Identification of RIFL, a novel adipocyte-enriched insulin target gene with a role in lipid metabolism

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Submitted 16 February 2012; accepted in final form 7 May 2012

Ren G, Kim JY, Smas CM. Identification of RIFL, a novel adipocyte-enriched insulin target gene with a role in lipid metabolism. Am J Physiol Endocrinol Metab 303: E334–E351, 2012. First published May 8, 2012; doi:10.1152/ajpendo.00084.2012.—To identify new genes that are important in fat metabolism, we utilized the Lexicon-Genentech knockout database of genes encoding transmembrane and secreted factors and whole murine genome transcriptional profiling data that we generated for 3T3-L1 in vitro adipogenesis. Cross-referencing null models evidencing metabolic phenotypes with genes induced in adipogenesis led to identification of a new gene, which we named RIFL (refeeding induced fat and liver). RIFL-null mice have serum triglyceride levels approximately one-third of wild type. RIFL transcript is induced $>100$-fold during 3T3-L1 adipogenesis and is also increased markedly during adipogenesis of murine and human primary preadipocytes. siRNA-mediated knockdown of RIFL during 3T3-L1 adipogenesis results in an $\sim35\%$ decrease in adipocyte triglyceride content. Murine RIFL transcript is highly enriched in white and brown adipose tissue and liver. Fractionation of WAT reveals that RIFL transcript is exclusive to adipocytes with a lack of expression in stromal-vascular cells. Nutritional and hormonal studies are consistent with a prolipogenic function for RIFL. There is evidence of an approximately eightfold increase in RIFL transcript levels in WAT in ob/ob mice compared with wild-type mice. RIFL transcript level in WAT and liver is increased $\sim80$- and 12-fold, respectively, following refeeding of fasted mice. Treatment of 3T3-L1 adipocytes with insulin increases RIFL transcript $\leq35$-fold, whereas agents that stimulate lipolysis downregulate RIFL. Interestingly, the 198-amino acid RIFL protein is predicted to be secreted and shows $\sim30\%$ overall conservation with the NH$_2$-terminal half of angiopoietin-like 3, a liver-secreted protein that impacts lipid metabolism. In summary, our data suggest that RIFL is an important new regulator of lipid metabolism.

triglyceride; adipogenesis; differentiation

WHITE ADIPOCYTES OF WHITE ADIPOSE TISSUE (WAT) are of major importance in lipid metabolism because they are involved in storage and release of triacylglycerol (10, 30, 31, 42, 58, 81). Adipogenesis is the conversion of fibroblast-like preadipocytes to rounded lipid-filled mature adipocytes (5, 35, 47, 48). Peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear hormone receptor family member, functions as a master transcriptional regulator of adipogenesis (29, 33, 64, 72). Multiple additional transcriptional and other signaling mechanisms positively or negatively regulate the process of adipogenesis (15, 35, 53, 69). Because adipocytes are highly specialized for lipid metabolism, genes that are markedly upregulated during adipogenesis are strong candidates as new regulators of lipid metabolism. First established as an in vitro model of adipogenic conversion in the 1970s, the 3T3-L1 cell line has remained the dominant in vitro model of adipogenesis and has been proven to be pivotal in the identification of genes and pathways key to adipogenesis and adipocyte function as occurring in vivo (52). While proliferating, these cells appear as fibroblasts, but upon treatment with the adipogenic-inducing agents dexamethasone and methylisobutylxanthine, these cells undergo an approximately 7- to 10-day process of differentiation to mature fat cells.

White adipocytes also impact systemic metabolism via production and secretion of a number of adipokines and other secreted factors (3, 75), e.g., leptin (9), adiponectin (58), and TNFα. Leptin is an adipocyte-specific hormone that is secreted by mature fat cells and signals through hypothalamic receptors to regulate energy intake and expenditure (9). Adiponectin is an adipocyte-specific secreted factor that exerts important regulatory effects on glucose uptake via interaction with adiponectin receptors in muscle and liver (58). Although macrophages are a key source of TNFα in obese WAT, adipocytes also produce this proinflammatory cytokine. TNFα is regarded as a major factor underlying the proinflammatory state present in obesity. Among its various effects, TNFα stimulates lipolysis of adipocyte triacylglycerol stores to lead to the release of nonesterified fatty acid into the circulation with accompanying deleterious effects. Treatment of mature fat cells in vitro with TNFα leads to a type of “dedifferentiation” that is accompanied by a partial reversal of the adipocyte transcriptome to that resembling the preadipocyte (55).

Dysregulation of lipid storage and release by fat cells underlies the metabolic consequences of obesity and lipodystrophy and results in lipotoxicity, the deleterious uptake and storage of fat in nonadipose tissues (73, 74). Thus the identification of new adipocyte genes, including those that may encode secreted factors and/or be involved in lipid storage, is of major importance to fully understand the development and function of fat cells and how fat cells may impact homeostasis within WAT as well as systemically.

We searched for new genes that may be important in lipid metabolism using a combination of in silico and experimental approaches. This led us to focus on a novel and uncharacterized murine gene known to date as Gm6484 and also as EG624219. The human version of this novel gene is designated as LOC55908, C19ORF80, or TD26. We have named this new gene RIFL (refeeding induced fat and liver). Based on our data, which include regulation of RIFL gene expression by nutritional and hormonal factors, restricted tissue expression to fat and liver, the reduction of serum triglyceride levels in RIFL-null mice, and siRNA-mediated knockdown studies in 3T3-L1 cells, we posit that RIFL is an important new regulator of lipid metabolism.

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MATERIALS AND METHODS

Cell culture and in vitro differentiation of preadipocytes to adipocytes. 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. For adipocyte differentiation, 3T3-L1 cells were typically treated at 2 days postconfluence with DMEM supplemented with 10% FCS and the adipogenic inducers 0.5 mM methylisobutylxanthine (MIX) and 1 μM dexamethasone (DEX) for 48 h. Adipogenic agents were then removed, and growth of cultures continued in DMEM containing 10% FCS. At 5–7 days postinduction of differentiation of naive 3T3-L1 preadipocytes, adipocyte conversion had generally occurred in ~90% of the cells, as assessed by lipid accumulation and rounded cell morphology.

ScAP-23 cells (26) were maintained in DMEM with 10% calf serum and passaged before confluence. For adipocyte differentiation, preconfluent ScAP-23 preadipocytes were treated with DMEM supplemented with 10% FCS in the presence of 0.5 mM MIX, 1 μM DEX, 17 nM insulin, and 0.2 mM indomethacin for 70 h. These agents were then removed, and cultures were maintained in DMEM with 10% FCS and 17 nM insulin for 4 more days.

For culture and differentiation of murine primary white preadipocytes, white adipose tissue was collected from C57BL6 mice and digested with 1 mg/ml type I collagenase by shaking for 40 min at 37°C. Digestion product was filtered through a 300-μm pore nylon mesh (Sefar America, Depew, NY) and filtrate centrifuged at 2,000 rpm for 5 min. The floating adipocyte fraction was removed and the stromal-vascular cell pellet was resuspended in DMEM containing 10% FCS and cells plated. Upon confluence, cells were either collected as preadipocytes or exposed to differentiation medium consisting of DMEM containing 10% FCS, 0.25 mM MIX, 0.1 μM DEX, and 17 nM insulin for 3 days. Differentiation medium was removed, and cultures were grown in DMEM containing 10% FCS and 17 nM insulin for 4 additional days. Human primary preadipocytes were purchased from Zen-Bio (Chapel Hill, NC) and cultured or differentiated to adipocytes exactly according to their provided protocol and reagents. Human cells were harvested as confluent preadipocytes or on day 14 postinduction of adipogenesis.

