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(54) **Title:** REGULATION OF LIPID DROPLET FORMATION BY MODULATION OF FIT1 AND FIT2 AND USES THEREOF

(57) **Abstract:** This invention provides methods of regulating fat storage in tissue by modulating the levels of Fibrate Induced Transcript 1 (FITl) and/or Fibrate Induced Transcript 2 (FIT2), as well as diagnostic screens for disorders and conditions involving regulation of fat storage in tissue, assays for identifying agents that can regulate fat storage in tissue through modulating the levels of FITl and/or FIT2, and genetically altered mammals in which expression of FITl and/or FIT2 is altered in one or more tissue.

REGULATION OF LIPID DROPLET FORMATION BY MODULATION OF FIT1 AND FIT2 AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 60/880,279, filed January 12, 2007.

STATEMENT OF GOVERNMENT SUPPORT

[0002] The invention disclosed herein was made with U.S. Government support under grant number P30-DK42196 from the National Institutes of Health. Accordingly, the U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The invention is directed to methods of regulating fat storage in tissue by modulating the levels of Fibrate Induced Transcript 1 (FIT1) and/or Fibrate Induced Transcript 2 (FIT2), as well as to assays for identifying agents that can regulate fat storage in tissue through modulating the levels of FIT1 and/or FIT2. Preferred forms of the invention are directed to methods of reducing fat storage in tissue by decreasing the levels of FIT1 and/or FIT2, as well as to assays for identifying agents that can reduce fat storage in tissue through decreasing the levels of FIT1 and/or FIT2. The reduction of fat storage may aid in treatment of any disease that involves storage of cytoplasmic fat in droplets, such as obesity, diabetes, metabolic syndrome, fatty liver disease (steatosis and steatohepatitis), atherosclerosis, diabetic nephropathy, and hepatitis C infection. Also provided are diagnostic screens for disorders involving regulation of fat storage in tissue, which involve assaying the level of expression or activity of FIT1 and/or FIT2 in a blood, fluid, tissue or cell sample from a subject. The invention also provides genetically altered mammals in which expression of FIT1 and/or FIT2 is altered in one or more tissue.

BACKGROUND OF THE INVENTION

[0004] Throughout this application various publications are referred to in parenthesis. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference in their entireties into the subject application to more fully describe the art to which the subject application pertains.

[0005] The ability to store energy in the form of triglyceride (TG) is conserved from *S. cerevisiae* to humans. Triglycerides are stored in the cytoplasm surrounded by a monolayer of phospholipid in distinct structures or organelles given numerous names such as lipid particles, oil bodies, adiposomes, eicosasomes, and more commonly lipid droplets (*1*). Under normal physiological conditions, lipid droplets are involved in maintaining energy balance at the cellular and organismal level; but under conditions of extreme lipid droplet acquisition as in obesity, type II diabetes and cardiovascular diseases may ensue (*2-6*).

[0006] Recently, a great body of information has been generated revealing details of the composition and functions of many of the components of lipid droplets from *S. cerevisiae* (*7*), drosophila and mammalian cells (*8-10*). In mammalian cells, the catabolism of lipid droplets is a highly regulated process involving hormonal signals, droplet structural proteins and lipases (*2, 11, 12*). While much has been learned about the components and catabolism of lipid droplets, the molecular mechanism of lipid droplet biogenesis remains unknown. The identification of many components of lipid droplets has not revealed obvious proteins that would be involved in their formation. A widely accepted model of lipid droplet biogenesis involves the budding of a single leaflet of the endoplasmic reticulum (ER) membrane with a newly forming core or "lens" of triglyceride (*1*). The evidence that cytosolic lipid droplets are derived from the ER is based on the findings that some lipid droplet associated proteins are also associated with the ER membrane, and in many instances lipid droplets have been shown to be in close association with the ER membrane (*8, 13-16*).

[0007] The occurrence of obesity is approaching epidemic proportions. Many of the diseases associated with obesity, namely diabetes, hepatic steatohepatitis and atherosclerosis are the result of inappropriate storage of fat. Thus, there is a need for means that can effectively reduce fat storage in human tissues.

SUMMARY OF THE INVENTION

[0008] The present invention provides methods of reducing fat storage in a subject by administering to the subject an amount of an agent that is effective to decrease the level of FIT1 and/or FIT2 in the subject.

[0009] The invention also provides methods of increasing fat storage in a subject by administering to the subject an amount of an agent that is effective to increase the level of FIT1 and/or FIT2 in the subject.

[0010] The invention also provides methods of screening for a disorder involving regulation of fat storage in tissue, the methods comprising determining the level of expression

and/or activity of FIT1 and/or FIT2 in a blood, body fluid, tissue biopsy or cell sample from a subject, wherein alternation from normal of the level of expression or activity of FIT1 and/or FIT2 is indicative of a disorder involving regulation of fat storage in tissue.

[0011] The invention provides methods for screening for candidate agents that can reduce fat storage in tissue by determining whether or not an agent is effective to decrease the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting an agent with a cell line or tissue culture that expresses FIT1 and/or FIT2, and wherein reduction in expression and/or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for reducing fat storage in tissue.

[0012] The invention also provides methods for screening for candidate agents that can increase fat storage in tissue by determining whether or not an agent is effective to increase the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting an agent with a cell line or tissue culture that expresses FIT1 and/or FIT2, and wherein an increase in expression and/or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for increasing fat storage in tissue.

[0013] The invention also provides transgenic mammals in which FIT1 and/or FIT2 is overexpressed in one or more tissue. The invention further provides mammals that have been genetically altered so that expression of FIT1 and/or FIT2 is reduced in one or more tissue.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Figure 1A-1D. Analysis of FIT gene expression. (A) Total RNA (15μg) from mouse tissues were subjected to Northern blot analysis for murine FIT1 and FIT2 (*mfit1 and mfit2*). The tissues analyzed were as follows: 1, brain; 2, eye; 3, heart; 4, lung; 5, liver; 6, kidney; 7, adrenal; 8, spleen; 9, skeletal muscle; 10, white adipose; 11, brown adipose; 12, ovary; 13, testis; 14, stomach. *Gapdh* served as a positive hybridization probe. (B) Total cell lysates (80μg) from selected mouse tissues were subjected to Western blot analysis. The following tissues were analyzed: 1, lung; 2, heart; 3, skeletal muscle; 4, liver; 5, kidney; 6, adrenal gland; 7, white adipose tissue; 8, brown adipose tissue; 9, HEK293 cell positive control expressing FIT1 or FIT2. Calnexin served as a loading control. (C) A human RNA blot containing 2μg of poly(A) RNA per lane (FirstChoice Human Blot 1 from Ambion) was analyzed for both *fit1 and fit2* (*hfit1 and hfit2*) expression in the following tissues: 1, brain; 2, placenta; 3, skeletal muscle; 4, heart; 5, kidney; 6, pancreas; 7, liver; 8, lung; 9, spleen; 10, colon. (D) Amino acid sequences of murine FIT1 (SEQ ID NO:3) and FIT2 (SEQ ID NO:4)

and hydropathy plots. The horizontal line (slightly below +2) indicates the average hydrophobicity.

[0015] Figure 2A-2B. Subcellular localization of FIT proteins. (A) Total post-nuclear membranes from mouse hearts were separated by continuous sucrose gradients. Fractions were subjected to Western blot analysis using antibody markers for the plasma membrane (Na K-ATPase), Golgi apparatus (Golgi 58kDa), endoplasmic reticulum (calnexin), and FIT1 and FIT2. (B) Immunofluorescence localization of mouse FIT1-V5 and FIT2-V5 with the ER marker protein RFP-KDEL in HEK293 cells.

[0016] Figure 3A-3C. Lipid droplet formation induced by FIT1 and FIT2. (A) HEK293 cells were transiently transfected with mouse FIT1, FIT2, or DGAT1 and lipid droplets were visualized using fluorescence microscopy by staining with BODIPY 493/503. (B) Triglyceride mass measurements from transiently transfected cells. M, mock transfected; F1, FIT1 transfected; F2, FIT2 transfected; D1, DGAT1 transfected; D2, DGAT2 transfected. Data are represented as the average ± SD. N = 4 transfections per construct. (C) Triglyceride (TG) biosynthesis was determined by incubating transiently transfected HEK293 cells with the indicated constructs with ^{14}C glycerol for the indicated times. ^{14}C TG were separated using TLC analysis and quantified using phosphorImager analysis. DGATs served as positive controls for TG biosynthesis.

[0017] Figure 4A-4C. shRNAi mediated knockdown of FIT2. NIH-3T3 or RAW246.7 cells were infected with lentivirus expressing shRNA sequence targeting murine FIT2 (FIT2shRNA), or control shRNA (contshRNA), or no lentivirus (control). (A) FIT2shRNA reduced lipid droplet formation during NIH-3T3 cell differentiation (days of differentiation are shown above image) as visualized by BODIPY493/503 staining. (B) Northern blot analysis shows that FIT2shRNA significantly reduced FIT2 mRNA levels compared to control or contshRNA infected cells. Expression of differentiation markers Ap2 and PPARgamma, were not changed in FIT2shRNA treated cells compared to controls. (C) Western blot analysis shows that FIT2 protein is reduced in FIT2shRNA cells compared to controls.

[0018] Figure 5A-5B. Sequence alignments of FIT orthologs in multiple species. (A) Cladogram generated with ClutalW showing the amino acid sequence homologies among FIT proteins. Accession numbers for each FIT ortholog is indicated next to the cladogram. (B) Amino Acid sequence alignment of some FIT orthologs shown in A. Alignment was generated using the ClustalW algorithm. *S. cerevisiae* (YDR319-C, U32517, AAB64755)

(SEQ ID NO:5), *C. elegans* (SEQ ID NO:7), *D. melanogaster* (SEQ ID NO:8), *D. rerio* (SEQ ID NO:11), *X. laevis* (SEQ ID NO:12), *G. gallus* (SEQ ID NO:13), mFIT2 (NM173397, NP775573) (SEQ ID NO:4), hFIT2 (XM371399, XP371399) (SEQ ID NO:2), hFIT1 (NM203402, NP981947) (SEQ ID NO:1), and mFIT1 (NM026808, NP081084) (SEQ ID NO:3). Additional FIT sequences are shown in the Sequence Listing: *S. cerevisiae* (SCS3, NP011389, NC001139) (SEQ ID NO:6), *A. gambiae* (SEQ ID NO:9), *T. nigroviridis* (SEQ ID NO:10), *P. troglodytes* FIT2 (SEQ ID NO:14), *C. familiaris* FIT2 (SEQ ID NO:15), *B. taurus* FIT2 (SEQ ID NO:16), *P. troglodytes* FIT1 (SEQ ID NO:17), *C. familiaris* FIT1 (SEQ ID NO:18), and *B. taurus* FIT1 (SEQ ID NO:19).

[0019] Figure 6. Fenofibrate induction of FIT1 and FIT2 in mouse liver and heart. Mice were fed fenofibrate (FF) as described in materials and methods. Total RNA (15μg) was subjected to Northern blot analysis. PPARalpha deficient mice (α-/-) served to demonstrate dependence of induction on PPARalpha. *GAPDH* served as a loading control.

[0020] Figure 7. Titration of FIT expression plasmids in HEK293 cells. HEK293 cells were transiently transfected with the concentrations of plasmid DNA indicated for each construct shown above the Western blots. M, mock transfected; F1V5, mouse FIT1-V5; F2V5, mouse FIT2-V5; F1, mouse FIT1; F2, mouse FIT2. β-actin served as a loading control. The Western blot on the far left was probed with anti-V5 antibody, and blots to the right were probed with either anti-FIT1 or anti-FIT2 antibodies. 2.4μg of FIT1-V5 and 0.9μg of FIT2-V5 plasmid consistently gave similar expression levels and were used thought this study.

