Inhibitor of Differentiation-3 Mediates High Fat Diet-Induced Visceral Fat Expansion

Alexis Cutchins, Daniel B. Harmon, Jennifer L. Kirby, Amanda C. Doran, Stephanie N. Oldham, Marcus Skaflen, Alexander L. Klibanov, Nahum Meller, Susanna R. Keller, James Garmey, Coleen A. McNamara

Objective—Inhibitor of differentiation-3 (Id3) has been implicated in promoting angiogenesis, a key determinant of high-fat diet (HFD)-induced visceral adiposity. Yet the role of Id3 in HFD-induced angiogenesis and visceral adipose expansion is unknown.

Methods and Results—Id3−/− mice demonstrated a significant attenuation of HFD-induced visceral fat depot expansion compared to wild type littermate controls. Importantly, unlike other Id proteins, loss of Id3 did not affect adipose depot size in young mice fed chow diet or differentiation of adipocytes in vitro or in vivo. Contrast enhanced ultrasound revealed a significant attenuation of visceral fat microvascular blood volume in HFD-fed mice null for Id3 compared to wild type controls. HFD induced Id3 and VEGFA expression in the visceral stromal vascular fraction and Id3−/− mice had significantly lower levels of VEGFA protein in visceral adipose tissue compared to wild type. Furthermore, HFD-induced VEGFA expression in visceral adipose tissue was completely abolished by loss of Id3. Consistent with this effect, Id3 abolished E12-mediated repression of VEGFA promoter activity.

Conclusion—Results identify Id3 as an important regulator of HFD-induced visceral adipose VEGFA expression, microvascular blood volume, and depot expansion. Inhibition of Id3 may have potential as a therapeutic strategy to limit visceral adiposity. (Arterioscler Thromb Vase Biol. 2012;32:00-00.)

Key Words: Obesity ▪ Id3 ▪ VEGFA ▪ helix-loop-helix motif ▪ visceral adiposity

Obesity, an important contributor to atherosclerosis and diabetes, is increasing in an epidemic manner. Yet, not all obese individuals are at the same risk of developing these diseases. Individuals with central fat accumulation, or visceral obesity, are at higher risk.1–7 As such, understanding the molecular and cellular mechanisms mediating visceral adiposity may have important implications for future therapies to limit morbidity and mortality due to obesity.

Angiogenesis is tightly linked with adipogenesis.8 New blood vessel formation contributes to adipose tissue growth by delivering nutrients, growth factors, and progenitor and inflammatory cells. Recent evidence suggests that modulation of adipose tissue angiogenesis may be a novel therapeutic option for treating obesity and preventing obesity-related morbidity.9

The helix-loop-helix (HLH) factors, Id1 and Id3, regulate tumor angiogenesis,10 yet their role in adipose angiogenesis and diet-induced obesity are unknown. The inhibitor of differentiation or inhibitor of DNA binding (Id) proteins (Id1, Id2, Id3, and Id4), belong to the family of HLH transcription regulators. Id proteins lack a DNA-binding domain but act as dominant negative inhibitors of gene regulation by associating with the broadly expressed E proteins and preventing them from forming homo- or heterodimers with other bHLH factors and binding DNA.11 Id proteins are negative regulators of cell differentiation and play key roles in the regulation of lineage commitment, cell fate decisions, and in the timing of differentiation.12 Ids 1 to 4 are all expressed in cultured preadipocytes13–14 and in adult adipose tissue in vivo,15 yet expression in response to differentiation media or high-fat feeding suggests unique roles for each of the Id proteins in the regulation of adipocyte differentiation and adipose depot development.16–18 Recent studies provide evidence that Id2 and Id4 promote adipocyte differentiation and adipose development. Mice null for Id2 have smaller inguinal and intrascapular adipose depot sizes at 4 days of age,15 whereas data from Id4 null mice demonstrate smaller epididymal and brown depots than their litter mate controls at birth and on chow diet.13 Previous studies reveal conflicting data on the impact of Id3 overexpression on adipocyte differentiation of cultured preadipocytes.14,16 In vivo studies demonstrate that Apoe−/− mice null for Id3 have increased adiponectin protein in visceral...
adipose and in serum16; however, the effect of Id3 on the development of adiposity is unknown. The present study is the first to demonstrate that mice null for Id3 have a significant attenuation of high-fat diet (HFD)-induced obesity. Furthermore, the effect of loss of Id3 on adiposity was more marked in the visceral depots. Consistent with these findings, Id3 was more abundantly expressed in visceral compared to subcutaneous (SC) fat, and Id3 expression was induced by HFD in visceral but not SC adipose tissue. Mice null for Id3 had no differences in adipocyte differentiation markers or enzymes involved in lipid metabolism when fed either a chow or HFD. Instead, mice null for Id3 had significantly reduced visceral depot VEGFA expression and microvascular blood volume compared to C57BL/6 (wild type [WT]) controls. Consistent with the paradigm of Id3 function, the HLH factor E12 significantly repressed VEGFA promoter activation; an effect antagonized by expression of Id3. Collectively, these results provide evidence that, in mice fed an HFD, Id3 promotes VEGFA expression, microvascular blood volume, and depot expansion in visceral adipose tissue, suggesting a potential new target to limit visceral adiposity.