For differentiation of the murine brown preadipocyte cell line, referred to here as WT-BAT and obtained from C. R. Kahn (Joslin Diabetes Foundation, Harvard Medical School, Boston, MA), the method was as described previously (23, 27). Briefly, cells were grown to confluence in DMEM with 10% FCS, 20 nM insulin, and 1 nM triiodothyronine. Confluent cells were incubated in this medium with 0.5 mM MIX, 0.5 μM DEX, and 0.125 mM indomethacin for 48 h, and cultures were then maintained in DMEM with 10% FCS, 20 nM insulin, and 1 nM triiodothyronine over the time course of full differentiation.

Treatment of adipocytes with hormones and other agents. All chemicals for the treatment of cultured cells were obtained from Sigma-Aldrich. For insulin regulation studies, 3T3-L1 adipocytes were cultured for 6 h in serum-free DMEM with 0.5% BSA. Cultures were then switched to serum-free DMEM containing 0.5% BSA or 0.25% BSA and with or without 100 nM insulin, or other concentration as shown, for the indicated periods. For studies of regulation by insulin and glucose in combination, 3T3-L1 adipocytes were first cultured for 16 h in serum-free DMEM with 0.5% BSA, 1 mM pyruvate, and 1 mM lactate. Cultures were then changed to serum-free DMEM containing 0.5% BSA supplemented with 2 μM insulin or 4.5 g/l d-glucose, separately or in combination as indicated, for 24 h. For treatment of 3T3-L1 adipocytes with TNFα, cells were incubated with or without 10 ng/ml TNFα in DMEM with 10% FCS for 24 or 72 h. Human adipocytes were treated with 10 ng/ml TNFα in DMEM-F-12 (1:1) under serum-free conditions for 36 h. For treatment of 3T3-L1 adipocytes with isoproterenol (10 μM), dibutylry cAMP (1 mM), or forskolin (10 μM), agents were added to cultures in DMEM with 10% FCS. For treatment with DEX, adipocytes were cultured for 6 h in serum-free DMEM prior to the addition of vehicle control or 1 μM DEX for 24 h.

siRNA-mediated knockdown studies. For knockdown of either PPARγ or RIFL in 3T3-L1 adipocytes, siRNA was used at a final concentration of 25 nM. siRNA was purchased from Dharmacon (Lafayette, CO). For RIFL siGenome SMARTpool catalog no. 072227-00 was used, for PPARγ a single siRNA species was used (catalog no. D-040712-01), and for siControl nontargeting siRNA catalog no. D-00121-01-05 was used. siRNA was introduced into 3T3-L1 cells using a Neon electroporation system (Life Technologies) according to the manufacturer’s instructions, with a protocol of two pulses at 1400 V and pulse width of 20 ms and utilization of 100-μl size electroporation tips. Following electroporation, cells were plated at a density of ~5 × 10^4 cells/well of 12-well plates. Cultures attained confluence by the next day and were subjected to adipogenic differentiation treatment with 0.25 mM MIX, 0.25 μM DEX, and 86 nM insulin. Studies of the effect of RIFL knockdown in 3T3-L1 cell differentiation were conducted three times independently with triplicate cell culture wells assessed, per each type of specific siRNA treatment, each time.

To assess effectiveness of the RIFL SMARTpool siRNA to knockdown RIFL transcript, 3T3-L1 adipocytes were electroporated, and RNA was harvested 2 days later. To assess effectiveness of RIFL knockdown at the protein level, since antibody for endogenous RIFL protein was not available, we assessed the effectiveness of protein knockdown using X-treme GENE HP (Roche Diagnostics, Indianapolis, IN) reagent to transfect an HA-tagged RIFL construct into 293T cells in the presence of either control siRNA or siRNA for RIFL, followed by Western blot analysis for HA-tagged RIFL protein. To assess the effectiveness of the knockdown of PPARγ with siRNA, PPARγ protein level in either PPARγ knockdown or control knockdown 3T3-L1 adipocytes was assessed by Western blot.

Oil Red O staining and measurement of intracellular triglyceride content. Cells were rinsed with PBS, fixed with paraformaldehyde for 1 h, and stained with Oil Red O for 1 h using the standard reagent and protocol. After thorough rinsing with water, cells were photographed using an Olympus IX70 microscope and Spot Advanced software; this was also used to capture images of live cells. To document views of complete culture wells, plates were scanned using a Hewlett-Packard HP7730A scanner to capture digital images. Minor adjustments to images were made for better visualization. When this was done, the same brightness and contrast settings were applied to the complete figure in the case of Fig. 6C and to each of the sets of three horizontal panels for Fig. 6B, with no information lost from the images. For quantification, Oil Red O was extracted from wells of fixed cells with 100% isopropanol, and the extracted material was diluted in 100% isopropanol and read at 490 nm using a Molecular Dynamics SpectraMax Plus384 plate reader. Absorbance readings for Oil Red O-stained PPARγ knockdown cultures, which contained essentially no adipocytes/lipid, were set to background for calculation of values for siRNA control and siRNA RIFL. All optical density (OD) readings were within the linear range for the assay, which we had determined previously by measuring OD at 490 nm of serial dilutions of Oil Red O in isopropanol. The effects of RIFL knockdown were tested in at least three independent studies, with three individual culture wells assessed for adipocyte/lipid content per study, for siRNA and siCon each, and for lipid content by photography and Oil Red O staining. To quantify lipid content, for two such independent experiments Oil Red O was extracted and measured spectrophotometrically, with n = 3 wells, for either siRIFL or siCon cultures. Assessment of adipocyte marker transcripts in siRIFL or siCon cultures by real-time PCR was done in two independent studies. One of these studies utilized three individual culture wells of siRIFL or siCon cells, and in the other study we pooled two culture wells together prior to RNA harvest and used two such pools per either siRIFL or siCon.

Animal treatments. For studies of RIFL transcript expression in murine tissues, 8-wk-old C57BL6 male mice or ob/ob mice were
purchased from Jackson Laboratories. For fasting and refeeding studies, 8-wk-old male C57BL/6 mice were subjected to overnight (16 h) food deprivation. The next morning animals were refed with a high-carbohydrate fat-free diet, and tissue was harvested 8 h later. Studies were conducted in duplicate. All procedures and animal treatments were carried out with protocols approved by the University of Toledo Institutional Animal Care and Use Committee.

**RNA preparation, real-time PCR, and transcriptional profiling.** RNA was purified using TriZol Reagent (Life Technologies) according to the manufacturer’s instructions. Human adipose tissue, testis, and heart RNA were from Clontech (Mountain View, CA), and human liver RNA was from Life Technologies. Human adipocyte RNA was generated by differentiation of human preadipocytes obtained from Zen-Bio. Fractionation of whole murine adipose tissue into adipocyte and stromal-vascular fraction was as described previously (14, 23, 24). For real-time PCR analyses, total RNA was purified from lysates with either an RNeasy RNA purification kit (Qiagen, Valencia, CA) or a DNA-Free RNA kit (Zymo Research, Irvine, CA); in all instances a DNease I treatment step was employed. Five micrograms of total RNA was used in first-strand cDNA synthesis using SuperScript II RNase H-reverse transcriptase (Life Technologies) and an oligo(dT)-22 primer. Real-time PCR was carried out with an ABI 7500 Real-Time PCR System. Target cDNA levels were determined by SYBR green-based real-time PCR in 25-μl reactions containing 1× SYBR or 1× Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 400 nM forward and reverse primers, and 10 ng of cDNA. Expression was normalized against GAPDH transcript signal. Sequences of real-time PCR primers are available on request, and in all cases primer pairs were designed to anneal such that they were separated by at least one intron. Cycle threshold value was generated using ABI PRISM 7500 SDS software version 1.2. Dissociation plots were generated after 40 cycles and showed a single distinct sharp peak. Statistical analyses were conducted using single-factor ANOVA. Additional details on graphical presentation of data are provided in the respective figure legends.

