Materials and Methods

Primary preadipocyte culture

The Institutional Animal Care and Use Committee of Creighton University approved the animal research protocol. The EAT adipose tissue samples around coronary arteries of adult Yucatan microswine were pooled, and minced with scissors into small pieces (2-3 mm^3) under an aseptic condition. The minced tissue samples were washed twice using PBS containing penicillin/streptomycin, placed in digestion solution (0.25% type 1 collagenase in DMEM/F12 medium) and incubated in a shaker (250 rpm) at 37°C for 60-80 minutes. The undigested tissue was separated from collagenase-digested samples by filtration through a 100-μm mesh fabric. Matured fat cells were separated from the pellets containing preadipocytes by centrifugation at 400 g at 4°C for 10 minutes. The preadipocytes were subsequently washed two times, and then resuspended in the growth medium (DMEM/F12 medium containing 10% FBS). The primary cultured preadipocytes (70–80 % confluent cells) were arrested by replacing 10% FBS medium with 0.2% FBS DMEM/F12. After 24 hours, cells were incubated with 10 nmol/L VD3 (calcitriol) (D1530 Sigma-Aldrich, St. Louis, MO) or 25 ng/ml recombinant TNF-α or IL-1β (PeproTech, Inc. NJ) at a different time period. The control group was treated with 1/1000 medium volume of ethanol or water. Cells were used for immunofluorescent analysis, or harvested for preparation of total RNA, total protein, or nuclear extract to do real-time PCR or Western blot analysis, respectively. Some cells were used for transfection experiments.

Animal study

Swine were housed and cared for according to NIH and USDA guidelines in the Animal Resource Facility of Creighton University under IACUC-approved protocol number 0831. Adult Yucatan™ miniature swine (30-40 lbs) were purchased from Sinclair Bio-resources, Windham, MA, and fed with 1–1.5 lb/swine/day of atherogenic vitamin D-sufficient or atherogenic vitamin D-deficient diets for one year. Swine were fed with two different vitamin D-deficient high-cholesterol diets. The first group of animals was randomly divided into two subgroups. The control subgroup was fed with a mixture of Teklad swine Diets 8753 and 9487 (Envigo, Madison, Wisconsin USA) and *8% chocolate.* This is an atherogenic vitamin D-sufficient control (C-1) diet that provides 1,500 IU of vitamin D3 per day. The vitamin D-deficient subgroup was fed a mixture of TD*8753*, 10125, 0.55% sodium cholate and 9% chocolate an atherogenic diet (Diet-1), which provides about 500 IU of vitamin D3/ day. The second group of animals was similarly divided into two subgroups. The control subgroup (C-2) was fed with mixed TD150250 *(Envigo, Madison, Wisconsin USA),* an atherogenic diet that provides 1,300 IU of vitamin D3 per day. The vitamin D-deficient subgroup was fed with TD150038 (Diet-2), which is completely devoid of vitamin D. Serum calcium and parathyroid hormone (PTH) levels were measured every 4-8 weeks in the vitamin D-deficient animals. The PTH levels started to rise above the normal level after 5 months and the total serum calcium levels started to decrease below the normal level after 6 months of feeding with the vitamin D-deficient diet. Once the PTH rose above the normal level (>65 pg/ml), the pigs were supplemented with oral calcium in the form of calcium citrate tablets (Puritan's Pride, USA) 0.5 to 1 g/ day according to their PTH and calcium levels, to maintain normal or near-normal PTH and calcium levels. After one year of the diets, the animals were euthanized and venous blood was collected from each animal for 25(OH) D measurement and complete metabolic profile.

VDR protein knockdown, whole cell and nuclear protein preparation and Western blot

VDR levels were down-regulated in preadipocytes using VDR small inhibitory RNA (siRNA) as a standard technique. VDR and control siRNAs were purchased from Cell Signaling Technology. The siRNAs were used at 100 nM concentrations with a standard transfection protocol using lipofectamine RNAiMAX (Invitrogen). Whole cell protein was extracted as a standard procedure and nuclear protein preparation was done using a nuclear extract kit per the manufacturer's protocol (Active Motif, CA). Western blot analysis was done as the standard protocol. The following primary antibodies were used in this study: VDR (D-6, sc-13133), CYP24A1 (ab175976, Abcam), CYP27B1 (ab95047), KPNA4 (ab176585), NF-κB p65 (C-20, sc-37372), Lamin B (C-20, sc-6216), GAPDH (6C5, sc-32233). The results were normalized to GAPDH for the protein from whole-cell lysates and lamin B for the nuclear protein.

