THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 34, Issue of August 23, pp. 30998–31004, 2002
Printed in U.S.A.

Regulation of Wnt Signaling during Adipogenesis*

Received for publication, May 8, 2002, and in revised form, May 31, 2002
Published, JBC Papers in Press, June 7, 2002, DOI 10.1074/jbc.M204527200

Christina N. Bennett‡§, Sarah E. Ross‡, Kenneth A. Longo‡, Laszlo Bajnok‡, Nahid Hemati‡, Kirk W. Johnson¶, Stephen D. Harrison¶, and Ormond A. MacDougald‡‖

From the ‡Department of Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0622 and ¶Chiron Corporation, Emeryville, California 94608

We have identified Wnt10b as a potent inhibitor of adipogenesis that must be suppressed for preadipocytes to differentiate *in vitro*. Here, we demonstrate that a specific inhibitor of glycogen synthase kinase 3, CHIR 99021, mimics Wnt signaling in preadipocytes. CHIR 99021 stabilizes free cytosolic β-catenin and inhibits adipogenesis by blocking induction of CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor γ. Preadipocyte differentiation is inhibited when 3T3-L1 cells are exposed to CHIR 99021 for any 24 h period during the first 3 days of adipogenesis. Consistent with this time frame of inhibition, expression of Wnt10b mRNA is suppressed upon induction of differentiation, with a 50% decline by 6 h and complete inhibition by 36 h. Of the agents used to induce differentiation, exposure of 3T3-L1 cells to *methyl*-isobutylxanthine or cAMP is sufficient to suppress expression of Wnt10b mRNA. Inhibition of adipogenesis by Wnt10b is likely mediated by Wnt receptors, Frizzled 1, 2, and/or 5, and co-receptors low density lipoprotein receptor-related proteins 5 and 6. These receptors, like Wnt10b, are highly expressed in preadipocytes and stromal vascular cells. Finally, we demonstrate that disruption of extracellular Wnt signaling by expression of secreted Frizzled related proteins causes spontaneous adipocyte conversion.

White adipose tissue is an important depot for short and long term energy storage. In addition, adipose tissue is an endocrine organ that regulates energy homeostasis through secretion of leptin, Acrp30/adiponectin, resistin, and other factors (1). The study of adipocyte biology has been greatly facilitated by development of immortalized preadipocyte lines (*e.g.* 3T3-L1) (2) that differentiate into adipocytes and recapitulate many of the metabolic and endocrine functions of adipocytes *in vivo*. Analysis of differentiation in preadipocyte lines and in animals has led to a paradigmatic model of the genetic program of adipogenesis (3–5). In response to stimulators of adipogenesis, two transcription factors, CCAAT/enhancer-binding protein β (C/EBPβ)[1] and C/EBPδ are rapidly and transiently induced. These proteins then stimulate expression of the key adipogenic transcription factors, C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), which together induce expression of the complement of genes necessary to create the adipocyte phenotype. Investigations of factors that regulate progression through this adipogenic program are important for our understanding of adipocyte differentiation.

Differentiation of preadipocytes into adipocytes is regulated by a balance of local and endocrine factors that either stimulate or inhibit differentiation (6). Well known factors that stimulate differentiation of preadipocyte lines include glucocorticoid agonists, high concentrations of insulin to stimulate the insulin-like growth factor 1 receptor, PPARγ agonists, and agents that elevate cAMP (3). Factors that counter these positive stimuli include Wnt10b, tumor necrosis factor α (TNFα), transforming growth factor β (TGF-β), epidermal growth factor, and prostaglandin F2α (6). Of these, Wnt signaling may be of particular physiological importance because its disruption results in spontaneous differentiation, suggesting that endogenous Wnt signaling activity inhibits adipogenesis. The endogenous activator of Wnt signaling is likely to be Wnt10b because expression of Wnt10b is elevated in preadipocytes and declines upon induction of differentiation. Moreover, ectopic expression of Wnt10b activates the Wnt signaling pathway and potently blocks differentiation (7).

Wnts are secreted glycoproteins that signal through their Frizzled (Fz) receptors, (8) and low density lipoprotein receptor-related protein (LRP) co-receptors (9) to have profound autocrine and paracrine effects on cellular differentiation and growth. There are >16 mouse Wnts, nine Fz receptors, and two LRP co-receptors, LRP5 and LRP6, and specificity of cellular effects may be influenced by the subset of Wnts and signaling molecules expressed (9–11). When Wnt signaling is active, glycogen synthase kinase 3 (GSK3) is inhibited. This loss of GSK3 activity allows cytosolic β-catenin to accumulate and translocate to the nucleus where it binds to the T-cell factor (TCF)/lymphoid-enhancing factor family of transcription factors and activates transcription of Wnt-regulated target genes (12). However, when Wnt signaling is suppressed, GSK3 phosphorylates β-catenin and targets it for ubiquitin-mediated degradation (13). Activity of Wnt proteins is regulated by secreted inhibitors. For example, Wnt inhibitory factor-1 (14) and the family of secreted Frizzled-related proteins (sFRPs) (15) appear to bind and sequester Wnts from their receptors. Another family of Wnt inhibitors, dickkopf, binds to the Wnt co-receptor, LRP6, and inhibits interactions with the Wnt-Fz complex (16, 17). Thus, preadipocyte differentiation could be influenced not

* This work is supported National Institutes of Health Grant DK51563 (to O. A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Cellular & Molecular Approaches to Systems and Integrative Biology Training Grant T32-GM08322.

‖ To whom correspondence should be addressed: Dept. of Physiology, 7620 Medical Science II, 1301 E. Catherine St., Ann Arbor, MI 48109-0622. Tel.: 734-647-4880; Fax: 734-936-8813; E-mail: macdouga@umich.edu.

[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor γ; TNFα, tumor necrosis factor α; TGF-β, transforming growth factor β; Frizzled, Fz; LRP, low density lipoprotein receptor-related protein; GSK3, glycogen synthase kinase 3; TCF, T-cell factor; sFRP, secreted frizzled related protein; MDI, methylisobutylxanthine dexamethasone and insulin.