[0021] Figure 8. V5 epitope tagged FIT proteins produce lipid droplets. HEK293 cells were transiently transfected with FIT1-V5 and FIT2-V5 and lipid droplets were visualized by staining cells with BODIPY493/503. Control cells were mock transfected.

[0022] Figure 9. FIT proteins cause lipid droplet formation in HeLa cells. HeLa cells were transiently transfected with mouse FIT1 and FIT2 and lipid droplets were visualized by staining cells with BODIPY493/503. Control cells were mock transfected.

[0023] Figure 10. Expression analysis of genes involved in TG biosynthesis in FIT expressing cells. Northern blot analysis was performed on total RNA (15μg) from HEK293 cells transfected with mouse FIT1 or FIT2, and hybridized to human DNA probes that listed. Acl, citrate lyase; acc, acteyl-CoA carboxylase; fas, fatty acid synthase; gpat, glyceraldehyde phosphate acyltransferase; dgat2, diacylglycerol acyltransferase 2; dgat1, diacylglycerol

acyltransferase 1; adrp, adipocyte differentiation related protein; fit, either human fit1 or fit2. β-actin was used as a loading control.

[0024] Figure 11A-11B. Triglyceride biosynthesis and lipolysis assays in FIT2 knockdown cells. (A) Triglyceride synthesis measurements were performed at each time point during differentiation using ^{14}C glycerol and TLC analysis of triglyceride levels quantified by PhosphorImager analysis. Each time point contained 3 independent samples for each condition, and represented as mean ± std, $p < 0.001$. 3 independent samples were used for each condition, and represented as mean ± std, $p < 0.001$. (B) Lipolysis assays performed on cell lysates from NIH 3T3-L1 cells at day 4 and day 6 of differentiation. No differences were observed between control and shRNAcont and FIT2shRNA adipocytes.

[0025] Figure 12. FIT1 is overexpressed in steatotic livers of *ob/ob* mice. RNA was isolated from wild-type and *ob/ob* mouse livers and adipose tissue and analyzed by Northern blot (20μg of RNA per lane). Figure shows results from two independent mice per genotype. FIT1 is upregulated in both livers and adipose tissue of *ob/ob* mice compared to wild-type lean controls.

[0026] Figure 13A-13B. FIT1 and FIT2 overexpression in mouse liver increases lipid deposition. (A) Control adenovirus injected mice had normal lipid deposition and liver morphology, while mice injected with the AdFIT1 and AdFIT2 adenovirus showed an accumulation of lipid in livers. Left column, Ad control; middle column, Ad FIT1; right column Ad FIT2. (B) FIT1 and FIT2 protein levels are increased in mouse livers injected with FIT1 and FIT2 adenoviruses compared to control injected mice.

[0027] Figure 14. Rosiglitazone induces mFIT2 expression. NIH 3T3 preadipocytes were differentiated for the days indicated and treated or not treated with 1μM rosiglitazone for 24hrs. FIT2 mRNA was increased at all time points of differentiation by treatment with rosiglitazone (-, no rosiglitazone; + 1μM rosiglitazone). All lanes are from duplicate wells of cells.

[0028] Figure 15. Lipid droplet formation in HEK 293 cells expressing wild-type or mutant FIT2. Mock, mock transfected control; WT, wild-type FIT2; FLL 157-9 AAA mutation, L164A mutation, E169A mutation.

[0029] Figure 16. Western blot of FIT2 protein levels in HEK 293 cells expressing wild-type or mutant FIT2. M, mock transfected control; WT, wild-type FIT2; FLL, 157-9FLLAAA mutation, L, L164A mutation, E, E169A mutation. All mutants and wild-type are c-terminally tagged with the V5 epitope.

[0030] Figure 17. Human FIT2 expression produces lipid droplets. Overexpression of human FIT2 (hFIT2) and human FIT2 with a C-terminal V5 epitope tag (hFIT2-V5) in HEK293 cells resulted in numerous lipid droplets compared to mock transfected control cells.

DETAILED DESCRIPTION OF THE INVENTION

[0031] The invention provides a method of reducing fat storage in a tissue in a subject by administering to the subject an amount of an agent that is effective to decrease the level of FIT1 and/or FIT2 in the tissue in the subject. Preferably, the reduced fat storage involves reduced storage of cytoplasmic fat.

[0032] The subject can be a mammal, such as a mouse, rat, cat, dog, horse, sheep, cow, steer, bull, livestock, or monkey or other primate. Preferably, the subject is a human. The subject can have a disease involving storage of cytoplasmic fat in droplets. For example, the subject can have one or more of obesity, diabetes, metabolic syndrome, fatty liver disease, steatosis, steatohepatitis, atherosclerosis, diabetic nephropathy, and hepatitis C infection.

[0033] An examination of human tissues showed that FIT1 is primarily expressed in heart and skeletal muscle, while FIT2 is expressed in all tissues investigated (Fig. 1). Accordingly, agents that reduce FIT1 would be expected to decrease fat storage primarily in the heart and skeletal muscle, while agents that reduce FIT2 would be expected to decrease fat storage in a wide range of tissues including, for example, heart, lung, liver, kidney, brain and adipose tissue. Decreasing fat storage especially in adipose tissue would be expected to result in loss of body weight.

[0034] The methods can involve intervention at the level of DNA, RNA, and/or protein. For example, the presence or activity of FIT1 and/or FIT2 can be reduced by addition of an antisense molecule, a ribozyme, or an RNA interference (RNAi) molecule such as short hairpin RNA (shRNA), where the antisense molecule, ribozyme or RNAi molecule specifically inhibits expression of FIT1 or FIT2. The antisense molecule, ribozyme, or RNAi molecule can be comprised of nucleic acid (e.g., DNA or RNA) or nucleic acid mimetics (e.g., phosphorothionate mimetics) as are known in the art. Methods for treating tissue with these compositions are also known in the art. The antisense molecule, ribozyme or RNAi molecule can be added directly to the tissue in a pharmaceutical composition that preferably comprises an excipient that enhances penetration of the antisense molecule, ribozyme or RNAi molecule into the cells of the tissue. The antisense molecule, ribozyme or RNAi can be expressed from a vector that is transfected into the tissue. Such vectors are known in the art.

[0035] The presence or activity of FIT1 and/or FIT2 can be reduced by addition of an antibody or aptamer to the tissue, wherein the antibody or aptamer specifically binds to and reduces the activity of FIT1 or FIT2 in the tissue. The antibody or aptamer can be added directly to the tissue, preferably in a pharmaceutical composition comprising an agent that enhances penetration of the antibody or aptamer into the tissue. The antibody or aptamer can be encoded on a vector that is used to transfect the tissue. The antibody can be a polyclonal antibody or a monoclonal antibody. The antibody can be a humanized antibody. Preferably, the antibody is an internalizing antibody that is taken up by cells. The agent can be a small molecule.

[0036] The invention also provides a method of increasing fat storage in a tissue in a subject by administering to the subject an amount of an agent that is effective to increase the level of FIT1 and/or FIT2 in the tissue in the subject. The commercial compounds TZDs (e.g. rosiglitazone) can be used to increase FIT2. Increasing fat storage in tissues may be useful for treating malnutrition or for creating animal models for disease. In addition, since the anti-diabetic drugs TZDs also increase body weight and data presented herein show that they increase FIT2 in adipocytes, increased FIT2 expression in adipocytes might be part of the anti-diabetic effect of TZDs.

[0037] In different forms of the methods disclosed herein, the agent can target amino acids phenylalanine-leucine-leucine (FLL), corresponding to amino acids 157 through 159 of mouse FIT2 (mFIT2); amino acid leucine (L), corresponding to amino acid 164 of mFIT2; and/or amino acid glutamic acid (E), corresponding to amino acid 169 of mFIT2.

[0038] The invention also provides a method of screening for a disorder involving regulation of fat storage in tissue, the method comprising determining the level of expression and/or activity of FIT1 and/or FIT2 in a blood, body fluid, tissue biopsy or cell sample (e.g., skin cells) from a subject, wherein alternation from normal of the level of expression or activity of FIT1 and/or FIT2 in the sample is indicative of a disorder involving regulation of fat storage in tissue. For example, the assays can involve measuring the level of FIT1 and/or FIT2 mRNA expression, the level of FIT1 and/or FIT2 protein, or FIT1 and/or FIT2 protein activity levels. In different examples, the expression or activity of FIT1 and/or FIT2 is increased or decreased in comparison to the level found in subjects not having a disorder involving regulation of fat storage. The disorder may be; for example, obesity, type 2 diabetes, metabolic syndrome, fatty liver disease (steatosis and steatohepatitis), atherosclerosis, diabetic nephropathy, hepatitis C infection, viral infection, or a disorder of cardiac, kidney, muscle or liver function.

[0039] The invention also provides a method for screening for a candidate agent that can reduce fat storage in tissue by determining whether or not the agent is effective to decrease the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting the agent with cells or tissue that express FIT1 and/or FIT2, and wherein reduction in expression and/or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for reducing fat storage in tissue.

[0040] The invention further provides a method for screening for a candidate agent that can increase fat storage in tissue by determining whether or not the agent is effective to increase the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting the agent with a cells or tissue that express FIT1 and/or FIT2, and wherein an increase in expression and/or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for increasing fat storage in tissue.

[0041] To screen for activators or inhibitors of FIT1 or FIT2, mammalian, insect, or yeast cells can be stably or transiently transfected with FIT1 or FIT2 gene under regulated control (such as the tet-on system) or constitutively active regulation. FIT1 and FIT2 expressing cells can be treated with molecules such as chemicals, proteins, nucleic acids (e.g., RNAi, antisense oligonucleotides (ASO), aptamers) and then or prior to treatment FIT1 and FIT2 expression is induced. Cells can be screened for fluorescence of lipid droplets (automated using a fluorimeter, facs sorter, microscope with camera, or manually visibly) by incubating cells with BODIPY 492/503 or Nile red, or other lipophilic dye (specific for neutral lipids such as oil-red-o). Alternatively, cellular neutral lipids can be screened by mass measurements of lipids (e.g., using TLC, mass spec, gas chromatography). Compounds that result in cells having enhanced fluorescent signals or neutral lipid mass will be considered as activators and further studied/verified. Compounds that result in cells having decreased fluorescent signals or neutral lipid mass will be considered as inhibitors and further studied/verified.

[0042] The following is a description of a specific example of a protocol that can be used to screen for small molecule inhibitors of FIT proteins. Infect NIH-3T3 L1 preadipocytes in 96 well plates with adenoviruses ($1X10^8$ particles per ml) expressing either FIT1 or FIT2 for 2 hours in normal growth media (DMEM). Remove virus and incubate cells in normal growth conditions (37deg C, 5%CO_2 chamber) overnight. Next day, a single species of chemical will be added to individual wells and incubated for 24hrs. The next day the presence of lipid droplets will be detected by adding 20μg/ml of BODIPY 492/503 lipid

stain for 20 minutes. Cells will be washed and imaged using a fluorimeter or fluorescent microscope. Drugs or agents that are effective in blocking lipid droplet formation are those that result in a decrease in fluorescence signal at a wavelength of 510-665nm. Drugs or agents that are identified through this screen can be further analyzed for specificity toward FIT proteins and cytotoxicity.

[0043] In addition, mouse embryo fibroblasts isolated from FIT2 knockout embryos may also serve as a suitable model for the screening methods described herein. These fibroblasts can be stably or transiently transfected with FIT1 and/or FIT2 under the control of regulated or constitutively active promoters. These cells may have the advantage over other cell types because they do not have endogenous FIT2 or FIT1 expression and thus have lower "background" lipid droplet formation.