Transcriptional profiling was carried out for 3T3-L1 adipogenesis using RNA from day 0 3T3-L1 preadipocytes and day 10 3T3-L1 adipocytes. Array hybridizations were done on quadruplicate samples for each of these two time points using Illumina MouseWG-6 version 2.0 Expression BeadChips for mouse whole genome expression profiling of >45,200 transcripts. Following background subtraction, the average signal intensity for the quadruplicate day 0 and the quadruplicate day 10 samples was calculated. Probes for which averages were less than a value of 25 for both of the averages (day 0 and day 10) were excluded from consideration. Array hybridization and data analysis were on a fee-for-service basis through the University of Chicago Genomics Core at the Knapp Center for Biomedical Discovery, with array data analysis carried out by core director Dr. Pieter Faber.

**Mammalian expression constructs for RIFL and introduction of plasmid DNA into mammalian cells.** A murine RIFL expression construct, in which a COOH-terminal HA epitope tag was fused in-frame to the RIFL coding sequence, termed RIFL-HeA, was generated by PCR using murine WAT cDNA as template. Primers were based on the GenBank sequence of EG624219, NM_001080940.1. Purified PCR fragment was cloned into pcDNA3.1(+) (Invitrogen) and the insert fully sequence verified.

**Western blot analysis.** Cells were harvested from culture dishes by scraping into TTN (+) buffer (10 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM EDTA supplemented with a protease inhibitor cocktail). Lysates were incubated on ice for 30 min with intermittent vortexing, supernatant collected via centrifugation, and protein content determined (Bio-Rad Laboratories, Hercules, CA). Proteins were size-fractionated on SDS-PAGE gels for Western analysis and transferred onto Immobilon membrane (Millipore, Bedford, MA). For signal detection, membranes were blocked by incubation for 1 h in 5% nonfat milk-0.1% Tween-20 in PBS. For RIFL-HeA studies, this was followed by incubation with a 1:1,000 dilution of Y-11 anti-HA rabbit polyclonal antibody catalog no. SC-505 (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h, followed by three 10-min washes. Secondary antibody was goat anti-rabbit antibody (Bio-Rad Laboratories) at a dilution of 1:1,000 for 1 h, followed by three 10-min washes. For PPARγ studies, following blocking as described above, membranes were incubated with a 1:1,000 dilution of mouse monoclonal antibody E-8 for PPARγ catalog no. SC-7273 (Santa Cruz Biotechnology) for 2 h, followed by three 10-min washes. Secondary antibody was goat anti-mouse at a dilution of 1:1,000 for 1 h, followed by three 10-min washes. Loading controls for Western blot utilized either a rabbit polyclonal antibody against peptidylprolyl isomerase A-cyclophilin catalog no. 07-313 (Millipore) used at a 1:1,000 dilution or the mouse monoclonal DR0388 antibody against β-tubulin used at a dilution of 1:1,000, catalog no. SC-58882 (Santa Cruz Biotechnology). All washes were in 0.1% Tween-20 in PBS. Signal was detected by ECL Plus enhanced chemiluminescence (GE Healthcare) and image captured using an Alpha Innotech FluorChem HD Imaging System. Western blot images derived from distinct antibodies are demarcated by a black horizontal line. Minor adjustments of brightness and contrast were carried out to better visualize data, but in all cases the same manner of such changes were applied to the complete image panel as a whole, and no information was lost from the image.

**RESULTS AND DISCUSSION**

Identification of RIFL as a new regulator of lipid metabolism.

To identify candidate genes that may act as important regulators of adipocyte function, we conducted whole genome transcriptional profiling of in vitro adipogenesis. We compared transcript expression levels in 3T3-L1 preadipocytes with 3T3-L1 adipocytes at 10 days post-induction of adipogenic differentiation using Illumina BeadChip arrays. Probes with average signal intensities of <25 for both preadipocyte and adipocyte samples were not considered for data analysis to assess fold inductions. Because we wished to focus on genes uniquely expressed in adipocytes and essentially absent in preadipocytes, we also removed from further consideration probes showing average signal intensity in preadipocytes of >100. We set the lower limit of fold increase in adipocytes vs. preadipocytes to 10-fold and prioritized for assessment genes not already studied in adipogenesis or those not described in detail in other settings. Genes meeting these criteria were interrogated against the BioGPS database (http://biogps.org) generated by Novartis (32, 77) to assess tissue expression profiles across a wide array of murine tissues. Genes were also searched against the null mouse model phenotype database for secreted and transmembrane proteins generated by Lexicon-Genentech (70) and were accessible in silico through the National Institutes of Health-supported KOMP project phenotyping data repository (http://www.kompphenotype.org). The latter contains phenotypic data on null mouse models generated by the KOMP pilot project as well as that of 472 null mouse models generated by Lexicon-Genentech (70). We focused on those genes where the null mouse models evidenced effects primarily on metabolism and not of a more wide-ranging nature. These studies led us to further investigate a predicted murine gene that was to date previously unstudied. This gene was variously designated as Gm6484 or EG624219 in mouse and LOC55908, TD26, or C19ORF80 in human. Based on our subsequent studies of the expression and regulation of the transcript of this gene, we have named this new gene RIFL for refeeding induced fat and liver. This name was chosen to
indicate the tissue expression pattern for this gene as well as its nutritional regulation, which we report on below.

Data conducted and assembled by Lexicon-Genentech for RIFL-null mice reveal that these mice had markedly reduced serum triglyceride levels (70). The database web page containing all available data on the phenotypic assessment of this null model by the Lexicon-Genentech knockout model phenotyping effort is http://www.kompphenotype.org/summary-tab.php?gene=EG624219&project=#. A graph of the data points reported in the Lexicon-Genentech phenotyping database for serum triglyceride level is shown in Fig. 1A. According to this information, mice null for RIFL have decreased levels of serum triglyceride of approximately one-third that of wild-type (WT) littermates. This holds for the population of combined males and females as well as for either males or females. These data suggested to us that RIFL has the potential to be a major new regulator of lipid metabolism; as such, we further investigated its expression, regulation, and function.

The RIFL gene encodes a predicted protein of 22 kDa with homology to the liver-secreted factor angiopoietin-like 3. The murine RIFL gene product encodes a novel 198-amino acid protein with a calculated primary translation product of ∼22 kDa and is predicted to contain an NH2-terminal signal peptide. The nucleotide and corresponding amino acid sequence is shown in Fig. 1B. We are not aware of any publications on the RIFL gene, transcript, or protein in any species. The original identification of the human transcript appears in a 2000 submission to Genbank (Dong X, Pang X, and Cheng W, unpublished work), where it was termed hepatocellular carcinoma-associated protein TD26. Human RIFL protein is also 198

![Fig. 1. Phenotype of RIFL (refeeding-induced fat and liver-null) mice and sequence of murine RIFL gene and protein.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00084.2012)

**A**: reduced serum triglyceride phenotype. Data taken from the Lexicon-Genentech null mouse phenotyping project was plotted to demonstrate levels of serum triglyceride (mg/dl) in wild-type (WT) littermates [n = 2 females (F) and 2 males (M)] and in RIFL-null mice (n = 4 F and 4 M). Data is shown as means ± SE. *P < 0.005 vs. WT mice.