TABLE I
Forward and reverse primers used for amplification of Wnt, Fz, and LRP PCR products

Probe	Forward Primer	Reverse Primer
Wnt10b	GGGGAATTCCAXGAXTGYAAXTGYCA*	AAAATCTAGAXCACCAXTGXAA*
Fz1	CCGGCCGGCTGAGCTTGGAACT	CAGGCGCGTACATGGAGCACAGGA
Fz 2	AGCACCCTTTCCACTGTCC	TGCACCGTGCGATAACCGT
Fz 5	GGTAGCACGCAGACAAGAAGATGA	TATAACCGAAGCGAAGCCACCAAC
LRP5	TCGAGCGGGCAGGGATGGATGGCAGA	TCGGGGACAGCAGGCACAGGTGGGAACAC
LRP6	TGAAACAAATATACTGGCGAGGGTCTG	CAATGGCATGCCGGATATCTTCTAACTG
GAPDH	TCACGGCAAATTCAACGGCACAGT	TCGGCAGAAGGGGCGGAGATGAT

*(X= A or G, Y= C or T)

only by Wnts but also through regulation of Fz, LRPs, Wnt inhibitors, or downstream signaling intermediates.

In this paper we further investigate the role of Wnt10b and Wnt signaling components in preadipocyte differentiation. We demonstrate that activation of Wnt signaling during the first days of adipogenesis blocks differentiation and that disruption of extracellular Wnt signaling causes spontaneous adipocyte conversion. Wnt10b is the best candidate for the endogenous inhibitory Wnt, and expression of this gene declines during adipogenesis in response to cAMP. Wnt10b likely signals through Fz1, Fz2, and/or Fz5 receptors, and LRP5 and/or LRP6 co-receptors, because these genes are expressed in preadipocytes and stromal vascular cells. These data further our understanding of the mechanism whereby active Wnt signaling within preadipocytes restrains these cells from undergoing adipogenesis.

EXPERIMENTAL PROCEDURES

Reagents—Forskolin and 8-Bromo-cAMP (Calbiochem) were dissolved in Me_2SO. The soluble Wnt inhibitors sFRP-1 and sFRP-2 were kind gifts from R&D Systems, Inc. The GSK3 inhibitor, CHIR 99021, the inactive control, CHIR GSKIA (Chiron),[2] and troglitazone (Pfizer) were diluted in Me_2SO.

Cell Culture—Maintenance and adipogenesis of 3T3-L1 preadipocytes were as described previously using methylisobutylxanthine dexamethasone and insulin (MDI) (18). Lipid accumulation in adipocytes was visualized by staining with Oil Red-O (19). To quantify retention of Oil Red-O, stained adipocytes were extracted with 1 ml of 4% Igepal CA-630 (Sigma) in isopropanol for 15 min, and absorbance was measured by spectrophotometry at 520 nm. To evaluate triacylglycerol in 3T3-L1 cells, Infinity triglyceride reagent (Sigma) was used following the protocol provided by the manufacturer. C2C12 cells (ATCC) were cultured in 20% fetal calf serum supplemented with chick embryo extract (Invitrogen), and G8 cells (ATCC) were cultured in 10% fetal calf serum and 10% horse serum as described previously (20, 21).

Kinase Assays—Kinases (see Fig. 1*A*) were purified from SF9 cells through use of their His or Glu tag. Glu-tagged proteins were purified as described (22), and His-tagged proteins were purified according to the manufacturer's instructions (Qiagen). Kinase assays were performed in 96-well plates with appropriate peptide substrates in a 300-μl reaction buffer (variations on 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 25 mM β-glycerophosphate, 1 mM NaF, and 0.01% bovine serum albumin). Peptides had K_m values from 1 to 100 μM. CHIR 99021 or CHIR GSKIA was added in 3.5 μl of Me_2SO, followed by ATP to a final concentration of 1 μM. After incubation, triplicate 100-μl aliquots were transferred to Combiplate 8 plates (Thermo LabSystems) containing 100 μl/well of 50 μM ATP and 20 mM EDTA. After 1 hour, the wells were rinsed five times with phosphate-buffered saline, filled with 200 μl of scintillation fluid, sealed, and counted in a scintillation counter 30 min later. All of the steps were at room temperature. The percentage of inhibition was calculated as 100 × (inhibitor − no enzyme control)/(Me_2SO control − no enzyme control).

β-Catenin Assay—To evaluate levels of free cytosolic β-catenin, confluent 3T3-L1 preadipocytes were treated for 4 h with Me_2SO, 3 μM CHIR 99021, 25 mM NaCl, or 25 mM LiCl, and cellular fractionation was performed as described (23). Cytosolic and membrane fractions were separated by SDS-PAGE on an 11.5% gel and transferred to polyvinylidene difluoride membrane, and β-catenin was evaluated by immunoblot analysis with a monoclonal antibody against β-catenin (Transduction Laboratories). All data analysis was performed using Bio-Rad multi-analyst software.

RNA Isolation—Epididymal fat pads from at least 12-week-old FVB mice were surgically removed and placed in ice-cold Krebs-Ringer HEPES buffer (pH 7.4) for isolation of stromal vascular cells and adipocytes as described (24, 25). White adipose tissue (1.5 g) was digested at 37 °C for 1 h in 6 ml of Krebs-Ringer HEPES buffer (pH 7.4) containing 10 mg of collagenase (Worthington) with continuous agitation. Digested adipose tissue was filtered through a mesh to separate cells from connective and undigested tissues. The cells were centrifuged at 500 × *g* for 5 min to separate by buoyant density, adipocytes, which are found at the top of the supernatant, from stromal vascular cells, which are found in the pellet. Adipocytes were transferred equally to two 12-ml polypropylene tubes, each containing 8 ml of RNA Stat60 (Tel-Test), and were mixed thoroughly by vortexing. Likewise, 1 ml of RNA Stat60 was added to the pellet and was mixed by trituration. Total RNA was isolated from stromal vascular cells, primary adipocytes, and 3T3-L1 cells by following the manufacturer's protocol for RNA Stat60.

RNase Protection Analysis—Wnt10b riboprobe template was amplified from 3T3-L1 preadipocyte RNA by reverse transcription-PCR with *pan*-specific Wnt primers (Table I) as described (7, 26). Fz1, Fz5, and glyceraldehyde-3-phosphate dehydrogenase templates were amplified from preadipocyte RNA by reverse transcription-PCR with specific primers (Table I). Fz2, LRP5, and LRP6 riboprobe templates were PCR-amplified from Fz2 (kind gift of T. Malik, Dana-Farber Cancer Institute) (11), LRP5, and LRP6 cDNAs (kind gift of F. Hess, Merck), respectively. PCR products for Wnt10b (384 bp), Fz1 (474 bp), Fz2 (373 bp), Fz5 (388 bp), LRP5 (417 bp), LRP6 (343 bp), and glyceraldehyde-3-phosphate dehydrogenase (250 bp) were cloned into pCRII-Topo (Invitrogen) and sequenced. The vectors were linearized for *in vitro* transcription reactions with T7 or Sp6 RNA polymerases (Promega) to produce ^{32}P-labeled riboprobes. RNase protection assays were performed essentially as described (27) except that 150 mM NaCl was used in the digestion buffer.

