



ARHGEF3 coordinates adipocyte hypertrophy and differentiation through dual YAP-RhoA and PPAR γ activation

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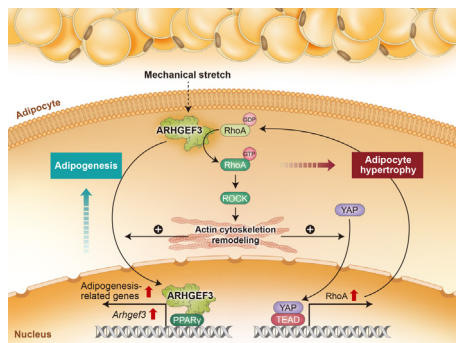
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HIGHLIGHTS

- ARHGEF3 regulates adipogenesis through RhoA-YAP signaling for hypertrophy while promoting differentiation via PPAR γ activation.
- ARHGEF3 deletion reduces fat accumulation by decreasing RhoA, impairing cytoskeletal reorganization and limiting adipocyte expansion.
- ARHGEF3 enables YAP nuclear transport to bind TEAD motifs on RhoA promoter, creating a feedback loop for cytoskeletal remodeling.
- ARHGEF3 and PPAR γ form a regulatory circuit: ARHGEF3 enhances PPAR γ activity while PPAR γ upregulates ARHGEF3, boosting adipogenesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Obesity presents a significant global health burden, necessitating insights into the molecular drivers of adipogenesis and adipose tissue regulation.

Objectives: This study investigates the role of Rho guanine nucleotide exchange factor 3 (ARHGEF3) in adipocyte differentiation and hypertrophy, focusing on its influence on adipogenesis and body weight regulation under high-fat diet conditions.

Methods: ARHGEF3^{-/-} mice and littermate controls were subjected to a high-fat diet (HFD) and underwent comprehensive metabolic phenotyping. In vitro studies in C3H10T1/2 cells were conducted to assess ARHGEF3's role in adipogenesis, utilizing quantitative PCR, western blotting, chromatin

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PPAR γ
 RhoA
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 Adipocyte hypertrophy

immunoprecipitation (ChIP), immunoprecipitation (IP), immunostaining, and luciferase reporter assays. **Results:** ARHGEF3 expression increased in white adipose tissue (WAT) of HFD-fed mice and during adipogenic differentiation in C3H10T1/2 cells. ARHGEF3-deficient mice exhibited reduced weight gain and adipocyte size, correlating with decreased RhoA expression and altered cytoskeletal dynamics. Additionally, ARHGEF3 facilitated yes-associated protein (YAP) nuclear translocation and its direct binding to the RhoA promoter, an effect reliant on ARHGEF3. ARHGEF3 also enhanced the transcriptional activity of peroxisome proliferator-activated receptor gamma (PPAR γ), establishing a reciprocal activation loop to drive adipocyte differentiation and hypertrophy.

Conclusion: ARHGEF3 emerges as a pivotal regulator of adipocyte dynamics by coordinating YAP-RhoA signaling and enhancing PPAR γ activity. These findings offer novel therapeutic insights for addressing obesity and related metabolic disorders.

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Introduction

Adipose tissue, once considered merely a site for energy storage, is now recognized as an endocrine organ crucial for maintaining metabolic homeostasis and overall health. To understand the origins of obesity and associated metabolic disorders, it is essential to investigate the complex processes governing adipogenesis, lipid metabolism, and systemic inflammation.

Rho guanine nucleotide exchange factor 3 (ARHGEF3), also known as XPLN (exchange factor found in platelets, leukemic, and neuronal tissues), plays a central role in facilitating the conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), specifically in Ras homolog family members A (RhoA) and B [1]. As a distinct member of the Dbl family, comprising over 70 Rho guanine nucleotide exchange factors (RhoGEFs), ARHGEF3 is composed of 526 amino acids and features a characteristic tandem arrangement of a Dbl homology (DH) domain at its N-terminus and a pleckstrin homology (PH) domain at its C-terminus [1]. In RhoGEFs, the DH domain typically binds Rho GTPases and functions as the catalytic domain [2], though in ARHGEF3, this domain is specifically tailored for interactions with RhoA and RhoB. The PH domain, primarily responsible for subcellular localization, is adapted for interacting with phosphoinositides such as PI(4,5)P2 [3]. Notably, the combined presence of both the DH and PH domains enhances its affinity for PI(3,5)P2, underscoring ARHGEF3's specificity in phosphoinositide binding and functional versatility [4].

Additionally, the C-terminus of ARHGEF3 seems to exert regulatory control by restricting lipid binding, a function that may be modulated by the N-terminal domain. Beyond its GEF activity, ARHGEF3 independently influences the mammalian target of rapamycin complex 2 (mTORC2)-Akt signaling pathway [5]. Notably, ARHGEF3 deficiency accelerates muscle regeneration after injury and improves strength recovery, a process mediated by ARHGEF3's GEF activity and autophagy, independent of Akt signaling [6]. Recent findings emphasize the significance of the ARHGEF3-Rho-associated protein kinase (ROCK)-autophagy pathway in addressing muscle weakness associated with Duchenne muscular dystrophy [7], underscoring its role in muscle physiology. Moreover, ARHGEF3 has emerged as a novel regulator of ATP-citrate lyase, an enzyme critical in converting citrate into acetyl-CoA and oxaloacetic acid in non-small cell lung cancer, thus promoting tumor growth by stabilizing ATP-citrate lyase through decreased acetylation and ubiquitination [8].

In adipose tissue development, GEFs and mTORC2 play integral roles, both regulated by ARHGEF3. The RhoA effector ROCK is a key regulator of the actin cytoskeleton dynamics [9], with inhibition of the RhoA/ROCK pathway enhancing early adipocyte progenitor recruitment and promoting preadipocyte differentiation into mature adipocytes [10]. Conversely, heightened RhoA/ROCK activity in adipose tissue leads to excessive F-actin assembly and altered actin-regulating protein expression, triggering adipocyte

stretching due to lipid accumulation and perpetuating a cycle of hypertrophy and compromised function [11]. Furthermore, reduced mTORC2 activity in adipocytes is associated with insulin resistance, largely due to diminished Akt signaling [12] and reduced lipid synthesis, which partly results from decreased carbohydrate response element-binding protein (ChREBP) β expression, a key transcription factor in lipid regulation [13]. As a dual regulator of RhoA and mTORC2, ARHGEF3 is likely to have an uncharted role in adipose tissue.

Given the link between adipogenesis and metabolic disorders like obesity, uncovering the mechanisms by which ARHGEF3 regulates adipocyte differentiation and hypertrophy may present new therapeutic targets. In this study, we aimed to investigate the multifaceted functions of ARHGEF3 in adipocyte biology using ARHGEF3 knockout mice. Our results demonstrate that ARHGEF3 facilitates the translocation and activation of yes-associated protein (YAP) to the nucleus, mediated by the RhoA pathway, which subsequently regulates RhoA expression. Additionally, we observed a reciprocal enhancement between ARHGEF3 expression and peroxisome proliferator-activated receptor gamma (PPAR γ) activity, underscoring the pivotal role of ARHGEF3 as a GEF in adipogenesis. These findings reveal the intricate mechanisms by which ARHGEF3 governs adipose size and differentiation, offering potential avenues for therapeutic interventions in obesity-related metabolic disorders.

Materials and methods

Antibodies and other reagents

The primary antibodies and reagents used in this study are listed in Table S1.

Animals and diet

The mice were housed under controlled and pathogen-free conditions maintained on a 12-h light/dark cycle. They were given ad libitum access to either a standard chow diet (PicoLab[®] Rodent Diet 20, 5053; LabDiet, St. Louis, MO, USA), which consisted of 24.651 % protein, 13.205 % fat, and 62.144 % carbohydrates by caloric content, or a high-fat diet (HFD) containing 20 % protein, 60 % fat, and 20 % carbohydrates (D12492, Research Diets, New Brunswick, NJ, USA). ARHGEF3 knockout (ARHGEF3^{-/-}) mice were characterized as previously reported [6]. Control mice for these experiments were littermate controls, homozygous for the wild-type ARHGEF3 allele.

Study approval

All animal care and treatments were conducted following the guidelines of the Institutional Animal Care and Use Committee of

the Center of Animal Care and Use at Lee Gil Ya Cancer and Diabetes Institute, Gachon University (approval numbers: LCDI-2017-0133 and LCDI-2020-0068).

Metabolic phenotyping

Body composition was assessed using the 1H-Minispec system (LF90II; Bruker Biospin, Billerica, MA, USA) at baseline and following 34 weeks of high-fat diet (HFD) feeding. Hepatic triglyceride and plasma non-esterified fatty acid levels were quantified utilizing colorimetric assay kits (279-75401, Wako Pure Chemical Industries, Osaka, Japan). Plasma triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, alanine transaminase, and aspartate transaminase levels were measured using a Cobas c111 analyzer (Roche, Basel, Switzerland). Glucose tolerance tests were conducted after a 16-h fast by administering an intraperitoneal glucose injection (1.5 g/kg body weight), followed by blood glucose measurements at 0, 15, 30, 60, 120, and 180-min post-injection using a glucose analyzer (GM9; Analox Instruments Ltd., Stokesley, UK).

Adipose tissue analysis

Perigonadal adipose tissues were fixed in 10 % neutral-buffered formalin (BBC Biochemical, Mount Vernon, WA, USA), followed by standard paraffin embedding. Sections were cut at a thickness of 5 μ m and mounted onto slides. Hematoxylin and eosin (H&E) staining for histological analysis was performed by the Core Facility at Gachon University. The stained sections were then digitized using the PANNORAMIC SCAN II scanner (3D Histech Ltd., Budapest, Hungary). The Panoramic Viewer software captured six randomly selected images under a 10 $\times$ objective lens for each animal. Adipocyte size and number were subsequently quantified using the Adiposoft plug-in (version 1.15) within ImageJ software.

Cell culture and adipogenic differentiation

C3H10T1/2 preadipocytes were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose and 10 % fetal bovine serum (FBS) (M1) at 37 °C in 5 % CO₂. The cells reached confluence two days after seeding (designated as day 0). Differentiation was initiated in the M2 medium (DMEM with 10 % FBS, 1.0 μ M dexamethasone, 0.5 mM IBMX, and 1.5 μ g/mL insulin). After 48 h, the medium was replaced with M3 (DMEM with 10 % FBS and 1.5 μ g/mL insulin), which was replenished every two days. Samples were collected at specific time points for real-time PCR, western blotting, and Oil Red O staining.

Protein extraction and western blot analysis

Total protein was extracted from inguinal white adipose tissue (iWAT) (100 mg) using a lysis buffer specifically formulated for adipose tissue (50 mM Tris-HCl pH 8.0, 0.25 M NaCl, 5 mM EDTA, 1 % Triton X-100). Other tissues (30 mg) were lysed using the T-PERTM Tissue Protein Extraction Reagent (#78510, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease (#04693159001, Roche, Basel, Switzerland) and phosphatase inhibitors (Roche, #04906837001). Tissue samples were homogenized using steel beads and a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 1–2 min. C3H10T1/2 cells were rinsed with phosphate-buffered saline (PBS) and lysed using a lysis buffer (#9303, Cell Signaling Technology, Danvers, MA, USA). Lysates were centrifuged at 13,000 $\times$ g for 10 min at 4 °C, and the supernatants were collected

for further analysis. Subsequently, the samples were boiled for 3 min at 100 °C before loading onto SDS-polyacrylamide gels for electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (PVDF) (Millipore, Billerica, MA, USA) and blocked with 5 % (w/v) skim milk in Tris-buffered saline with 1 % Tween-20 for 30 min at room temperature. Primary antibodies were incubated with membranes overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies. Detection was carried out using Immobilon western Chemiluminescent HRP Substrate (Millipore), and the signals were captured using an iBright 1500 imaging system (Thermo Fisher Scientific). Band intensities were quantified using ImageJ software [14].