**B**: nucleic acid and protein sequence for murine RIFL. The italicized and underlined region indicates the predicted signal peptide. The 2 gray downward arrows demarcate the region of homology with murine angiopoietin-like 3 (ANGPTL3). Nos. at left indicate amino acid positions.

**C**: alignment of murine RIFL protein sequence with that of murine ANGPTL3 and ANGPTL4. Nos. at left indicate amino acid positions, and dashes indicate discontinuity of sequence alignment. +Amino acid similarity.

**D**: Western blot analysis of ectopic RIFL protein expression in 3T3-L1 adipocytes. Adipocytes were electroporated with either pcDNA3.1 [empty vector (EV)] or the RIFL-HA expression construct. Cell lysate was collected at 48 h post-transfection and analyzed using an HA primary antibody (top) or a peptidylpropyl isomerase A (PPIA) antibody for loading control (bottom). Digital images of enhanced chemiluminescence data are shown. Nos. on right are protein mass markers in kDa. The arrow shows the major species of RIFL-HA protein. *A slightly larger RIFL-HA protein species.
amino acids and shows 73% identity and 82% similarity to the murine protein. Human RIFL is located at chromosomal position 19p13.2, and the murine gene is present on chromosome 9. RIFL appears to be a relatively rare transcript, with only 22 and 17 ESTs present in the respective human and murine NCBI Unigene databases. A BLAST (Basic Local Alignment Search Tool) search of the RIFL protein sequence against translated NCBI nucleotide databases indicates transcript for RIFL also in genus Rattus, Macaca, Bos, Canis, and Sus. A BLAST search of the NCBI protein database showed that the full, predicted RIFL protein sequence has a statistically significant 22% identity and 50% similarity with the NH2-terminal half of the amino acid sequence for angiopoietin-like 3 (ANGPTL3), a well-studied liver secreted factor that, among its various biological activities (13, 16, 40, 43–45), can regulate lipid metabolism (11, 28, 34, 46, 61, 63). The RIFL protein sequence does not contain any structural regions or motifs, such as the α/β hydrolase fold, an esterase/lipase signature GXSXG, or other elements indicative of an enzymatic function.

The alignment of RIFL and ANGPTL3 is shown in Fig. 1C, top, and comprises nearly the full sequence of the RIFL protein. ANGPTL3 acts to diminish plasma TG clearance by reducing the action of lipoprotein lipase (LPL) (11, 28, 34, 46, 61). Structure/function studies of the effects of ANGPTL3 on LPL protein have reported that the NH2-terminal half of the protein, which is comprised of the coiled-coiled region, is necessary and sufficient for the ability of ANGPTL3 to inhibit LPL activity (34, 46). This is the same region of ANGPTL3 with protein sequence homology to RIFL. A shorter region of the RIFL protein, shown in the lower portion of Fig. 1C, has significant homology to ANGPTL4. ANGPTL4 also acts to reduce plasma triglyceride clearance by inhibiting LPL activity reportedly by a mechanism differing from that of ANGPTL3 (36, 61). The functions of ANGPTL3 and ANGPTL4 are not restricted to effects on lipid metabolism; they also exert numerous effects in other settings (13, 16, 40, 43–45). Whether the degree of protein similarity between ANGPTL3 and ANGPTL4 and RIFL represents a similarity of function or merely the fact that each of these proteins has a coiled-coiled structural region remains to be determined in future studies. However, ANGPTL3 expression has been reported as essentially not affected by nutritional status (12) and to be negatively regulated by insulin (62). ANGPTL4, also known as fasting-induced factor, is induced in WAT and liver by fasting (21). In contrast, our data presented in the remainder of this report show very clearly the dramatic positive regulation of RIFL transcript by refeeding and its positive regulation by insulin. We suggest that this argues against closely related physiological roles or modes of action for RIFL and ANGPTL3 or ANGPTL4.

Signal peptide analysis for the RIFL protein using the SignalP 4.0 program (51) reveals a strong prediction of a signal peptide, with cleavage between amino acid positions 15 and 16 of RIFL. No other regions of hydrophobicity are predicted, suggesting that RIFL may function as a secreted factor. We readily detect protein of the predicted size of RIFL-HA in the cell lysate of 3T3-L1 adipocytes transfected with RIFL-HA expression construct, which is shown in Fig. 1D. We also consistently note in Western blots for RIFL-HA the presence of a protein band slightly larger than the major product detected, depicted by an asterisk in Fig. 1D. We speculate that this may be the form of RIFL that has not undergone cleavage of the signal sequence. We have attempted to detect RIFL protein in conditioned media of 293T, COS, and 3T3-L1 adipocyte cells that we transiently transfected with RIFL expression constructs tagged with Flag or HA at the RIFL COOH terminus. For these studies, we followed the methods we reported on in our prior identification of a novel adipocyte-secreted factor, Wdnm1-like, and its detection in culture media (78). To date, we have not demonstrated such for RIFL (data not shown). This may mean that, despite the presence of a strong prediction for signal peptide, RIFL is not secreted. The RIFL protein sequence does not contain the canonical KDEL motif, or variations thereof, for retention of soluble proteins in the endoplasmic reticulum (50). However, the possibility that a novel sequence element or protein interaction might act to retain RIFL in the endoplasmic reticulum can also not be ruled out at this time. An additional possibility is that the RIFL protein, if secreted, is very tightly associated with the ECM or cell surface via strong protein-protein interactions and as such does not freely enter the culture media. It may also indicate that RIFL undergoes regulated rather than constitutive secretion or that addition of epitope tags to the protein alters its processing. Detailed studies on the nature of the RIFL protein will be the subject of future work by our laboratory.