RESULTS

CHIR 99021 Inhibits GSK3 and Mimics Wnt Signaling in 3T3-L1 Preadipocytes—CHIR 99021 is a small organic molecule that inhibits GSK3α and GSK3β by competing for their ATP-binding sites. *In vitro* kinase assays reveal that CHIR 99021 specifically inhibits GSK3β (IC_{50} = ~5 nM) and GSK3α (IC_{50} = ~10 nM), with little effect on other kinases (Fig. 1*A*). Because inhibition of GSK3 mediates a subset of the cellular effects of insulin, it is not surprising that CHIR 99021 potentiates and mimics the actions of insulin.[2] The fact that inhibition of GSK3 also plays a critical role in the Wnt/β-catenin pathway suggests that CHIR 99021 may activate Wnt signaling as well. To determine whether inhibition of GSK3 by CHIR

[2] D. Ring, K. Johnson, E. Henriksen, J. Nuss, D. Goff, T. Kinnick, S. Ma, J. Reeder, I. Samuels, T. Slabiak, A. Wagman, M. Wernette-Hammond, and S. Harrison, manuscript in preparation.

A

Kinase	IC50
GSK3-β	5
GSK3-α	10
CDC2	8,800
AKT/PKB	>10,000
IGF RTK	>10,000
PI3 Kinase	>10,000

B

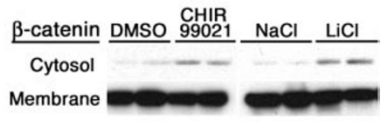


FIG. 1. **CHIR 99021 inhibits GSK3 and mimics Wnt signaling in 3T3-L1 preadipocytes.** *A*, specificity of CHIR 99021 as an inhibitor of GSK3α and GSK3β was evaluated using *in vitro* kinase assays with the indicated purified protein kinases and peptide substrates as described under "Experimental Procedures." IC_{50} is the concentration of CHIR 99021 required for half-maximal inhibition. *CDC2*, cyclin-dependent kinase 2; *AKT1/PKB*, protein kinase B; *IGF1 RTK*, insulin-like growth factor 1 receptor tyrosine kinase; *PI3 Kinase*, phosphatidylinositol 3-kinase. *B*, free cytosolic β-catenin was evaluated in 3T3-L1 preadipocytes treated for 4 h with dimethyl sulfoxide (*DMSO*), 3 μM CHIR 99021, 25 mM NaCl, or 25 mM LiCl. After cellular fractionation (23), cytosolic and membrane fractions were evaluated for β-catenin by immunoblot analysis. CHIR 99021 and LiCl increased free cytosolic β-catenin by 1.9- and 2.4-fold, respectively. These data are representative of three independent experiments.

99021 mimics stimulation of the Wnt signaling pathway, confluent 3T3-L1 preadipocytes were treated with CHIR 99021 or vehicle for 4 h, at which time cells were separated into cytosolic and membranous fractions. As a positive control for pharmacological activation of Wnt signaling, the cells were also treated with LiCl or a control (NaCl). Immunoblot analysis revealed that CHIR 99021, like LiCl, stabilizes free cytosolic β-catenin (Fig. 1*B*), without substantial change to β-catenin associated with membranes (23). Thus, it appears that inhibition of GSK3 by CHIR 99021 mimics the canonical Wnt signaling pathway in 3T3-L1 preadipocytes.

CHIR 99021 Inhibits Adipogenesis by Blocking Induction of C/EBPα and PPARγ—We have shown previously that Wnt signaling potently inhibits preadipocyte differentiation (7). To determine whether activation of Wnt signaling by CHIR 99021 also blocks adipogenesis, we treated 3T3-L1 preadipocytes with CHIR 99021 or CHIR GSKIA at days 0 and 2 of the standard differentiation protocol. Although adipogenesis of 3T3-L1 cells is not affected by treatment with the inactive control molecule, treatment with 1 μM CHIR 99021 results in a complete block of differentiation as assessed by Oil Red-O staining, with partial inhibition observed with 0.3 μM CHIR 99021 (Fig. 2*A*). Extraction and quantification of Oil Red-O indicates that half-maximal inhibition of preadipocyte differentiation is observed with ~0.3 μM CHIR 99021 (Fig. 2*B*). Thus, inhibition of GSK3 by CHIR 99021 activates Wnt signaling and blocks adipocyte conversion.

Differentiation of preadipocytes has been extensively studied, and a model for the cascade of genetic events involved in adipocyte conversion has emerged (3–5). Although the rapid and transient induction of adipogenic transcription factors, C/EBPβ and C/EBPδ, does not appear to be affected by treatment of differentiating 3T3-L1 cells with CHIR 99021, induction of the master adipogenic transcription factors C/EBPα and PPARγ is completely blocked by this molecule (Fig. 2*C*). Furthermore, induction of the adipocyte lipid-binding protein, 422/aP2, which is downstream of C/EBPα and PPARγ, is also suppressed (Fig. 2*C*). These results are consistent with our prior findings on the mechanism whereby Wnt signaling inhibits preadipocyte differentiation (7).

Differentiating Preadipocytes Are Sensitive to Inhibition by CHIR 99021 for the First 3 Days of Adipogenesis—Treatment of 3T3-L1 cells with CHIR 99021 for the first 4 days of differentiation completely blocks adipogenesis (Figs. 2*A* and 3). To determine the window of time during which differentiating preadipocytes are sensitive to CHIR 99021, we treated 3T3-L1 preadipocytes with the GSK3 inhibitor for the intervals of time indicated (Fig. 3). Exposure of differentiating preadipocytes to CHIR 99021 for 2- or 3-day intervals suppressed differentiation. Indeed, treatment of preadipocytes during any of the first 3 days of adipogenesis is sufficient to completely block the process. In contrast, exposure to CHIR 99021 during the fourth day resulted in only partial inhibition of adipogenesis, and subsequent treatments did not repress differentiation compared with controls (data not shown). Thus, the adipogenesis program is sensitive to inhibition by Wnt signaling during any of the first 3 days of differentiation.