**Research Design and Methods**

**Animals**

Id3<−/− mice used in these studies were on a pure C57BL/6 background (confirmed by microsatellite testing). Male Id3<−/− and C57BL/6 (WT) litter-mate controls were used for all experiments. Epididymal, SC, and retroperitoneal fat depots were harvested for analysis according to the technique of Hausman et al.17 Details of the materials and methods used for the evaluation of body composition by DEXA, measurement of serum parameters, isolation of adipocytes, quantitative PCR, Western blotting, promoter-reporter assays, and metabolic cage measurements are provided in the Supplemental Data, available online at http://atvb.ahajournals.org.

**Statistics**

Details are provided in the Supplemental Data.

**Results**

Id3 Expression Is Induced by High Fat Feeding in Visceral But Not in Subcutaneous Fat

C57BL/6 (WT) male mice were fed either a chow diet or HFD containing 60% kcal from fat for 4 weeks starting at 3 to 4 weeks of age. After 4 weeks, there was a significant increase in both visceral (Figure 1A) and SC (Figure 1C)
adipose depots with HFD, although the effect on the visceral depot was more marked. Western blot analysis revealed a clear HFD-induced increase in Id3 protein expression in the visceral, but not SC depots (Figure 1B and 1D), suggesting a preferential role for Id3 in HFD-induced visceral adiposity. Evaluation of Id3 mRNA and protein levels in the 2 depots from the same animals fed 4 weeks of HFD revealed 2- to 3-fold higher Id3 expression in the visceral depot at both the mRNA and protein levels (Figure 1E and 1F). To determine if the HFD-induced changes in Id3 expression were due to increased Id3 in the stromal vascular fraction (SVF) or adipocytes, Western blot analysis for Id3 protein was performed on both fractions. Notably, HFD induced a 2-fold increase in Id3 protein in the SVF but had no effect on Id3 expression in isolated visceral adipocytes (Figure 1G and H).

Mice Null for Id3 Have Reduced Visceral Adiposity in Response to HFD
To evaluate the consequences of loss of Id3 on adiposity in vivo, we fed Id3<sup>-/-</sup> and WT littermates either chow or HFD. Mice null for Id3 had reduced HFD-induced weight gain (Figure 2A) and percent body fat (Figure 2B) but no difference in lean body mass (Figure 2C) compared to littermate controls. Body length was not different between genotypes. However, Id3<sup>-/-</sup> mice had a smaller waist circumference compared to controls (Supplemental Figure 1A and 1B, available online at http://atvb.ahajournals.org, and representative photo, Figure 1C). In contrast, there were no differences in the weight of Id3<sup>-/-</sup> mice when compared to their WT littermate controls at baseline or with chow diet feeding (Figure 2A). Collectively, these results suggest that Id3 may be important in regulating processes specifically involved in HFD-induced visceral adipose tissue accumulation.

Examination of specific adipose tissue depot weights after 20 weeks of HFD demonstrated significantly smaller visceral (epididymal and retroperitoneal) depot weights in mice null for Id3 (Figure 2D and 2E). In contrast, although there was a trend to smaller SC depots in Id3<sup>-/-</sup> mice fed 20 weeks of HFD, there was no significant difference in SC (Figure 2F) or brown fat (data not shown) depot weights between genotypes fed chow or HFD.

**Id3<sup>-/-</sup> Mice Have Smaller Visceral Adipocytes in Response to HFD Compared to WT Controls**
To determine if the decreased visceral fat pad size in mice null for Id3 could be secondary to reduced visceral adipocyte size, we performed H&E staining on the epidid-
Id3-dependent Visceral Depot Expansion and Regulation of Microvascular Blood Volume Occurs at Early Time Points

Differences in metabolism of Id3−/− and WT mice fed 5 weeks of HFD and the increase in Id3 expression in visceral adipose tissue after 4 weeks of HFD (Figure 1) suggested that the effect of loss of Id3 on visceral adipose depot size may occur sooner than 20 weeks and be mediated by early responses of the adipose depots to HFD. Indeed, similar to mice fed a HFD for 20 weeks, mice fed 4 weeks of HFD displayed significantly reduced visceral adipose depots: epididymal (Figure 4A) and retroperitoneal (data not shown). While, as expected, HFD induced an increase in SC depot weight, there was no difference between genotypes in SC depot weights after HFD. The SC depot was slightly greater in the Id3−/− mice fed Chow diet (Figure 4B). Together, data suggests that loss of Id3 attenuates the early development of diet-induced obesity through mechanism(s) independent of adipocyte differentiation.