[0044] The cell culture screens for activator and inhibitors of FIT 1 and FIT2 can use, for example, human FIT1 and/or human FIT2 genes or mouse FIT1 and/or TIF2 genes.

[0045] The invention also provides a transgenic mammal in which FIT1 and/or FIT2 is overexpressed in one or more tissue. Preferably, fat storage is increased in the tissue compared to a wild-type mammal. For example, FIT1 and/or FIT2 can be overexpressed in the liver, heart, and/or skeletal muscle. As another example, FIT2 can be overexpressed in adipose tissue. The adipose tissue can be white and/or brown adipose tissue. The invention further provides a mammal that has been genetically altered so that expression of FIT1 and/or FIT2 is reduced in one or more tissue. Preferably, fat storage is reduced in the tissue in comparison to a wild-type mammal. For example, the mammal can be a FIT1 knockout or FIT1 conditional knockout or can be heterozygous for FIT1. The mammal can also be heterozygous for FIT2 or be a FIT2 conditional knockout. The mammal can be a non-human mammal such as a mouse.

[0046] This invention will be better understood from the Experimental Details that follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Materials and Methods

[0047] *Reagents.* FirstChoice Human Blot 1 (purchased from Ambion, cat#3140). Mouse total RNA was extracted from C57BL/6J mice and 15μg of total RNA was used for Northern blot analysis. Rabbit polyclonal antibodies were raised against peptides

corresponding to the C-terminal 15 amino acids of murine FIT1 and FIT2. Anti-V5 antibodies were purchased from Invitrogen Corporation. Full length FIT1 and FIT2 cDNAs were amplified from cDNA generated from mouse liver RNA using Thermoscript RT with polyDT primers (Invitrogen Corporation), followed by subcloning into pcDNA3.1 Directional TOPO (Invitrogen Corporation). BODIPY 493/503 and Nile Red were purchased from Invitrogen Corporation and used at 10μg/ml.

[0048] *Mice and gene arrays.* 3 male wild-type mice and 3 male PPARalpha-/- mice (purchased from Jackson labs, Bar Harbor, ME) were fed a chow diet containing 0.2% fenofibrate (purchased from Sigma-Aldrich) for 7 days. After day 7, mice were euthanized and liver RNA extracted. RNA from the 3 wild-type mice and 3 PPARalpha-/- mice were pooled in equal proportions to create 2 pools of RNA (wild-type versus PPARalpha-/-). RNA was processed by standard Affymetrix protocols to screen the MG_430 2.0 mouse gene expression array.

[0049] Manual analysis of the gene array data revealed the presence of sequence 1451488 on the array (See Affymetrix data file at Gene Expression Omnibus) to be induced in the wild-type mice relative to the PPARalpha-/- mice. 1451488 corresponds to gene bank accession number NM_026808. Using the BLAST search algorithm by NCBI, FIT2 (Affymetrix number 1454935 and accession number NM_173397) and many other FIT orthologs were identified (Fig. 6). Sequence alignments were made using the publicly accessed program ClustaW.

[0050] *Cell culture.* HEK293, and HeLa cells were grown in DMEM 10% FBS. NIH-3T3 L1 adipocytes were a kind gift from Philip Scherer and grow and differentiated by standard methods (*24*).

[0051] *Membrane fractionation.* Mouse hearts were homogenized with 25 strokes using a dounce homogenizer in a buffer containing 10mM Hepes pH7.4, 1mM MgCl2 and proteinase inhibitors (Complete EDTA free, Roche) followed by 3 brief sonication pulses on ice. Nuclei were removed by a 2000 X g spin for 5min and samples were loaded on the top of a continuous sucrose gradient and centrifuged in a SW41 rotor at 100,000 X g for 18 hrs. The continuous sucrose gradient was prepared by mixing a 1.1M sucrose solution made in the above buffer used for homogenization with a 0.58M sucrose solution made in the same buffer using a standard 12ml gradient maker. After centrifugation, the top 3 fractions (800μl each) were discarded because of visible cellular debris. All subsequent 800μl fractions were collected for Western blot analysis.

[0052] *Confocal immunofluorescence microscopy.* HEK293 cells were transiently transfected with expression plasmids for mFIT1 and mFIT2 with C-term V5 tags and RFP-KDEL. Cells were fixed with 10% formalin for 15 minutes at room temperature, followed by permeablization with 0.1% triton X-100. Cells were then blocked with Goat serum (5%) followed by consecutive incubations with anti-V5 antibody, and anti-mouse Alexa 488. After several post-antibody washes, cells were visualized by confocal microscopy using a BioRad Radiance 2000 Laser Scanning Confocal Microscope.

[0053] *Human FIT expression.* Human FIT2 cDNA (hFIT2) was amplified from the human hepatic cell line HepG2 using primers forward primer: 5'-CACCATGGAGCATCTGGAGCGCTGCGAGTGGTTGTTGCG-3' (SEQ ID NO:56) and reverse primer: 5'-TTATTTCTTGTAACTATCTTGCTTCAAATTCAAAC-3' (SEQ ID NO:57). To produce a V5-epitope tagged version of hFIT2 (hFIT2-V5) the following reverse primer was used instead of the one described above: 5'-TTTCTTGTAACTATCTTGCTTCAAATTCAAACTAC-3' (SEQ ID NO:58). The amplified hFIT2 cDNA was subcloned into pcDNA3.1 topo directional plasmid (Invitrogen Corp.). To test if hFIT2 and hFIT2-V5 can produce droplets, HEK293 cells were transfected with 2.4μg of the above described plasmid expressing hFIT2 and stained 36hr posttransfection with the lipid droplet stain BODIPY493/503. Lipid droplets were visualized by fluorescence microscopy.

[0054] *TLC assays.* Lipids were extracted from cells in culture using HIP: Hexane:isopropanol (3:2 V/V). Triglyceride and cholesterol ester mass was quantified using TLC according (*21, 25*). The TLC solvent system used was Hexane:Ethyl ether:Acetic acid (80:20:1).

[0055] *Triglyceride biosynthesis and DGAT assays.* Cells in culture were incubated with 7.5 μCi/mL of 14C-glycerol (Specific activity: 125-180 mCi/mmol, NEN-Amersham) for the indicated times, followed by extraction with HIP and separation by TLC as referenced above. Radioactive triglycerides were quantified using PhosphorImager analysis and represented as a rate of triglyceride production using arbitrary units obtained from PhosphorImager analysis.

[0056] *Lipase activity assay.* Total cellular lipase activity was measured using the established method of Holm and Osterlund in Methods in Molecular Biology 1999; 109, p109-121.

[0057] *Lentivirus shRNA.* The following short hairpin RNA (shRNA) sequences (sense strand shown) were used to knockdown mouse FIT2 expression: shRNAcont, CACCGAATTCTCCGAACGTGTCACGCGAACGTGACACGTTCGGAGAA (SEQ ID NO:20); shRNAFIT2, (SEQ ID NO:21) CACCGCACCATGTTTGGTTTGTTGGCGAACCAACAAACCAAACATGGTGC.

[0058] Double stranded shRNA DNAs were used to generate lentiviruses using the U6 promoter driven BLOCK-iT Lentivirus RNAi expression system (Invitrogen Corporation). Knockdown of human FIT2 in HEK293 cells was performed by stably transfection using pEntryU6 vector (Invitrogen Corp). NIH-3T3 L1 pre-adipocytes and HepG2 cells were infected with an MOI of 10-50.

[0059] *FIT2 mutations.* FLL157-9 and single 164L and 169E residues were changed to alanines using standard PCR mutagenesis methods on the mouse FIT2 cDNA. Mutants were subcloned into pcDNA3.1 Topo Directional plasmid (Invitrogen corp.) for expression in HEK 293 cells. HEK 293 cells were transfected with 2μg of DNA from each construct and cells were stained 36hrs after transfection with BODIPY493/503 (10μg/ml for 10 minutes) to visualize lipid droplets.

[0060] *Mouse transgenic models of FIT1 and FIT2 overexpression: liver-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 and FIT2 specifically in liver using the murine albumin promoter (Albe/p vector , kind gift from Ronald Kahn, Harvard University). FIT1 and FIT2 located in pcDNA3.1 Directional vector were digested with HindIII-EcoRV and blunt ended using Klenow enzyme. A bovine growth hormone polyadenylation sequence was amplified by PCR from pcDNA3.1 using primers containing a 5' BamH1 site and a 3' EcoRV site. This PCR product was digested with the aforementioned restriction enzymes and subcloned into Albe/p vector digested with the same enzymes and now designated Albe/p-BGH. This step introduced a necessary polyadenylation sequence. Next, the FIT1 and FIT2 blunt ended DNA fragment was subcloned into Albe/p-BGH digested with EcoRV yielding FIT1-, FIT2-Albe/p-BGH construct. This construct was then used to create transgenic mice at the Albert Einstein College of Medicine.

[0061] *Mouse transgenic models of FIT1 and FIT2 overexpression: muscle-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 specifically in skeletal muscle using the muscle creatine kinase promoter (MCK pCK4800, kind gift from Rhonda Bassel-Duby, UT Southwestern Medical Center). The transgene was constructed by subcloning HindIII-EcoRV blunt ended FIT1 cDNAs into MCK pCK4800 digested with EcoRV to yield MCK-FIT1.

[0062] *Mouse transgenic models of FIT1 and FIT2 overexpression: heart-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 specifically in cardiac myocytes using the alpha myosin heavy chain promoter enhancer vector (alpha-MHC vector, a kind gift of Ira Goldberg, Columbia University). To generate the transgene construct alpha-MHC vector was digested with SalI and made blunt using klenow enzyme. A HindIII-EcoRV blunt ended fragment of FIT1 was subcloned into the digested alpha-MHC vector to generate alpha-MHC-FIT1.

[0063] *Mouse transgenic models of FIT1 and FIT2 overexpression: adipose-specific expression.* Transgenic mice were generated that overexpress mouse FIT2 specifically in adipose tissue (white and brown adipose) using the Ap2 promoter/enhancer (kind gifts from Philip Scherer, Albert Einstein College of Medicine). To produce this transgene the 3'UTR Ap2 enhancer was digested with HindIII-EcoRV. FIT2 was digested with HindIII-EcoRV (from pcDNA3.1) and ligated into the vector containing the 3'UTR Ap2 enhancer to yield FIT2-3'UTR. The Ap2 promoter was digested with XmaI then made blunt, followed by digestion with SalI. FIT2-3'UTR was digested with HindIII and made blunt, then cut with SalI. The Ap2 digested fragment was then ligated into FIT2-3'UTR to make the final transgene Ap2-FIT2-3'UTR.

[0064] *Mouse deficiency models for FIT1 and FIT2: FIT1 gene targeted mice.* BAC recombineering methods were utilized according to the protocols found in Warming et al. (26) and publicly available at http://recombineering.ncifcrf.gov/. All materials obtained to perform BAC recombineering were obtained free of charge from NIH Division of Technology Development and Transfer Office (Phone: 301-435-5502). BAC RP23-186J4 (referred to as BAC J4) was purchased from Children's Hospital Oakland Research Institute. BAC J4 was electroporated into SW102 cells for further use in recombineering. Plasmid pl452 was used as template to add on 50 base pairs of homology flanking the NEO cassette located in pl452. The primer sequences are as follows: forward primer: CATTAGCCCCTCCTCAGCCTCCAGCAGAGCAGACAGTTAGTGGGGAGGGGCCAT GGccgatcatattcaataaccc (SEQ ID NO:22); reverse primer: TGTTTGCGCATTGAGCGGGATCGAGGGAAGAGCCCGTGGCCTGGGATCCCATAA CTTCGTATAGCATAC (SEQ ID NO:23). Plasmid pl452 has the sequence set forth in SEQ ID NO:54.