Spontaneous Differentiation of Preadipocytes Exposed to Soluble Wnt Inhibitors, sFRP-1 or sFRP-2—Our previous finding that inhibition of Wnt signaling blocks adipogenesis implicates endogenously produced Wnt proteins in the regulation of adipocyte differentiation (7). However, this conclusion was never proven directly, and it remained possible that endogenous Wnts are not involved in the regulation of adipogenesis. For instance, our previous results are also consistent with the possibility that TCF activity is constitutive in preadipocytes or alternatively that TCF activity is regulated by another uncharacterized signal. It was critical, therefore, to test directly the involvement of endogenously produced Wnts in the inhibition of adipogenesis. To determine whether sequestration and inactivation of extracellular Wnts causes differentiation in the absence of inducing agents, we exposed preadipocytes to sFRP-1 or sFRP-2, which appear to inhibit Wnt signaling by interfering with Wnt-Fz receptor interactions (15). Preadipocytes grown in 10% fetal calf serum were exposed to 100 nM sFRP-1 or sFRP-2 every 2 days for 8 days with treatments initiated at 70% confluence. Fourteen days after the initial treatments, ~20% of preadipocytes exposed to Wnt inhibitors spontaneously differentiated, whereas less than 1% of control cells differentiated under these conditions as assessed by phase contrast microscopy (Fig. 4*A*). As expected, adipogenesis in response to sFRP-1 or sFRP-2 was associated with induction of the adipogenic transcription factor, C/EBPα, as well as the adipocyte protein, 422/aP2 (Fig. 4, *bottom panel*). Although less dramatic, preadipocyte differentiation in the absence of inducing agents was also observed with the addition of purified recombinant sFRP-3, Wnt inhibitory factor-1, and dickkopf-1 (data not shown). Spontaneous differentiation of preadipocytes in response to extracellular Wnt inhibitors provides strong evidence that endogenously produced Wnts inhibit adipogenesis.

Wnt10b Is Suppressed during Adipogenesis by Elevated cAMP—Wnt10b is the best candidate for the endogenous inhibitory Wnt because this protein activates the canonical Wnt signaling pathway and blocks adipogenesis (7). Moreover, Wnt10b mRNA is expressed in dividing and confluent 3T3-L1 cells and is largely suppressed within 24 h of induction of differentiation (7). To determine how rapidly Wnt10b is suppressed, 3T3-L1 preadipocytes were induced to differentiate, and RNA was isolated at the times indicated. RNase protection analyses revealed that expression of Wnt10b declines by ~50% within 6 h and is undetectable 36 h after induction of differentiation (Fig. 5*A*). Rapid suppression of Wnt10b during adipogenesis appears to be required for relieving inhibition of GSK3 during this critical period of differentiation, thus allowing adipocyte conversion to occur.

Treatment of 3T3-L1 preadipocytes with inducers of differentiation is sufficient to suppress expression of Wnt10b. To identify which component or components of the differentiation mixture (*i.e.* MDI) suppress Wnt10b, we treated confluent 3T3-L1 preadipocytes with 10% fetal calf serum, *methyl*-isobutylxanthine, dexamethasone, insulin, or combinations thereof.

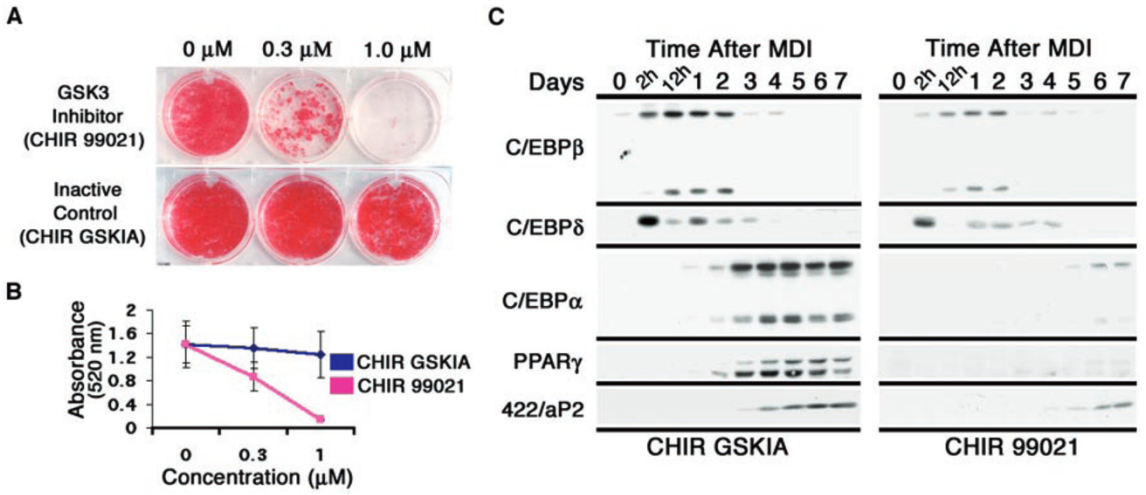


FIG. 2. **CHIR 99021 inhibits adipogenesis by blocking induction of C/EBPα and PPARγ.** *A*, confluent 3T3-L1 preadipocytes were treated with CHIR 99021 or the inactive control, CHIR GSKIA, at days 0 and 2 of the standard differentiation protocol. Control or GSK3 inhibitor was added at 0, 0.3, or 1.0 μM. Two weeks after induction of differentiation, adipocytes were stained with Oil Red-O and photographed. The data are representative of three independent experiments. *B*, to estimate the accumulation of neutral lipid in *A*, Oil Red-O was extracted with 4% Igepal in isopropanol and quantified with spectrophotometry at 520 nm. The results are from two independent experiments and are presented as the means ± range. *C*, confluent 3T3-L1 preadipocytes were treated with 1 μM CHIR 99021 or CHIR GSKIA at day 0 and day 2 of the standard differentiation protocol. Cells were lysed at the times indicated and expression of C/EBPβ, C/EBPδ, C/EBPα, PPARγ, and 422/aP2 were determined by immunoblot analyses. The data are representative of two independent experiments.

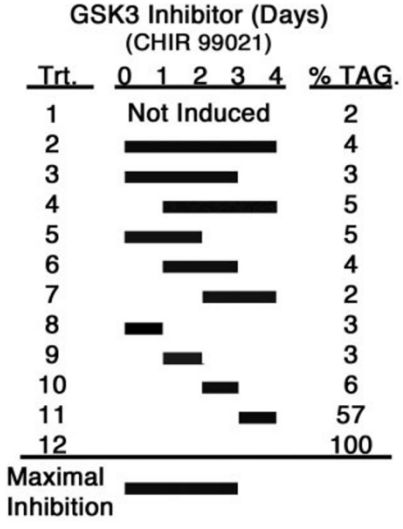
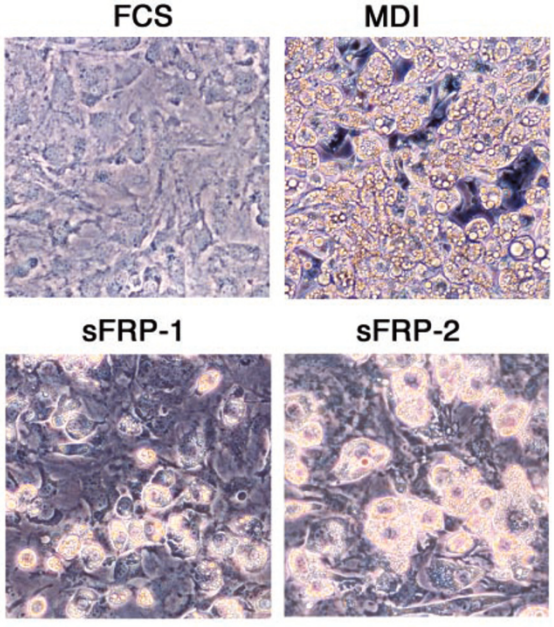


