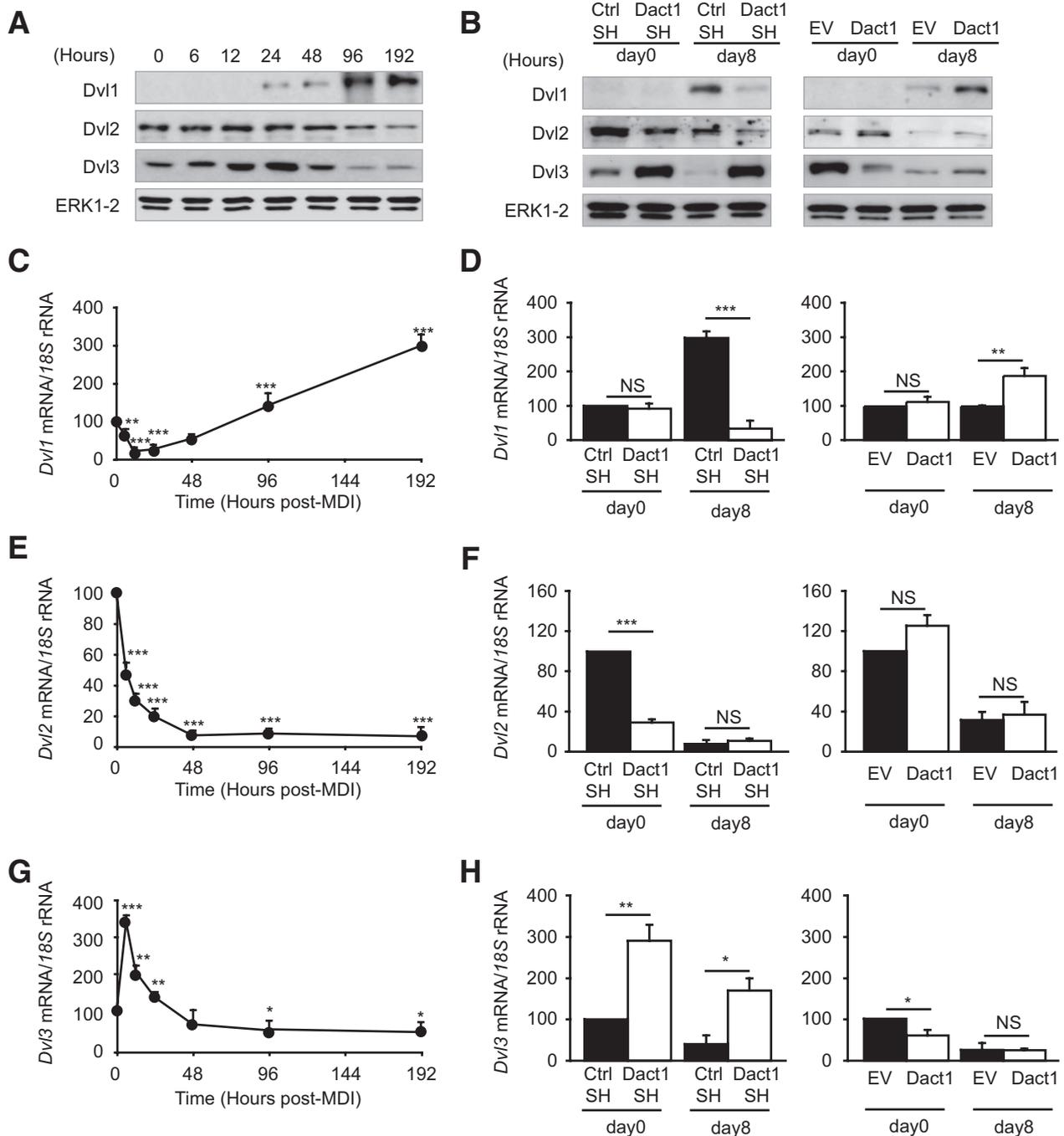
FIG. 4. Dact1 regulates the expression of Dvl in 3T3–L1 cells. *A*: Whole-cell lysates were extracted at indicated times after induction of 3T3–L1 adipogenesis. Representative immunoblots of Dvl1, Dvl2, Dvl3, and ERK1/2 (loading control) from three separate experiments are shown. *Dvl1* (*C*), *Dvl2* (*E*), and *Dvl3* (*G*) mRNA levels, normalized to 18S rRNA levels, were measured using real-time RT-PCR at indicated times after induction, *n* = 3 experiments performed in duplicate. *B*: Whole-cell lysates were extracted at days 0 and 8 of differentiation from Dact1SH, CtrlSH, EV, and Dact1 cells and analyzed by immunoblotting. Representative immunoblots of Dvl1, Dvl2, Dvl3, and ERK1/2 from three separate experiments are shown. *Dvl1* (*D*), *Dvl2* (*F*), and *Dvl3* (*H*) mRNA levels, normalized to 18S rRNA levels, were measured at days 0 and 8 of differentiation in Dact1SH and CtrlSH cells differentiated with MDI and in differentiation in Dact1 and EV cells differentiated with DI. Complete time course data for experiments presented in *D*, *F*, and *H* can be found in supplementary Fig. 1, available in the online appendix. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. CrtlSH or EV, *n* = 3 experiments performed in duplicate.