[0065] Purified PCR product (200ng) was used to electroporate SW102 cells harboring BAC J4, and Kan and Chlor resistant colonies were selected for on plates (designated as BAC

J4-NEO). Verification of correct targeting was carried out using the following pairs of PCR primers:

1) FIT1F-TVP, GTTGACCGTCAGTCCTCAAACTGGCCCCTTGC (SEQ ID NO:24);
PGK-R-TVP, GCTTGGCTGGACGTAAACTCCTCTTCAGACC (SEQ ID NO:25);
2) FIT1R-TVP, GAAAAGAATCGGAGGAGACAGAGCCAGGCCTGG (SEQ ID NO:26);
BGH-F-TVP, GAACCAGCTGGGGCTCGACTAGAGCTTGCGG (SEQ ID NO:27).

[0066] In addition, Southern blot analysis was performed to confirm proper targeting. BAC J4-NEO DNA was digested with NcoI and probed with a radiolabeled PCR fragment (300bp fragment) generated using the following two PCR primers and BAC J4 as template:
NcoI-F: Acacaagttttgcacagacatagatgcagg (SEQ ID NO:28);
Nco-R: Caacttcacacagcctagaatcatctgagaggagaacc (SEQ ID NO:29).

[0067] Following confirmation that exon 1 and exon 2 were replaced by the NEO cassette from plasmid pl452, a diptheria toxin cassette with Amp resistance, DTA (kind gift of Chingwen Yang, Rockefeller University) was homologously recombined into BAC J4-NEO. 50bp homology arms were add by PCR to the DTA cassette using the following pair of primers:

FIT-DTA-F:
ACACAAGTTTTGCACAGACATAGATGCAGGTAAAATATACATAAGTAAGCGAAC
AACTCCGCCGCGCGCGCTC (SEQ ID NO:30);
FIT-DTA-R:
CAACTTCACACAGCCTAGAATCATCTGAGAGGAGAACCTCAATGGAGGAAGAGT
AAACTTGGTCTGACAG (SEQ ID NO:31).

[0068] PCR product (200ng) was electroporated into SW102 cells harboring the BAC J4-NEO and colonies were selected that were Chlor, Kan and Amp resistant. Targeting of the DTA cassette was verified using the following two sets of PCR primer pairs:

1) FIT3F-TVP, CAGGCTGCTCTTCAGGAGTATATCTGGGTTC (SEQ ID NO:32);
POLIIP1R-TVP, CCGGGAGCCACCTTCTTCTCCAACCGTCCCGG (SEQ ID NO:33);
2) FIT3R-TVP, AGTTACAGACAATTGTGAGTTGCCATGTGGC (SEQ ID NO:34);
AMP1F-TVP, CTGAGATAGGTGCCTCACTGATTAAGCATTGG (SEQ ID NO:35).

[0069] 10μg of BAC J4-NEO-DTA was cut with NotI to linearize and then electoporated into embryonic stem (ES) cells at the Columbia University Transgenesis facility. 45 ES cells were screened for homologous recombination using the following two PCR primers:

FIT-ES1-TVP-F, GTACCCCACAGTCACATCCATAGGACAATCC (SEQ ID NO:36);

FIT-ES1-R , CTAACTGTCTGCTCTGCTGGAGGCTGAGGAGG (SEQ ID NO:37).

[0070] Targeted ES cells were then confirmed by Southern blot analysis using the NcoI probe as described above. The targeted D5 ES cell line was used to generate chimeric mice. The FIT1 Knockout mice are viable and fertile.

[0071] *Mouse deficiency models for FIT1 and FIT2: FIT2 gene targeted mice.* BAC recombineering methods were utilized according to the protocols found in Warming et al. (26) and publicly available at http://recombineering.ncifcrf.gov/. All materials obtained to perform BAC recombineering were obtained free of charge from NIH Division of Technology Development and Transfer Office (Phone: 301-435-5502). BAC RP23-36P22 (referred to as BAC P22) was purchased from Children's Hospital Oakland Research Institute. BAC P22 was electroporated into SW105 cells for further use in recombineering to specifically delete exon2. Plasmid pl452 was used as template to add on 50 base pairs of homology flanking the NEO cassette located in pl452. The primer sequences are as follows:

pL452FIT2a-F,

GCTCAGAGGAGGCAGACATGGCAGATGTTGCTGTATCTGGCCTAATGAACccgatcat attcaataaccc (SEQ ID NO:38);

pL452FIT2a-R,

ACGTGGGAATCCTATTAGCCATTGTCCTCCTGTCCCTATGTCCTTCTCTTataacttcgta tagcatac (SEQ ID NO:39).

[0072] Purified PCR product (200ng) was used to electroporate SW105 cells harboring BAC P22, and Kan and Chlor resistant colonies were selected for on plates (designated as BAC P22-NEO). Verification of correct targeting was carried out using the following pairs of PCR primers:

1) FIT2-TVP1c-F, TAAGAATAGAGTGTAAGGGTGGTAGTTGTTCC (SEQ ID NO:40);

PGK-R-TVP, GCTTGGCTGGACGTAAACTCCTCTTCAGACC (SEQ ID NO:25);

2) FIT2-TVP1d-F, CCTTCATCCTTCCCCACTCGTAGTGGCTGGTC (SEQ ID NO:41);

BGH-F-TVP, GAACCAGCTGGGGCTCGACTAGAGCTTGCGG (SEQ ID NO:27).

[0073] Following confirmation that exon 2 were replaced by the NEO cassette from plasmid pl452, a diptheria toxin cassette with Amp resistance, DTA (kind gift of Chingwen Yang, Rockefeller University) was homologously recombined into BAC P22-NEO. 50bp homology arms were add by PCR to the DTA cassette using the following pair of primers:

FIT2-DTA-F,

ACAGTCCCAGCTCCCATGGCACTCCTCACTGTAAATATAACTCGCCGATGgaacaact ccgccgcgcgcgctc (SEQ ID NO:42);

FIT2-DTA-R,
GCTGGCCTGGAACTCACTTTATAGAGAGGGCTGGTTTTGAACCTGTGTTGgagtaaact tggtctgacag (SEQ ID NO:43).

[0074] 200ng of PCR product was electroporated into SW102 cells harboring the BAC P22-NEO and colonies were selected that were Chlor, Kan and Amp resistant. Targeting of the DTA cassette was verified using the following two sets of PCR primer pairs:
1) FIT2-TVP5-F, CTTCGTGCACACTAGTCAAGCATCCTACCAAC (SEQ ID NO:44);
POLIIP1R-TVP, CCGGGAGCCACCTTCTTCTCCAACCGTCCCGG (SEQ ID NO:33);
2) FIT2-TVP7-R, CTATTTTGAAACAGGATCTCTTACGTGACCC (SEQ ID NO:45);
AMP1F-TVP, CTGAGATAGGTGCCTCACTGATTAAGCATTGG (SEQ ID NO:35).

[0075] 10μg of BAC P22-NEO-DTA was cut with NotI to linearize and then electoporated into ES cells at the Columbia University Transgenesis facility. 48 ES cells were screened for homologous recombination by Southern blot analysis. ES cell DNA was digested in 96 well plates with NcoI and probed with a 300bp fragment, which is the same fragment replaced by the DTA cassette on the P22 BAC. Wild-type alleles yielded a 2.8kb fragment and targeted alleles resulted in the appearance of a 3.5kb fragment.

[0076] The targeted A3 ES cell line was used to generate chimeric mice. It was been determined that deletion of FIT2 results in embryonic lethality and therefore conditional FIT2 targeted mice were generated as described below.

[0077] *FIT2 Conditional targeted mice.* Based on the finding described herein that gene targeted deletion of FIT2 results in embryonic lethality, BAC recombineering was used to produce a "floxed" allele of FIT2 to conditionally knock it out in adult mice.

[0078] The same BAC, P22 was used for generating an engineered targeting vector. For insertion of a single loxP site within intron 1 upstream of exon 2, pL451 was PCR amplified to introduce 50bp homology arms to this region using the following primers (uppercase letters correspond to vector sequences of pL451):
FIT2pL451a-F,
taatgaaccaggagcccagcagcctggcacgccaattgtttaggggtagcGATATCGAATTC (SEQ ID NO:46);
FIT2pL451a-R,
gaaaaaaagaaagaaagaaagcgagcaagctttagttcaagctacaagacAGTGGATCCACC (SEQ ID NO:47).
Plasmid pl451 has the sequence set forth in SEQ ID NO:55.

[0079] 200ng of PCR product was electroporated into SW105 cells harboring the P22 BAC and cells were selected for kanamycin resistance yielding P22-NEO. Homologous recombination was verified using the following primers:

1) FIT2-TVPC1-R, accacatggtggctcataaccatctgtcatg (SEQ ID NO:48);
BGH-F-TVP, GAACCAGCTGGGGCTCGACTAGAGCTTGCGG (SEQ ID NO:27);
2) FIT2-TVP1c-F, TAAGAATAGAGTGTAAGGGTGGTAGTTGTTCC (SEQ ID NO:49);
PGK-R-TVP, GCTTGGCTGGACGTAAACTCCTCTTCAGACC (SEQ ID NO:25).

[0080] The Neo cassette was then removed from BAC-NEO by treating cells with arabinose to induce the endogenous flip recombinase resulting in a single loxP and Frt site left behind in intron 1, and also sensitivity to kanamycin. This construct is called BAC-Frt-loxP.

[0081] Next, insertion of a second loxP site downstream of exon 2 was made by PCR amplification of pL451 with the following primers:
FIT2pL451b-F,
acaagcttagttggcagactctgctggcggtgccaccatgttgccagcagGATATCGAATTC (SEQ ID NO:50);
FIT2pL451b-R,
acgatgaggcaggctgctctccctctgctgagcctcagctctccctctggAGTGGATCCACC (SEQ ID NO:51).

[0082] 200ng of PCR product was electroporated into SW105 cells harboring BAC-Frt-loxP and cells were again selected for kanamycin resistance and the new recombined BAC was designated BAC-Frt-Loxp-exon2-NEO-loxP.

[0083] Homologous recombination was verified using the following primers:
1) FIT2-TVPC-4R, agtgagttccagaacacgcagggctacacag (SEQ ID NO:52);
BGH-F-TVP, GAACCAGCTGGGGCTCGACTAGAGCTTGCGG (SEQ ID NO:27);
2) FIT2-TVPC5-F, gctcagcagagggagagcagcctgcctgatcgt (SEQ ID NO:53);
PGK-R-TVP, GCTTGGCTGGACGTAAACTCCTCTTCAGACC (SEQ ID NO:25).

[0084] Following confirmation that exon 2 was flanked by both an upstream loxp-frt site and downstream loxp-neo cassette from pL451, a diptheria toxin cassette with Amp resistance, DTA (kind gift of Chingwen Yang, Rockefeller University) was homologously recombined into BAC P22-NEO. 50bp homology arms were add by PCR to the DTA cassette using the following pair of primers:
FIT2-DTA-F:
ACAGTCCCAGCTCCCATGGCACTCCTCACTGTAAATATAACTCGCCGATGgaacaact ccgccgcgcgcgctc (SEQ ID NO:42);
FIT2-DTA-R:
GCTGGCCTGGAACTCACTTTATAGAGAGGGCTGGTTTTGAACCTGTGTTGgagtaaact tggtctgacag (SEQ ID NO:43).