FIG. 3. **Differentiating preadipocytes are sensitive to inhibition by CHIR 99021 for the first 3 days of adipogenesis.** Confluent 3T3-L1 preadipocytes were exposed to 3 μM CHIR 99021 for the times indicated during the standard differentiation protocol. For removal of CHIR 99021 at days 1 and 3 (*Treatments* (*Trt.*) *3*, *6*, *8*, and *10*), the media were replaced with that from preadipocytes differentiating in the absence of CHIR 99021. The cells were lysed 9 days after induction of differentiation, and the amount of triacylglycerol (*TAG*) was determined. Triacylglycerol is expressed as a percentage of control 3T3-L1 adipocytes (*Treatment 12*). The data are representative of two independent experiments.

RNA was prepared after 48 h, and Wnt10b mRNA was analyzed by RNase Protection assay. Although Wnt10b mRNA is not suppressed by 10% fetal calf serum, dexamethasone, and/or insulin, the addition of *methyl*-isobutylxanthine is sufficient to repress Wnt10b (Fig. 5*B*). Furthermore, activation of PPARγ with troglitazone is not sufficient to repress expression of Wnt10b. Suppression of Wnt10b by *methyl*-isobutylxanthine does not appear to be cell type-specific because this compound also causes a similar decline in expression of Wnt10b in C2C12 and G8 myoblast cell lines (Fig. 5*C*). Suppression of Wnt10b RNA by the phosphodiesterase inhibitor *methyl*-isobutylxanthine suggests that elevation of intracellular cAMP is responsible for the decline of Wnt10b during adipogenesis. To confirm that cAMP regulates expression of Wnt10b, confluent preadipocytes were treated with a phosphodiesterase inhibitor (*methyl*-isobutylxanthine), a cell-permeable analog of cAMP (8-bromo-cAMP), or an activator of adenylate cyclase (forskolin). After 48 h, RNA was isolated, and repression of Wnt10b in response to these treatments was observed by RNase protection assay (Fig. 5*D*). Thus, elevated cAMP appears to be the primary signal for repression of Wnt10b during adipogenesis.

Regulation of Wnt Receptors and Co-receptors during Adipogenesis—The Fz family of Wnt receptors is comprised of nine members, and the specificity of interactions between individual Wnts and Fz remains largely uncharacterized. As an initial step toward identifying the Fz through which inhibitory effects of Wnt10b are transduced, we have determined which Fz are expressed within preadipocytes. After cloning and sequencing fragments complementary to each mouse Fz mRNA, RNase protection assays were used to assess the level and pattern of expression of individual Fz during the course of adipogenesis. Fz1, Fz2, and Fz5 mRNAs are expressed at high levels in preadipocytes and are suppressed during differentiation (Fig. 6*A*), suggesting that one or more of these Wnt receptors could mediate the inhibition of adipogenesis by Wnt10b. Although suppression of Wnt10b during adipogenesis is almost exclusively due to elevated cAMP, repression of Fz1, Fz2, and Fz5 mRNAs requires more than one component of the differentiation mixture and appears to be correlated with whether adipogenesis occurs (data not shown). The decline of Fz during adipogenesis will render preadipocytes refractory to secretion of Wnts from neighboring cells, and thus suppression of Fz may be required for differentiation to occur.

Wnt signaling is mediated through Wnt receptors, Fz, and Wnt co-receptors LRP5 and LRP6 (28, 29). To determine which LRP is expressed in 3T3-L1 preadipocytes, RNase protection analyses of LRP5 and LRP6 were performed. Our data indicate that both LRP5 and LRP6 are expressed constitutively throughout differentiation (Fig. 6*B*). Although the relative affinity of Wnts for LRPs is unknown, based upon the specific activity of our probes, LRP5 is more highly expressed in preadipocytes than LRP6 and thus may be more important for mediating Wnt signals. In contrast to Wnt10b and Fz receptors



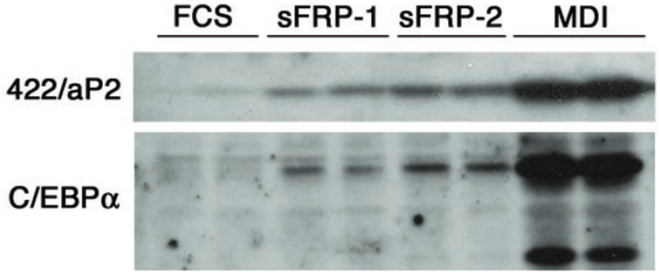


FIG. 4. **Spontaneous differentiation of preadipocytes exposed to soluble Wnt inhibitors sFRP-1 or sFRP-2.** *A*, 3T3-L1 cells were grown in 10% fetal calf serum (*FCS*). Starting at 70% confluence, the cells were cultured in the presence of 100 nM sFRP-1 or sFRP-2 for 8 days. Wnt inhibitors were added with medium changes every 2 days. The degree of differentiation in control and sFRP-treated cells, 14 days after the initial exposure is shown by phase contrast photomicroscopy. *B*, cells from *A* were lysed, and expression of 422/aP2 and C/EBPα was evaluated by immunoblot analyses.