Marked increase in RIFL transcript expression occurs during in vitro adipocyte conversion. We initially identified upregulation of RIFL transcript in adipogenesis as a result of our Illumina BeadChip whole mouse transcriptome profiling. To confirm and expand on these data, we analyzed expression of RIFL transcript throughout a 7-day time course of in vitro adipogenesis of 3T3-L1 cells. In this model system, lipid droplets first appear on days 3 and 4, and the majority of cells show evidence of the hallmark lipid droplets and rounded morphology of adipocytes by day 7. Data in Fig. 2A, top, show that a significant increase in RIFL transcript is first detected on day 3, with a 30-fold upregulation with respect to day 0 preadipocytes, and reaches a maximum of 100- to 200-fold higher than preadipocytes on days 4–7. Validation of differentiation is documented in Fig. 2A with real-time PCR analysis for two well-established markers of mature fat cells, PPARγ and fatty acid-binding protein 4 (FABP4). Figure 2B documents expression of RIFL transcript during in vitro adipocyte differentiation of a second preadipocyte cell line that was generated previously in this laboratory, ScAP-23 (26). In this adipogenesis culture model, an ~120-fold upregulation of RIFL transcript is detected from day 4 through day 7 compared with day 0. Figure 2B, bottom, shows validation of adipocyte differentiation in this model via assessment of transcripts for the differentiation markers PPARγ, FABP4, and patatin-like phospholipase domain containing 2 (PNPLA2) in ScAP-23 preadipocytes vs. ScAP-23 adipocytes. To determine whether upregulation of RIFL transcript is tied closely to the adipocyte phenotype, we also assessed transcript regulation of RIFL during in vitro adipogenesis of brown preadipocytes to brown adipocytes using a cell line termed herein as WT-BAT, which was generated by Dr. C. R. Kahn from neonatal brown adipose tissue (BAT) (27). Figure 3, top, shows that a robust upregulation of RIFL transcript is also observed during adipocyte conversion in this brown adipogenesis culture model. The real-time PCR data presented in Fig. 3, bottom, validate transcriptional conversion of these cells to brown adipocytes. This is shown by marked induction of transcripts for the brown adi-
preadipocytes. Fig. 2. Dramatic increase in RIFL transcript with in vitro adipogenesis of 3T3-L1 adipogenesis. Real-time PCR was carried out on cDNA prepared from RNA collected from day 0 (D0) and daily through day 7 (D7). Top: RIFL transcript. Bottom: transcripts for 2 adipogenesis marker genes. The bracketed area marked as NS (not significant) indicates lack of significant change in RIFL transcript level between D0 and D1 or D2. One of 2 representative analyses is shown for the D0 –D7 time course. A: increase in RIFL transcript expression during ScAP-23 adipogenesis (D indicates days postinduction of adipogenesis). B: adipogenesis marker transcripts. For A and B the respective values in each graph are normalized to the D0 value, which is set to 1. Bars represent means of measurement triplicates ± SD. *$P<0.001$ and $\#P<0.05$ with respect to the value on D0. PPARγ, peroxisome proliferator-activated receptor-γ; FABP4, fatty acid-binding protein 4.

Although in vitro models of established cell lines are very useful in defining genes and pathways that are functional in adipogenesis, it is important to assess whether this appropriately reflects the in vivo setting. In some cases, distinctions between in vivo and in vitro adipogenesis have been noted (66). We thus assessed regulation of RIFL in primary cultures of preadipocyte-containing cell populations. The nonadipocyte portion of adipose tissue cell population is designated as the stromal vascular fraction (SVF), which includes presumed preadipocytes. Murine SVF cells prepared from WAT were plated as primary cultures and harvested either as preadipocytes or following their in vitro differentiation to adipocytes. Figure 4A shows that adipocyte differentiation of murine primary preadipocyte cultures is accompanied by a significant increase in the expression of a RIFL transcript. Here, we also assessed transcripts for three markers of adipocytes to validate adipogenic conversion in this model system, namely PPARγ, stearoyl-CoA desaturase 1 (SCD1), and PNPLA2. To ensure that our observations on upregulation of RIFL transcript in adipogenesis are not particular to murine models, but also applicable to human, we determined the level of RIFL transcript via real-time PCR analysis of human primary preadipocytes obtained from human subcutaneous WAT and following in vitro conversion of these cells to human adipocytes. Figure 4B indicates a 150-fold higher level of the RIFL transcript in human adipocytes compared with human preadipocytes. Also in Fig. 4B is assessment of three marker genes to validate adipogenesis in this human cell culture model, namely PPARγ, SCD1, and PNPLA2. Thus we have shown that a robust increase in RIFL transcript expression accompanies adipocyte differentiation of both rodent and human primary preadipocyte cultures.

Regulation of adipocyte RIFL transcript level by TNFα. TNFα is a cytokine that is well characterized to both inhibit adipogenesis and to induce a dedifferentiation response in adipocytes. In this regard, it has been studied primarily in the 3T3-L1 adipocyte model. TNFα treatment of these cells down-regulates adipocyte-expressed genes and results in increased expression of preadipocyte-enriched genes (55–57). TNFα treatment of fat cells also stimulates lipolysis (56). To determine whether TNFα modulates expression of RIFL transcript in adipocytes, we exposed day 7 3T3-L1 adipocytes to 10 ng/ml TNFα for 24 h and assessed expression of RIFL transcript by real-time PCR. As shown in Fig. 5A, TNFα treatment results in a dramatic decrease in RIFL transcript in 3T3-L1 adipocytes to <10% of untreated time-matched cultures. As a control for the TNFα treatment, we assessed effects of TNFα on PPARγ, SCD1, small adipocyte factor 1 (SMAF1), and CIDEC, transcripts established to be decreased markedly in TNFα-treated adipocytes (22, 24, 25, 55, 57, 79). As is apparent in the right four graphs in Fig. 5A, TNFα resulted in highly effective decreased expression of each of these transcripts. A 72-h exposure of 3T3-L1 adipocytes resulted in a similar level of decrease for RIFL transcript and further decrease in the expression in levels of PPARγ, SCD1, SMAF1, and CIDEC transcripts (data not shown). These TNFα treatment times are
in line with those used in numerous studies of 3T3-L1 cells (7, 67, 68, 71, 79). The decrease in transcript levels upon 24- or 72-h TNFα treatment was not due to general effects on viability of cultures. We observed an essentially similar pattern and level of total protein expression in control and treated conditions, similar expression levels of two additional control housekeeping transcripts, β-actin and rplp0, and no visual indications of alterations in viability (data not shown). We also determined the effects of TNFα on expression of RIFL transcript in human adipocytes. As shown in Fig. 5B, treatment with 10 ng/ml of TNFα for 36 h decreased levels of RIFL transcript in these cells 50-fold.

The downregulation of adipocyte genes by TNFα has been attributed, at least in part, to its rapid effects on decreasing expression of the master regulator of adipogenesis, PPARγ. However, studies using siRNA-mediated knockdown of PPARγ in mature 3T3-L1 adipocytes have found that PPARγ knockdown did not dramatically affect the appearance or lipid content or markedly diminish expression of some genes that define the function and phenotype of mature adipocytes (37, 59). Thus it appears that only a subset of those genes markedly upregulated during in vitro adipocyte differentiation are dependent on PPARγ for their transcriptional expression in adipocytes. To ensure that we were not affecting the overall process of differentiation of preadipocytes to adipocytes in this study, due to the requirement of PPARγ for effective adipogenesis, we utilized late-stage adipocytes on day 14 rather than adipocytes from an earlier time point. This obviates concerns that a reduction of adipocyte gene expression is due to abrogated adipocyte differentiation resulting from PPARγ knockdown. We also employed Ces3 as a control to further rule out effects resulting from ongoing adipogenesis even in these late-stage fat cells. Ces3, a gene markedly induced during adipogenesis (6, 76), was reported by Schupp et al. (59) to not be appreciably affected upon PPARγ knockdown of day 14 3T3-L1 adipocytes. Figure 5D, top, shows transcript levels for PPARγ, RIFL, and Ces3. Figure 5D, bottom, shows transcript levels for two genes shown by Schupp et al. (59) to be decreased in siPPARγ knocked-down late-stage adipocytes, FABP4 and RETN, and for the known direct PPARγ target gene SMAF1 (14, 24). As anticipated, siRNA for PPARγ reduced levels of PPARγ transcript, whereas those for Ces3 were not dependent on PPARγ. Each of the three known PPARγ-dependent genes (FABP4, RETN, and SMAF1) showed reduced transcript level upon PPARγ knockdown. The fact that Ces3 levels, which serve here as a marker of adipocyte conversion, did not change with PPARγ knockdown indicates that the day 14 cultures were no longer undergoing further differentiation. Figure 5D reveals that the level of RIFL transcript is decreased significantly with PPARγ knockdown, indicating that in fully differentiated adipocytes RIFL transcript is dependent, at least in part, on PPARγ.