[0085] 200ng of PCR product was electroporated into SW105 cells harboring the BAC-P22 Frt-Loxp-exon2-NEO-loxP and colonies were selected that were Chlor, Kan and Amp resistant. The completed vector is now designated BAC-P22 Frt-Loxp-exon2-NEO-loxP-DTA. Targeting of the DTA cassette was verified using the following two sets of PCR primer pairs:

1) FIT2-TVP5-F, CTTCGTGCACACTAGTCAAGCATCCTACCAAC (SEQ ID NO:44);
POLIIP1R-TVP, CCGGGAGCCACCTTCTTCTCCAACCGTCCCGG (SEQ ID NO:33);
2) FIT2-TVP7-R, CTATTTTGAAACAGGATCTCTTACGTGACCC (SEQ ID NO:45);
AMP1F-TVP, CTGAGATAGGTGCCTCACTGATTAAGCATTGG (SEQ ID NO: 35).

[0086] 10μg of BAC-P22 Frt-Loxp-exon2-NEO-loxP-DTA was cut with NotI to linearize and then electoporated into ES cells at the Columbia University Transgenesis facility. ES cells will be screened for homologous recombination by Southern blot analysis. ES cell DNA will be digested in 96 well plates with NcoI and probed with a 300bp fragment which is the same fragment replaced by the DTA cassette on the BAC-P22 Frt-Loxp-exon2-NEO-loxP-DTA. Wild-type alleles yield a 2.8kb fragment and Neo-floxed exon 2 allele will result in the appearance of a 5.6kb fragment. Removal of the Neo cassette by crossing Neo-floxed mice with a flip-recombinase expressing mouse line (e.g. constitutive expression driven by the beta-actin promoter available for purchase from Jackson Laboratories, Bar Harbor Maine) will result in a floxed exon 2 allele (without the neo cassette downstream of exon 2) of 6kb. Exon 2 can be deleted in a tissue- and stage-specific manner by crossing mice to cre or flip recombinase transgenic mice with the recombinase under the control of a tissue or developmentally regulated promoter. Many of these transgenic lines expressing cre or flip are available for purchase.

[0087] *Regulated promoter for FIT1 and FIT2 expression.* To generate an inducible expression system for murine FIT1 and FIT2, FIT1-V5 and FIT2-V5 (both having c-terminal V5 epitopes) have been subcloned from pcDNA3.1 topo directional (plasmid from Invitrogen corp.) using restriction enzymes HindIII and PmeI into pcDNA5/TO cut with restriction enzymes HindIII and EcoRV. pcDNA5/TO contains two tetracycline operator sites in the promoter region of the plasmid that serve as binding sites for the tetracycline repressor. Induction of either FIT1 or FIT2 expression will be achieved by treating cells with 1μg/ml of tetracycline or doxycycline. All cell lines that will be used must express the tetracycline repressor. Tetracycline repressor expressing cells will be generated by stably transfecting cells with pcDNA6/TR (from Invitrogen corp.) and selecting for G418 resistance.

pcDNA6/TR harboring cells will also be made to stably harbor FIT1 and FIT2 pcDNA5/TO constructs by selection on hygromycin.

Results and Discussion

[0088] *Identification of FIT genes.* The initial goal was to identify genes involved in intracellular fatty acid transport toward either storage in lipid droplets as triglyceride or catabolism via beta-oxidation in peroxisomes and mitochondria. Fenofibrate and other fibrate drugs are agonists for the peroxisome proliferator-activated receptor alpha (PPARalpha) nuclear hormone receptor, and activation of PPARalpha leads to the induction of the genes coding for proteins involved in the entire biochemical repertoire of beta-oxidation (*17-19*). To carry out this goal, genotype matched wild-type and PPARalpha deficient mice were fed a diet containing fenofibrate for 7 days. mRNA from livers were used to query gene arrays. Since most of the genes activated by PPARalpha likely have been identified, the focus was exclusively on genes that were listed as expression sequence tags (ESTs) or having unknown function. By performing BLAST searches against the mouse genome, many of the ESTs to the 3 prime end of the final exons of genes were located. This analysis was followed by both an examination using the Signal P algorithm to determine the putative cellular location of the gene product and using Novartis SymAltas (http://symatlas.gnf.org/SymAtlas/) to determine the putative tissue expression pattern. Attention was directed toward a particular unknown transcript, named Fibrate Induced Transcript 1 (FIT1, Affymetrix number 1451488). FIT1 potentially has multiple transmembrane domains (Fig. 1D), was putatively located in the secretory pathway (determined by Signal P), and highly expressed in heart and skeletal muscle by gene array studies (determined by Novartis Gene Atlas). However, FIT1 did not have homology to known proteins or known protein domains found in any species. These characteristics indicated a protein potentially involved in lipid transport in oxidative tissues.

[0089] Performing a BLAST search of the full-length mouse FIT1 amino acid sequence against the expressed data base, a second FIT1 homolog was identified in mammals that was designated FIT2. Mammals have two FIT genes, FIT1 and FIT2, while amphibians, birds, fish, insects, worms have a single FIT gene exhibiting higher homology to FIT2 (Fig. 5B) *S. cerevisiae* is an exception because it has two FIT2 orthologs. A second FIT2 ortholog not shown in the cladagram of Fig. 5A is called SCS3. FIT2 was found on the same Affymetrix gene array used to identify FIT1 (Affymetrix number 1454935) and was also increased by fenofibrate on the gene array. The expression of both mouse FIT1 and FIT2 were confirmed to be induced by fenofibrate in a PPARalpha-dependent fashion in liver (Fig. 6). Northern

blot analysis of mouse tissues indicated that FIT1 is highly expressed in heart and skeletal muscle, and to lower levels in liver, kidney, brown adipose, testes, and eye (Fig. 1A). Western blot analysis of mouse tissues indicated that FIT1 protein was detected primarily in heart and at low levels in liver and skeletal muscle (Fig. 1B). Mouse FIT2 mRNA was detected as two transcripts in most tissues examined with highest levels in heart, skeletal muscle and brown adipose tissue (Fig. 1A). Note that mFIT2 but not mFIT1 is expressed in white adipose tissue (Fig. 1A). Western blot analysis indicated FIT2 protein highest in heart, and lower levels in lung, liver, and kidney (Fig. 1B) An examination of human tissues showed that FIT1 was primarily expressed in heart and skeletal muscle, while FIT2 was expressed in all tissue represented on the Northern blot (Fig. 1C). Together, these analyses indicated that both mouse and human FIT1 have a more restricted expression pattern to oxidative tissues, while FIT2 has a broader tissue expression pattern.

[0090] *FIT1 and FIT2 are localized to the endoplasmic reticulum.* In order to define a specific function to FIT proteins, both subcellular fractionation of mouse heart membranes and confocal immunolocalization studies were carried out. Sucrose density fractionation of mouse heart membranes indicated that both FIT1 and FIT2 were co-localized exclusively with endoplasmic reticulum (ER) membrane fractions (Fig. 2A). Expression of a c-terminal V5 tagged FIT1 and FIT2 in HEK 293 cells co-localized with the ER resident protein calnexin (Fig. 2B). Importantly, the V5 tag on FIT1 and FIT2 did not abolish its activity (Fig. 8). Together, the data indicated that FIT1 and FIT2 are localized in the ER.

[0091] *Overexpression of FIT1 and FIT2 results in the formation of lipid droplets.* In order to determine if FIT proteins play a role in lipid metabolism, experiments were designed to overexpress FIT proteins in cells. FIT1 and FIT2 were overexpressed in HEK293 cells and examined using the fluorescent lipid droplet stain BODIPY493/503. Figure 3A shows that overexpression of FIT1 or FIT2 resulted in the accumulation of lipid droplets. FIT1 overexpression led to the accumulation of multiple droplets per cell with one or two larger droplets per cell, while FIT2 expressed to similar levels as FIT1 (Fig. 7) led to the frequent appearance of a single large droplet. Cells expressing DGAT1, one of two acyltransferases important in the committed step in TG biosynthesis (*20*) showed multiple lipid droplets smaller than those produced in FIT1 and FIT2 expressing cells (Fig. 3). Overexpression of FIT1 and FIT2 in HeLa cells resulted in similar findings except that FIT2 expression resulted in multiple large droplets per cell, together indicating that FIT1 and FIT2-induced lipid droplet accumulation is not cell-type specific (Fig. 9). Compared to mock transfected cells, cellular TG mass was significantly increased in cells expressing FIT1 and FIT2 (Fig. 3B).

These data are consistent with the fluorescent images indicating the presence of lipid droplets in FIT1 and FIT2 expressing cells (Fig. 3A). As a positive control, cells were transfected with DGAT1 or DGAT2, a second acyltransferases important in the committed step in TG biosynthesis (*20*). Overexpression of DGAT1 or DGAT2 resulted in increased levels of cellular TG that were significantly greater than in FIT1 and FIT2 cells (Fig. 3A).

[0092] *Human FIT expression produces lipid droplets.* Overexpression of human FIT2 (hFIT2) and human FIT2 with a C-terminal V5 epitope tag (hFIT2-V5) in HEK293 cells resulted in numerous lipid droplets compared to mock transfected control cells (Figure 17).

[0093] *FIT proteins do not enhance triglyceride biosynthesis.* It was also determined whether FIT1 and FIT2 enhance TG biosynthesis causing lipid droplets to form by quantifying the rate of TG biosynthesis using radiolabeled glycerol as a precursor for TG. Cells expressing FIT1 or FIT2 showed similar rates of TG biosynthesis compared to mock transfected cells, indicating that the accumulation of lipid droplets in FIT1 and FIT2 expressing cells is not the result of enhanced TG biosynthesis. The mRNAs of genes important in fatty acid and TG biosynthesis were not changed in cells expressing FIT1 or FIT2 (Fig. 10). As expected from previous studies, DGAT1 and DGAT2 overexpression led to a significant increase in TG biosynthetic rate (*20*) (*21*). To test if increased cellular TG is the result of partitioning of TG into lipid droplets, cells were incubated with ^{14}C glycerol tracer for 24hrs and radiolabel in lipid droplets were quantified. Figure 3C shows that cells expressing FIT1 and FIT2 had a significant increase in the percent of labeled glycerol in lipid droplets compared to mock transfected cells. As a positive control, DGAT1 and DGAT2 expressing cells exhibited a significant increase in radiolabeled glycerol in lipid droplets compared to mock, FIT1 and FIT2 expressing cells (Fig. 3C). Taken together, these results indicate that FIT1 and FIT2 do not directly mediate triglyceride biosynthesis, but lipid droplet formation leading to the accumulation of cellular neutral lipids.

[0094] *shRNA suppression of FIT2 abolishes lipid droplet formation during adipogenesis.* To provide a direct test if FIT proteins are essential for the formation of lipid droplets, knockdown of FIT expression was sought in established cellular models of lipid droplet formation. If FIT genes are essential for lipid droplet formation, then the NIH-3T3 L1 cell line, a classic adipocyte differentiation cell model that produces large amounts of lipid droplets during differentiation of pre-adipocytes into adipocytes, should express FIT genes during adipogenesis at the onset of lipid droplet accumulation. Figure 4B and 4C show that high levels of FIT2 at both mRNA and protein levels, but not FIT1 (data not shown), were detected during adipogenesis at the onset of formation of visible lipid droplets (Fig.

4A). This is in line with the observation that FIT2 and not FIT1 are expressed in white adipose tissue in vivo (Fig. 1A,B). If FIT2 is indeed essential for droplet formation, then suppression of FIT2 expression should abolish lipid droplet formation. Pre-adipocytes cells infected with lentivirus expressing shRNA against FIT2, a control shRNA, or no virus were induced to differentiate. FIT2 mRNA and protein were significantly suppressed in adipoctyes infected with lentivirus expressing FIT2 shRNA. Examination of these cells for lipid droplets showed that cells having suppressed FIT2 expression had an almost complete absence of lipid droplets in pre-adipocytes (day 0) and in mature adipocytes (between day 4 and 9) (Fig. 4A). Only a low level of cells expressing the FIT2shRNA exhibited multiple lipid droplets per cell between day 6 and 9 (Fig. 4A). Further examination of FIT2 suppressed cells showed that the expression of PPARgamma and AP2, genetic markers of adipocyte differentiation, were not changed (Fig. 4B). Triglyceride biosynthesis or triglyceride lipase activities were similar in adipocytes having a knockdown of FIT2 compared to controls (Fig. 11-11B). Taken together, the data indicate that knockdown of FIT2 specifically inhibited lipid droplet formation.