Immunoprecipitation

C3H10T1/2 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % NP-40) supplemented with a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA). The lysates were incubated at 4 °C with anti-Flag M2 affinity gels (A2220, Sigma-Aldrich) for 1 h for immunoprecipitation. Alternatively, lysates were incubated overnight with specific antibodies, followed by incubation with protein G-agarose (16–266, Millipore) for 1 h. After centrifugation at 10,000 rpm for 1 min, the beads were washed three times with RIPA buffer and resuspended in 30 μ L of 2 $\times$ SDS sample buffer. Protein samples were then boiled for 3 min at 100 °C and subjected to western blot analysis as described previously.

Cytosolic and nuclear Fractionation

We followed previously established methods to isolate cytosolic and nuclear fractions [14]. C3H10T1/2 cells were washed three times with cold PBS and then lysed in 300 μ L of cytoplasmic protein extraction buffer (5 mM KCl, 5 mM HEPES pH 7.9, 0.05 mM ethylene glycol-bis (β -aminoethyl ether)-N, N, N, N-tetra acetic acid, 0.05 mM EDTA, and 0.075 % NP-40). The lysates were incubated on ice for 30 min, shaking every 10 min. After centrifugation at 3,000 $\times$ g for 5 min at 4 °C, the cytosolic extract was collected, and the remaining nuclear pellet was washed three times with cytoplasmic extraction buffer. The nuclear pellet was then resuspended in 100 μ L of nuclear extraction buffer (0.25 M EDTA, 0.5 M Tris-HCl pH 7.4, 2.5 M NaCl, 5 % sodium deoxycholate, 5 % SDS, and 5 % Triton X-100), and incubated on ice for 30 min with shaking every 10 min. Nuclear extracts were collected by centrifugation at 13,000 $\times$ g for 30 min at 4 °C. The resulting supernatant was mixed with an SDS sample buffer, boiled for 3 min at 100 °C, and then subjected to SDS-PAGE. Proteins were analyzed by western blotting.

Preparation of F-actin and G-actin fractions

F-actin and G-actin fractions were prepared using the G-Actin/F-actin In Vivo Assay Kit (BK037; Cytoskeleton, Denver, CO, USA) following the manufacturer's protocol. Cells were treated with LAS01 buffer (1 mL of Lysis and F-actin Stabilization Buffer, 10 μ L of ATP stock solution (100 mM), and 10 μ L of 100 $\times$ protease cocktail inhibitor) to facilitate lysis. The mixture was gently homogenized by pipetting, incubated at 37 °C for 10 min, and centrifuged at 350 $\times$ g for 5 min to pellet unbroken cells. After discarding cell debris, supernatants were centrifuged at 100,000 $\times$ g at 37 °C for 1 h to separate F-actin (pellet) from soluble G-actin (supernatant). A F-actin depolymerization buffer was added to the pellet to induce actin depolymerization. As previously described [14], both pellet and supernatant samples were mixed with 5 $\times$ SDS sample buffer and analyzed by western blotting.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from C3H10T1/2 cells, iWAT (100 mg), and other tissues (30 mg) using the TRIzol reagent (CC0926-928, Favorgen Biotech Corp., Ping-Tung, Taiwan). Subsequently, 25 ng/ μ L RNA was used to synthesize cDNA employing TOPscript RT Dry-MIX (dT18 plus), according to the manufacturer's instructions (Enzynomics, Daejeon, South Korea). qRT-PCR was conducted using TOPreal qPCR 2X PreMIX (SYBR Green with high ROX; Enzynomics) and a CFX384 C1000 thermal cycler (Bio-Rad, Hercules, CA, USA). Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclophilin A (CypA) expression levels. The primers used are listed in Table S2.

Transduction with shRNA and transfection

Lentiviral shRNA constructs targeting mouse YAP and the transcriptional coactivator with a PDZ-binding domain (TAZ) were obtained from Sigma-Aldrich;

YAP shRNA#1 (shY#1; TRCN0000238432; GAAGCGCTGAGTTC CGAAATC),

YAP shRNA#2 (shY#2; TRCN0000095866; CGGTGAAACAAC AGGAATTA),

TAZ shRNA (shTAZ; TRCN 0000121047; CCAGGATTCAAGCA CAGCTAA).

ARHGEF3 shRNA#1 (shA#1; TRCN0000110045, CCAGTTTCAT-TAGCTCTCATT), ARHGEF3 shRNA #2 (shA#2; TRCN 0000047546, CGCAAAGTAGATCTCTGGAAT), along with a negative control shRNA containing a scrambled hairpin sequence, were used as previously described [5]. These constructs were packaged into lentiviruses using 293L cells, following established protocols [14]. Transduction of C3H10T1/2 cells was carried out in a growth media supplemented with 8 μ g/mL polybrene and 2 μ g/mL puromycin for a selection period of five days. For plasmid transfection, C3H10T1/2 cells were transfected using the Lipofectamine 3000 reagent (Invitrogen, Thermo Fisher Scientific) and the 4D-Nucleofector-X kit (Lonza-V4XP-2012, AAF-1002X, Lonza, Basel, Switzerland) according to the manufacturer's protocol.

Fluorescence imaging

C3H10T1/2 cells were cultured in 8-well chamber plates (154941; Thermo Fisher Scientific) and fixed with a 3.7 % formaldehyde solution. Cells were permeabilized with 0.1 % Triton X-100 for 10 min, followed by blocking with 3 % bovine serum albumin for 30 min. The cells were incubated overnight at 4 °C with an anti-Flag M2 antibody (F1804, Sigma-Aldrich) in a blocking solution. The next day, cells were treated with Zenon mouse IgG and Alexa Fluor 488 (Z25202; Basel, Switzerland) or Alexa Fluor 555 (A21424; Invitrogen, Thermo Fisher Scientific). To visualize stress fibers, cells were incubated with rhodamine-phalloidin (ab235138; Abcam, Cambridge, UK) at a dilution of 1:2000. Nuclei were counterstained using Vectashield antifade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories, Burlingame, CA, USA). Images were captured using Zeiss-LSM-700 (Carl Zeiss, Oberkochen, Germany) and Leica Thunder imager (Leica Microsystems, Wetzlar, Germany).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [14]. Briefly, cells were treated with 0.75 % formaldehyde for 30 min, then sonicated at 20 Hz using ChIP lysis buffer (50 mM Tris-HCl pH 7.4, 1 % Igepal CA-630, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 0.5 mM dithiothreitol, 5 mM sodium butyrate, protease inhibitor cocktail (P8340, Sigma-Aldrich), phosphatase inhibitor

cocktail I (P2850, Sigma-Aldrich), and phosphatase inhibitor cocktail II (P5726, Sigma-Aldrich). Nuclei were collected, sonicated, and then centrifuged at 14,000 $\times$ g for 10 min. The supernatant was incubated overnight with anti-YAP or anti-PPAR γ antibodies. Protein A/G-PLUS-agarose beads (SC-2003, Santa Cruz Biotechnology, Dallas, TX, USA) were added for precipitation. Beads were washed, and the DNA was eluted with a buffer containing 1 % SDS and 100 mM NaHCO₃. Following RNA and protein removal with RNase and proteinase K, DNA was purified using phenol–chloroform extraction and analyzed by PCR targeting PPRE and TEAD. The primers are listed in Table S3.

Oil Red O staining

Lipid accumulation in C3H10T1/2 cells was evaluated via Oil Red O (ORO) staining (O0625, Sigma-Aldrich). A stock solution of ORO in isopropanol (3.5 mg/mL) was prepared and filtered. The working solution was made by diluting the stock solution 2:3 with distilled water, filtered through a 0.2- μ m filter. Cells were cultured on coverslips, washed with PBS, fixed with 3.7 % formaldehyde in PBS for 10 min at room temperature, and further incubated with fresh formalin for 1 h. After washing with distilled water and 60 % isopropanol, cells were stained with ORO solution for 30 min at room temperature. Following four-time washes, images were taken using an Imager Z1 (Zeiss) microscope (Carl Zeiss). To quantify staining, 100 % isopropanol was used to elute the dye, and absorbance was measured at 492 nm using a Victor NivoTM plate reader (PerkinElmer, Waltham, MA, USA).

Cloning

We replaced the YAP sequence from HA-YAP WT (Addgene, #27007) with pCMV-Flag-YAP5SA mutant (Addgene, #2731) with KpnI and XbaI restriction enzymes to construct Flag-YAP WT. For the promoter analysis of ARHGEF3 and RhoA, we amplified DNA fragments encoding the RhoA promoter region containing TEAD binding site (GGAATC) from –80 to –74, as well as the ARHGEF3 promoter region containing the PPRE binding site (AGGTA) from –157 to –151. Additionally, we generated a construct of the ARHGEF3 promoter region with the PPRE site deleted. These DNA fragments were amplified using the Q5 High-fidelity DNA polymerase kit (M0491, New England BioLabs, Ipswich, MA, USA) and then inserted into the pGL4.20 luciferase reporter vector (Promega, Madison, WI, USA) using KpnI and XhoI restriction enzymes. Ligation was performed using the EZ-Fusion™ HT Cloning Kit (EZ015TM, Enzynomics). For ARHGEF3 overexpression, we subcloned wild-type ARHGEF3 and its two mutants, W440L and L269E [5], from previously generated constructs into the pLVX-puro vector under the control of the CMV promoter region using XhoI and BamHI restriction enzymes. Ligation was again performed using the EZ-Fusion™ HT Cloning Kit. The EGFP-ARHGEF3-wild type (WT) and its mutant EGFP-ARHGEF3-K342E/K348E were described in prior studies [4].

Luciferase reporter assay

C3H10T1/2 cells were co-transfected with a luciferase promoter-reporter and a vector expressing β -galactosidase alongside a DNA plasmid, as the figure legend details. Following cell lysis, the luciferase activity was assessed using the Luciferase Assay System Kit (E1500, Promega) according to the manufacturer's instructions. Concurrently, β -galactosidase activity was measured using the β -galactosidase Enzyme Assay System (E2000, Promega). Luminescence intensity was recorded with a Victor Nivo™ multimode microplate reader (PerkinElmer). To ensure accuracy, luciferase activity was normalized against β -

galactosidase activity to account for variations in transfection efficiency.

Statistical analysis

Data are expressed as means $\pm$ standard deviation (SD), with n indicating the number of independent experiments or individual mouse phenotypes used. Random allocation was applied to the experimental groups. Each experiment, whether in vitro or in vivo, was replicated in at least three independent trials (ranging from three to six across the figures). In the quantified graphs, individual data points are represented as dots. Statistical significance was evaluated using a two-tailed paired Student's t -test with GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA) for Microsoft Windows, with a significance threshold set at $p < 0.05$.

Results

ARHGEF3 is predominantly expressed in adipose tissue and upregulated during adipogenesis

To investigate ARHGEF3's role in weight gain, we examined its expression in the adipose tissue of mice on a high-fat diet (HFD). ARHGEF3 mRNA levels were significantly elevated in inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) of HFD-fed mice compared to those on a regular chow diet, whereas no significant changes were observed in the liver, tibialis anterior muscle, or gastrocnemius muscle (Fig. 1A). Similarly, Western blot analysis confirmed increased ARHGEF3 protein levels in iWAT and eWAT of HFD-fed mice, while expression in other tissues remained unchanged (Fig. 1B). Given the well-documented effects of long-term HFD feeding on hepatic lipid accumulation, we further assessed whether ARHGEF3 expression is altered in the liver under these conditions. We reanalyzed ARHGEF3 mRNA expres-

sion in the liver of C57Bl/6J mice fed an HFD for 4 or 8 weeks. Our results showed a mild but not statistically significant upregulation of ARHGEF3, despite a significant increase in body weight, liver weight, and triglyceride content (Fig. S1). These findings suggest that ARHGEF3 expression remains largely unchanged in the liver, even in the presence of HFD-induced fatty liver development, further reinforcing its primary role in adipose tissue. To further characterize ARHGEF3 expression in adipose tissue, we analyzed publicly available single-nucleus RNA sequencing (snRNA-seq) data from human adipose tissue [15], which confirmed ARHGEF3 expression in adipocytes and fibroblast-like adipose progenitors (FAPs) (Fig. S2). Additionally, we examined ARHGEF3 expression during adipogenesis in vitro. ARHGEF3 mRNA and protein levels increased significantly during the 8-day differentiation of C3H10T1/2 cells into adipocytes, further supporting its involvement in adipogenesis (Fig. 1C and D).