**Fig. 3.** Upregulation of RIFL transcript in a brown adipogenesis model. Top: real-time PCR data for RIFL transcript level during in vitro adipocyte conversion of a brown preadipocyte cell line, WT-BAT (brown adipose tissue). Bottom: marker genes for brown adipogenesis [uncoupling protein 1 (UCP1) and cell death-inducing DNA fragmentation factor-α-like effector A (CIDEA)] and for general adipogenesis (PPARγ and FABP4). D indicates days postinduction of adipocyte differentiation. Bars represent means of measurement triplicates ± SD. For PCR data the respective values in each graph are normalized to the D0 value, which is set to 1. *P < 0.001 compared with respective value for D0.
Knockdown of RIFL leads to reduced adipocyte lipid content.

The above observations show that RIFL transcript is markedly upregulated in multiple models of adipogenesis and that this increase is first noted at a time point when adipocytes are developing lipid droplets to store triglyceride, at around day 3 of differentiation. We hypothesized that RIFL expression may be functionally tied to lipid accumulation. To address this, we conducted knockdown studies by transfecting 3T3-L1 preadipocytes with siRNA for RIFL or control siRNA, subjecting these cells to an adipogenic differentiation protocol and determining triglyceride content with Oil Red O staining. Cultures were photographed for Oil Red O-stained fat cells, and Oil Red O was extracted from stained cultures to quantify relative level of triglyceride content in siControl and siRIFL transfected cultures. We also used siPPARγ treatment of 3T3-L1 preadipocytes as a positive control because this is well-documented to fully inhibit adipose conversion of these cells. We hypothesized that RIFL expression may be functionally tied to lipid accumulation. To address this, we conducted knockdown studies by transfecting 3T3-L1 preadipocytes with siRNA for RIFL or control siRNA, subjecting these cells to an adipogenic differentiation protocol and determining triglyceride content with Oil Red O staining. Cultures were photographed for Oil Red O-stained fat cells, and Oil Red O was extracted from stained cultures to quantify relative level of triglyceride content in siControl and siRIFL transfected cultures. We also used siPPARγ treatment of 3T3-L1 preadipocytes as a positive control because this is well-documented to fully inhibit adipose conversion of these cells. First, we assessed the efficacy of the siRNA for murine RIFL by electroporating either siRIFL or siControl into 3T3-L1 adipocytes and comparing levels of RIFL transcript. As is shown in Fig. 6A, top, knockdown of RIFL led to a >90% decrease in RIFL transcript level compared with siControl. Because there is no available antibody for RIFL, we could not assess knockdown for endogenous RIFL protein. In place of this, we assessed the effects of siRNA-mediated RIFL knockdown at the protein level using cotransfection of HA-tagged RIFL and siRNA into 293T cells. Figure 6A, bottom, shows that cells transfected with RIFL-HA and assessed by Western blot with anti-HA antibody express a protein band of the correct size and that this signal is absent from the empty vector control transfection (top lane). Moreover, SMARTpool siRNA for RIFL markedly diminished the intensity of the RIFL-HA protein band, indicating effectiveness of the RIFL siRNA for knockdown at the protein level.

Because siRNA targeting of RIFL proved effective, we next assessed the effect of RIFL knockdown on lipid accumulation in 3T3-L1 adipogenesis. For this, 3T3-L1 preadipocytes were electroporated with either siControl, siRIFL, or siPPARγ, the latter serving as a positive control. The effectiveness of the siPPARγ for knockdown was illustrated in Fig. 5, C and D. Following electroporation to introduce the respective siRNA into 3T3-L1 preadipocytes, cells were replated such that they
attained confluence the following day. The next day, cells were subjected to adipogenic induction and cultures assessed at 7 days postinduction of differentiation. To document adipocyte conversion and intracellular lipid content, cells were photographed either live (Fig. 6B, top) or following fixation and staining with Oil Red O (Fig. 6B, middle and bottom, with representative microscopic fields of culture dishes shown).

RIFL knockdown resulted in diminished numbers of lipid-laden adipocytes compared with siControl knockdown 3T3-L1 adipocytes. A view of duplicate samples of the entire well of cultures stained with Oil Red O is shown in Fig. 6C, where a marked reduction in Oil Red O staining is evident in siRIFL-transfected cultures compared with siControl cultures. The degree of staining seen for the wells of the siPPARγ-trans-
fected cultures is essentially background, as attested to by images in Fig. 6B, right. Close microscopic inspection of the entirety of the culture dishes reveals only a very rare Oil Red O-stained cell in siPPARγ cultures. The fact that PPARγ knockdown resulted in essentially no fat cells in the cultures despite adipogenic agent treatment attests to effectiveness of the knockdown methodology. To quantify the effect of RIFL knockdown on lipid content, Oil Red O was extracted with isopropanol and dye intensity measured by reading absorbance at 490 nm. As shown for two independent RIFL knockdown studies in Fig. 6D, RIFL knockdown led to an ~35% reduction in lipid content. We used real-time PCR to compare transcript levels for a number of typical adipocyte marker genes in RIFL knockdown vs. control knockdown cultures. We observed no significant differences in transcript level ($P > 0.05$; Fig. 6E). On the other hand, for the PPARγ knockdown cultures, each of these transcripts was decreased by >90% (data not shown). These data suggest to us that the effect of RIFL on lipid content does not appear to be due to an overall diminished adipocyte differentiation, but may rather affect aspects of lipid accumu-
RIFL transcript expression was altered in WAT tissue during liver.

Assessed, RIFL transcript expression is enriched in WAT and liver. Figure 7 important to illustrate expression of this transcript in human where the RIFL transcript is present in humans, we thought it was regulation of genes differ in murine and human systems (60).

Cell function, in some cases the adipose tissue expression and defining genes and pathways important in adipogenesis and fat derived from in vivo WAT (Fig. 4).

Dipocytes differentiate into adipocytes in either the established restricted to the adipocyte fraction. This is in line with our data, B, RIFL transcript expression is essentially PPARY and the preadipocyte gene Pref-1 shown in Fig. 7B. As shown in Fig. 7B, RIFL transcript expression is essentially restricted to the adipocyte fraction. This is in line with our data, described above, that RIFL transcript is upregulated as preadipocytes differentiate into adipocytes in either the established culture models (Figs. 2 and 3) or primary SVF cultures derived from in vivo WAT (Fig. 4).

Although studies in rodent models have been invaluable in defining genes and pathways important in adipogenesis and fat cell function, in some cases the adipose tissue expression and regulation of genes differ in murine and human systems (60). Although database assessments we conducted indicated that the RIFL transcript is present in humans, we thought it was important to illustrate expression of this transcript in human WAT and liver. Figure 7C shows that, of the human tissues assessed, RIFL transcript expression is enriched in WAT and liver.

Upregulation of RIFL in adipose and liver in genetic obesity and by a nutritional refeeding regimen. To investigate whether RIFL transcript expression was altered in WAT tissue during genetic obesity, we used the ob/ob mouse model. We also included assessment of two marker genes already defined as altered in ob/ob WAT. Adiponectin (ADIPOQ) (18) transcript level is well established to be decreased in expression in ob/ob compared with WT mice, and HMOX1 transcript level was reported previously to be more highly expressed in ob/ob than in WT WAT (41, 65). Figure 8A shows that RIFL transcript levels in WAT are elevated an average of approximately eightfold in ob/ob compared with WT. The two marker genes assessed in Fig. 8A, ADIPOQ and HMOX1, were each regulated in the anticipated manner. We also examined expression of RIFL transcript in liver in these mice and observed a significant increase, ~4.5-fold, in RIFL transcript level in ob/ob compared with WT (Fig. 8B, left). Two transcripts established to be upregulated in ob/ob liver, PNPLA3 (1) and CD36 (22), are shown in Fig. 8B, middle and right.