[0095] *FIT is overexpressed in steatotic livers of ob/ob mice.* *Ob/ob* mice are insulin resistant, have fatty livers and are obese. RNA was isolated from wild-type and *ob/ob* mouse livers and adipose tissue and analyzed by Northern blot (20μg of RNA per lane). Figure 12 shows results from two independent mice per genotype. Figure 12 indicates that FIT1 is upregulated in both livers and adipose tissue of *ob/ob* mice compared to wild-type lean controls. This data suggests that FIT1 overexpression in livers of obese animals might be responsible for the formation of fatty liver.

[0096] *FIT1 and FIT2 overexpression in mouse liver increases lipid deposition.* Overexpression of mouse FIT1 and FIT2 in mouse liver causes triglyceride accumulation. Mouse FIT1 and FIT2 were first subcloned into adenovirus shuttle vector (pVW-CVM K-NpA) and adenovirus was synthesized (service provided by Viraquest, Iowa). Two groups of 6 wild-type male C57BL6J mice were injected intravenously with $1X10^8$ pfu with empty adenovirus control (adEmpty) or adenovirus expression FIT1 (adFIT1). All mice were fed a standard chow diet for the first 3 days, then switched to a high fat diet (cat# TD.93075 Harlan Teklad) for 4 days. Perfused livers from mice were either frozen in OTC for producing frozen sections for oil-red-o staining, or fixed in 10% formalin and embedded in paraffin for H&E staining. Figure 13A shows that control adenovirus injected mice had normal lipid deposition and liver morphology, while mice injected with the AdFIT1 and AdFIT2

adenovirus showed an accumulation of lipid in livers. Figure 13B shows increased protein levels of FIT1 and FIT2 in mouse livers injected with FIT1 and FIT2 adenoviruses compared to control injected mice.

[0097] *Rosiglitazone induces mFIT2 expression.* NIH 3T3 preadipocytes were differentiated as in Figure 14 for the days indicated (0, 1, 2, or 3 days) and treated or not treated with 1μM rosiglitazone for 24hrs. mRNA was isolated and analyzed by Northern blot analysis. FIT2 mRNA was increased at all time points of differentiation by treatment with rosiglitazone. Importantly, rosiglitazone increased FIT2 at time point zero indicating that the effect is likely independent of differentiation of the preadipocytes into adipocytes.

[0098] *Lipid droplet formation in cells transfected with wild-type and mutant FIT2.* The effects of mutations in amino acids comprising a highly conserved domain in mouse FIT2 were observed on lipid droplet formation. Amino acids 157 phenylalanine (F), 158 leucine (L), 159 leucine (L), 164 leucine (L), and 169 glutamic acid (E) are among the most highly conserved amino acids found in all species having a FIT2 homolog. Mutations in the tandem amino acids FLL (amino acids 157 through 159) substituted with alanine (A) residues resulted in a FIT2 protein that produced more and brighter droplets than the wild-type FIT2 (FIT2-V5) transiently expressed in HEK 293 cells (Figure 15). FIT2 mutants having either the 164L or 169E residue substituted by alanine resulted in a greatly diminished ability to produce droplets. Moreover, the L164A and E169A mutants both produced multiple small droplets, a phenotype distinct from the large droplets produced by wild-type and FLL157-9AAA mutant FIT2 proteins (Figure 15). Importantly, the phenotypes observed were not due to changes in FIT2 protein levels, although L164A and E169A mutants had a minor decrease in levels as well as minor cleavage of the signal peptide as shown in Western blot Figure 16. Together, the results indicate that the FLL domain plays a role to limit FIT2 activity, while the 164L and 169E residues are essential for FIT2 to form droplets. Thus, these amino acid domains on FIT2 can serve as targets for small molecule activators and inhibitors that may interact with these amino acid domains to either augment FIT2 activity or inhibit FIT2 activity.

[0099] *Mouse transgenic models of FIT1 and FIT2 overexpression: liver-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 and FIT2 specifically in liver. Based on the finding that overexpression of FIT1 and FIT2 in mouse liver using adenovirus results in fatty liver on a high fat diet, these transgenic mouse models are expected to have fatty liver on high fat diets. These animal models should be suitable to study the progression of fatty liver diseases (alcoholic and non-alcoholic) and steatohepatitis

as well as the role of fatty liver in type 2 diabetes and insulin resistance. In addition, these models will be useful in testing therapies for these diseases, such as screening therapeutic compounds (small molecules, proteins, RNAi, aptamers).

[00100] *Mouse transgenic models of FIT1 and FIT2 overexpression: skeletal muscle-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 specifically in skeletal muscle. This animal model is expected to be useful in studying type 2 diabetes and obesity, and muscle metabolism and function. This model will be useful in testing therapies or screening therapeutic compounds (small molecules, proteins, RNAi, aptamers) for diseases (rhabomyolysis, myostitis, type 2 diabetes, lipotoxycity) as well as improving normal physiology (i.e. muscle function, lipid and glucose metabolism).

[00101] *Mouse transgenic models of FIT1 and FIT2 overexpression: heart-specific expression.* Transgenic mice were generated that overexpress mouse FIT1 specifically in cardiac myocytes. These mice are expected to be suitable models to study cardiomyopathy, and defects in cardiac function related to altered cardiac metabolism. This model will be useful in testing therapies or screening therapeutic compounds (small molecules, proteins, RNAi, aptamers) for diseases such as cardiomyopathy, cardiac dysfunction, cardiac ischemia, and altered cardiac metabolism, as well as improving normal physiology (i.e. cardiac function, lipid and glucose metabolism).

[00102] *Mouse transgenic models of FIT1 and FIT2 overexpression: adipose-specific expression.* Transgenic mice were generated that overexpress mouse FIT2 specifically in adipose tissue (white and brown adipose). These transgenic mice are expected to be useful as a model of obesity and type 2 diabetes and metabolic syndrome. These transgenic mice can be used to test therapeutic compounds or other therapies to treat obesity, type 2 diabetes and metabolic syndrome.

[00103] *Mouse deficiency models for FIT1 and FIT2: FIT1 gene targeted mice.* FIT1 Knockout mice have been generated and found to be viable and fertile. These mice might be a suitable model for defects in cardiac, skeletal muscle, liver and kidney dysfunction and therefore are expected to also be useful to test therapeutic agents that regulate muscle, heart, liver, and kidney function.

[00104] *Mouse deficiency models for FIT1 and FIT2: FIT2 gene targeted mice.* The targeted A3 ES cell line was used to generate chimeric mice. It was determined that deletion of FIT2 results in embryonic lethality. Mouse embryonic fibroblasts from FIT2 knockouts may be useful as a model to screen for small molecule inhibitors of FIT genes. In addition, mice heterozygous for FIT2, which are viable, might be a suitable model to study the effects

of inhibitors or activators of FIT2, as well as assessing the effects of having reduced levels of FIT2 on cardiac, muscle, liver, kidney and brain function. These mice having 50% less FIT2 might mimic humans that are heterozygous for mutations in FIT2, since homozygous mutations would be expected to be extremely rare.

[00105] *FIT2 Conditional targeted mice.* Based on the finding that gene targeted deletion of FIT2 results in embryonic lethality, BAC recombineering was used to produce a "floxed" allele of FIT2 to conditionally knock it out in adult mice. Conditional knockout mice for FIT2 are expected to be useful for studying organ specific effects of FIT2 inhibition on normal and pathophysiology. Conditional knockout mice will be an important model to test the safety and efficacy of inhibiting FIT2 in specific cell types and organs. For example, knockdown of FIT2 specifically in white adipose tissue should result in resistance to obesity and enhanced insulin sensitivity.

[00106] The present study describes for the first time the identification of a highly conserved family of proteins that are essential in mammalian cells for the formation of lipid droplets, independent of TG biosynthesis. FIT genes encode for multi-transmembrane, endoplasmic reticulum localized proteins. Overexpression of FITs in cell culture and mouse liver resulted in the formation of lipid droplets without enhancing triglyceride biosynthesis. Moreover, shRNA silencing of FIT genes in white adipocytes prevented lipid droplet formation during adipogenesis. Reagents that regulate FIT expression or activity are expected to impact the many diseases associated with excessive lipid droplet formation, such as obesity, diabetes, and atherosclerosis. Regulation of energy metabolism, feeding behavior and hepatic glucose output is highly regulated by lipids and energy status (ATP, AMP) in the brain. Inhibiting or activating FIT1 or FIT2 genes or protein activity in the brain (using small molecules, or nucleic acids, ASO, RNAi, aptamers) may beneficially alter energy metabolism, feeding behavior and hepatic glucose output in humans.

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What is claimed is:

1. A method of reducing fat storage in a tissue in a subject by administering to the subject an amount of an agent that is effective to decrease the level of FIT1 and/or FIT2 in the tissue in the subject.

2. The method of Claim 1, wherein the subject has a disease involving storage of cytoplasmic fat in droplets.

3. The method of Claim 1, wherein the subject has one or more of obesity, diabetes, metabolic syndrome, fatty liver disease, steatosis, steatohepatitis, atherosclerosis, diabetic nephropathy, and hepatitis C infection.

4. The method of any of Claims 1-3, wherein the level of FIT2 is decreased in adipose tissue in the subject.

5. The method of Claim 4, wherein the subject loses body weight.

6. The method of any of Claims 1-3, wherein the level of FIT1 and/or FIT2 is decreased in the heart, muscle or liver in the subject.

7. The method of any of Claims 1-6, wherein the agent is an antisense molecule, a ribozyme, an RNA interference (RNAi) molecule, a short hairpin RNA (shRNA), an antibody or an aptamer.

8. A method of increasing fat storage in a tissue in a subject by administering to the subject an amount of an agent that is effective to increase the level of FIT1 and/or FIT2 in the tissue in the subject.

9. The method of any of Claims 1-8, wherein the agent affects amino acids FLL, corresponding to amino acids 157 through 159 of mFIT2; amino acid L, corresponding to amino acid 164 of mFIT2; or amino acid E, corresponding to amino acid 169 of mFIT2.

10. A method of screening for a disorder involving regulation of fat storage in a tissue, the method comprising determining the level of expression or activity of FIT1 and/or FIT2 in a blood, fluid, tissue or cell sample from a subject, wherein alternation from normal of the level of expression or activity of FIT1 and/or FIT2 in the sample is indicative of a disorder involving regulation of fat storage in a tissue.

11. The method of Claim 10, wherein the expression of FIT1 and/or FIT2 is increased from normal.

12. The method of Claim 10, wherein the expression of FIT1 and/or FIT2 is decreased from normal.

13. The method of Claim 10, wherein the activity of FIT1 and/or FIT2 is increased from normal.

14. The method of Claim 10, wherein the activity of FIT1 and/or FIT2 is decreased from normal.

15. The method of any of Claim 10-14, wherein expression and/or activity of FIT1 is altered from normal.

16. The method of any of Claim 10-14, wherein expression and/or activity of FIT2 is altered from normal.

17. The method of any of Claim 10-14, wherein expression and/or activity of FIT1 and FIT2 is altered from normal.

18. The method of any of Claims 10-17, wherein the disorder involving regulation of fat storage is obesity, type 2 diabetes, metabolic syndrome, fatty liver disease (steatosis or steatohepatitis), atherosclerosis, diabetic nephropathy, hepatitis C infection, viral infection, or a disorder of cardiac, kidney, muscle or liver function.