signals. We speculate that the net effect of these is likely to be important for titrating the actual target gene expression and hence adipogenic potential of whole adipose tissue in vivo.

Dact1, Wnt10b, and Sfrp5 are all regulated during dietary and genetic models of obesity and by PPARγ activators. To further investigate the pathophysiological regulation of *Dact1*, we measured the expression profiles of *Dact1*, *Wnt10b*, and *Sfrp5* in adipose tissue collected

from *ob/ob* mice. This is a mouse model of severe positive energy balance characterized by marked hyperphagia and early-onset obesity. By 5 weeks of age, *ob/ob* mice are in a phase of rapid adipose accretion and are already significantly heavier than their lean wild-type littermates (2). In contrast, by 16 weeks of age the growth rate of *ob/ob* mice has begun to plateau, a phase that coincides with these animals being overtly obese and diabetic (29). Adipose tissue taken from 5-week-old *ob/ob* mice show elevated
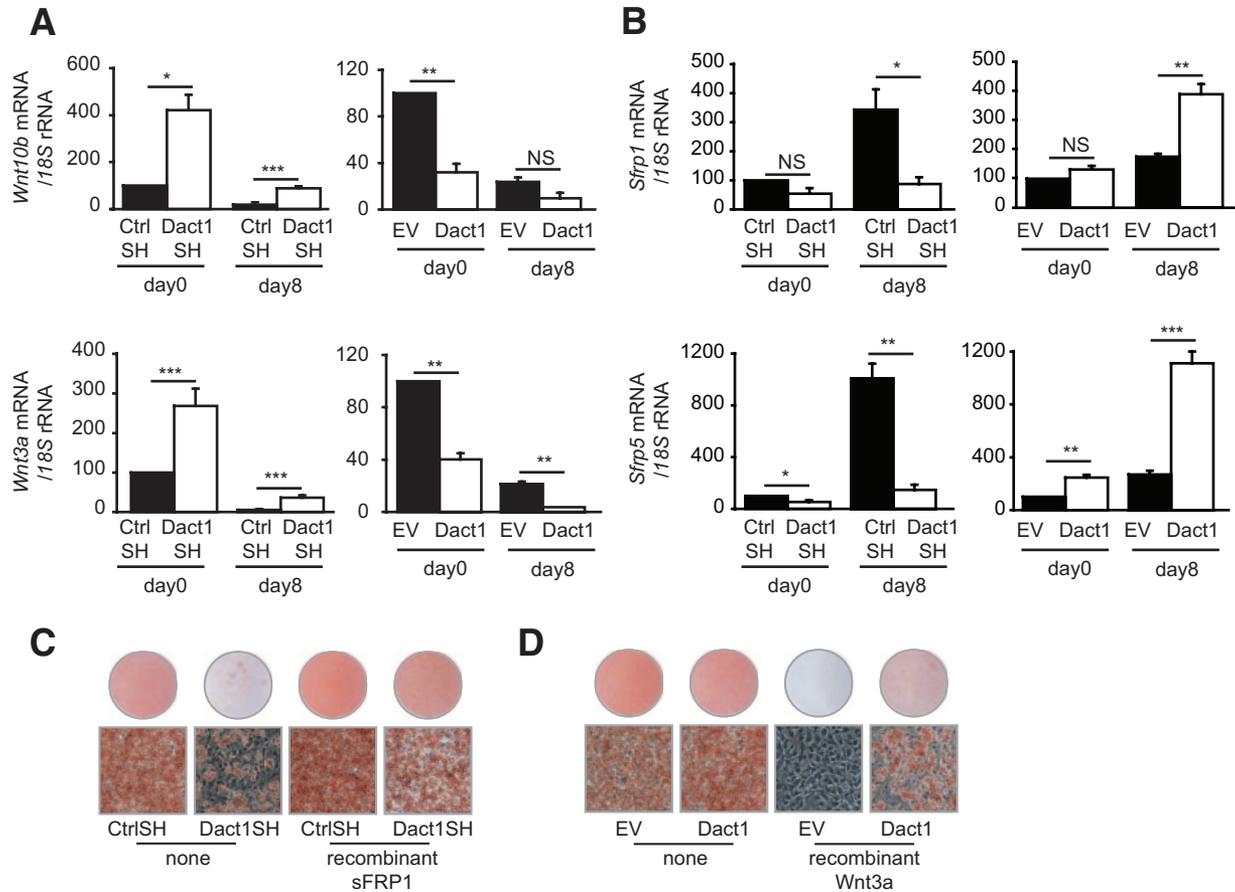
FIG. 5. Dact1 regulates paracrine/autocrine Wnt signaling in 3T3–L1 adipocytes. *Wnt10b* and *Wnt3a* (*A*) and *Sfrp1* and *Sfrp5* (*B*) mRNA levels, normalized to *18S* rRNA levels, were measured at the indicated hours of differentiation in Dact1SH and CtrlSH cells differentiated with MDI and in Dact1 and EV cells differentiated with DI. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. CtrlSH or EV, $n = 3$ experiments performed in duplicate. *C*: Dact1SH and CtrlSH 3T3–L1 cells were differentiated with MDI in the presence or absence of recombinant sFRP1 (75 nmol/l) from days 0–8 and stained with Oil Red-O to visualize lipid droplets 8 days after induction. *D*: Dact1 and EV 3T3–L1 cells were differentiated with MDI in the presence or absence of recombinant Wnt3a (0.25 nmol/l) from days 0–8 and stained with Oil Red-O at day 8, $n = 3$. (Please see http://dx.doi. org/10.2337/db08-1180 for a high-quality digital representation of this figure.)

levels of proadipogenic *Dact1* (Fig. 7*A*) and *Sfrp5* (Fig. 7*C*) and decreased levels of antiadipogenic *Wnt10b* (Fig. 7*B*), resulting in a steady state that could be considered highly proadipogenic. In contrast, *Dact1* levels in the metabolically compromised 16-week-old *ob/ob* animals are significantly lower than their age-matched wild-type littermates. Interestingly, these older *ob/ob* mice also express reduced *Wnt10b*, but their levels of *Sfrp5* continued to rise (Fig. 7*A*, *D*, and *G*).