Because our data pointed to a role for RIFL upregulation in settings of lipid accumulation, we hypothesized that RIFL transcript may be increased during nutritionally or hormonally regulated lipogenic states. We assessed expression of RIFL transcript in WAT and liver during a fasting and high-carbohydrate refeeding nutritional regimen in mice. RNA from WAT and liver from fasted and fasted/refed animals was subject to real-time PCR analysis for RIFL transcript. We also included in these analyses two control transcripts known to be oppositely regulated by this dietary regimen. Transcript expression of the lipase PNPLA2 is increased in fasting and decreased upon refeeding (20, 23), and that for PNPLA3 is decreased in fasting and upregulated during refeeding (2, 20).

As shown by real-time PCR analysis in Fig. 9A, top, we detected an ~80-fold upregulation for RIFL transcript in WAT following refeeding of fasted animals and an ~12-fold upregulation for RIFL transcript in liver following refeeding of fasted animals (Fig. 9B). The regulation of transcripts for the control genes PNPLA2 and PNPLA3 each showed the correct directionality of regulation during the fasting and refeeding dietary regimen.

An assessment of the PubMed and Google Scholar databases reveals several dietary studies, including one in humans wherein RIFL transcript level in WAT was observed as responsive to nutritional status. In these reports, RIFL was not the focus of study but rather one of a multitude of genes listed in tables of differentially expressed genes resulting from large-

Fig. 6. siRNA-mediated knockdown of RIFL during 3T3-L1 adipogenesis decreases lipid content. A: assessment of effectiveness of siRNA-mediated knockdown of RIFL transcript and protein. Top: real-time PCR analysis of RNA prepared from 3T3-L1 adipocytes electroporated with either siRNA for nontargeting control siRNA (siCon) or siRNA for RIFL (siRIFL). Bars represent means of measurement triplicates ± SD. Level in control was set to a value of 1; *P < 0.001. One of 2 representative experiments is shown. Bottom: Western blot using HA-tag antibody conducted on lysates of 293T cells transiently transfected with either EV or the RIFL-HA expression construct. For RIFL-HA transfections, cells were cotransfected with either siCon or siRIFL. Western blot for tubulin is shown as loading control. Results of 2 independent siRNA knockdowns are shown for siCon and siRIFL. B: effect of RIFL knockdown on intracellular lipid content; microscopic view. Microscopic view of day 7 adipocytes following electroporation of indicated siRNAs into 3T3-L1 preadipocytes, which were then subjected to an adipogenic differentiation protocol. Top: live, unstained cells. Middle and bottom: paraformaldehyde fixed, Oil Red O (ORO)-stained cultures. Relative magnifications are indicated in parentheses. C: effect of RIFL knockdown on intracellular lipid content; view of whole culture wells. Cells harboring indicated siRNA were fixed with paraformaldehyde and stained with ORO. Culture plate was scanned, and resultant digital image is shown. For B and C, images are representative of 3 independent experiments. D: quantification of culture neutral intracellular lipid content. Triclipate independent wells (n = 3) each were transfected and analyzed for siCon or siRIFL. Following differentiation, cultures were stained with ORO and extracted with isopropanol, and absorbance was measured at an optical density of 490 nm. Data are shown as means of triplicate wells ± SD; *P < 0.01. Results of 2 independent studies are shown (black bars, experiment 1; gray bars, experiment 2). For each experiment, level in the siCon was set to a value of 1. E: assessment of adipocyte marker transcripts. Real-time PCR was used to assess level of indicated mature adipocyte marker gene expression in control siRNA cultures (C; black bars) or in RIFL siRNA knock-down cultures (R; gray bars). For each indicated transcript, the value of the respective control was set to 1. *Nonsignificant difference (P > 0.05) for control siRNA vs. RIFL siRNA. Bars represent means ± SD of n = 3 culture dishes for either control siRNA or RIFL siRNA. The study was carried out in 2 independent times with essentially the same results; 1 of the 2 studies is shown.
scale microarray profiling. Although these data have not been
validated beyond the microarray level, taken together they lend
support to our experimental findings of alteration of RIFL
transcript in WAT in response to refeeding. Microarray data
reported by Luo et al. (38) on comparison of fed vs. fasted
states in mice showed a decrease to 13 and 6% for RIFL
transcript expression in liver compared with that of fed controls
after a 6- and 12-h fast, respectively. Franck et al. (8) assessed
transcriptional alterations in human subcutaneous WAT in
response to dietary manipulation. Participants were subjected
to 8 wk of a very low-calorie diet, followed by a 2-wk
refeeding period. During the caloric restriction period, RIFL
transcript expression decreased to 41% of the level in the
control subjects that did not undergo caloric restriction.

Fig. 7. Tissue and cell type enrichment of RIFL transcript expression. A: real-time PCR assessment of RIFL transcript expression in a panel of murine tissues. Level in heart was set to a value of 1. B: in white adipose tissue (WAT), RIFL transcript expression is restricted to adipocytes. Real-time PCR analysis was carried out on murine WAT tissue that had been subjected to cellular fraction into the preadipocyte-containing stromal vascular fraction (SVF) or the adipocyte fraction (AF). Signal in SVF was set to a value of 1. Left: data for the RIFL transcript; middle left and middle right: data for adipocyte marker transcripts; right: level of a preadipocyte marker transcript. C: relative expression level of RIFL transcript in human liver, adipocytes, and WAT. Human adipocytes obtained from in vitro differentiation of human primary preadipocytes and human WAT, liver, testis, and heart RNA were analyzed for relative transcript expression for human RIFL using real-time PCR. Level in human heart sample was set to a value of 1. For A–C, data shown are means of measurement triplicates ± SD; *P < 0.001 vs. unmarked samples (i.e., no asterisk) within the same graph.

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00084.2012 • www.ajpendo.org
tionally, a subsequent increase of RIFL transcript level in subcutaneous WAT to 148% of that of control subjects occurred following a 2-wk refeeding period (8). In this same report, an overfeeding diet for 4 wk led to a reported 3.88-fold increase in the expression of RIFL transcript in subcutaneous WAT (8). A microarray profiling study found RIFL to be among those genes whose transcript level decreased upon treatment of primary human hepatocytes with the PPAR ligand WY-14643 (54). Because PPAR functions in activating the transcriptional response to fasting in liver, it might be anticipated that an apparent prolipogenic gene such as RIFL would be decreased in expression as a result of PPAR activation.