ARHGEF3 deficiency mitigates weight gain and adiposity in mice fed a high-fat diet

To assess ARHGEF3's function in adipose tissue under HFD, we utilized ARHGEF3^{-/-} mice, which carry a 17-base pair deletion in exon 3, generated via transcription activator-like effector nucleases (TALEN) technology [6]. We confirmed the absence of ARHGEF3 mRNA expression in ARHGEF3^{-/-} mice (Fig. 2A). After 14 weeks on an HFD, ARHGEF3^{-/-} mice exhibited significantly reduced body weight compared to ARHGEF3^{+/+} mice, a trend that persisted throughout the 34-week feeding period (Fig. 2B). Additionally, ARHGEF3^{-/-} mice had significantly lower fat mass while maintaining similar lean mass compared to ARHGEF3^{+/+} mice (Fig. 2C and Fig. S3A). However, ARHGEF3^{-/-} mice under regular chow diet did not show significant differences in body weight, fat mass, or lean mass compared to ARHGEF3^{+/+} mice (Fig. S3B), suggesting that ARHGEF3's role in body composition is specific to

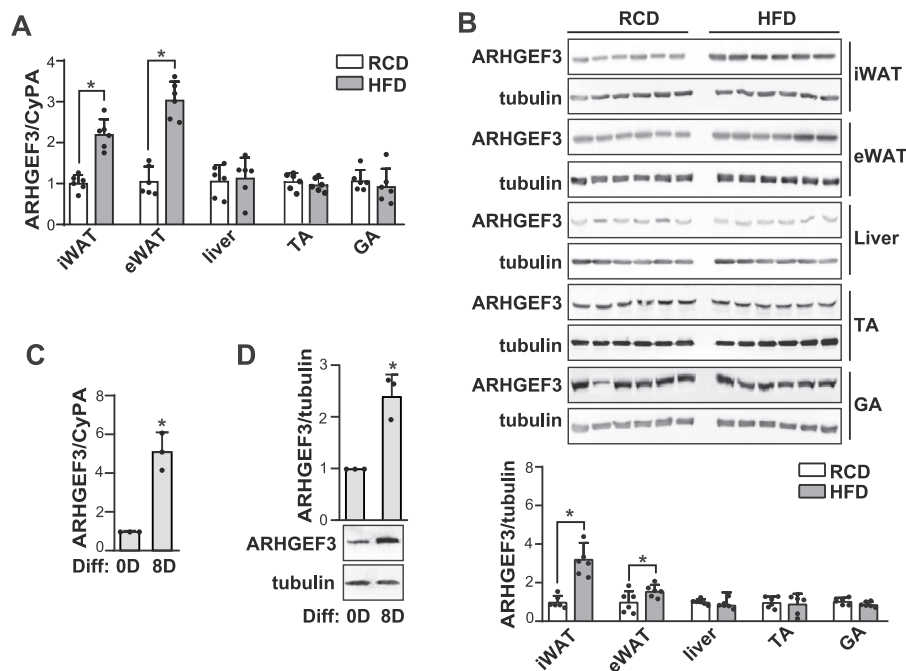


Fig. 1. ARHGEF3 expression is upregulated in adipose tissue under high-fat diet conditions and during adipogenic differentiation. (A) ARHGEF3 mRNA expression was analyzed in inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), liver, tibialis anterior (TA), and gastrocnemius (GA) tissues of mice fed an HFD for 15 weeks compared to regular chow diet (RCD)-fed controls ($n = 6$) using qRT-PCR. (B) Protein levels of ARHGEF3 in iWAT, eWAT, liver, TA, and GA were evaluated by western blotting ($n = 6$). (C–D) ARHGEF3 expression during the 8-day differentiation of C3H10T1/2 preadipocytes was assessed by qRT-PCR (C) and western blotting analysis (D) ($n = 3$). Band intensities were quantified using ImageJ software for panels (B) and (D). Data are presented as mean $\pm$ standard deviation. Statistical significance was determined by unpaired Student's t -test, with $*p < 0.05$ indicating significant differences compared to RCD-fed mice or undifferentiated controls (Day 0).

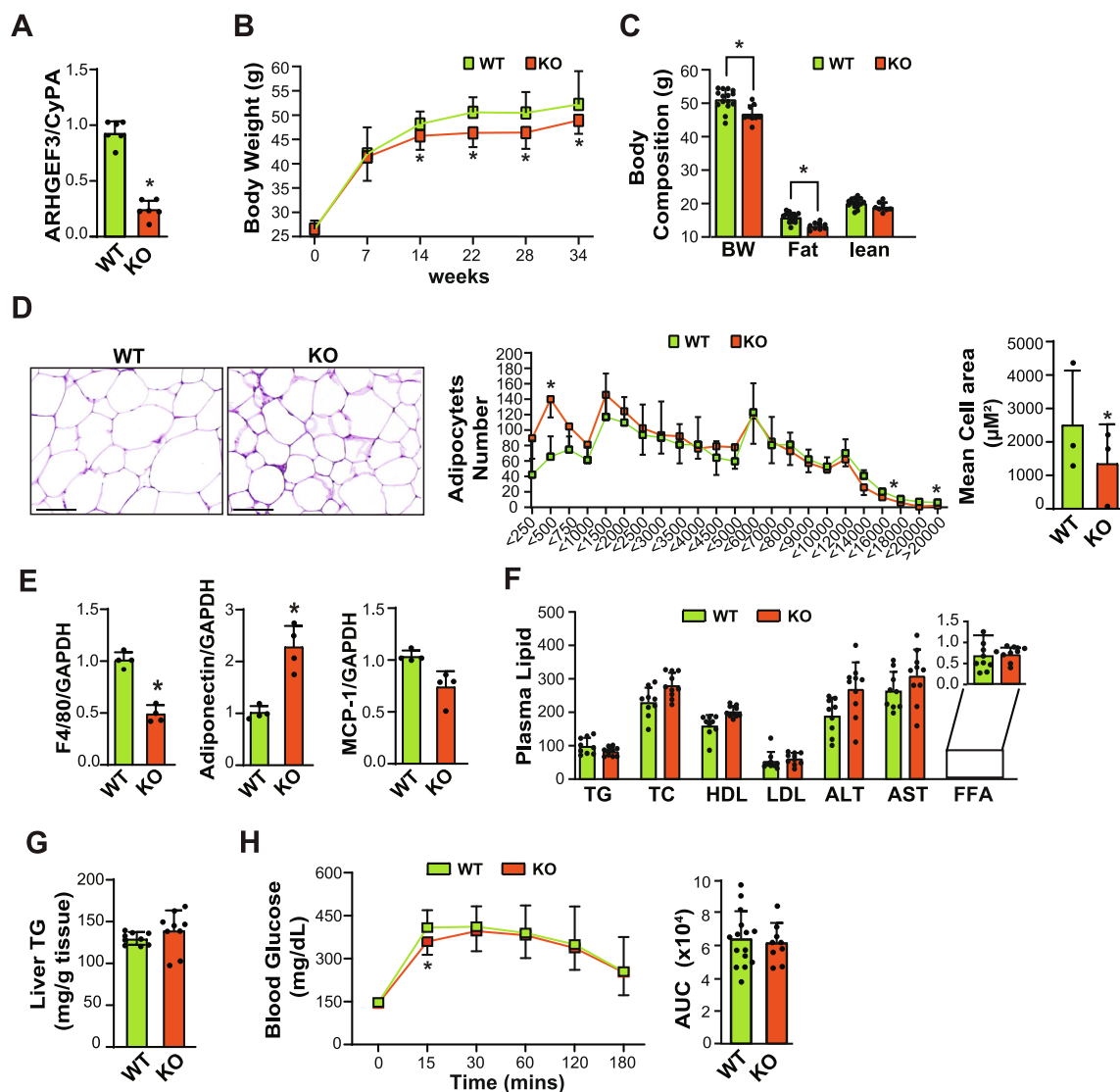
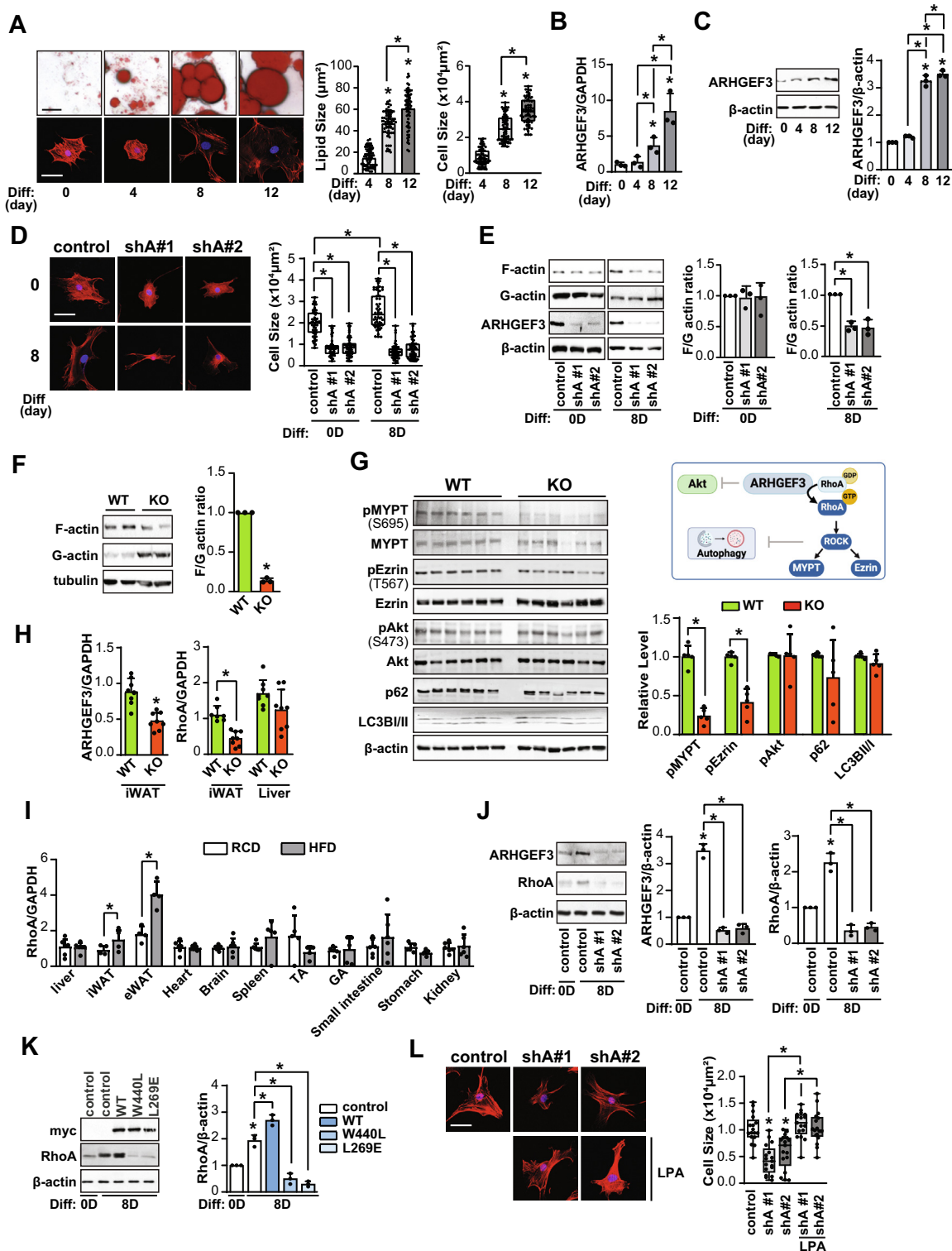