We also investigated *Dact1* expression in mice challenged with a short- and long-term high-fat diet. Similarly to the young *ob/ob* mice, mice fed high-fat diet for 4 weeks show significant increases in WAT mass compared with chow-fed and age- and sex-matched littermates. Epididymal WAT weights from 4-week high-fat diet– and chow-fed mice were 1.01 ± 0.14 g compared with 0.33 ± 0.03 g, respectively, $P < 0.002$. At this time, adipose tissue levels of proadipogenic *Dact1* (Fig. 7*B*) and *Sfrp5* (Fig. 7*H*) were increased, whereas the levels of antiadipogenic *Wnt10b* were decreased (Fig. 7*E*). Furthermore, in agreement with the tendencies observed in older metabolically compromised *ob/ob* mice, *Dact1* and *Wnt10b* levels were reduced (Fig. 7*B–E*), albeit to a lesser extent, in these wild-type mice that have become obese and metabolically compromised as a result of long-term high-fat diet (6 months).

We tested whether this Wnt-related network was modulated by pharmacological challenge with thiazolidinedio-

nes (TZDs), drugs known to promote adipogenesis and improve insulin sensitivity. TZD treatment increased the expression of adipogenic markers in adipose tissue (aP2 was increased by 1.63 ± 0.14-fold, $P = 0.047$). Strikingly, this was accompanied by increases in *Dact1* and *Sfrp5* levels (Fig. 7*C* and *I*) and decreases in *Wnt10b* levels (Fig. 7*F*). Further studies on SVFs and adipocyte fractions demonstrated that both *Dact1* and *Wnt10b* expression was primarily altered in SVFs and preadipocytes. Furthermore, TZD feeding significantly increased Sfrp5 and Fabp4 levels primarily in adipocytes fractions (supplementary Fig. 2, available in an online appendix at http://dx.doi.org/10. 2337/db08-1180). TZD-induced changes in Sfrp1 were the same as those of Sfrp5 (data not shown). Taken together, these findings suggest that *Dact1* expression and key extracellular components of the Wnt/β-catenin signaling network are coordinately regulated to facilitate adipose tissue adaptation during anabolic states characterized by active adipose tissue expansion. However, in predominantly catabolic states, such as insulin resistance and diabetes, some aspects of this network may become uncoupled.

DISCUSSION

Dact1 was first identified as an evolutionarily conserved, Dvl-interacting protein (19,20); however, its mechanism of
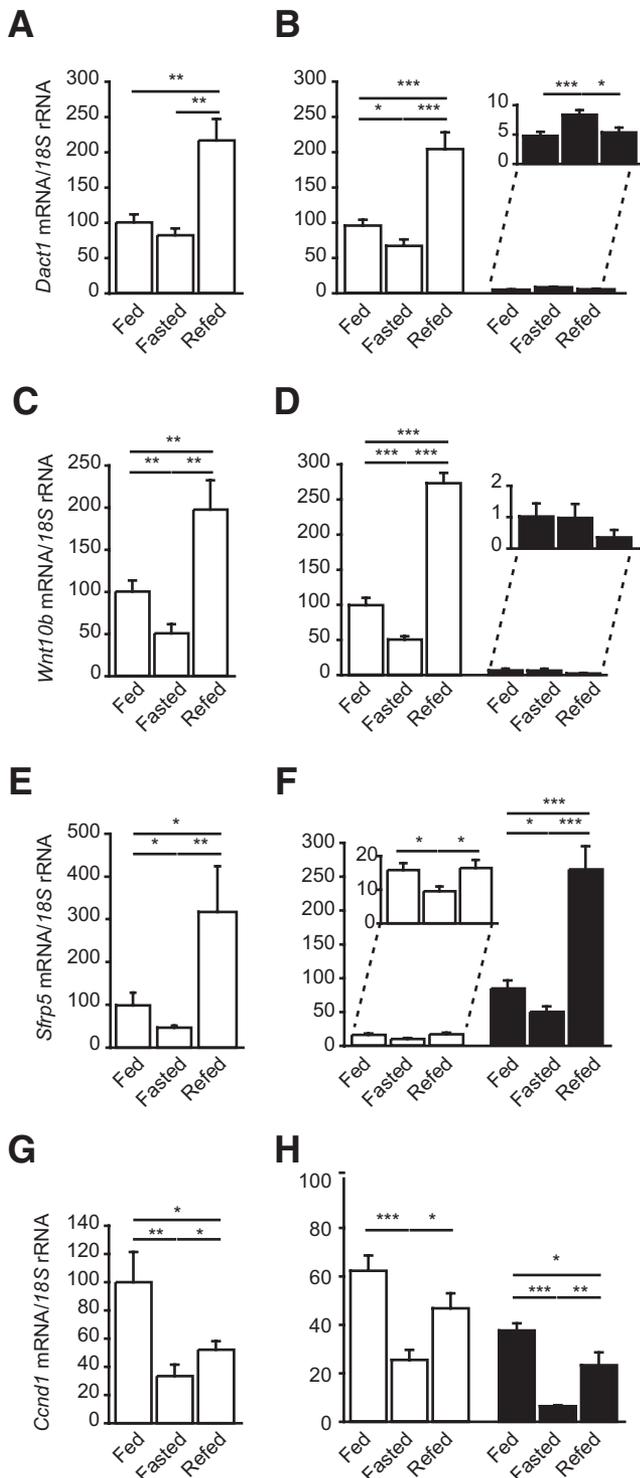
FIG. 6. Wnt/β-catenin signals and Dact1 are regulated by nutritional status in adipose tissue in vivo. *Dact1* (*A* and *B*), *Wnt10b* (*C* and *D*), *Sfrp5* (*E* and *F*), and *Ccnd1* (*G* and *H*) mRNA levels, normalized to *18S* rRNA levels, were measured in whole WAT (*A*, *C*, *E*, and *G*) and fractionated adipose tissue (*B*, *D*, *F*, and *H*) from 8-week-old male C57/Bl6 mice under the following conditions: fed (*n* = 8–9), fasted (24 h) (*n* = 7–8), and refed (24 h) (*n* = 7–8). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