Given the established role of angiogenesis in modulating visceral adiposity and the role of Id3 in regulating tumor angiogenesis, we sought to determine if mice null for Id3 had reduced visceral depot microvasculature by performing contrast enhanced ultrasound of the visceral depot. Contrast enhanced ultrasound has been shown to be an efficient and accurate noninvasive imaging modality to assess angiogenesis in mice.18 Results demonstrated that compared to WT, Id3−/− mice fed 4 weeks of HFD had significantly lower visceral adipose tissue microbubble accumulation, a measure of microvascular blood volume as an index of microvascular density (Figure 4C, representative picture with quantitation). Consistent with a role for Id3 in regulating microvascular density, coimmunostaining of visceral adipose tissue from WT mice fed HFD with anti-CD31 and anti-Id3 antibodies revealed CD-31 positive vessels coexpressing Id3 (Supplemental Figure VI, available online at http://atvb.ahajournals.org).

Id3−/− Mice Have Reduced Expression of VEGFA in Visceral Fat

To determine a potential mechanism whereby Id3 may regulate angiogenesis in visceral adipose, we evaluated the expression of the potent angiogenic factor, VEGFA, in visceral adipose tissue of WT and Id3−/− mice. In fact, in vitro analysis of MEFs as well as stromal vascular cells isolated from WT and Id3−/− visceral adipose tissue demonstrated no difference in oil-red-O staining after differentiation with dexamethasone, 3-isobutyl-1-methylxanthine, and insulin (Supplemental Figure IV, available online at http://atvb.ahajournals.org). Consistent with the findings of smaller visceral adipocytes, Id3−/− mice had significantly lower insulin levels compared to WT controls. Glucose levels measured at the same time and circulating lipid levels were equivalent between the 2 groups (Supplemental Figure VA–VC). Evaluation of metabolic parameters in mice fed 5 weeks of Chow showed no difference in food intake, total activity, or oxygen consumption between WT and Id3 null mice (data not shown). In contrast, Id3 null mice fed 5 weeks of HFD had significantly higher average and resting oxygen consumption (Supplemental Table II, available online at http://atvb.ahajournals.org). In addition, Id3−/− mice had a trend toward lower serum free fatty acid levels compared to WT controls (1886±217 versus 2472±197 μmol/L, P=0.076, n=5–6 mice per group).
expression in the SVF in response to HFD, VEGFA is also increased in the SVF after HFD (Figure 5D).

The VEGFA promoter contains several E-boxes, CANNTG consensus binding sites for bHLH factors. To more fully elucidate the molecular mechanism whereby Id3 may promote VEGFA expression, we transiently cotransfected a 5.2-kb VEGFA promoter-luciferase reporter construct (Figure 6A) with expression plasmids encoding Id3 and the Id3-binding partner E12 into NIH 3T3 cells. Results demonstrated a significant repression of VEGFA promoter activation by cotransfected E12; an effect significantly antagonized by cotransfection of increasing amounts of Id3 (Figure 6B).

**Discussion**

Many genes that regulate embryonic development are reexpressed in the adult animal in disease states, such as cancer and cardiovascular disease. These genes make particularly appealing targets of therapy, as they are generally not expressed in normal adult tissues where modulation of expression could lead to untoward effects. Id3 is expressed early in embryonic development, with expression declining as the embryo matures. Previous studies have implicated Id3 in cancer and cardiovascular disease; however, the present study provides the first evidence implicating Id3 in visceral adipose depot expansion in response to HFD. Although loss of Id3 did not alter visceral depot sizes at baseline, it did result in a significant attenuation of HFD and age-induced visceral depot expansion. This suggests that Id3, independent of its developmental role, functions in the adult animal to modulate the response to HFD leading to visceral adiposity.

Decreased visceral adiposity of Id3 null mice on HFD was associated with lower serum insulin levels and higher metabolic rates. This is consistent with published reports demonstrating an inverse relationship between visceral adipose tissue mass and whole body insulin sensitivity and metabolic rates. In Id3 null mice, the smaller visceral adipose tissue depots contain smaller adipocytes, which have been associated with reduced basal lipolysis. Consistent with decreased adipocyte lipolysis, Id3 null mice showed a trend toward lower free fatty acid levels under HFD conditions. Note that less fat storage in adipose tissue in Id3 null mice does not lead to increased circulating lipid levels and, as indicated by improved insulin sensitivity, increased lipid deposition in...
other tissues like liver and skeletal muscle. Instead, free fatty acids may be burned at an increased rate to cover increased energy expenditure. These observations are consistent with a hypothesis that Id3 regulates adipose angiogenesis and consequently adipose tissue expansion and whole body metabolism. Indeed, when angiogenesis was inhibited pharmacologically in mouse models of obesity, smaller adipose tissue mass, improved whole body metabolism, and increased use of fatty acids as energy substrates were observed. However, we cannot rule out the possibility that the metabolic phenotype of our Id3 null mice is due to changes in other tissues that play roles in the regulation of insulin sensitivity and energy homeostasis such as skeletal muscle, liver, and brain. The answer to this question will need to await the availability of mice with cell type-specific deletion of Id3.