Fig. 2. Deficiency of ARHGEF3 reduces weight gain and adiposity in mice fed a high-fat diet (HFD). (A) ARHGEF3 mRNA expression in inguinal white adipose tissue (iWAT) was analyzed using qRT-PCR in wild-type (WT, ARHGEF3^{+/+}) and ARHGEF3 knockout (KO, ARHGEF3^{-/-}) mice (n = 6) after 34 weeks on an HFD. (B) Weekly measurements of body weight were recorded for WT and ARHGEF3 KO mice throughout the 34-week HFD feeding period (n = 15). (C) Body composition analysis, including body weight (BW), whole-body fat mass (fat), and lean mass (lean), was performed in WT and ARHGEF3 KO mice after 34 weeks on an HFD (n = 15). (D) Representative hematoxylin-eosin (H&E) staining images (left) and mean cell area measurements (right) were obtained from iWAT of WT and ARHGEF3 KO mice after 34 weeks on an HFD. Scale bar = 100 μm. (E) qRT-PCR was used to assess the expression of F4/80 (macrophage marker), adiponectin (adipokine), and MCP-1 (inflammatory marker) in iWAT from WT and ARHGEF3 KO mice (n = 4). (F) Serum biochemical profiles were compared between WT and ARHGEF3 KO mice (n = 10). TG: triglyceride, TC: total cholesterol, HDL: high-density lipoprotein, LDL: low-density lipoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, FFA: free fatty acids. (G) Liver triglyceride (TG) levels were analyzed in WT and ARHGEF3 KO mice after 34 weeks of HFD feeding (n = 9). (H) An intraperitoneal glucose tolerance test (GTT) was performed in mice after 34 weeks on an HFD, with area under the curve (AUC) calculated (n = 15). Data are presented as mean ± standard deviation. Statistical analysis was performed using an unpaired Student's *t*-test with significance set at *p* < 0.05. Asterisks (*) indicate significant differences compared to WT mice.

Fig. 3. ARHGEF3 regulates cytoskeletal reorganization and adipocyte size by modulating RhoA expression through GEF-mediated activity. (A) C3H10T1/2 cells were differentiated for 4, 8, or 12 days, followed by Oil Red O staining for lipid droplets and phalloidin-rhodamine staining for F-actin visualization. Lipid droplet size and F-actin-positive areas were quantified using ImageJ. Scale bar = 100 μm. (B-C) C3H10T1/2 cells differentiated as described in (A) were harvested for qRT-PCR analyses (B) and western blotting (C). (D) C3H10T1/2 preadipocytes transduced with control and ARHGEF3-targeting shRNAs (shARHGEF3 #1, shA#1; shARHGEF3 #2, shA#2) were selected with 1.5 μM puromycin for 5 days and subsequently differentiated for 8 days, followed by phalloidin-rhodamine staining. Scale bar = 100 μm. (E) F-actin and G-actin fractions were isolated from undifferentiated or 8-day differentiated C3H10T1/2 cells and analyzed by western blotting. The F-actin/G-actin ratio was calculated as the band intensity of F-actin normalized to β-actin divided by the band intensity of G-actin normalized to β-actin. (F) F-actin and G-actin fractions were isolated from inguinal white adipose tissue (iWAT) of high-fat diet (HFD)-fed wild-type (WT, ARHGEF3^{+/+}) and ARHGEF3 knockout (KO, ARHGEF3^{-/-}) mice and analyzed by western blotting (n = 3). (G) Western blot analysis of iWAT from HFD-fed WT and ARHGEF3 KO mice (n = 6). Schematic illustration was created using BioRender.com. (H) qRT-PCR analysis of ARHGEF3 and RhoA mRNA levels in iWAT and liver tissues from WT and ARHGEF3 KO mice (n = 8). (I) qRT-PCR analysis of RhoA expression in various tissues from HFD-fed mice after 15 weeks (n = 4–6). (J) C3H10T1/2 preadipocytes transduced with control or ARHGEF3-targeting shRNAs were differentiated for 8 days and analyzed by western blotting (n = 3). (K) C3H10T1/2 cells were transfected with Myc-tagged wild-type ARHGEF3 (WT) or GEF activity-deficient mutants (W440L, L269E) and differentiated for 8 days, followed by western blotting. (L) C3H10T1/2 preadipocytes transduced with control and ARHGEF3-targeting shRNAs as described in (D) followed by treatment with 10 μM lysophosphatidic acid (LPA), a RhoA activator, for 6 h. The cells were stained with phalloidin-rhodamine. Scale bar = 100 μm. Protein band intensities were quantified using ImageJ, with phosphorylated forms normalized to corresponding total protein levels and other proteins normalized to β-actin as loading control. The size measurements of lipid droplet and adipocyte were obtained by manually tracing cell boundaries, followed by quantification using the 'Measure' function in ImageJ. Data are expressed as mean ± standard deviation. Statistical significance was determined using an unpaired Student's *t*-test, with *p* < 0.05 considered significant. Asterisks (*) indicate significant differences (*p* < 0.05) compared to WT mice, undifferentiated controls (Day 0), or 8-day differentiated controls as appropriate.

high-fat dietary conditions. In HFD-fed mice, further analysis revealed that iWAT adipocytes in HFD-fed ARHGEF3^{-/-} mice were smaller in both average size and cell size distribution compared to those in ARHGEF3^{+/+} mice (Fig. 2D). This reduction in adipocyte size was accompanied by decreased expression of the proinflammatory gene F4/80 and elevated levels of adiponectin, an adipokine inversely associated with adiposity [16], in the iWAT of ARHGEF3^{-/-} mice (Fig. 2E). However, MCP-1 expression, a chemokine involved in immune cell recruitment, remained unchanged.

We also examined metabolic parameters in both genotypes. Plasma lipid levels, along with alanine transaminase (ALT) and aspartate transaminase (AST) levels—markers of liver function [17]—were not significantly different between ARHGEF3^{-/-} and ARHGEF3^{+/+} mice (Fig. 2F). Similarly, hepatic triglyceride levels were comparable between the two groups (Fig. 2G), and ARHGEF3^{-/-} mice showed no differences in insulin sensitivity compared to wild-type mice, as indicated by glucose tolerance tests (Fig. 2H).



ARHGEF3 regulates adipocyte hypertrophy and cytoskeletal reorganization via RhoA-mediated F-actin dynamics

Adipocyte development and hypertrophy involve cell size and shape changes that exert mechanical stress on the cytoskeleton, promoting F-actin reorganization [18,19,20]. During adipogenesis, cytoplasmic F-actin stress fibers were reduced, as shown by rhodamine-phalloidin staining, particularly on days 8 and 12 of differentiation (Fig. 3A). This restructuring coincided with increased ARHGEF3 mRNA and protein levels (Fig. 3B and C). Proper cortical F-actin remodeling is essential for lipid droplet integrity, as disruptions can alter lipid droplet size and number, ultimately impairing adipocyte function [21–23]. Consistent with ARHGEF3's role in cytoskeletal organization, ARHGEF3-knockdown C3H10T1/2 adipocytes displayed a condensed cytoskeleton and reduced cell size compared to controls (Fig. 3D). This was accompanied by a reduction in F-actin / G-actin ratio 8 days after adipocyte differentiation, indicating decreased polymerized actin filaments and increased monomeric actin content (Fig. 3E). Importantly, adipose tissue from ARHGEF3^{-/-} mice exhibited similar alterations in F-actin to G-actin ratio (Fig. 3F), confirming that these cytoskeletal abnormalities occur both in vitro and in vivo. To investigate the molecular mechanism by which ARHGEF3 deficiency affects adipocyte cytoskeleton, we examined RhoA signaling in iWAT of HFD-fed ARHGEF3 knockout mice. We observed a significant decrease in phosphorylation of Ezrin at Thr567 and myosin phosphatase target (MYPT) at Ser695, key downstream targets of ROCK, indicating diminished RhoGEF signaling (Fig. 3G). However, Akt phosphorylation at Ser473—a key mTORC2 target—was unaffected, and autophagic flux remained stable, as evidenced by a mild decrease in p62 and unaltered LC3B II/I level (Fig. 3G). Similar changes in signaling were observed in ARHGEF3 knockdown C3H10T1/2 cells (Fig. S4). We next examined the relationship between ARHGEF3 and RhoA expression. ARHGEF3 knockout significantly reduced RhoA mRNA levels in iWAT but not in the liver (Fig. 3H). Under HFD conditions, RhoA expression was upregulated in iWAT and eWAT, but remained unchanged in other tissues (Fig. 3I). In C3H10T1/2 cells, ARHGEF3 knockdown reduced RhoA protein levels, during 8-day differentiation (Fig. 3J). Conversely, ARHGEF3 overexpression elevated RhoA levels, while expression of GEF-inactive ARHGEF3 mutants (W440L and L269E) markedly reduced RhoA levels (Fig. 3K), demonstrating the requirement for ARHGEF3's GEF activity. To establish a direct link between ARHGEF3 and RhoA in cytoskeletal regulation, we treated ARHGEF3 knockdown cells with lysophosphatidic acid (LPA), a known RhoA activator. LPA treatment restored actin fiber formation and cell size to levels comparable to control cells (Fig. 3L). These findings indicate that ARHGEF3 regulates adipocyte cytoskeletal organization and size through GEF-dependent modulation of RhoA expression and activity.

YAP regulates RhoA expression in an ARHGEF3-dependent manner

Building on our previous findings regarding ARHGEF3's role in adipocyte hypertrophy, we next investigated the relationship between mechanotransduction and RhoA regulation. During adipogenesis, cytoplasmic F-actin stress fibers undergo reorganization and reduction at the cortical region [20]. These mechanical alterations during hypertrophy development influence the mechanotransducers YAP and TAZ, which act as transcriptional coactivators, translating physical changes into cell-specific transcriptional responses [20,24]. As a transcriptional coactivator, YAP modulates the expression of target genes through interactions with transcription factors such as transcriptional enhanced association domain (TEAD) [25]. While YAP has been reported as down-

stream of RhoA [26], we identified a potential TEAD binding motif within the promoter region of RhoA at -80 to -74 (Fig. 4A). Reduced YAP expression led to a decrease in RhoA expression (Fig. 4B), whereas YAP overexpression enhanced RhoA levels (Fig. 4C). Consistently, the mRNA levels of YAP target genes—including Cysteine-rich angiogenic inducer 61 (CYR61), Ankyrin repeat domain 1 (ANKRD1), and connective tissue growth factor (CTGF), which are reported to be increased in hypertrophic adipocytes [20], were significantly reduced in YAP knockdown C3H10T1/2 adipocytes (Fig. S5). Conversely, TAZ knockdown did not affect RhoA protein levels (Fig. 4D), suggesting a specific role for YAP rather than TAZ in RhoA regulation. To investigate the potential role of YAP in regulating RhoA expression, we performed chromatin immunoprecipitation (ChIP) assays with an anti-YAP antibody. Our findings indicate that YAP directly binds to the TEAD-binding motif within the RhoA promoter, with this binding being dependent on ARHGEF3 in differentiated C3H10T1/2 cells (Fig. 4E). Consistent with this, ARHGEF3 knockdown led to reduced mRNA levels of YAP target genes (Fig. 4F), while overexpression of ARHGEF3 WT enhanced them (Fig. 4G). Notably, the ARHGEF3 mutant K342E/K348E, which lacks PI (4,5) P2 binding and GEF activity [4], decreased mRNA levels of YAP target genes (Fig. 4G), confirming that YAP regulation is dependent on ARHGEF3 with plasma membrane localization and GEF activity. Furthermore, YAP overexpression enhanced RhoA promoter activity, with co-expression of ARHGEF3 and YAP further amplifying this effect (Fig. 4H), aligning with the observed RhoA mRNA levels (Fig. 4I). In contrast, the ARHGEF3 mutant K342E/K348E did not influence RhoA promoter activity or RhoA mRNA levels (Fig. 4H and I), regardless of YAP overexpression, further supporting that YAP regulation is dependent on both plasma membrane localization and the GEF activity of ARHGEF3. Collectively, these findings reveal a novel regulatory mechanism in which ARHGEF3 functions as an upstream regulator of YAP, which in turn controls RhoA expression through direct binding to its promoter.