19. A method for screening for a candidate agent that can reduce fat storage in tissue by determining whether or not the agent is effective to decrease the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting the agent with cells or tissue that express FIT1 and/or FIT2, and wherein reduction in expression or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for decreasing fat storage in tissue.

20. A method for screening for a candidate agent that can increase fat storage in tissue by determining whether or not the agent is effective to increase the level of FIT1 and/or FIT2 in tissue or cells, wherein the method comprises contacting the agent with cells or tissue that express FIT1 and/or FIT2, and wherein an increase in expression or activity of FIT1 and/or FIT2 is indicative that the agent is a candidate agent for increasing fat storage in tissue.

21. The method of Claim 19 or 20, wherein the cells are mouse embryo fibroblasts isolated from FIT2 knockout embryos, and wherein the fibroblasts are stably or transiently transfected with FIT1 and/or FIT2 under control of a regulated or constitutively active promoter.

22. The method of any of Claims 19-21, wherein the agent affects expression of FIT1 and/or FIT2.

23. The method of any of Claims 19-21, wherein the agent affects activity of FIT1 and/or FIT2.

24. The method of any of Claims 19-21, wherein the agent affects FIT1.

25. The method of any of Claims 19-21, wherein the agent affects FIT2.

26. The method of any of Claims 19-21, wherein the agent affects FIT1 and FIT2.

27. A transgenic mammal in which FIT1 and/or FIT2 is overexpressed in one or more tissue.

28. The transgenic mammal of Claim 27, wherein fat storage is increased in the tissue compared to a wild-type mammal.

29. The transgenic mammal of Claim 27 or 28, wherein FIT1 and/or FIT2 are overexpressed in liver.

30. The transgenic mammal of Claim 27 or 28, wherein FIT1 and/or FIT2 are overexpressed in skeletal muscle.

31. The transgenic mammal of Claim 27 or 28, wherein FIT1 and/or FIT2 are overexpressed in heart muscle.

32. The transgenic mammal of Claim 27 or 28, wherein FIT2 is overexpressed in adipose tissue.

33. The transgenic mammal of Claim 32, wherein the adipose tissue is white adipose tissue.

34. The transgenic mammal of Claim 32, wherein the adipose tissue is brown adipose tissue.

35. A mammal that has been genetically altered so that expression of FIT1 and/or FIT2 is reduced in one or more tissue.

36. The genetically altered mammal of Claim 35, wherein fat storage is reduced in the tissue in comparison to a wild-type mammal.

37. The genetically altered mammal of Claim 35 or 36, wherein the mammal is a FIT1 knockout or a FIT1 conditional knockout or is heterozygous for FIT1.

38. The genetically altered mammal of Claim 35 or 36, wherein the mammal is heterozygous for FIT2 or is a FIT2 conditional knockout.

39. The mammal of any of Claims 27-38, wherein the mammal is a non-human mammal.

40. The mammal of Claim 39, wherein the mammal is a mouse.

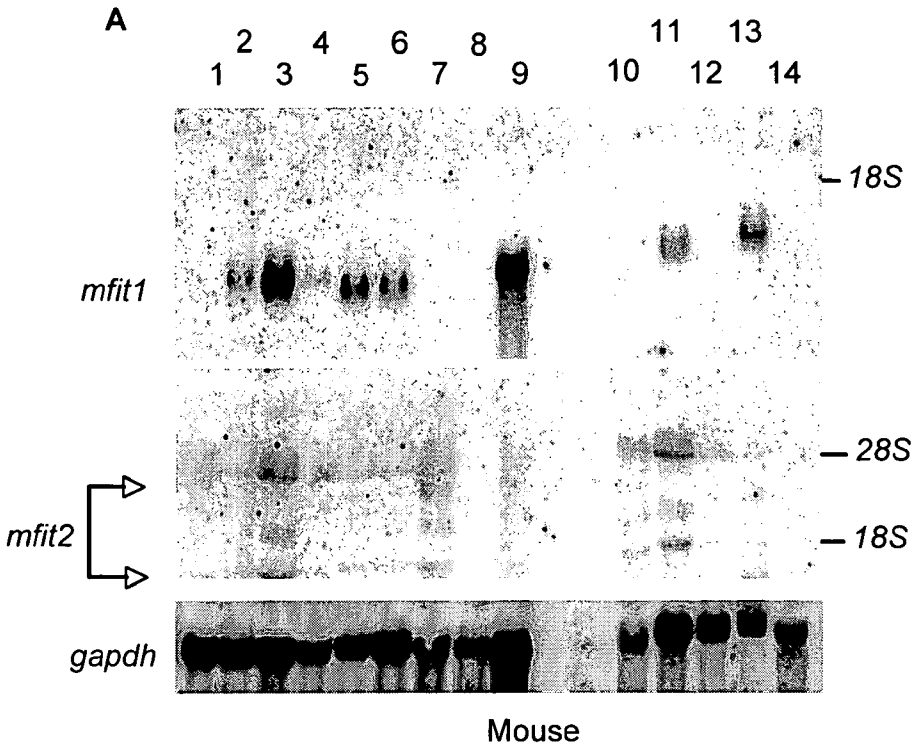
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
18S
mfit1
28S
mfit2
18S
gapdh
Mouse
B
1 2 3 4 5 6 7 8 9
FIT1
25
20
FIT2
25
20
calnexin
Mouse, Western

Figure 1A-1B

Figure 1C

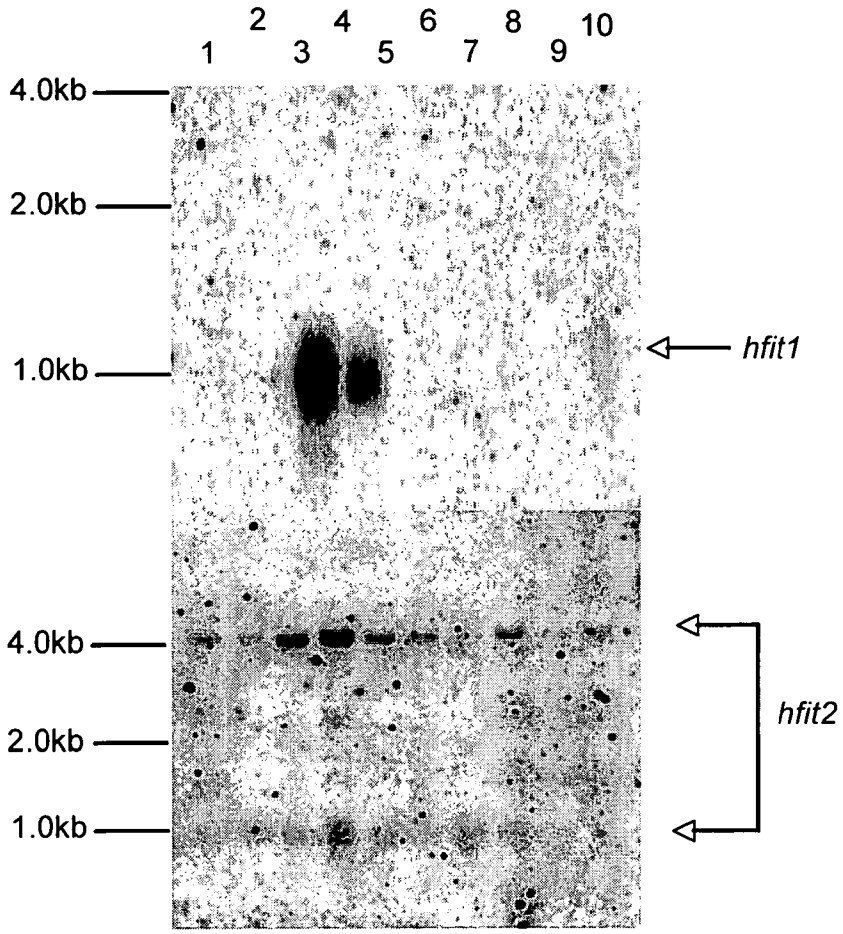
1
2
3
4
5
6
7
8
9
10
4.0kb
2.0kb
1.0kb
hfit1
4.0kb
2.0kb
1.0kb
hfit2
Human

Figure 1D

mFIT1

```
  1 MERGPTVGAG LGAGTRVRAL LGCLVKVLLW VASALLYFGS EQAARLLGSP CLRRLYHAWL 60
 61 AAVVIFGPLL QFHVNSRTIF ASHGNFFNIK FVNSAWGWIC TFLGGFVLLV VFLATRRVAV 120
121 TARHLSRLVV GAAVWRGAGR AFLLIEDLTG SCFEPLPQGL LLHELPDRKS CLAAGHQWRG 180
181 YTVSSHTFLL TFCCLLMAEE AAVFAKYLAH GLPAGAPLRL VFLLNVLLLG LWNFLLLCTV 240
241 LYFHQYTHKV VGAAVGTFAW YLTYGSWYHQ PWSPGIPGHG LFPRSRSMRK HN
```