In addition to a unique role in visceral versus SC adipose tissue, our data also demonstrate a unique role for Id3 in adipose tissue biology relative to other Id proteins. Both Id2 and Id4 have been implicated in promoting differentiation of adipocytes in vivo and in vitro. Overexpression of Id2 increased expression of PPARγ, aP2, C/EBPα, and adiponectin and promoted lipid accumulation in 3T3-L1 preadipocytes in response to an adipocyte differentiation cocktail. Conversely, compared to WT, MEFs null for Id2 had decreased expression of PPARγ, aP2, and adiponectin when treated with adipocyte differentiation media. Moreover, Id2−/− mice had decreased intrascapular and inguinal adipose tissue weights at birth. Similarly, mice lacking Id4 had decreased fat mass compared to control mice even on chow diet, and MEFs null for Id4 had decreased levels of similar markers of adipocyte differentiation. The role of Id3 in adipocyte differentiation has been less clear. Moldes et al demonstrated that Id1, Id2, and Id3 mRNA levels, abundant in multiplying 3T3-F422A cells, drop significantly when the cells are induced to differentiate. Coupled with data demonstrating that overexpression of Id3 resulted in decreased glycerol-phosphate dehydrogenase activity, a marker of adipocyte differentiation, the authors concluded that Id3 prevented differenti-
visceral adiposity in the Id3 null mice. Angiogenesis is necessary for adipose tissue expansion and studies have shown that inhibition of angiogenesis inhibits that growth. It has also been proposed that visceral fat secretes proangiogenic factors, suggesting visceral fat is better adapted for rapid expansion. In addition to promoting adipocyte hypertrophy, adipose tissue angiogenesis allows for inflammatory cell infiltration. Increased levels of lymphocytes are visible in murine visceral adipose tissue after just 3 weeks of high-fat feeding. Continued high-fat feeding leads to enhanced CD8⁺ T cell infiltration and promotes a CD4⁺ Th1 bias, both of which help recruit M1 macrophages to visceral adipose tissue—a key event in the development of insulin resistance. Macrophages and mast cells have also been shown to further enhance adipose tissue angiogenesis in the context of diet-induced obesity, suggesting a positive feedback loop that contributes to the systemic metabolic abnormalities observed in obese individuals. Id3 proteins have been established as necessary for tumor angiogenesis. Data from this study demonstrates not only that mice null for Id3 have less microvascular blood volume in their epididymal fat depots but also that Id3 regulates visceral adipose VEGFA expression at the protein and promoter level. VEGFA has been implicated as an important mediator of angiogenesis and serum VEGFA levels are significantly increased in obese compared to lean humans and mice. Taken together, results suggest that loss of Id3 attenuates visceral fat expansion by inhibiting HFD-induced visceral fat VEGFA expression and increased capillary density. Future studies using postmortem histology and other techniques to assess visceral adipose angiogenesis are needed to confirm and extend these findings. Nonetheless, as inhibition of Id3 has been proposed as a strategy to limit angiogenesis in vivo and inhibition of adipose angiogenesis is a current target to attenuate obesity, results hold promise to lead to novel approaches to limit visceral adiposity.

Figure 6. The bHLH protein E12 inhibits expression of the VEGFA promoter; an effect antagonized by Id3. A, The VEGFA promoter contains numerous E box sequences (CANNTG) where the promoter contains numerous E box sequences (CANNTG) where the bHLH protein E12 inhibits expression of the expression of the adipocyte differentiation marker, GLUT-4, in OP9 or 3T3-L1 cell lines treated with 0.05 μg of the 5.2 Kb VEGFA-promoter-luciferase reporter together with specified expression vectors (EV indicates empty vector, quantities transfected are in μg). Luciferase activity is normalized to protein levels. Data are the result of 3 separate experiments of duplicate samples.

B, NIH 3T3 cells were transfected with 0.05 μg of the 5.2 Kb VEGFA-promoter-luciferase reporter together with specified expression vectors (EV indicates empty vector, quantities transfected are in μg). Luciferase activity is normalized to protein levels. Data are the result of 3 separate experiments of duplicate samples.