ARHGEF3 facilitates YAP nuclear translocation through GEF-mediated RhoA activation

To explore the role of ARHGEF3 in YAP-mediated regulation of RhoA expression, we examined its effect on YAP localization during adipogenesis. YAP nuclear translocation relies on an active RhoA pathway, functioning independently of the Hippo pathway [26]. We observed enhanced nuclear localization of YAP after days 8 and 12 following adipogenesis induction (Fig. 5A). Correspondingly, the mRNA level of YAP target genes, such as CYR61, ANKRD1, and CTGF, significantly increased during this period (Fig. S6). Treatment with C3 exoenzyme, a Rho-GTPase inhibitor, or latrunculin B, an actin polymerization inhibitor, reduced the nuclear localization of YAP WT, as confirmed by anti-Flag immunostaining of Flag-YAP WT and Flag-YAP 5SA (Fig. 5B–E). However, the nuclear localization of the YAP5SA mutant, which primarily resides in the nucleus [27], remained unaffected by these treatments (Fig. 5B–E). EGFP-tagged ARHGEF3 WT was present in both the cytoplasm and the nucleus, whereas the ARHGEF3 K342E/K348E mutant, which lacks PI (4,5) P2 binding and GEF activity, localized exclusively to the nucleus (Fig. 5F and G), aligning with previous findings [4]. ARHGEF3 WT promoted YAP WT nuclear translocation, whereas the ARHGEF3 K342E/K348E mutant did not exert this effect (Fig. 5F and G). Furthermore, other GEF-deficient mutants (W440L, L269E) also resulted in reduced YAP nuclear localization (Fig. 5H). Supporting these results, ARHGEF3 overexpression further enhanced RhoA mRNA levels in cells overexpressing the YAP WT but did not enhance levels further in cells overexpressing the YAP5SA mutant, which was already localized in the nucleus (Fig. 5I), underscoring the importance of ARHGEF3's GEF activity

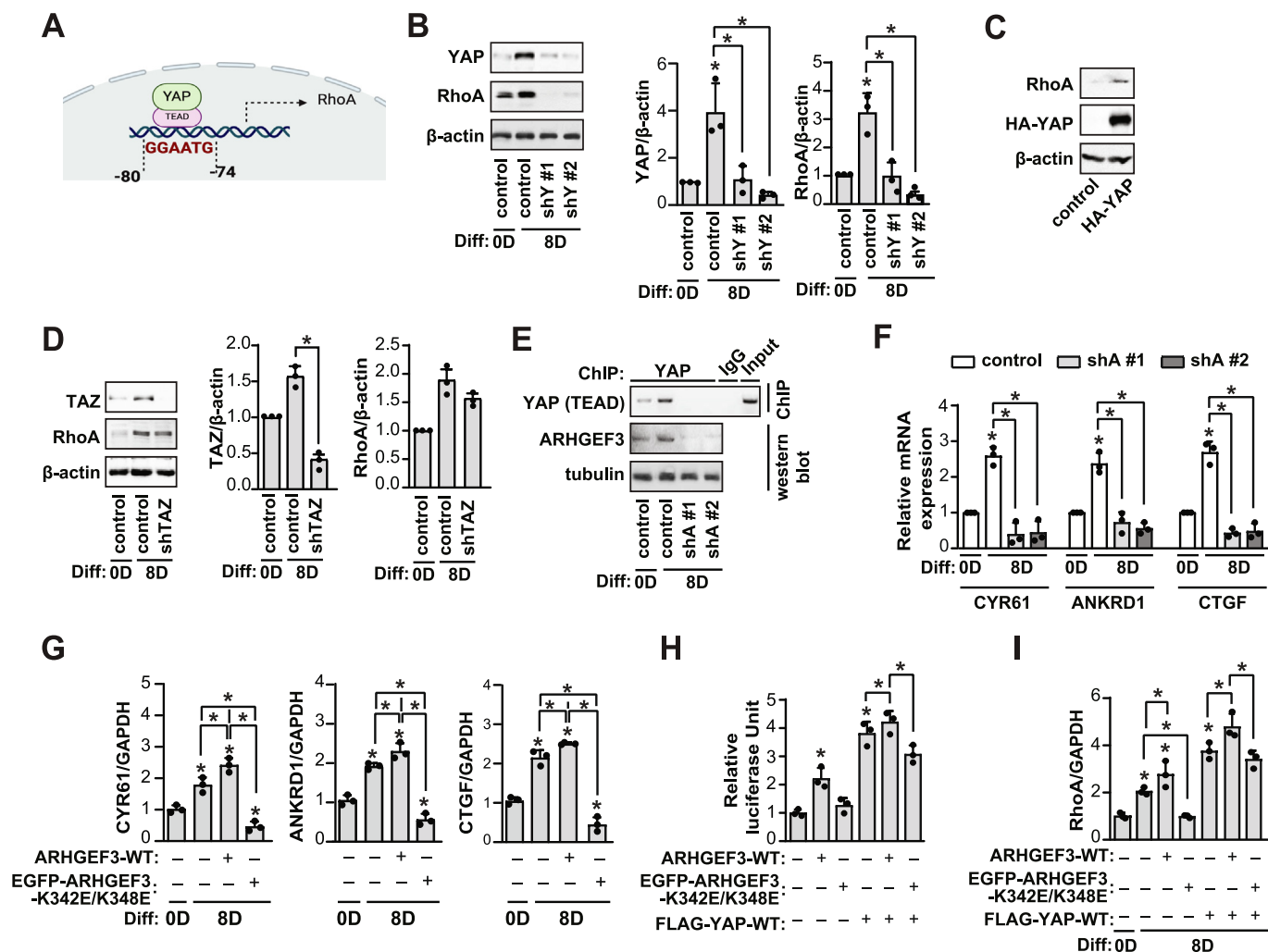


Fig. 4. YAP regulates RhoA expression dependent on ARHGEF3. (A) The promoter region of RhoA contains a predicted YAP/TEAD binding site at position –80 to –74 relative to the transcription start site. Schematic illustration was created using [BioRender.com](#). (B) C3H10T1/2 preadipocytes were transduced with control or YAP-targeting shRNAs (shYAP #1, shYAP #2, shYAP #3, shYAP #4), selected with 1.5 μ M puromycin for 5 days, and differentiated for 8 days. Cell lysates were analyzed by western blot for YAP and RhoA expression ($n = 3$). (C) C3H10T1/2 preadipocytes were transfected with HA-YAP WT, lysed, and subjected to western blot analysis to assess RhoA induction. (D) C3H10T1/2 preadipocytes were transduced with control and TAZ-targeting shRNAs, selected with puromycin for 5 days, differentiated for 8 days, and lysed for western blot analysis of TAZ and RhoA levels ($n = 3$). (E) C3H10T1/2 cells were transduced with control and ARHGEF3-targeting shRNAs (shARHGEF3 #1, shA#1; shARHGEF3 #2, shA#2), selected with puromycin for 5 days, and differentiated for 8 days. Chromatin immunoprecipitation (ChIP) was performed using anti-YAP antibodies, with input and IgG samples as controls to assess YAP binding to the RhoA promoter. PCR products were visualized by agarose gel electrophoresis. ARHGEF3 knockdown efficiency was confirmed by western blotting. (F) Cells treated as in (E) were lysed for qRT-PCR analysis of the expression of YAP target genes (CYR61, ANKRD1, and CTGF). (G) C3H10T1/2 cells were transfected with EGFP-ARHGEF3 WT or EGFP-ARHGEF3 K342E/K348E mutant, differentiated for 8 days, and analyzed by qRT-PCR for the expression of YAP target genes ($n = 3$). (H) C3H10T1/2 cells were transfected with a luciferase reporter containing the putative TEAD-binding site of RhoA along with Flag-YAP WT, EGFP-ARHGEF3 WT, or EGFP-ARHGEF3 K342E/K348E mutant. Cell lysates were subjected to luciferase assays to measure promoter activity ($n = 3$). (I) C3H10T1/2 cells were transfected with Flag-YAP WT, EGFP-ARHGEF3 WT, or EGFP-ARHGEF3 K342E/K348E, differentiated for 8 days, and analyzed by qRT-PCR for RhoA expression ($n = 3$). (B and D) Band intensities were quantified using ImageJ, normalized to β -actin levels. Data are presented as mean $\pm$ standard deviation. Statistical significance was assessed using an unpaired Student's t -test ($p < 0.05$). Asterisks (*) indicate significant differences ($p < 0.05$) compared with undifferentiated cells (Day 0) or differentiated controls (Day 8).

in facilitating YAP nuclear translocation. These findings emphasize the essential role of ARHGEF3's GEF activity in regulating YAP localization and subsequent control of RhoA expression.

ARHGEF3 deficiency suppresses genes associated with adipocyte differentiation

To investigate how ARHGEF3 deficiency impacts adipocyte size, we examined adipogenesis-related gene expression. In ARHGEF3^{-/-} mice, significant reductions in the expression of key adipogenic markers—including PPAR γ , CCAAT-enhancer-binding proteins α and β (C/EBP- α , C/EBP- β), and fatty acid binding protein 4 also known as adipocyte protein 2 (FABP4/aP2)—were observed in iWAT compared to ARHGEF3^{+/+} mice (Fig. 6A). Additionally, lipogenic genes, such as fatty acid synthetase (FAS) and lipoprotein

lipase (LPL), and transcription factors sterol regulatory element-binding protein-1c (SREBP1-c) and carbohydrate-responsive element-binding protein (ChREBP), showed decreased expression in the iWAT of HFD-fed ARHGEF3^{-/-} mice (Fig. 6A). Similarly, protein levels of adipogenic markers and PPAR γ -dependent genes, such as C/EBP- α , C/EBP- β , and FABP4/aP2, were significantly lower in the iWAT of HFD-fed ARHGEF3^{-/-} mice (Fig. 6B). In vitro knockdown of ARHGEF3 led to a reduction in the expression of adipogenesis-related genes (PPAR γ , C/EBP- α , C/EBP- β , and FABP4/aP2), as well as lipogenic genes (FAS and LPL) and transcription factors (SREBP-1c and ChREBP) (Fig. S6A). Correspondingly, protein levels of PPAR γ , FABP4/aP2, C/EBP- α , and C/EBP- β were lower in ARHGEF3-depleted C3H10T1/2 preadipocytes (Fig. S6B). Lipid accumulation was also significantly decreased in ARHGEF3-knockdown cells after 8 days of adipogenesis induction, while cell