both human and murine preadipocytes and in SVFs of adipose tissue. Furthermore, *Dact1* is downregulated during adipogenic conversion of primary cultures and cell lines. With this profile, *Dact1* could be considered a surrogate preadipocyte marker, however, we demonstrate that its biological action contrasts with other previously described "vanishing preadipocyte" genes (e.g., *Gata2/3*, *Pref1*, and *Wnt10b*). Through both gain- and loss-of-function approaches, we have shown that expression of *Dact1* confers proadipogenic potential and that its presence in the preadipocyte is required for efficient adipocyte differentiation.

Our observations of the effects of Dact1 on adipogenesis and on signaling in preadipocytes are most consistent with its role as an antagonist of Wnt/β-catenin signaling in this tissue. This has only previously been demonstrated in *Xenopus* embryos and mammalian embryonic fibroblasts (19,20,26). Resolution of the discrepancies in the apparent signaling function of Dact proteins may lie in our observations showing that manipulation of Dact1 in preadipocytes elicits a differential and specific effect on the levels of other proteins configuring a Wnt/β-catenin signaling network. This suggests that changes in Dact1 protein concentrations have downstream effects on a whole network of signaling proteins, which may contribute exquisite modulation to the strength of Wnt/β-catenin signaling tone. It is noteworthy that some of the effects of Dact1 (i.e., altering Dvl levels) are strictly cell-autonomous, whereas others (i.e., altering Wnt and sFRP levels) can also be expected to affect signaling in neighboring cells. The latter is likely to be highly relevant in vivo in an otherwise cellularly heterogeneous adipose tissue, where gene expression and differentiation state is likely to vary significantly between neighboring cells. Dact1 may play a significant role in determining not only the sensitivity of the intracellular Wnt/β-catenin pathway but also the strength of the extracellular autocrine/paracrine Wnt signals to neighboring cells. We speculate that this type of modulation, when regulated by nutrient availability, may be the basis of a local self-regulating mechanism to titrate the differentiation of an appropriate number of preadipocytes to specific storage demands.