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Disclosures

None.

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Research Design and Methods:

Animals – All animal protocols were approved by the Animal Care and Use Committee at The University of Virginia. Id3−/− mice used in these studies were on a pure C57BL/6 background (confirmed by microsatellite testing). Male C57BL/6 (WT) and Id3−/− litter-mates were given standard chow diet and water ad libitum until they were genotyped and were either continued on a standard chow diet or placed on a 60% fat diet (HFD) from Research Diets (catalog #D12492) starting at 3-4 weeks of age. Length of high-fat feeding ranged from four to 20 weeks as specified in each experiment. At sacrifice, the mice were given an overdose of ketamine/xylazine and blood was collected by left ventricular puncture prior to perfusion with PBS. Epididymal, subcutaneous and retroperitoneal fat depots as well as intrascapular brown fat were harvested for analysis according to the technique of Hausman et al1. Specifically, the epididymal depot was designated as visceral fat (unless otherwise noted in the figure legend) and the subcutaneous fat obtained was dissected from the inguinal region up to a horizontal line parallel to the xiphoid cartilage on both sides. All lymph nodes were removed prior to analysis.

Isolation of Adipocytes and Stromal Vascular Fraction (SVF) – Epididymal and subcutaneous adipose tissue was minced with scissors and digested with collagenase type I (Worthington lot #47N9955) (1 mg/mL) in KRH (130 mM NaCl, 4.7 mM KCl, 1.24 mM MgSO₄, 2.5 mM CaCl₂, 1 mM HEPES, 2.5 mM NaH₂PO₄, 5 mM D-glucose, and 200 nM adenosine, pH 7.4) + 2.5% BSA, pH 7.4, for 60 minutes in a shaking water bath.
at 37°C. After filtration through a nylon mesh (400μm), samples were centrifuged at 400 x g for 5 minutes. SVF pellets and floating adipocytes were separated. Both the SVF and adipose cell suspensions were washed two times with KRH, then one time in PBS to ensure removal of all BSA in the digestion medium. The SVF and adipocytes were either snap-frozen in liquid nitrogen or immediately homogenized in lysis buffer for evaluation by western blot.

Western Blot Analysis – Whole adipose tissue was homogenized in lysis buffer (100 mmol/L Tris pH 6.8, 11% glycerol, 7.7% SDS), incubated at room temperature for ten minutes, and centrifuged for five minutes at 1,310 x g. The infranatants were separated from the lipid layer and re-centrifuged at 1,310 x g for ten minutes. Stromal vascular fraction cells and isolated adipocytes were homogenized in Triton lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Hepes, 10% glycerol, protease inhibitor cocktail- Sigma, catalog #P8340) and samples were assayed for protein concentration with the Bio-Rad DC protein assay (catalog #500-0111), adjusted to equal concentration with lysis buffer, and supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad). Equal protein loading was confirmed by staining the membrane with Amido Black or by immunodetection of β-tubulin. Western blotting was carried out using an antibody to Id3 (0.1 μg/ml, CalBioreagents, catalog #M097), e-IF5 (40 μg/ml Santa Cruz, catalog #sc-282), AP2 (0.04 μg/ml, R&D Systems, catalog #AF1443), PEPCK (0.08 μg/ml, Santa Cruz, catalog #sc-32879), CEBPα (0.4μg/ml, Santa Cruz, catalog #sc-61), β-actin (0.05 μg/ml, Santa Cruz, catalog #sc-
47778), GAPDH (0.02 μg/ml, Chemicon, catalog #MAB374), β-tubulin (16 μg/ml, Cell Signaling Technology, Inc. catalog #2146), or VEGFA (1μg/ml, Santa Cruz catalog #sc-507) followed by horseradish peroxidase-linked secondary antibody (Jackson).

Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL reagent (Amersham Pharmacia Biotech).

**Real-Time PCR Analysis** – Adipose tissue was snap-frozen in liquid nitrogen and total cellular RNA was collected using TRIzol Reagent (Invitrogen) as per the manufacturer’s instructions. Genomic DNA was removed by Nase I (Invitrogen), and cDNA was then synthesized using an iScript cDNA synthesis kit (Bio-Rad). Total cDNA was diluted 1:5 in water and 2 μl were used for each real-time PCR reaction using a Bio-Rad iCycler and iQ SYBR Green Supermix (Bio-Rad). Each primer was used at 60º C annealing temperature for 30 seconds for 40 cycles. Analysis was performed by normalizing to cyclophilin using the standard curve method to approximate the amount of starting material. Primers for the detection of mouse Id3, perilipin, fatty acid synthase (FAS), hormone sensitive lipase (HSL), stearoyl-CoA-desaturase-1 (SCD-1), adipose triglyceride lipase (ATGL), and liver x-receptor-α (LXR-α) are provided in Table I.