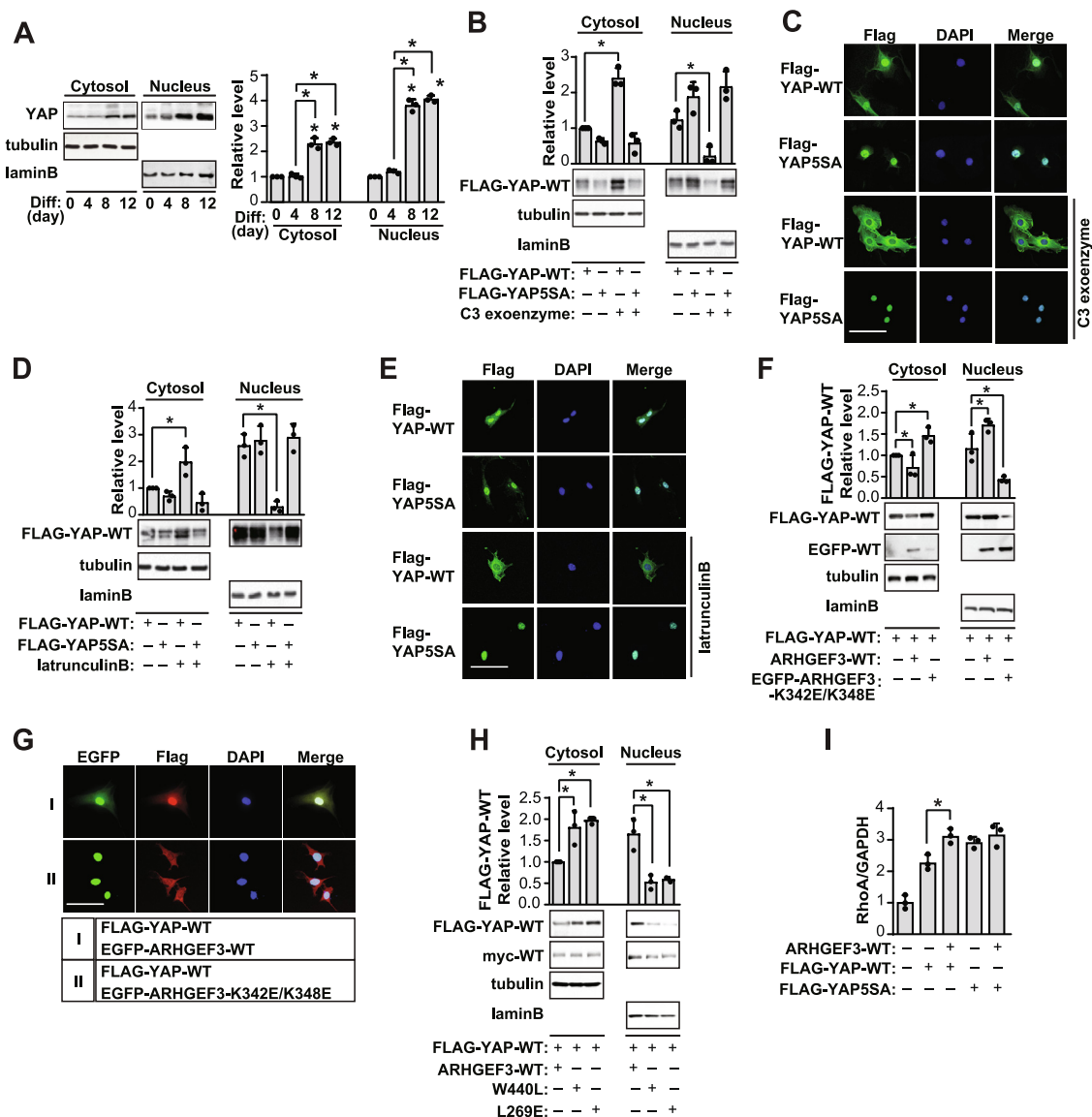


Fig. 5. The GEF activity of ARHGEF3 promotes YAP nuclear translocation. (A) C3H10T1/2 cells were differentiated for 4, 8, and 12 days, and nuclear extracts were prepared for western blot analysis of YAP localization. Tubulin and lamin B were used as cytoplasmic and nuclear markers respectively (n = 3). (B) C3H10T1/2 cells were transfected with FLAG-YAP-WT or FLAG-YAP5SA (constitutively active YAP), pre-treated with 1 μ g/ml of C3-exoenzyme for 24 h, and fractionated into cytosolic and nuclear fractions, followed by western blot analysis (n = 3). (C) Immunofluorescence staining was performed on cells treated as in (B), using anti-FLAG (green) and DAPI (blue) for nuclear visualization. Scale bar = 100 μ m. (D) C3H10T1/2 cells were transfected as in (C), pre-treated with 2.5 μ M latrunculin B for 24 h, fractionated into cytosolic and nuclear fractions, and subjected to western blot analysis (n = 3). (E) Cells treated as in (D) were subjected to immunofluorescence staining using anti-FLAG (green) and DAPI (blue). Scale bar = 100 μ m. (F) C3H10T1/2 cells were co-transfected with Flag-YAP WT and either EGFP-ARHGEF3 WT or EGFP-ARHGEF3 K342E/K348E mutant (PI(4,5)P₂ binding and GEF activity –deficient mutant), followed by western blot analysis of nuclear and cytosolic fractions (n = 3). (G) Immunofluorescence staining was performed on cells co-transfected as in (F) using anti-FLAG (red) and DAPI (blue). Scale bar = 100 μ m. (H) C3H10T1/2 cells were co-transfected with Flag-YAP-WT and either Myc-ARHGEF3 WT or GEF-deficient mutants (W440L and L269E), followed by western blot analysis of nuclear and cytosolic fractions to assess YAP localization. (I) C3H10T1/2 cells were transfected with Myc-ARHGEF3 WT, FLAG-YAP, or FLAG-YAP5SA alone or in combination, lysed, and analyzed by qRT-PCR for RhoA expression (n = 3). Band intensities were quantified using ImageJ, with tubulin and lamin B serving as reference proteins for cytosolic and nuclear fractions respectively. Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using an unpaired Student's t-test (p < 0.05). Asterisks (*) denote significant differences (p < 0.05) compared to undifferentiated controls (Day 0), differentiated controls (Day 8), or vehicle-treated controls.

proliferation remained unaffected (Fig. 6C and Fig. S6C). In contrast, overexpression of ARHGEF3 WT enhanced the expression of adipogenic and lipogenic genes, as well as the transcription factors during adipogenesis in C3H10T1/2 preadipocytes (Fig. 6D). Notably, overexpression of two GEF-inactive ARHGEF3 mutants (W440L and L269E) markedly suppressed these genes, suggesting a dominant negative effect (Fig. 6D). Additionally, protein levels of PPAR γ and FABP4/aP2, along with fat accumulation, increased with ARHGEF3 WT overexpression during adipogenesis but remained unchanged in cells overexpressing the two GEF-inactive ARHGEF3 mutants (W440L and L269E) (Fig. 6E and F).

These findings underscore the importance of ARHGEF3's GEF activity in regulating adipogenesis and suggest a mechanistic link between ARHGEF3 function and adipocyte differentiation through modulation of key adipogenic pathways.

ARHGEF3 is a reciprocal activator of PPAR γ in a GEF activity-dependent manner

ARHGEF3 deficiency led to decreased PPAR γ expression in both in vivo and in vitro models (Fig. 6). These models included ARHGEF3 knockout mice and shRNA-mediated knockdown in