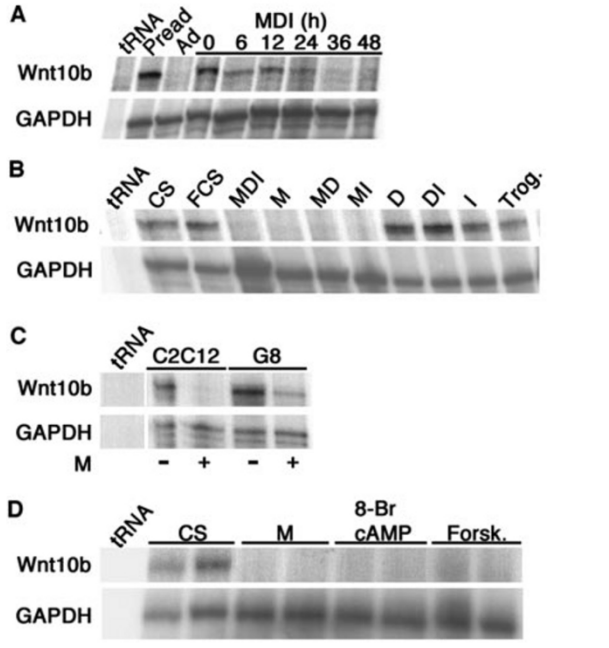


FIG. 5. **Wnt10b is suppressed during adipogenesis by elevated cAMP.** *A*, RNA was prepared when cells were confluent (*Pread*), fully differentiated (*Ad*), and at the hours indicated after MDI for analysis of Wnt10b by ribonuclease protection assay. Yeast tRNA was used as a negative control for background protection. Glyceraldehyde-3-phosphate dehydrogenase was an internal control for the RNase protection assay and for the quality of RNA preparation. The data are representative of three independent experiments. *B*, 3T3-L1 preadipocytes were not treated (calf serum, *CS*) or treated with fetal calf serum (*FCS*), 11.5 μg/ml *methyl*-isobutylxanthine (*M*), 1 μM dexamethasone (*D*), 167 nM insulin (*I*), 5 μM troglitazone (*Trog.*), or combinations thereof, as indicated. RNA was isolated 2 days after treatment for analysis of Wnt10b by ribonuclease protection assay. The data are representative of three independent experiments. *C*, C2C12 and G8 myoblasts were treated (+) or not (−) with 11.5 μg/ml *methyl*-isobutylxanthine (*M*) as indicated. RNA was collected 2 days later for ribonuclease protection analyses of Wnt10b. The data are representative of two independent experiments. *D*, 3T3-L1 preadipocytes were not treated (CS) or treated with 11.5 μg/ml *methyl*-isobutylxanthine (*M*), 500 μM 8-bromo-cAMP (*8-Br cAMP*), or 10 μM forskolin (*Forsk.*). RNA was prepared 2 days later, and the expression of Wnt10b was determined. The data are representative of three independent experiments.

(Fig. 6*A*), whose expression declines during adipogenesis, the constitutive expression of LRP5 and LRP6 suggests that suppression of these proteins is not required for adipogenesis and perhaps that these proteins serve other functions within fully differentiated 3T3-L1 adipocytes.

Expression of Wnt Signaling Components in Vivo—We have used 3T3-L1 preadipocytes to characterize the regulation of Wnt10b, Fz receptors, and LRP co-receptors during adipogenesis. To determine whether these members of the Wnt signaling pathway could play a role in regulation of preadipocyte differentiation *in vivo*, we examined expression of each gene in RNA from stromal vascular cells and primary adipocytes. After collagenase treatment of mouse white adipose tissue and fractionation of cell types by buoyant density centrifugation (24, 25), RNA was prepared and analyzed by RNase protection assay. Expression of Wnt10b is high in stromal vascular cells and is undetectable in primary adipocytes (Fig. 7*A*), which is consistent with the decline observed during differentiation of 3T3-L1 cells (Fig. 5*A*) and with Wnt10b playing a regulatory role for adipogenesis *in vivo*. Furthermore, expression of Fz1, Fz2, and Fz5 in stromal vascular cells but not primary adipocytes suggests that these Wnt receptors may mediate the inhibitory effect of Wnt10b *in vivo* (Fig. 7*B*). Unlike the constitutive expression observed in differentiating 3T3-L1 preadipocytes, suppression of LRP5 and LRP6 in primary adipocytes compared with stromal vascular cells suggests that decline of these co-receptors during adipogenesis requires *in vivo* signals.

DISCUSSION

Differentiation of preadipocytes is controlled by factors that either repress or stimulate adipogenesis (6). Of the inhibitory forces, Wnts appear to be particularly important. Although activation of Wnt signaling during the first days of adipogenesis blocks differentiation (Figs. 2 and 3), disruption of extracellular Wnt signaling results in spontaneous adipogenesis (Fig. 4). Taken together, these data provide strong evidence that preadipocytes secrete a Wnt or Wnts, which feedback to restrain preadipocytes from undergoing adipogenesis. Wnt10b is an excellent candidate for the endogenous inhibitory Wnt because it blocks adipogenesis through activation of the canonical Wnt/β-catenin signaling pathway (7) and because expression of Wnt10b declines during adipogenesis in a time frame consistent with subsequent differentiation. Of the standard inducers of differentiation, elevated intracellular cAMP is the primary stimulus for decreasing expression of Wnt10b mRNA (Fig. 5). Inhibition of adipogenesis by Wnt10b is likely mediated by Wnt

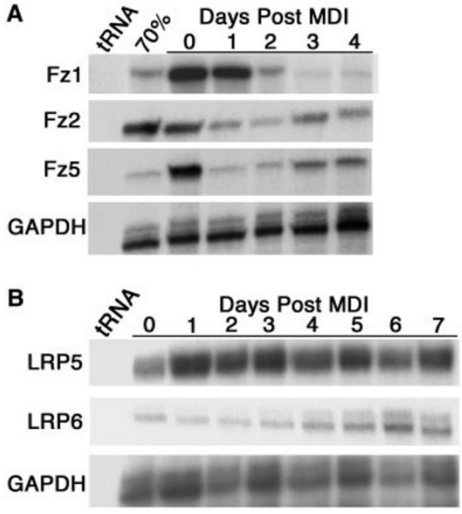


FIG. 6. **Regulation of Wnt receptors and co-receptors during adipogenesis.** Confluent 3T3-L1 preadipocytes were induced to differentiate and RNA was purified at the times indicated for ribonuclease protection assays using Fz1, Fz2, and Fz5 (*A*) or LRP5 and LRP6 (*B*) probes. The data are representative of two to five independent experiments.

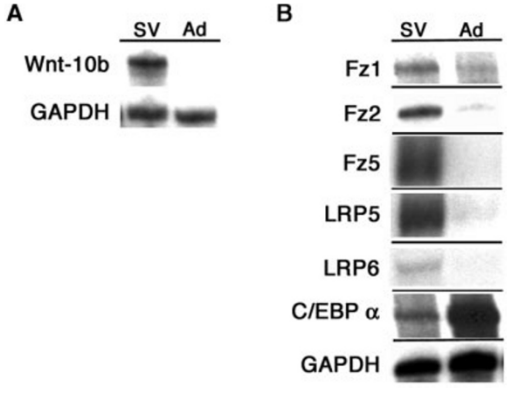


FIG. 7. **Expression of Wnt signaling components *in vivo*.** Stromal vascular cells (*SV*) and adipocytes (*Ad*) were isolated from mouse adipose tissue, and RNA was prepared. Expression of Wnt10b (*A*) Fz1, Fz2, Fz5, LRP5, and LRP6 (*B*) were evaluated by RNase protection assay. C/EBPα was used as a positive control for fractionation of adipocytes from stromal vascular cells. The data are representative of two to four independent experiments.

receptors Fz1, Fz2, and Fz5 because these receptors, like Wnt10b, are expressed in preadipocytes and stromal-vascular cells but not in 3T3-L1 and primary adipocytes (Figs. 6 and 7). In addition, the Wnt co-receptors LRP5 and LRP6 may mediate the Wnt10b signal because both of these genes are expressed in 3T3-L1 preadipocytes and stromal vascular cells. In summary, these data indicate that Wnt signaling through Fz receptors and LRP co-receptors is critical for regulation of adipogenesis and potentially for development of white adipose tissue.