**Body composition by DEXA** – The mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), introduced into the DEXA machine (GE Lunar PIXImus II densitometer) and subjected to total body imaging. Body composition, lean and fat mass, was then determined using
PIXiMus software. DEXA analysis was performed after four, eight, 12, and 16 weeks of HFD.

*Cell Culture* – MEFs were isolated from day 13 WT or Id3−/− embryos. MEFs were cultured in DMEM with high glucose, 10% fetal bovine serum (FBS- purchased from ZenBio), 2mM L-glutamine and penicillin/streptomycin. Once confluent (D0), the media was changed for an additional two days (D+2). On D+2, the cells were then cultured in differentiation media (DM1- DMEM with high glucose, 10% FBS, 2mM L-glutamine, 6µg/ml dexamethasone, 0.5mM IBMX, and 10µg/ml insulin, penicillin/streptomycin) for seven days at which time they were incubated in post-differentiation media (DM2- DMEM with high glucose, 10% FBS, 2mM L-glutamine, 10µg/ml insulin and penicillin/streptomycin) for an additional seven days. For the stromal vascular cell (SVF) differentiation, epididymal fat pads were removed from 3 WT and 3 Id3−/− littermates. The SVF was isolated as previously described5. The entire SVF from each depot was placed in a single well of a 12-well plate. Cells were cultured in DM1 two days then switched to DM2 for an additional seven days until fully differentiated. For both MEFs and SVFs lipid accumulation as a marker of adipocyte differentiation was evaluated by quantifying oil-red-o staining, after elution with isopropanol, at 510nm absorption. NIH 3T3 cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% Penicillin/Streptomycin (Invitrogen).
**Histology** – Adipose tissue from the proximal portion of the epididymal depot was paraffin embedded and then sectioned and stained with hematoxylin and eosin. To assess adipocyte sizes, three sections per mouse of tissue separated by 100 micrometers were analyzed, and the perimeter of every cell was measured using Image Pro software. Data for adipose cell perimeters are presented in micrometers.

**Measurement of Serum Parameters** – Non-fasting glucose measurements were obtained from tail vein whole blood using an AccuCheck advantage glucometer. Insulin levels were obtained from mouse serum obtained through retro-orbital bleed at the same time as the glucose measurements, and determined by radioimmunoassay per the manufacturer’s instructions (Millipore, catalog #SRI-13K). Serum lipid values were obtained by the Clinical Pathology lab at the University of Virginia from serum collected after 20 weeks of diet. Free Fatty Acid (FFA) levels were assessed by colorimetric assay per the manufacturer’s instructions (ZenBio catalog #SFA-1) from serum of mice on HFD for 4 weeks. Mice were fasted overnight for 12 hours prior to serum collection. VEGFA levels were assessed by ELISA assay (R&D Systems, Inc. catalog #MMV000) in serum collected from mice on HFD for 4 weeks according to manufacturer’s instructions.

**Metabolic Cage Measurements** – Both Id3−/− and WT mice were placed on HFD at four weeks of age. They were transferred into the metabolic cages at nine weeks of age. Mice were maintained on a 14-hour light/10-hour dark cycle in a pathogen-free animal facility. Food intake, ambulatory activity, VO₂ (oxygen consumption) and VCO₂ (carbon dioxide production) were simultaneously determined for four mice per experiment in an Oxymax
metabolic chamber system (Comprehensive Laboratory Animal Monitoring System from Columbus Instruments, Columbus, Ohio, USA). One reading per mouse was taken every 15 minutes over 72 hours. The mice were allowed to adjust to the cages during the first 24 hours and only the last 48 hours of each experimental run was used for data analysis.

**Immunofluorescence** – Serial 5 µm sections were cut from paraformaldehyde-fixed, paraffin-embedded epididymal adipose tissue. Samples were deparaffinized and subjected to antigen retrieval by microwaving for 20 minutes in 1x antigen unmasking solution (Vector Labs, Burlingame, CA). Slides were incubated for 30 minutes in 3% H₂O₂ followed by 1 hour in 10% horse serum in PBS. Primary antibodies and dilutions were rabbit anti-Id3, 1:50 and goat anti-Cd31, 1:50 (both from Santa Cruz Biotechnology, Santa Cruz, CA.). Immunodetection was accomplished using 1:100 dilutions of donkey anti rabbit Alexa Fluor 488 for Id3 and donkey anti goat Alexa Fluor 555 for CD31 (Invitrogen).