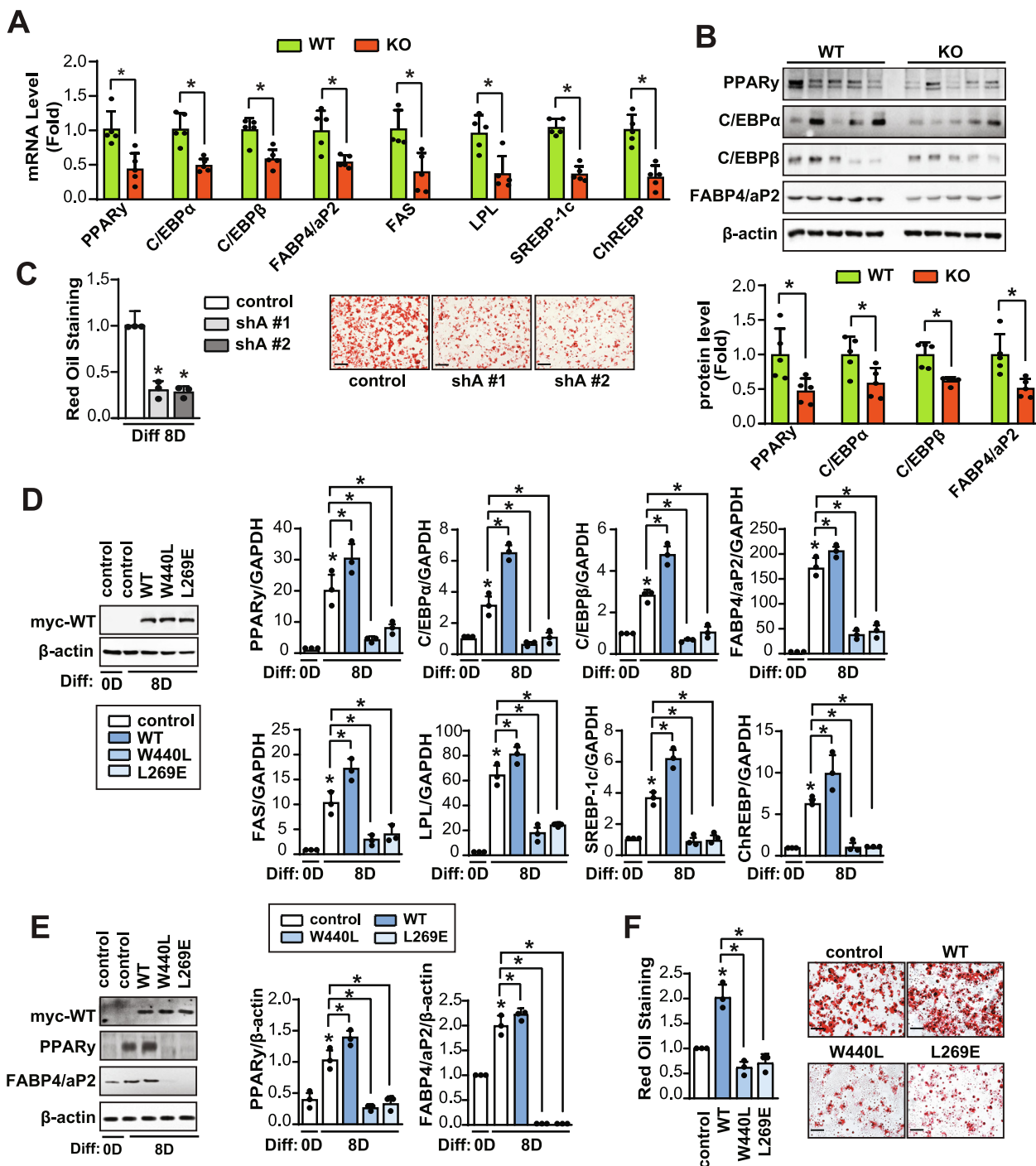


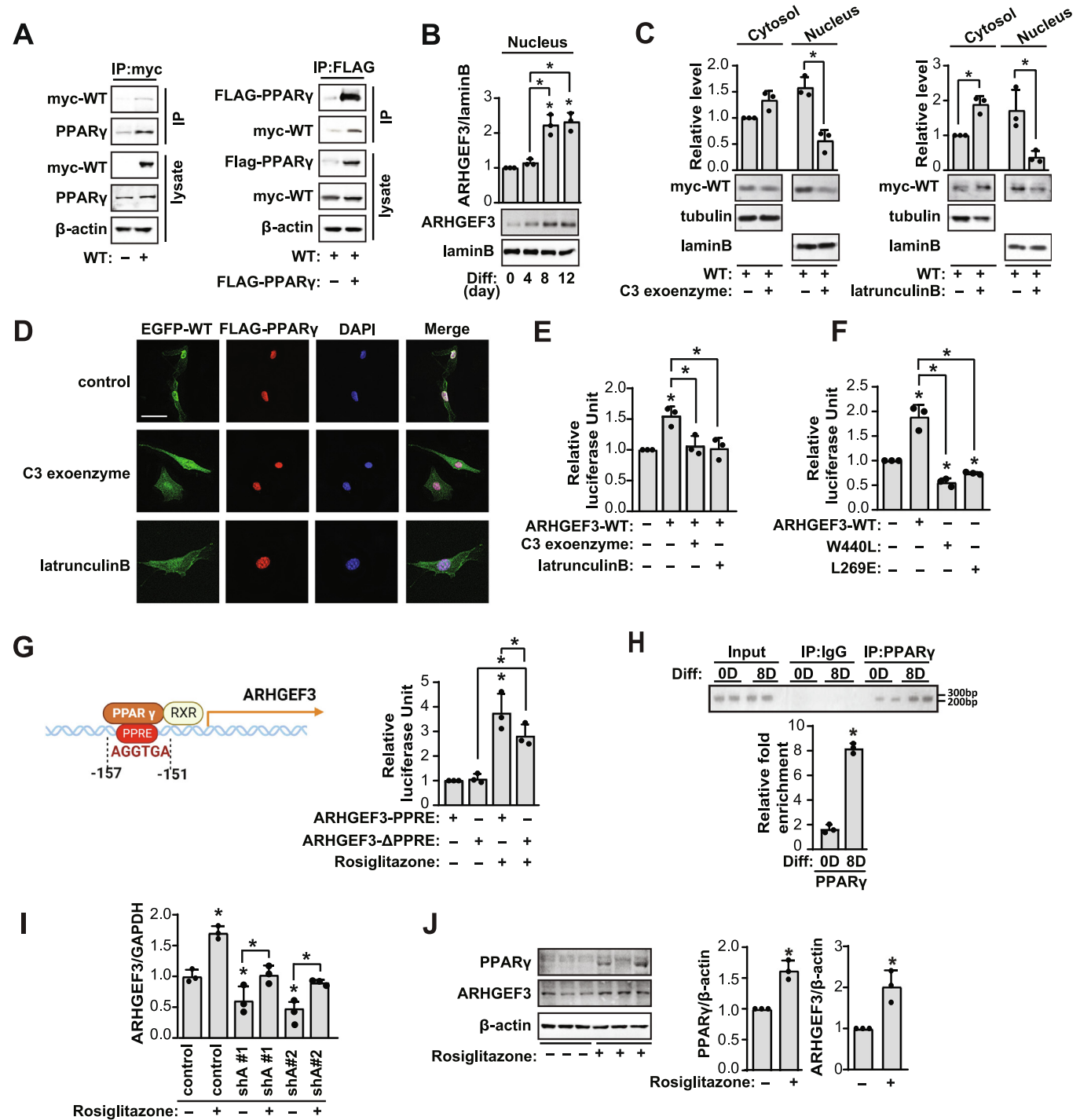
Fig. 6. ARHGEF3 deficiency suppresses the expression of adipogenic genes. (A) qRT-PCR analysis of adipogenic gene expression was performed in inguinal white adipose tissue (iWAT) from wild-type (WT, ARHGEF3^{+/+}) and ARHGEF3 knockout (KO, ARHGEF3^{-/-}) mice fed an HFD for 34 weeks (n = 5). (B) Protein levels of adipogenic markers in iWAT from HFD-fed WT and ARHGEF3 KO mice were analyzed by western blotting (n = 5). (C) C3H10T1/2 preadipocytes transduced with control or ARHGEF3-targeting shRNAs (shARHGEF3 #1, shA#1; shARHGEF3 #2, shA#2) were selected with puromycin for 5 days and induced to differentiate into adipocytes for 8 days, followed by Oil Red O staining (n = 3). Scale bar = 500 μ m. (D) C3H10T1/2 preadipocytes were transfected with Myc-ARHGEF3 WT or the GEF activity-deficient mutants Myc-ARHGEF3-W440L and Myc-ARHGEF3-L269E. After transfection, cells were differentiated for 8 days. Cells were harvested for qRT-PCR analysis to assess adipogenic gene expression and western blotting to confirm ARHGEF3 WT and mutants' expression (n = 3). (E) Cells treated as in (D) were lysed and subjected to western blotting to analyze the protein levels of PPAR γ and FABP4/aP2. (F) Cells treated as in (D) were stained with Oil Red O after 8 days of adipogenesis induction (n = 3). Scale bar = 500 μ m. (B, E) Band intensities were quantified using ImageJ and normalized to β -actin levels. Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using an unpaired Student's *t*-test, with *p* < 0.05 considered statistically significant. Asterisks (*) indicate significant differences (p < 0.05) compared to WT mice, undifferentiated controls (Day 0), or differentiated controls (Day 8). Abbreviations: FABP4/aP2 (Fatty Acid Binding Protein 4/Adipocyte Protein 2), FAS (Fatty Acid Synthase), LPL (Lipoprotein Lipase), PPAR γ (Peroxisome Proliferator-Activated Receptor γ), C/EBP α and C/EBP β (CCAAT/Enhancer-Binding Proteins α and β), SREBP-1c (Sterol Regulatory Element-Binding Protein 1-c), ChREBP (Carbohydrate-Responsive Element-Binding Protein).

C3H10T1/2 preadipocytes. Immunoprecipitation assays demonstrated that ARHGEF3 physically interacts with PPAR γ , as shown by co-immunoprecipitation of myc-ARHGEF3 with either endoge-

nous PPAR γ or Flag-PPAR γ in C3H10T1/2 adipocytes (Fig. 7A). During adipocyte differentiation, cellular morphological changes associated with lipid accumulation and cellular hypertrophy

enhanced the nuclear localization of ARHGEF3 after 8 and 12 days of adipogenesis induction (Fig. 7B). Given ARHGEF3’s role in RhoA signaling, we hypothesized that RhoA inhibition might affect ARHGEF3 localization and its interaction with PPAR γ . In C3H10T1/2 preadipocytes, treatment with C3 exoenzyme (a Rho-GTPase inhibitor) or latrunculin B (an actin polymerization inhibitor) reduced ARHGEF3 nuclear localization (Fig. 7C and D). While ARHGEF3’s nuclear localization was significantly decreased by these inhibitors, the nuclear localization of PPAR γ remained unaffected, as shown by Flag-PPAR γ immunostaining, suggesting that PPAR γ localization is independent of ARHGEF3 localization (Fig. 7D). Importantly, when ARHGEF3 and PPAR γ co-localized in the nucleus, PPAR γ transcriptional activity increased, as indicated by

the luciferase reporter assay containing a triple-repeat DR1 sequence, a potent PPAR γ -responsive element (PPRE) (Fig. 7E). Reducing ARHGEF3 nuclear localization via C3 exoenzyme or latrunculin B significantly downregulated PPAR γ transcriptional activity, indicating that ARHGEF3’s nuclear localization is necessary for full PPAR γ activation (Fig. 7E). To determine whether ARHGEF3’s enzymatic activity is also required for this regulation, we performed additional transcriptional assays using wild-type and mutant ARHGEF3 constructs. ARHGEF3 WT significantly increased PPAR γ transcriptional activity, while GEF-inactive mutants (W440L and L269E) showed markedly reduced effects (Fig. 7F), suggesting that the GEF function of ARHGEF3 is required for PPAR γ transcriptional activation. Interestingly, our data further revealed



that PPAR γ also regulates ARHGEF3 expression, establishing a reciprocal feedback mechanism. Notably, we identified a potential PPRE binding motif within the promoter region of ARHGEF3 at –157 to –151 (Fig. 7G). Treatment with rosiglitazone, a PPAR γ agonist, enhanced ARHGEF3 promoter activity, consistent with the presence of this putative PPRE site (Fig. 7G). A mutant ARHGEF3 promoter lacking this PPRE region retained partial responsiveness to rosiglitazone, although to a significantly lesser extent than the wild-type promoter (Fig. 7G). Chromatin immunoprecipitation (ChIP) assays confirmed direct PPAR γ binding to the PPRE site on the ARHGEF3 promoter (Fig. 7H). Furthermore, treatment with rosiglitazone upregulated ARHGEF3 mRNA expression and restored it in ARHGEF3-knockdown C3H10T1/2 cells (Fig. 7I). Consistently, ARHGEF3 protein levels also increased in rosiglitazone-treated C3H10T1/2 cells (Fig. 7J). Collectively, these findings establish a reciprocal regulatory feedback loop between ARHGEF3 and PPAR γ : ARHGEF3 enhances PPAR γ transcriptional activity through its GEF function, while PPAR γ , in turn, drives ARHGEF3 expression through direct binding to its promoter. This bidirectional regulation appears to be critical for normal adipogenesis and adipocyte hypertrophy. The mechanosensitive nature of this feedback loop suggests it may serve as a molecular mechanism linking physical forces to metabolic regulation in adipose tissue, potentially contributing to adipose tissue remodeling in response to obesity or other metabolic challenges.

Discussion

Obesity is a complex, multifactorial condition that necessitates a deeper understanding of the molecular mechanisms underlying adipocyte differentiation and hypertrophy. Our study identifies ARHGEF3 as a central regulator of these processes, integrating mechanical signals, cytoskeletal reorganization, and transcriptional control through the YAP-RhoA and PPAR γ pathways. By coordinating these pathways, ARHGEF3 exerts a multifaceted influence on adipocyte function, particularly in regulating cell size and lipid accumulation. Previous studies have demonstrated the individual roles of YAP and PPAR γ in adipogenesis [20,26,28–30], but our findings establish ARHGEF3 as a central integrator, supporting adipocyte hypertrophy via cytoskeletal rearrangement and transcriptional activation (Fig. 8).

During adipogenesis, cytoskeletal changes are essential for lipid droplet expansion and increasing cell volume [23,31]. ARHGEF3

facilitates this process by activating the RhoA-ROCK pathway, which drives F-actin reorganization—an essential step in lipid droplet formation and maintenance of adipocyte structure [23]. Actin dynamics are central to this process, and disruptions in actin polymerization can impair lipid droplet formation, ultimately compromising adipocyte function [23,31]. Furthermore, a recent study identified Zc3h10, a zinc finger transcription factor, as a critical regulator of early adipocyte differentiation through its control of F-actin remodeling and mitochondrial dynamics [32]. These findings highlight the importance of cytoskeletal remodeling not only in lipid droplet formation but also in metabolic regulation during adipogenesis.

While our study primarily focused on the cellular and molecular mechanisms of adipogenesis, the metabolic implications of ARHGEF3 modulation present an area for further exploration. Other studies have assessed metabolic outcomes, including insulin sensitivity and glucose tolerance [23,32]. These studies revealed that cytoskeletal dynamics, regulated by actin remodeling, influence metabolic function by affecting glucose uptake and insulin responsiveness. Although we did not observe a direct effect of ARHGEF3 on insulin sensitivity, it is plausible that ARHGEF3 may indirectly modulate glucose uptake through its regulation of cytoskeletal integrity. Given the role of actin remodeling in GLUT4 translocation [33] and cellular mechanical properties [34], prolonged alterations in cytoskeletal integrity may influence insulin sensitivity over time.

ARHGEF3's function is distinct from other Rho GEF family members, such as PDZ-RhoGEF, which predominantly regulates insulin/IGF-1 signaling [35]. Both GEFs contribute to adipogenesis, but their mechanisms are non-overlapping, underscoring ARHGEF3's unique role in adipocyte biology. Thus, our study expands the understanding of how different GEFs regulate adipogenesis and suggest that ARHGEF3 might indirectly influence insulin sensitivity through complementary pathways. Future studies should evaluate the long-term metabolic impacts of ARHGEF3 modulation, particularly regarding glucose homeostasis and insulin sensitivity, to comprehensively understand its role in metabolic disorders like obesity and diabetes.