Mechanistic analyses of Wnt signaling have been hindered by an inability to purify many members of the Wnt signaling pathway while maintaining their biological activity. To circumvent this problem, we have ectopically expressed Wnts or signaling intermediates to examine the effects of Wnt signaling on adipogenesis. A limitation of this method, however, is that constitutive expression of these signaling molecules does not permit analyses of the time frame during which Wnt signaling inhibits the differentiation program. This problem can be largely overcome with the use of pharmacological agents. Lithium is widely used to mimic Wnt signaling because it inhibits GSK3 and stabilizes free cytosolic β-catenin (30). However, the concentrations needed to reduce GSK3 activity have nonspecific effects on other signaling pathways (30). Herein we demonstrate that CHIR 99021 is a highly specific inhibitor of GSK3, and CHIR 99021 activates Wnt signaling as assessed by stabilization of free cytosolic β-catenin and inhibition of adipogenesis *in vitro* (Figs. 1 and 2). Thus, this small, lipid-soluble molecule provides an ideal tool with which to analyze kinetic and mechanistic aspects of Wnt signaling and its role in differentiation.

We have used CHIR 99021 to investigate the mechanism whereby the Wnt signaling pathway blocks preadipocyte differentiation. Activation of Wnt signaling with CHIR 99021 inhibits adipogenesis and the induction of C/EBPα and PPARγ, without detectable effects on transient induction of C/EBPβ and C/EBPδ (Fig. 2). Although C/EBPβ and C/EBPδ are regulated primarily by *methyl*-isobutylxanthine and dexamethasone, respectively, expression of C/EBPα and PPARγ is highly correlated with whether preadipocytes undergo adipogenesis (4, 31). Analyses of the mechanism whereby other molecules inhibit adipogenesis (*e.g.* retinoic acid, TGF-β, TNFα, and 2,3,7,8-tetrachloro dibenzo-P-dioxin) reveal a similar pattern of gene expression in which C/EBPβ and C/EBPδ are transiently induced, but induction of C/EBPα and PPARγ is inhibited (32–35). In addition, the time during adipogenesis in which preadipocytes are sensitive to inhibition by CHIR 99021 (Fig. 3) is similar to the time of sensitivity to TNFα, retinoic acid, TGF-β, and 2,3,7,8-tetrachloro dibenzo-P-dioxin. These similarities in mechanism of inhibition probably indicate that after day 3 of differentiation, expression of C/EBPα and PPARγ is self-supporting through positive feedback regulation, and thus, adipogenesis is not susceptible to inhibition by these factors. Based upon the paradigmatic view of the adipogenic program (3–5), the ability of C/EBPβ and C/EBPδ to induce expression of C/EBPα and PPARγ may be subject to inhibition by apparently disparate signaling pathways (*i.e.* TNFα, TGF-β, 2,3,7,8-tetrachloro dibenzo-P-dioxin, and retinoic acid), all of which result in inhibition of adipogenesis. Alternatively, these agents might act to disrupt the positive feedback loop between C/EBPα and PPARγ.

Although pharmacological agents are available to suppress GSK3 activity, there are no such agents that block the Wnt pathway upstream of this enzyme. Thus, to determine whether endogenous Wnts repress differentiation, we treated 3T3-L1 preadipocytes with soluble Wnt inhibitors, sFRP-1 and sFRP-2, and we observed considerable spontaneous adipogenesis (Fig. 4). Treatment with Wnt inhibitors consistently stimulated spontaneous adipocyte differentiation prior to background differentiation of control preadipocytes. Although adipogenesis in response to soluble Wnt inhibitors was dramatic in Fig. 4, differentiation in some experiments was only severalfold above background. This variability in the percentage of differentiation may be related to the confluence of the preadipocytes when inhibitor was added, the half-life of the inhibitor, and/or the specificity of Wnt inhibitors for endogenous Wnts. Although both sFRPs and cAMP mimetics are weak adipogenic agents when added alone, sFRPs, like cAMP, may synergize with insulin, glucocorticoids, or other positive effectors of adipogenesis to achieve more complete differentiation.

In response to adipogenic cues, preadipocytes suppress expression of Wnt10b to relieve the autocrine repression of differentiation (Fig. 5). These cells, however, could still respond to inhibitory effects of Wnt10b from paracrine sources. Thus, loss of Fz receptors during differentiation may be required for an individual cell to become refractory to Wnts secreted from neighboring cells (Fig. 6). In addition to Wnt10b, Fz1, Fz2, and Fz5, other members of the Wnt signaling pathway such as

Wnt5a, sFRP-2, dickkopf-3, dvl2, and TCF3 are also repressed (43). These data indicate that there is a loss of many Wnts, Wnt regulators, and Wnt signaling intermediates during the process of adipogenesis. This loss may be required because of cross-talk between the canonical Wnt signaling pathway and other signaling pathways. For example, recent evidence indicates that the signaling pathway for TGF-β, another well known inhibitor of adipogenesis, can interact with the Wnt signaling pathway intermediates such as axin (36) and members of the TCF/lymphoid-enhancing factor family of transcription factors. Thus, loss of Wnt signaling intermediates may be necessary to ensure that differentiating preadipocytes are refractory to inhibitors of adipogenesis that act by co-opting part of the canonical Wnt signaling pathway.

We have shown that Wnt signaling is an adipogenic switch that inhibits differentiation when on and that must be shut off for adipocyte conversion to proceed (Figs. 2–4 and Ref. 7). Work by Reusch *et al.* (37) has shown that expression of constitutively active CREB likewise causes spontaneous differentiation. Given that cAMP activates CREB and also causes repression of Wnt10b (Fig. 5), it is tempting to speculate that the spontaneous adipogenesis that results from expression of constitutively active CREB may be, in part, through repression of Wnt10b. Likewise, other genetic interventions that cause spontaneous adipogenesis (*e.g.* enforced expression of C/EBPα or PPARγ) will also inhibit Wnt10b. Although activators of adipogenesis are expected to repress expression of Wnt10b, it could also be that inhibitors of adipogenesis do so through enforced expression of Wnt10b. Consistent with this idea, a recent report indicates that TNFα stimulates Wnt10b expression in a human gastric cancer cell line (38).