Total RNA preparation, reverse transcription, and real-time PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA using the Improm-II reverse transcription kit (Promega, Madison, WI). The cDNA was subjected to real-time PCR for the KPNA4 gene using the SYBR Green PCR kit (Promega, Madison, WI) on the BioRad CFX detection system. Quantification was done by normalization against GAPDH. The following primer sequences were used: (KPNA4-sense: GACTGTCCCCTGTCACCAAC, KPNA4-antisense: CAGAGGAGCCCATTCTCTGT; GAPDH sense: AAACGGCTACCACATCCAAG, GAPDH: TCTTGGCAAATGCTTTCGC).

Transfection and luciferase assay

The 0.5 µg of NF-κB responsive firefly luciferase reporter (Origene, Rockville, MD) and 0.5 µg of Renilla-luciferase vector were transiently co-transfected with 0.1 or 0.3 µg of pCMV6-driven human KPNA4 expression vector (Origene) into quiescent preadipocytes using Human Preadipocyte Avalanche Transfection Reagent (EZ-Biosystems LLC, College Park, MD). Cells were then incubated with VD3 for 24 hours with or without TNF-α for the final 8 hours. At the end of the treatment, cells were lysed and luciferase activity was measured using the Dual-Luciferase® reporter assay system (Promega). NF-κB-luciferase activity was normalized for Renilla luciferase activity to control for transfection efficiency.

Generation of lentiviral KPNA4 expression construct

The full length 1566 bp coding sequence of human KPNA4 in the pCMV6-driven expression vector (Origene) was amplified using Phusion high-fidelity DNA polymerase with the forward primer: AATAGCGCTCATGGCGGACAACGAGAAACT and reverse primer: AAAGGATCCTAAAACTGGAACCCTTCTGTTG. The amplified DNA was purified from the agarose gel using a Qiagen Gel Extraction kit. About 1 µg of the amplified KPNA4 coding sequence and the lentiviral expression vector pLVXEF1α- AcGFP1 were independently digested with AfeI and BamHI restriction enzymes (NEB Biolabs). The digested samples were directly purified using a Qiagen Gel Extraction kit. Ligation reaction was carried out in a 20 µl reaction volume containing 2 µl of 10x ligation buffer, 50 ng of vector DNA, 25 ng of insert DNA, and 1 µl of T4 DNA ligase (10 Units) overnight at room temperature. Bacterial transformation was carried out using BioBlue chemically competent E. coli cells purchased from Bioline. Isolated single colonies were amplified by culturing overnight in an orbital shaker maintained at 37 ^{o}C and 220 rpm. Plasmid DNA was isolated and subjected to restriction

digestion with AfeI and BamHI to screen the recombinant clones containing KPNA4 gene fragments (pLVX-EF1α-KPNA4-AcGFP1), which were confirmed by sequencing.

Production of lentiviral particles expressing KPNA4

Lentiviral particles expressing KPNA4 were developed using the Lenti-X expression system from Clontech. Lenti-X 293T cells were cultured in DMEM medium containing 10% tetracycline-reduced serum in the presence of penicillin and streptomycin antibiotics. To generate lentivirus particles, about 7 µg of the plasmid DNA pLVX-EF1α-KPNA4-AcGFP1 was diluted with 557 µl of Xfect Reaction buffer along with 36 µl of Lenti-X HTX Packaging plasmids. The DNA mix was then vortexed briefly and allowed to incubate at room temperature for 2 minutes. In a separate tube, 7.5 µl of Xfect Polymer was diluted in 592.5 µl of Xfect Reaction Buffer, briefly vortexed and added to the tube containing the pLVX-EF1α-KPNA4-AcGFP1 and Lenti-X Packaging plasmids. The mixture was then incubated at room temperature for 10 minutes and directly added to Lenti-X 293T cells at a density of 70% confluence with 10 ml of culture medium. The cells were allowed to grow overnight and the media supernatant was discarded in 10% bleach solution. After adding fresh culture medium, the cells were allowed to grow for additional 24 hours and the supernatant containing lentiviral particles was collected and stored at -80 °C. About 20 µl of the supernatant was used to quantify the titer of the virus using Lenti-X GoStick, which shows two distinct bands on the GoStick if the titer is above 5×10^5 IFU/ml.

Cell transduction using KPNA4-expressing lentiviruses

Based on the intensity of the band that appears on the GoStick, about 100 to 500 µl of the viral supernatant was used to transduce cells grown in a 10 cm plate. Virus supernatant was mixed with Lenti-X accelerator (10:1) for 30 minutes, and the mixture was added directly to the cell culture medium to transduce overnight. The next day, medium supernatant was discarded in 10% bleach solution and fresh medium was added to the transduced cells. The cells were allowed to grow for one to two more days and the lentiviral transduction efficiency was evaluated by observing GFP expression. After visualizing the GFP, the cells were digested and subjected to puromycin selection (2 µg/ml) for 4 days prior to the experiments.