**Contrast Enhanced Ultrasound** – Animals were anesthetized with 1% isoflurane mixed with room air, then placed on a warming plate to maintain body temperature and minimize any temperature induced changes in blood flow. A small incision was made in the side of the abdominal wall and the epididymal depot was extracted and placed on an ultrasound barrier warmed to 37°C. The vasculature was kept intact and extreme care was taken to minimize tissue manipulation to avoid damaging the microvasculature. Ultrasound gel (Sonotech, Bellingham, WA) was warmed to 37°C, and then placed onto the depot. Data was acquired using the Sequoia 512 ultrasound apparatus with a 15 L8
transducer, operated in CPS mode at 7 MHz and MI=0.2 to avoid microbubble
destruction and obtain optimal signal-to-background ratio. The apparatus was placed
over the depot in the gel, centered off of the testis. Imaging focus was placed at the tissue
depth. Data was acquired at a 50dB dynamic range. The transducer was placed
approximately 1 cm over the depot. Baseline data was acquired prior to bubble
administration, then a bolus of 25 µl of the microbubble suspension (3x10⁹ bubbles/ml)
was delivered intravenously via a retro orbital sinus route². Data was acquired for a
minimum of 30 seconds before the bolus injection, and continuously for 5 minutes
following it. The data acquired by the system was exported as MOV files, and frames
analyzed using ImageJ software (NIH). Still captures were taken from the video file
every 5 seconds for 2 minutes for the vascular volume measurements, and once every
second for the perfusion rate analysis, as the bolus infused the depot. A consistent ROI
was drawn within the depot, approximately 0.25 cm from the testis, avoiding large
vessels to determine the microvasculature volume of the fat depot³.

*Microbubble Preparation* – Microbubbles were prepared by sonication from
decafluorobutane gas and stabilized with a lipid monolayer shell. Briefly, during
sonication at 20 KHz (Misonix XL2020 instrument, equipped with 1/2" tip horn and
operated at maximum power for 30 sec), the aqueous saline dispersion of 2 mg/ml
phosphatidylcholine (Avanti Lipids, Alabster, AL) and 2 mg/ml PEG-stearate (Stepan
Kessco, Northfield, IL) was bubbled with a decafluorobutane gas. Microbubbles were
purified from excess lipid and size-adjusted by multiple flotations in degassed saline,
then aliquotted in glass vials and stoppered in decafluorobutane atmosphere, for refrigerated storage.

**VEGFA ELISA Assay on Adipose Lysates** – Epididymal fat pads were removed from WT and Id3-/- mice on HFD for seven weeks and lysed in 2 mL RIPA buffer (1% NP-40, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, protease inhibitor cocktail). VEGFA ELISA assays (R&D Systems, Inc catalog #MMV000) were performed with 50 µL of lysate in duplicate per manufacturer’s instructions.

**VEGFA Promoter Reporter Assay**– NIH 3T3 cells (100,000 per well in 6 well plates) were transfected with 0.05 µg of the 5.2 Kb VEGFA-promoter-luciferase reporter together with 0.6 µg total of hE12 in pEF4, hId3 in pEF4, and/or pEf4 Empty Vector using Effectene reagent according to the manufacturer’s instructions (Qiagen). Twenty-four hours after transfection, cells were harvested, lysed and assayed for luciferase activity using a luciferase assay lit from Promega (Madison, WI). Protein concentrations from individual samples were quantified using the Pierce BCA Protein Assay Kit and luciferase values were normalized to protein.

**Statistics** – A Student’s t test was performed in the setting of equal variance, while a Mann-Whitney test was used if the variance was not equal to compare continuous variables. A paired t test was performed when comparing two continuous variables derived from the same animals. A p-value of < 0.05 was considered significant.
<table>
<thead>
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<th>Gene (mouse)</th>
<th>Primer Sequence</th>
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<tr>
<td>Id3</td>
<td>Forward 5’- TGT CGT CCA AGA GGC TAA GAG GCT -3’</td>
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<td>Reverse 5’- TGC TAC GAG GCG GTG TGC TG -3’</td>
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<tr>
<td></td>
<td>Reverse: 5’ - AAC AGC CTC AGA GCG ACA AT -3’</td>
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<td>Hormone Sensitive Lipase</td>
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<td>Reverse: 5’ – TCT CGT TGC GTT TGT AGT GC -3’</td>
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<td>Reverse: 5’ - GGG CAT CCT GGC TTC CTC -3’</td>
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Figure I.

A) Length

B) Waist Circumference

C) Images of WT and Id3−/−
Figure II.

A. AP2

B. PEPCK

C. CEBP-α

[Graphs showing densitometry in arbitrary units for WT and Id3-/- for each protein]
Figure III.
Figure IV.