Our studies indicate that Fz1, Fz2, and Fz5 are expressed in preadipocytes and thus serve as candidate Fz for mediating repressive effects of Wnt10b on adipogenesis. However, we[3] and others (39) have observed that Fz4 is expressed in primary adipocytes but not during differentiation of 3T3-L1 cells. Although mechanisms of Fz4 action have not been well characterized, this raises the possibility that Wnts play a role in regulation of adipocyte metabolism or other functions within the fully differentiated state.

Wnt signaling plays an integral role in differentiation and development of cells and tissues across a wide range of phylogeny. In addition, Wnts play critical roles in regulation of cell growth. Indeed, Wnt10b was originally cloned because of its ability to cause mammary tumor formation (40). Although function of Wnt10b has not been extensively characterized, it is believed to have functions in development of mammary gland and hair follicles (41, 42). Our data are consistent with Wnt10b playing an important role in adipogenesis *in vitro* and likely *in vivo*, based upon expression in stromal vascular cells but not in adipocytes. Further definition of the roles of Wnt10b in development of adipose tissues, and potentially as a modulator of obesity, await development and analyses of appropriate animal models.

REFERENCES

1. Steppan, C. M., and Lazar, M. A. (2002) *Trends Endocrinol. Metab.* **13,** 18–23
2. Green, H., and Kehinde, O. (1974) *Cell* **1,** 113–116
3. Rangwala, S. M., and Lazar, M. A. (2000) *Annu. Rev. Nutr.* **20,** 535–559
4. MacDougald, O. A., and Lane, M. D. (1995) *Annu. Rev. Biochem.* **64,** 345–373
5. Rosen, E. D., and Spiegelman, B. M. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 145–171
6. MacDougald, O. A., and Mandrup, S. (2002) *Trends Endocrinol Metab* **13,** 5–11
7. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. (2000) *Science* **289,** 950–953
8. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996) *Nature* **382,** 225–230
9. Bejsovec, A. (2000) *Curr. Biol.* **10,** R919–R922
10. Malbon, C. C., Wang, H., and Moon, R. T. (2001) *Biochem. Biophys. Res. Commun.* **287,** 589–593
11. Malik, T. H., and Shivdasani, R. A. (2000) *Biochem. J.* **349,** 829–834
12. Papkoff, J., and Aikawa, M. (1998) *Biochem. Biophys. Res. Commun.* **247,** 851–858
13. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16,** 3797–3804
14. Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B., and Nathans, J. (1999) *Nature* **398,** 431–436
15. Uren, A., Reichsman, F., Anest, V., Taylor, W. G., Muraiso, K., Bottaro, D. P., Cumberledge, S., and Rubin, J. S. (2000) *J. Biol. Chem.* **275,** 4374–4382
16. Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001) *Nature* **411,** 321–325
17. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001) *Nat. Cell Biol.* **3,** 683–686
18. Hemati, N., Ross, S. E., Erickson, R. L., Groblewski, G. E., and MacDougald, O. A. (1997) *J. Biol. Chem.* **272,** 25913–25919
19. Erickson, R. L., Hemati, N., Ross, S. E., and MacDougald, O. A. (2001) *J. Biol. Chem.* **276,** 16348–16355
20. Blau, H. M., Chiu, C. P., and Webster, C. (1983) *Cell* **32,** 1171–1180
21. Oderwald, H., Hughes, M. J., and Jost, J. P. (1996) *FEBS Lett.* **382,** 313–318
22. Rubinfeld, B., Munemitsu, S., Clark, R., Conroy, L., Watt, K., Crosier, W. J., McCormick, F., and Polakis, P. (1991) *Cell* **65,** 1033–1042
23. Young, C. S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998) *Mol. Cell. Biol.* **18,** 2474–2485
24. Rodbell, M. (1964) *J. Biol. Chem.* **239,** 375–380
25. Cushman, S. W. (1970) *J. Cell Biol.* **46,** 326–341
26. Gavin, B. J., McMahon, J. A., and McMahon, A. P. (1990) *Genes Dev.* **4,** 2319–2332
27. Kaestner, K. H., Flores-Riveros, J. R., McLenithan, J. C., Janicot, M., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1933–1937
28. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., III, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001) *Mol Cell* **7,** 801–809
29. Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000) *Nature* **407,** 535–538
30. Hedgepeth, C. M., Conrad, L. J., Zhang, J., Huang, H. C., Lee, V. M., and Klein, P. S. (1997) *Dev. Biol.* **185,** 82–91
31. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552
32. Schwarz, E. J., Reginato, M. J., Shao, D., Krakow, S. L., and Lazar, M. A. (1997) *Mol. Cell. Biol.* **17,** 1552–1561
33. Kurebayashi, S., Sumitani, S., Kasayama, S., Jetten, A. M., and Hirose, T. (2001) *Endocr. J.* **48,** 249–253
34. Choy, L., Skillington, J., and Derynck, R. (2000) *J. Cell Biol.* **149,** 667–682
35. Chen, C. L., Brodie, A. E., and Hu, C. Y. (1997) *Obes. Res.* **5,** 146–152
36. Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K., and Kato, M. (2001) *Mol. Cell. Biol.* **21,** 5132–5141
37. Reusch, J. E., Colton, L. A., and Klemm, D. J. (2000) *Mol. Cell. Biol.* **20,** 1008–1020
38. Saitoh, T., Kirikoshi, H., Mine, T., and Katoh, M. (2001) *Int. J. Oncol.* **19,** 1187–1192
39. Soukas, A., Socci, N. D., Saatkamp, B. D., Novelli, S., and Friedman, J. M. (2001) *J. Biol. Chem.* **276,** 34167–34174
40. Lee, F. S., Lane, T. F., Kuo, A., Shackleford, G. M., and Leder, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2268–2272
41. Lane, T. F., and Leder, P. (1997) *Oncogene* **15,** 2133–2144
42. Reddy, S., Andl, T., Bagasra, A., Lu, M. M., Epstein, D. J., Morrisey, E. E., and Millar, S. E. (2001) *Mech. Dev.* **107,** 69–82
43. Ross, S., Erickson, R., Gerin, I., DeRose, P., Bajnok, L., Longo, K., Misek, D., Kuick, R., Hanash, S., Atkins, K., Andresen, S., Nebb, H., Madsen, L., Kristiansen, K., and MacDougald, O. (2002) *Mol. Cell. Biol.*, in press