Mechanical stress is another crucial factor in adipocyte function, particularly in regulating RhoA activation [36]. Lipid accumulation induces mechanical stretching, activating the RhoA-ROCK pathway, which is essential for maintaining adipocyte structure and size. Our study demonstrates that this activation facilitates

Fig. 7. ARHGEF3 acts as a reciprocal activator of PPAR γ in a GEF activity-dependent manner. (A) C3H10T1/2 cells were transfected with Myc-ARHGEF3 WT or FLAG-PPAR γ , followed by co-immunoprecipitation with anti-Myc or anti-Flag antibodies to assess protein–protein interaction. (B) Nuclear extracts from C3H10T1/2 cells differentiated for 0, 4, 8, or 12 days were analyzed by western blotting to monitor ARHGEF3 nuclear localization, with lamin B used as a nuclear marker. (C) C3H10T1/2 cells transfected with Myc-ARHGEF3 WT were pre-treated with 1 μ g/ml C3-exoenzyme (Rho inhibitor) or 2.5 μ M latrunculin B (actin depolymerizing agent) for 24 h, then fractionated into cytoplasmic and nuclear extracts for western blot analysis. Tubulin and lamin B were used as cytoplasmic and nuclear markers, respectively. (D) C3H10T1/2 cells were co-transfected with FLAG-PPAR γ and EGFP-ARHGEF3 WT, pre-treated as described in (C), and subjected to immunofluorescence staining using anti-Flag (red) and DAPI (blue) to visualize subcellular localization. Scale bar = 100 μ m. (E) Luciferase assays were performed by co-transfecting C3H10T1/2 cells with Flag-PPAR γ and RXR α and a luciferase reporter containing three DR1 consensus PPAR γ -responsive elements (PPRE X3-TK-luc) along with Myc-ARHGEF3 WT. Cells were pre-treated with 1 μ g/ml C3-exoenzyme and 2.5 μ M latrunculin B for 24 h to assess the role of Rho signaling and actin cytoskeleton in ARHGEF3-mediated PPAR γ activation (n = 3). (F) C3H10T1/2 cells were co-transfected as described in (E) along with Myc-ARHGEF3 WT and GEF-inactive mutants (W440L and L269E) and subjected to luciferase assays to determine the requirement of GEF activity for PPAR γ activation (n = 3). (G) The ARHGEF3 promoter region contains a putative PPAR γ -responsive element (PPRE) between –157 and –151 base pairs upstream of the transcription start site. Schematic illustration was created using BioRender.com. C3H10T1/2 cells were transfected with either the ARHGEF3-PPRE luciferase reporter construct or its deletion mutant (ARHGEF3- Δ PPRE) in the presence or absence of 1 μ M rosiglitazone (PPAR γ agonist), and luciferase assays were performed (n = 3). (H) ChIP analysis using anti-PPAR γ was conducted on undifferentiated and 8-day-differentiated C3H10T1/2 cells. Input and IgG immunoprecipitated samples were used as positive and negative controls, respectively. The upper panel presents the PCR products of the ChIP assay, while the lower panel shows PPAR γ enrichment on the ARHGEF3 promoter relative to IgG quantified by densitometry. (I) C3H10T1/2 preadipocytes were transduced with control or ARHGEF3-targeting shRNAs (shARHGEF3 #1, shA#1; shARHGEF3 #2, shA#2) and selected with 1.5 μ M puromycin for 5 days, then treated with or without 1 μ M rosiglitazone for 24 h. Cells were lysed and subjected to qRT-PCR analysis for ARHGEF3 expression (n = 3). (J) Western blot analysis was performed on cells treated with or without 1 μ M rosiglitazone for 24 h (n = 3). Band intensities were quantified using ImageJ, with tubulin and lamin B serving as reference proteins for cytosolic and nuclear fractions, respectively (B–C), or β -actin serving as reference protein (J). Data are presented as mean $\pm$ standard deviation. Statistical significance was assessed using an unpaired Student's t -test, with p < 0.05 considered statistically significant. Asterisks (*) indicate significant differences (p < 0.05) compared to control conditions or as specified in individual panels.

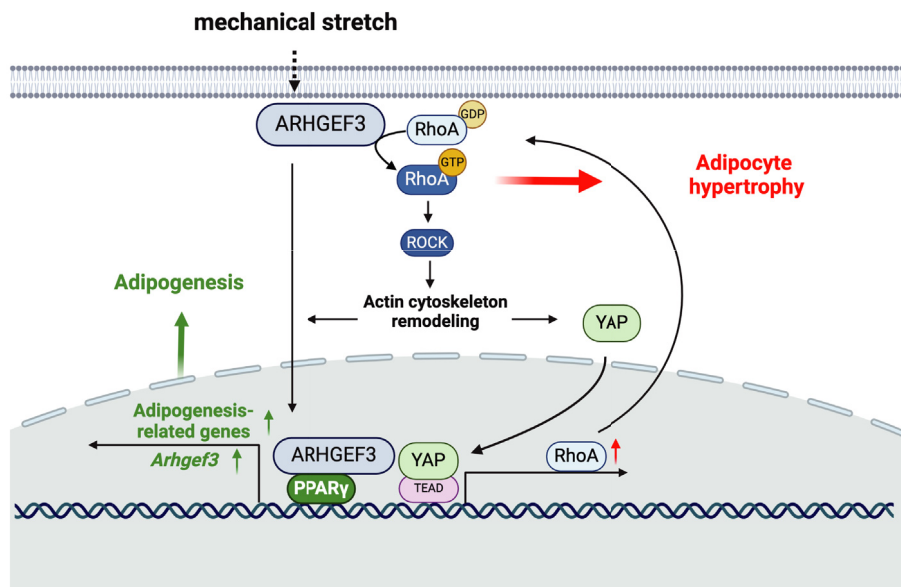


Fig. 8. Schematic representation of ARHGEF3's role in regulating adipocyte hypertrophy and differentiation. This diagram illustrates the dual regulatory pathways mediated by ARHGEF3 during adipogenesis. At the plasma membrane, ARHGEF3 activates RhoA through its guanine nucleotide exchange factor (GEF) activity, which drives YAP translocation into the nucleus. Once in the nucleus, YAP enhances transcriptional activities in complex with TEAD transcription factors to regulate RhoA expression through direct binding to the RhoA promoter region (–80 to –74 bp relative to the transcription start site), which promotes adipocyte hypertrophy and regulates cell size. Simultaneously, ARHGEF3 translocates to the nucleus, where it directly interacts with PPAR γ , a master regulator of adipocyte differentiation, thereby amplifying PPAR γ 's transcriptional activity on adipogenic target genes. Additionally, the presence of a PPAR γ -responsive element (PPRE) located between –157 and –151 base pairs in the ARHGEF3 promoter establishes that ARHGEF3 is also a downstream target of PPAR γ , creating a positive feedback loop. This network of interactions reveals ARHGEF3 as a critical mediator in adipogenesis, mechanistically linking hypertrophy and differentiation pathways. These findings suggest ARHGEF3 as a potential therapeutic target for managing obesity and associated metabolic disorders by modulating both adipocyte size and differentiation capacity. The schematic illustration was generated using BioRender.com.

the translocation of YAP to the nucleus, where it upregulates RhoA expression. We observed that ARHGEF3 promotes this process, establishing a feedback loop in which mechanical signals enhance RhoA expression and promote cytoskeletal changes. Notably, while RhoA activity is suppressed in the early stages of adipocyte differentiation [37,38], its activation during hypertrophy underscores its dual role in both adipocyte commitment and maturation.

Recent studies have emphasized the role of Hippo-YAP/TAZ signaling in adipose tissue plasticity, particularly by decoupling leptin expression from fat mass [39]. YAP/TAZ has been identified as a negative regulator of fat accumulation, promoting the dedifferentiation of mature adipocytes into progenitor-like cells [39]. Intriguingly, our findings indicate that ARHGEF3 facilitates YAP nuclear translocation, leading to adipocyte hypertrophy, suggesting a context-dependent role for YAP in fat regulation. Both our work and previous studies underscore YAP's critical and context-dependent effects on adipogenesis and metabolic homeostasis. These findings suggest that the YAP-RhoA signaling axis could be a viable therapeutic target for modulating fat accumulation, with outcomes potentially varying based on metabolic conditions.

Beyond RhoA modulation, ARHGEF3 has a broader role in mechanotransduction by linking cytoskeletal changes to transcriptional regulation through YAP nuclear translocation. We also confirmed that ARHGEF3's own nuclear translocation is regulated by RhoA and actin polymerization, suggesting that, in addition to the nuclear localization signal (NLS), active RhoA and actin polymerization are required for ARHGEF3's effective nuclear translocation. Furthermore, ARHGEF3's dual localization—both at the membrane and within the nucleus [4] implies it may have distinct functions based on its subcellular distribution. The failure of the K342E/K348E mutant to induce YAP nuclear translocation further supports this hypothesis. Future studies should focus on elucidating the specific roles of ARHGEF3 within these cellular compartments and their implications for adipocyte biology and function.

Our study also reveals a reciprocal regulatory relationship between ARHGEF3 and PPAR γ . ARHGEF3 enhances PPAR γ transcriptional activity, promoting the expression of key adipogenic genes such as C/EBP- α , C/EBP- β , and AP2. In turn, PPAR γ binds directly to the PPRE site on the ARHGEF3 promoter, further amplifying ARHGEF3 expression and reinforcing this regulatory loop. This relationship is analogous to other coactivator-driven transcriptional networks, such as the PPAR γ -PGC-1 α interaction [40], supporting the hypothesis that ARHGEF3 functions as a coactivator in a GEF-dependent manner. Upon ligand activation, PPARs undergo conformational changes, displacing co-repressors and recruiting coactivators like PGC-1 α to initiate transcription [40,41]. Given the central role of PPAR γ in adipogenesis, exploring whether other PPAR family members, such as PPAR α , regulate ARHGEF3 expression could enhance our understanding of how ARHGEF3 modulates lipid metabolism.

In summary, our findings demonstrate that ARHGEF3 is essential for maintaining adipose tissue homeostasis by coordinating cytoskeletal rearrangement, transcriptional regulation, and adipocyte hypertrophy. These insights provide a novel understanding of the molecular mechanisms through which ARHGEF3 regulates adipogenesis and suggest potential therapeutic strategies for managing obesity-related metabolic disorders. Future research should investigate the in vivo effects of ARHGEF3, particularly its impact on insulin sensitivity and interactions with other metabolic tissues.

Conclusion

This study establishes that ARHGEF3 as a pivotal regulator of adipocyte differentiation and hypertrophy by coordinating the YAP-RhoA and PPAR γ pathways. Our findings indicate that ARHGEF3 is essential for cytoskeletal reorganization and adipogenic

gene expression, which are essential for maintaining adipose tissue homeostasis in diet-induced obesity. Targeting ARHGEF3 may represent a promising therapeutic approach for treating obesity-related metabolic disorders. Further research is needed to elucidate the mechanisms through which ARHGEF3 regulates these pathways across different metabolic tissues.

Ethics approval and content to participate

All animal care and treatments were conducted following the guidelines of the Institutional Animal Care and Use Committee of the Center of Animal Care and Use at Lee Gil Ya Cancer and Diabetes Institute, Gachon University (approval numbers: LCDI-2017-0133 and LCDI-2020-0068).

CRediT authorship contribution statement

Sana Abdul Khaliq: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Shi-Young Park:** Methodology, Investigation, Formal analysis. **Syeda Maham:** Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Yoonil Cho:** Investigation, Data curation, Writing – review & editing. **Miseon Lee:** Investigation, Data curation, Writing – review & editing. **Seungyeon Nam:** Investigation, Data curation, Supervision. **Je Kyung Seong:** Writing – review & editing, Funding acquisition. **Jie Chen:** Visualization, Supervision, Project administration, Writing – review & editing. **Cheol Soo Choi:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Mee-Sup Yoon:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

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Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2025.04.010>.

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