Histology

Whole heart was removed after the swine was euthanized. Right coronary arteries from the aorta to the ventricular branch with EAT were excised (some samples from left anterior descending arteries and left circumflex arteries). The arteries were fixed in 10% buffered formalin for 24 hours. The arteries were sectioned into 4 to 5 mm portions and placed into tissue cassettes numbered 1 to 5 (1 being the larger portion of the right coronary, the origin at the aorta, and 5 being the smaller distal region at the branch with the acute marginal artery). The sections in the tissue cassettes were processed with ethanol and xylene and embedded into paraffin blocks. The paraffin blocks (1 through 5) were sectioned and stained with H&E. The percent stenosis was determined for each section (1 through 5) and the mean of these values was obtained to reflect the mean percent stenosis of the right coronary artery for each swine. The luminal and internal elastic lamina area was measured by NIH ImageJ software. Briefly, percent area stenosis was calculated as [1-(luminal area/internal elastic lamina area)]*100. Some slides were used for immunofluorescent assay.

Immunofluorescent assay

After fixing the cultured cells with 4% paraformaldehyde or after deparaffinization and rehydration of the slides containing swine EAT, antigen retrieval was performed prior to immunostaining. Fixed cells on the slides were incubated for 2 hours in block/permeabilizing solutions containing PBS, 0.25 % Triton X-100, and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with primary antibodies including rabbit anti-p65, rabbit anti-KPNA4, at 4°C overnight. After washing with PBS four times for 5 minutes each, a secondary antibody (affinity purified goat anti-rabbit cyanine 3 (cy3) antibody, 1:300) (Jackson ImmunoResearch, Westgrove, PA) was applied to the sections for 2 hours in dark. Negative controls were run in parallel with normal host IgG including ChromPure rabbit IgG or complete omission of primary antibody. Sections were washed with PBS for 5 minutes four times. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). A single layer of nail polish was placed around the edge of slide to prevent escape of mounting media from the coverslip. Pictures were taken within 1 to 24 hours of mounting using an Olympus DP71 camera. All parameters during image acquisition were the same between the groups. To measure the fluorescent intensities of KPNA4 and nuclear NF-κB in EAT, a region was drawn around each nuclei which were confirmed by DAPI co-staining for the assessment, and the identical region near it without a fluorescent object was drawn and measured for the background subtraction. The Nuclear net-integrated intensities = integrated density – (area of selected nucleus × mean fluorescence of background readings), was calculated using NIH Image J software. The Nuclear net-integrated intensities from 150 cells of EAT each group were quantified (n= 3 per group).

Statistical Analysis

The data were analyzed using the GraphPad Prism 5.0 (GraphPad Software, Inc). The values are presented as mean $\pm$ SD. ANOVA with Bonferroni's multiple comparison test or two-tailed unpaired student t-test were used to analyze statistically significant differences between the groups.

Supplementary Figures and Table:

Suppl. Figure I. Pro-inflammatory cytokines stimulated activation of NF-κB p65. (**A**) Effect of different doses of TNF-α on p65 nuclear translocation: the optimum dose of TNF-α was 20 ng/ml in mediating p65 nuclear translocation. The experiments were repeated twice. The representative images and pooled data in the graph are shown. (**B**) VD3 prevented IL-1β–induced p65 nuclear translocation. The cells were fixed for immunofluorescent assay after being treated with 10^{-8} mol/L VD3 for 24 hours and 20 ng/ml IL-1β for 25 minutes. The experiments were repeated three times and the representative immunofluorescent images are shown.

Suppl. Figure II. Effect of VD3 on vitamin D signaling molecules or prohibitin. (**A** and **B**) VD3 had no effect on the expression of 1-α-hydroxylase and 24-hydroxylase in the preadipocytes. The experiments were repeated twice. (**C** and **D**) VD3 had no significant effect

on prohibitin mRNA and protein levels in the preadipocytes. The experiments were repeated three times using different samples.

Suppl. Figure III. Effect of TNF-α on KPNA4 and VDR expression. (**A**) TNF-α (20 ng/ml) did not affect KPNA4 expression. (**B**) Treatment with 20 ng/ml TNF-α for 24 hours had no significant effect on VDR expression. The experiments were repeated three times.

Suppl. Figure IV. GFP positive cells selected by 2mg/ml puromycin represent the successful expression of exogenous KPNA4 by lentivurus-KNPA4-GFP expression vector in the preadipocytes.

Suppl. Figure V. Representative picture identified by H&E staining shows the characterization of EAT with adipocytes in the fat tissue connected tightly with a coronary artery.

Suppl. Table 1. The characterization of swine prior to euthanization

	VD-Def Diet-1	VD-Suf C-1	VD-Def Diet-2	VD-Suf C-2
Age (months)	21 ± 0.6	20.1 ± 1.3	16.5 ± 1.9	15 ± 1.9
Body weight (kg)	48.5 ± 4.9	57.5 ± 3.5	36 ± 11	47 ± 6.1
Plasma cholesterol (mg/dL)	380 ± 20.6	359 ± 123	352 ± 33.8	406 ± 32.8