![Graph showing OD510 (fold over WT) for MEF and visceral SVF samples with WT and Id3-/- comparisons.](image-url)
A. Serum Insulin

B. Serum Glucose

C. Serum Lipid Levels
Figure VI
<table>
<thead>
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<th>Table II.</th>
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<tr>
<td>Weight in (g)</td>
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<td>Weight out (g)</td>
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<td>VO$_2$ (ml/kg BW/h)</td>
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<td>Food intake (g/48 h)</td>
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<td>Water intake (ml/48 h)</td>
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Supplemental Figure Legends

**Figure I.** Mice null for Id3 are smaller than WT controls after 20 weeks on high fat diet. (A.) Length (nose to base of tail) and (B.) waist circumference (measured above the iliac crest) of mice after 20 weeks on HFD (n = 9-10). (C.) A representative photograph of Id3<sup>+/-</sup> and WT mice after 20 weeks on HFD.

**Figure II.** Established markers of differentiation were evaluated at the protein level and showed no differences in expression between the WT and Id3<sup>−/−</sup> groups. Protein lysates (n = 3-5 for each blot) were obtained from epididymal adipose tissue of WT and Id3<sup>−/−</sup> mice fed a HFD for 20 weeks. The histograms display densitometric measurements of (A.) AP2, (B.) PEPCK, and (C.) CEBP-α, and were normalized to either β-actin or GAPDH as indicated. A representative blot is displayed below each histogram. No significant differences were seen between the WT and Id3<sup>−/−</sup> groups for any of the markers evaluated.

**Figure III.** Real time PCR performed on WT and Id3<sup>−/−</sup> visceral adipose tissue show no differences in structural and lipolytic markers. Total RNA was harvested from epididymal adipose tissue of WT and Id3<sup>−/−</sup> mice fed HFD for 4 weeks fed mice (n = 5 in each group) and analyzed by quantitative PCR. Target transcripts were normalized to the corresponding cyclophilin signal. Transcripts analyzed were for (A.) perilipin, (B.) fatty acid synthase (FAS), (C.) hormone sensitive lipase (HSL), (D.) adipose triglyceride lipase (ATGL), (E.) stearoyl-CoA desaturase-1 (SCD-1), and (F.) liver X receptor-α (LXR-α). No significant differences were seen between the WT and Id3<sup>−/−</sup> groups for any of the markers evaluated.

**Figure IV.** WT and Id3<sup>−/−</sup> MEFs and visceral SVF show no differences in Oil Red O staining after treatment with differentiation media. MEFs were isolated from day 13 embryos of WT and Id3<sup>−/−</sup> mice (n = 4 separate isolations for each genotype) and plated in triplicate. At confluence, the cells were differentiated with dexamethasone, IBMX and insulin (as described in the methods section). Stromal vascular cells were isolated from the epididymal depot of three separate pairs of WT and Id3<sup>−/−</sup> mice as described in the methods section. All of the stromal vascular cells isolated from one mouse were plated, then differentiated with dexamethasone, IBMX and insulin, as described. Lipid content is shown as density of Oil Red O staining and shown as fold change compared to WT MEFs.

**Figure V.** Id3<sup>−/−</sup> mice have significantly lower circulating insulin levels than WT controls while maintaining equivalent glucose levels and lipid levels after 20 weeks of HFD. (A.) Serum was obtained via retro-orbital bleed at random times and insulin levels are shown as ng/mL. (B.) Glucose levels were obtained from whole blood via tail snip at the same time blood for determination of insulin levels were drawn. (C.) Serum was obtained at sacrifice from WT and Id3<sup>−/−</sup> animals fed a HFD for 20 weeks and was sent to the Clinical Pathology lab at the University of Virginia for analysis. Each circle represents one mouse with the line representing the mean value, n.s. indicates a p-value of ≥ 0.05.
Figure VI. Immunolocalization of Id3 and CD31 in visceral adipose tissue.
Immunostaining for Id3 and CD31 was performed on sections of visceral fat from 8 week old C57BL/6 mice. (A.) Bright-field image showing adipocytes and small blood vessel (arrow). Co-staining for Id3 and CD31 was performed on the same slide using antibodies for CD31 (B.) and Id3 (C.). (D.) Image merging to demonstrate co-expression Arrowhead denotes area of CD31 staining only. Magnification is 40X.

Table II. Metabolic cage data obtained on WT and Id3<sup>−/−</sup> mice fed HFD. Nine week old WT and Id3<sup>−/−</sup> fed HFD for 5 weeks were placed in metabolic cages as described in the methods section (n = 5-6 mice in each group). VO2 = total oxygen consumption, VCO2 = total carbon dioxide production.

References:

2. Phillips P: Contrast-agent detection and quantification, European radiology 2004, 14 Suppl 8:P4-10
4. Wible JH, Galen KP, Wojdyla JK, Hughes MS, Klibanov AL, Brandenburger GH: Microbubbles induce renal hemorrhage when exposed to diagnostic ultrasound in anesthetized rats, Ultrasound in Medicine & Biology 28:1535-1546