These studies also present several new components of the Wnt/β-catenin network not previously described in adipose tissue and/or studied during adipogenesis. These include four proteins expressed primarily in preadipocytes (Dact1 Dvl2, Dvl3, and Wnt3a) and three expressed primarily by mature adipocytes (Dvl1, sFRP1, and sFRP5). Importantly, we also demonstrate that these molecules are coordinately regulated in vivo in response to nutritional and dietary challenges, in genetic forms of obesity and to pharmacological agents typically producing adipose tissue expansion and improving insulin sensitivity.

Given their intrinsic peculiarities, it is not unexpected that our studies have revealed some differences between the expression profiles obtained from in vivo and in vitro models of adipogenic regulation. One important factor that is worth considering is that the in vitro systems used here represent a homogenous population of preadipocytes that are simultaneously exposed to and synchronously respond to defined adipogenic stimuli and/or genetic manipulation, thus allowing construction of detailed temporal profiles. In contrast, whole adipose tissues as used for in vivo studies represent a heterogeneous population of cell types each with distinct gene expression profiles and sensitivities to nutritional and pathological cues and con-
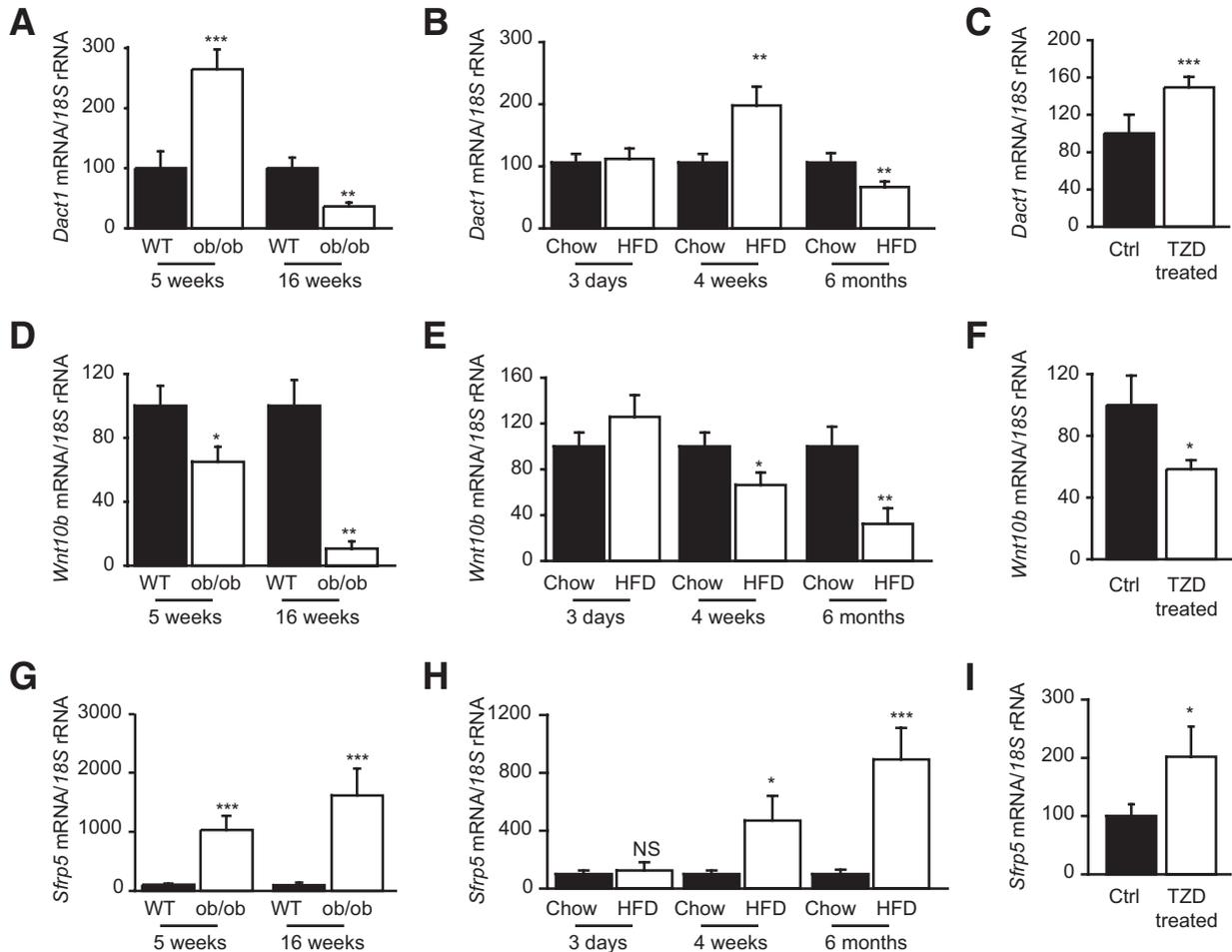
action and function remained ambiguous (19–23,25) and was reported to be cell/tissue and context dependent (23,26–28). Before this study, the presence and role of Dact1 in adipogenesis/adipose tissue was unexplored. Here, we demonstrate that *Dact1* is primarily expressed in