RIFL transcript expression level in adipocytes is dramatically induced by insulin. The above real-time PCR data are consistent with the idea that RIFL transcript is increased in expression during conditions that favor energy storage in the form of triglyceride and/or during lipogenesis. This is also consistent with our observations in multiple adipogenesis models with respect to massive induction of RIFL transcript expression as cells convert from fibroblastic preadipocytes to lipid-filled adipocytes. With regard to fat cell function and energy metabolism, insulin is the key lipogenic hormonal signal. Therefore, we tested whether insulin had effects on RIFL transcript level in adipocytes. 3T3-L1 adipocytes were serum-starved for 16 h, followed by stimulation with 100 nM insulin for 48 h. In this study, in addition to assessing RIFL transcript level, we also examined transcripts for two genes known to be positively regulated by insulin, CIDEA (22) and PNPLA3 (2, 20), and also PNPLA2, which is negatively regulated by insulin (20, 23). Figure 10A shows that insulin addition to adipocytes cultured in serum-free, high glucose-containing media increased transcript levels for CIDEA and PNPLA3 as predicted and decreased levels for PNPLA2 transcript. Insulin treatment resulted in a striking ~35-fold increase in levels of RIFL transcript in adipocytes. Upregulation of RIFL transcript by insulin was also observed for human adipocytes (Fig. 10B). To expand on this observation, we conducted time course and dose response studies in 3T3-L1 adipocytes. The time course data shown in Fig. 10C indicate that a significant increase in RIFL transcript level occurs by 2 h post-exposure to insulin, with subsequent increases in RIFL transcript level occurring throughout later time points. No significant alteration of RIFL transcript level was noted for cultures not subjected to insulin treatment but continued in culture and assessed at time points of 24 and 48 h (data not shown). This upregulation of RIFL transcript level by insulin is reinforced by the dose response study in Fig. 10D. Here, we find that even the lowest concentration of insulin tested, 0.1 nM, yielded near-maximal upregulation of RIFL transcript expression.

Because RIFL upregulation is noted upon refeeding (Fig. 9), similar to that found for PNPLA3, we further investigated whether regulation of RIFL transcript also mirrored that of PNPLA3 with regard to glucose responsiveness. PNPLA3 transcript expression in adipocytes is known to be positively regulated by glucose in the absence of insulin (2, 39), and assessment of PNPLA3 in these samples serves to validate our cell culture treatments in this study. 3T3-L1 adipocytes were cultured under glucose and insulin-free conditions or in...
ditions of added glucose, added insulin, or added glucose and insulin in combination. These data are shown in Fig. 10E and indicate that, whereas PNPLA3 transcript expression in 3T3-L1 adipocytes is increased with glucose treatment in the absence of insulin, the RIFL transcript is not responsive to alteration of glucose levels under these conditions.

RIFL transcript expression level in adipocytes is dramatically decreased by agents that stimulate lipolysis. We believe that the studies described above serve as a strong argument for a critical role for RIFL in lipogenic and/or lipid storage processes. We next asked whether agents that act to stimulate lipolysis and/or oppose lipogenesis in adipocytes might decrease expression of RIFL transcript. For these studies, we treated 3T3-L1 adipocytes with agents that stimulate the PKA-mediated pathway of lipolysis in fat cells using dibutyryl cAMP, forskolin, and isoproterenol. As shown in Fig. 11A, left, for dibutyryl cAMP and forskolin and in Fig. 11A, right, for isoproterenol, each of these agents led to a 50–80% decrease in levels of RIFL transcript levels in 3T3-L1 adipocytes when determined by real-time PCR. This is similar in magnitude to the response we show in this figure for levels of fatty acid synthase and GLUT4 transcripts, each previously reported to be decreased by lipolytic agents that act via cAMP (19, 49).

We also tested the effects of dexamethasone, another agent that promotes lipolysis in 3T3-L1 adipocytes (17, 80). As illustrated in Fig. 11B, dexamethasone treatment for 24 h reduced RIFL transcript level in 3T3-L1 adipocytes to ~10% of that of vehicle-treated controls. Thus, RIFL transcript level in adipocytes appears to be finely coordinated by the opposing nutritional/hormonal states of energy storage and energy utilization.

Summary. We have identified a novel gene product with specific expression in two highly metabolically active tissues, fat and liver. Whereas RIFL has a degree of amino acid similarity to ANGPTL3 and ANGPTL4, proteins that act via inhibition of LPL, we show that the nutritional and hormonal regulation of RIFL is very different from that reported for ANGPTL3 (12, 62) or ANGPTL4 (12, 21). The lack of sequence homologies, domains, or motifs indicative of enzymatic activity suggests a novel physiological role and mode of function for RIFL. The totality of the experimental data we report strongly supports positive and likely novel roles for RIFL in lipid storage. These include robust increases in RIFL transcript level in multiple in vitro adipogenesis models at a time point that coincides with onset of lipid accumulation, upregulation of RIFL transcript in fatty liver in the ob/ob genetic mouse model, upregulation by the lipogenic effectors of dietary refueling in adipose tissue and liver in mice, and insulin treatment of cultured adipocytes. The ability of RIFL to
influence lipid storage is also supported by our observations that siRNA-mediated knockdown of RIFL during adipogenesis quantitatively and significantly decreases the level of neutral lipid content in 3T3-L1 adipocytes. This potential importance of RIFL expression and regulation with respect to lipid balance in vivo is bolstered by the phenotypic data on RIFL-null mice. That RIFL-null mice show reduced triglyceride levels in serum argues that there may be a causal relationship between RIFL

**Fig. 10.** RIFL is a new insulin-induced gene in adipocytes. A: upregulation of RIFL transcript level by insulin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were untreated (control (C)) or treated with 100 nM insulin (Ins) for 48 h under serum-free conditions, as described in MATERIALS AND METHODS. Levels for RIFL transcript and for transcripts of established insulin target genes (CIDEC, PNPLA3, and PNPLA2) were analyzed by real-time PCR. Three independent experiments were conducted, with 1 representative study shown. B: effect of insulin on RIFL transcript in human adipocytes. In vitro-differentiated human adipocytes were treated with insulin for 36 h, and RNA was assessed for RIFL transcript level with real-time PCR. Bars represent means of Ins-treated (n = 2) and C (n = 2) ± SD; *P < 0.005 for Ins vs. C. C: time course of RIFL transcript upregulation by Ins. 3T3-L1 adipocytes were exposed to 100 nM Ins starting at time 0 and RNA harvested at indicated hourly intervals thereafter through 48 h. #P < 0.05, ^P < 0.01, and *P < 0.001 vs. the 0 h, which was set to a value of 1. D: dose response of RIFL transcript regulation by insulin. 3T3-L1 adipocytes were exposed from 0 to 1,000 nM insulin, as indicated, and samples analyzed by real-time PCR 24 h later. Level on day 0 is set to a value of 1. For A, C, and D, bars represent means of measurement triplicates ± SD; *P < 0.001 vs. either control (for A) or day 0 (for C and D), which was set to a value of 1. E: regulation of RIFL transcript level in 3T3-L1 adipocytes by insulin and glucose singly or in combination. 3T3-L1 adipocytes were cultured as described in MATERIALS AND METHODS; control (Con), glucose-treated only (Glu), insulin-treated only (Ins), and treatment with glucose and insulin combined (Glu + Ins). RNA was analyzed for either RIFL transcript level (left) or PNPLA3 transcript level (right) by real-time PCR. Bars represent means (n = 2) for each condition ± SD, with control set to a value of 1. For RIFL data, *P < 0.01 for Glu + Ins vs. Con, Glu, or Ins. For the PNPLA3 graph, *P < 0.01 for Glu or Glu + Ins vs. Con or Ins.
expression and regulation in liver and adipose tissue and whole body lipid homeostasis. The fact that our key observations herein on RIFL expression and regulation in the murine setting also hold with respect to human cells/tissues indicates that RIFL has the potential to play an important role in adipocyte function and/or lipid metabolism in humans. Work in this laboratory is currently underway to characterize the function and regulation of the RIFL protein and to investigate the

**ACKNOWLEDGMENTS**

We thank former and present laboratory staff (S. Zhou and P. Eskandari) for excellent technical assistance with RNA preparation. Present address of J. Y. Kim: Sanford-Burnham Medical Research Institute, La Jolla, CA.
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