FIG. 7. Dact1, Wnt10b, and Sfrp5 are regulated in adipose tissue by genetic and diet-induced models of obesity and by TZDs. *Dact1*, *Wnt10b*, and *Sfrp5* mRNA levels were measured in whole WAT from indicated murine models as described in experimental procedures. *A*, *D*, and *G*: Data are normalized to 18s mRNA and represented relative to wild-type (WT) levels ± SE. Expression data from 5-week-old *ob/ob* mice and from 16-week-old diabetic *ob/ob* mice ($n = 6$–8 per group) *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. wild-type mice. *B*, *E*, and *H*: Expression data from C57/Bl6 mice fed either chow diet (chow) or high-fat diet (HFD) for 3 days (acute), 4 weeks (short term), or 6 months (long term) ($n = 6$–7 per group) after weaning. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. chow-fed mice. *C*, *F*, and *I*: Expression data from 4-month-old C57/B6 mice fed rodent chow supplemented with or without rosiglitazone (TZD) for 3 weeks ($n = 9$–10 per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. untreated mice.

text-dependent feedback loops. These coupled with local paracrine signals are likely to affect the dynamic balance between mature adipocytes and preadipocytes. Furthermore, it is evident that adipose tissue expansion in vivo does not involve the simultaneous recruitment of all preadipocytes into the adipogenic program. Nonetheless, it is clear from our findings that key determinants of Wnt/β-catenin signaling tone are regulated in response to refeeding and that the sustained induction of *Dact1* in preadipocytes could be one mechanism facilitating, on one hand, the preselection of preadipocytes for subsequent differentiation and being the source of paracrine signals that prevents the differentiation of more distal preadipocytes, resulting in an appropriate titrated adipose tissue expansion in response to nutritional status.

That being said, our in vitro observation that changes in *Dact1* levels lead to reciprocal changes in Wnt10b in preadipocyte cell lines are recapitulated in adipose tissue obtained from three in vivo models that reflect actively expanding adipose tissue (short-term high-fat diet, young *ob/ob*, and TZD-treated wild-type mice). Conversely, our data also show that under conditions of insulin resistance when adipogenesis and fat deposition are compromised and fat accumulation plateaus, this network becomes uncoupled, but *Dact1* appears to be appropriately regu-

lated in the opposite direction. This suggests that Dact1 may act as a gate-keeper, facilitating the accurate adaptation of adipose tissue expansion to storage requirements based on nutritional and metabolic status.

In summary, we present evidence of a functional network formed by Dact1, sFRP, and Wnt ligands that facilitates cross talk in adipose tissue between preadipocytes and mature adipocytes, thereby ensuring appropriate titration of adipose tissue expansion in response to nutrient availability. We speculate that dysregulation of this network may be the pathogenic basis leading to an altered balance between the processes of adipocyte growth versus preadipocyte recruitment—ultimately leading to a spectrum of adipose tissue cellularity ranging from hypertrophy to hyperplasia. Similarly, modulation of this network by targeting Dact1 may be of therapeutic value to improve the metabolic efficiency of the adipose tissue and prevent obesity-associated metabolic complications.

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