mFIT2

```
  1 MEHLERCAWF LRGTLVRATV RRHLPWALVA AMLAGSVVKE LSPLPESYLS NKRNVLNVYF 60
 61 VKLAWAWTVC LLLPFIALTN YHLTGKTSLV LRRLSTLLVG TAIWYICTAL ESNIEHYTGS 120
121 CYQSPALEGI RQEHRSKQQC HREGGFWHGF DISGHSFLLT FCAIMIVEEM AVLHEVKTDR 180
181 GHHLHAAITT LVVALGFLTF IWVWMFLCTA VYFHDLTQKV FGTMFGLLGW YGTYGYWYLK 240
241 SFSPGLPPQS CSLTLKRDTY KK
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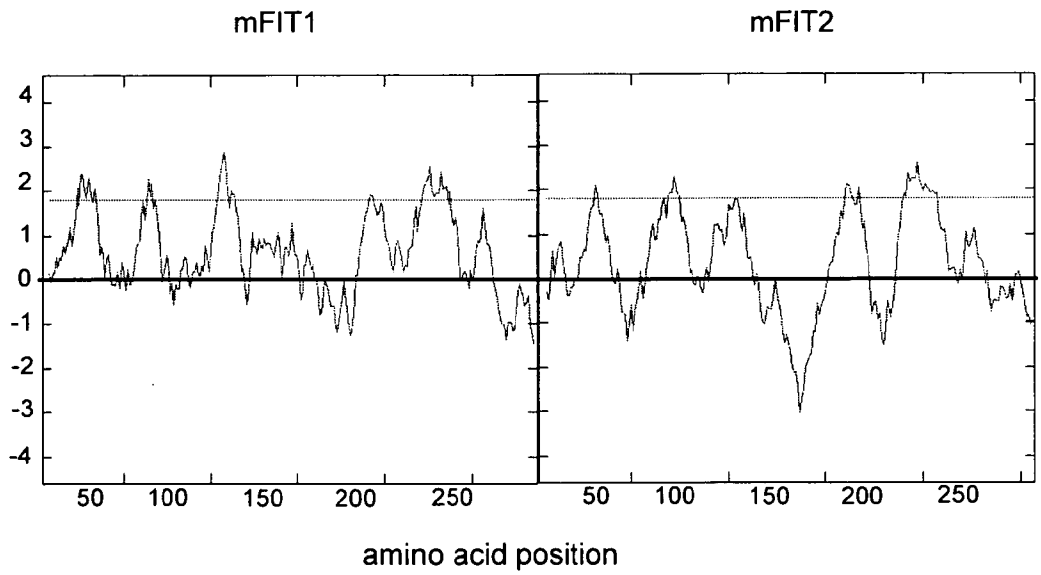


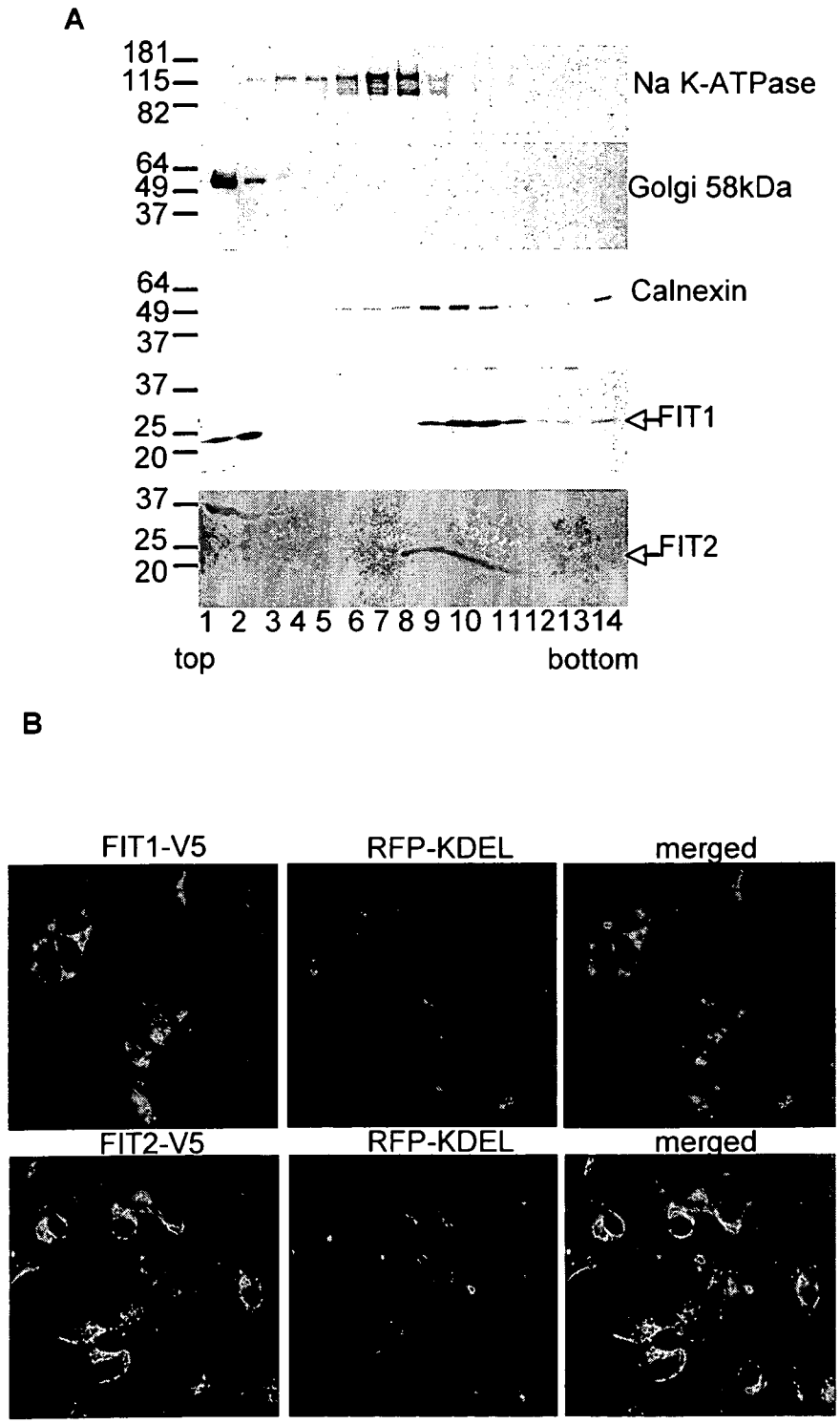

A
181
115
82
64
49
37
Na K-ATPase
Golgi 58kDa
64
49
37
Calnexin
37
25
20
FIT1
37
25
20
FIT2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
top
bottom
B
FIT1-V5
RFP-KDEL
merged
FIT2-V5
RFP-KDEL
merged

Figure 2A-2B

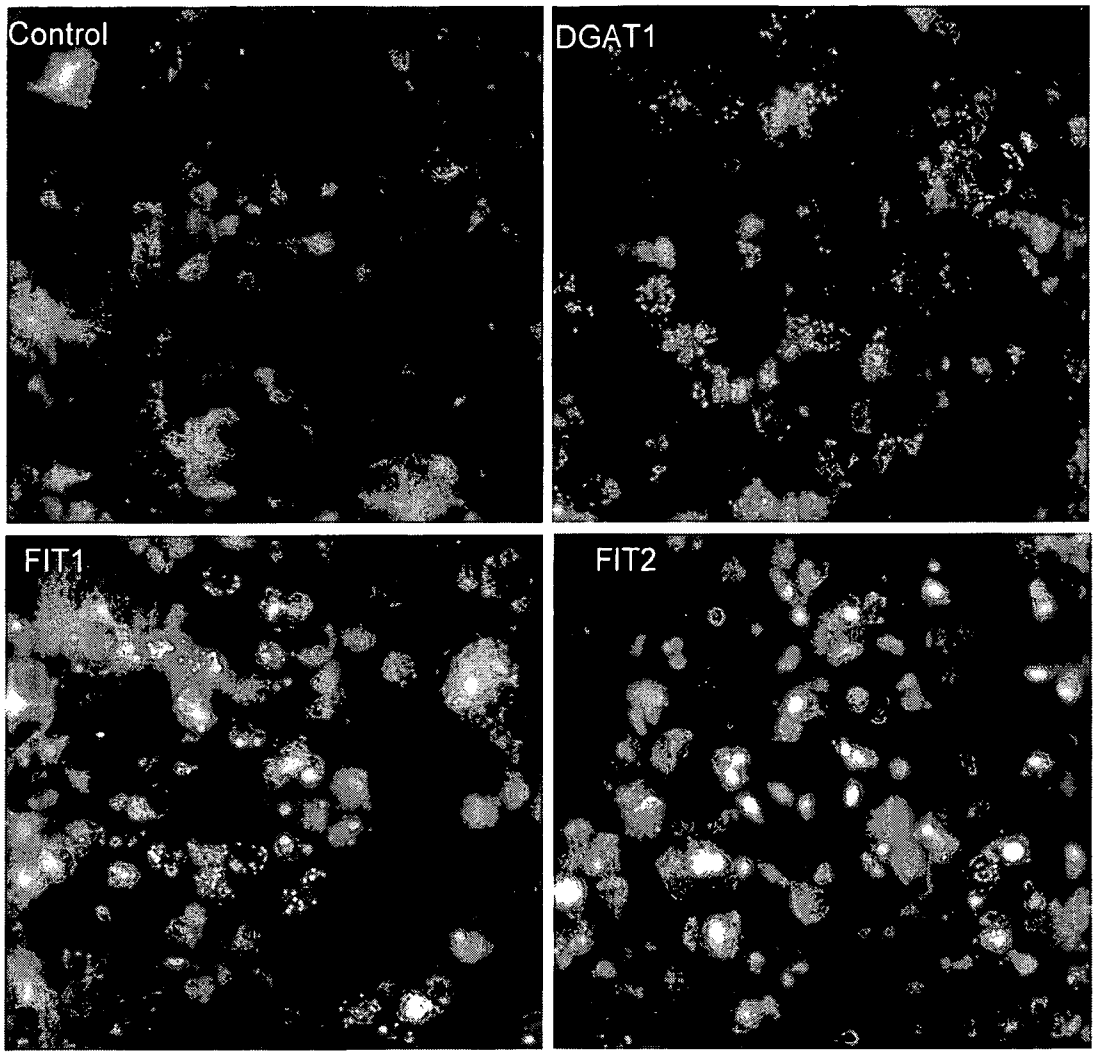


Figure 3A

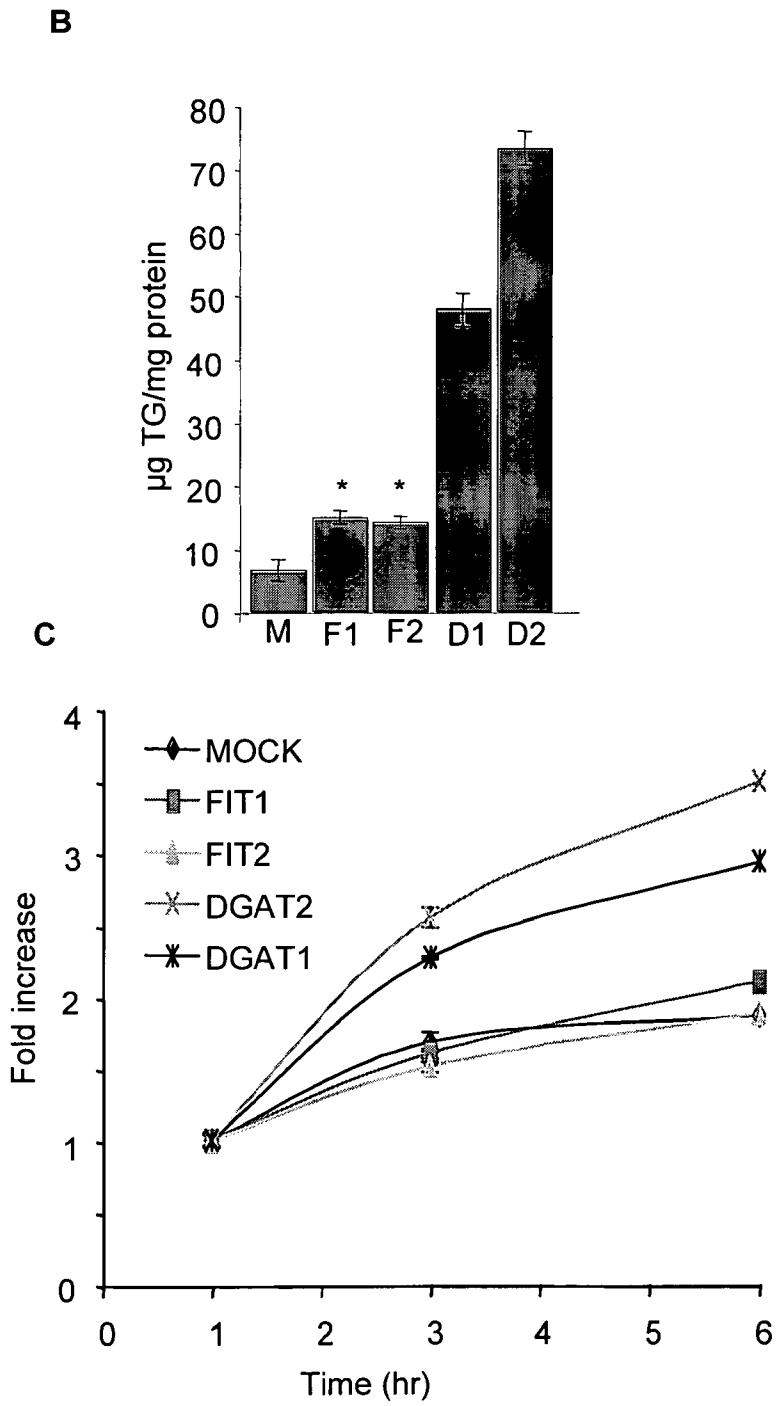

B
µg TG/mg protein
80
70
60
50
40
30
20
10
0
*
*
M
F1
F2
D1
D2
C
Fold increase
4
3
2
1
0
MOCK
FIT1
FIT2
DGAT2
DGAT1
0
1
2
3
4
5
6
Time (hr)

Figure 3B-3C

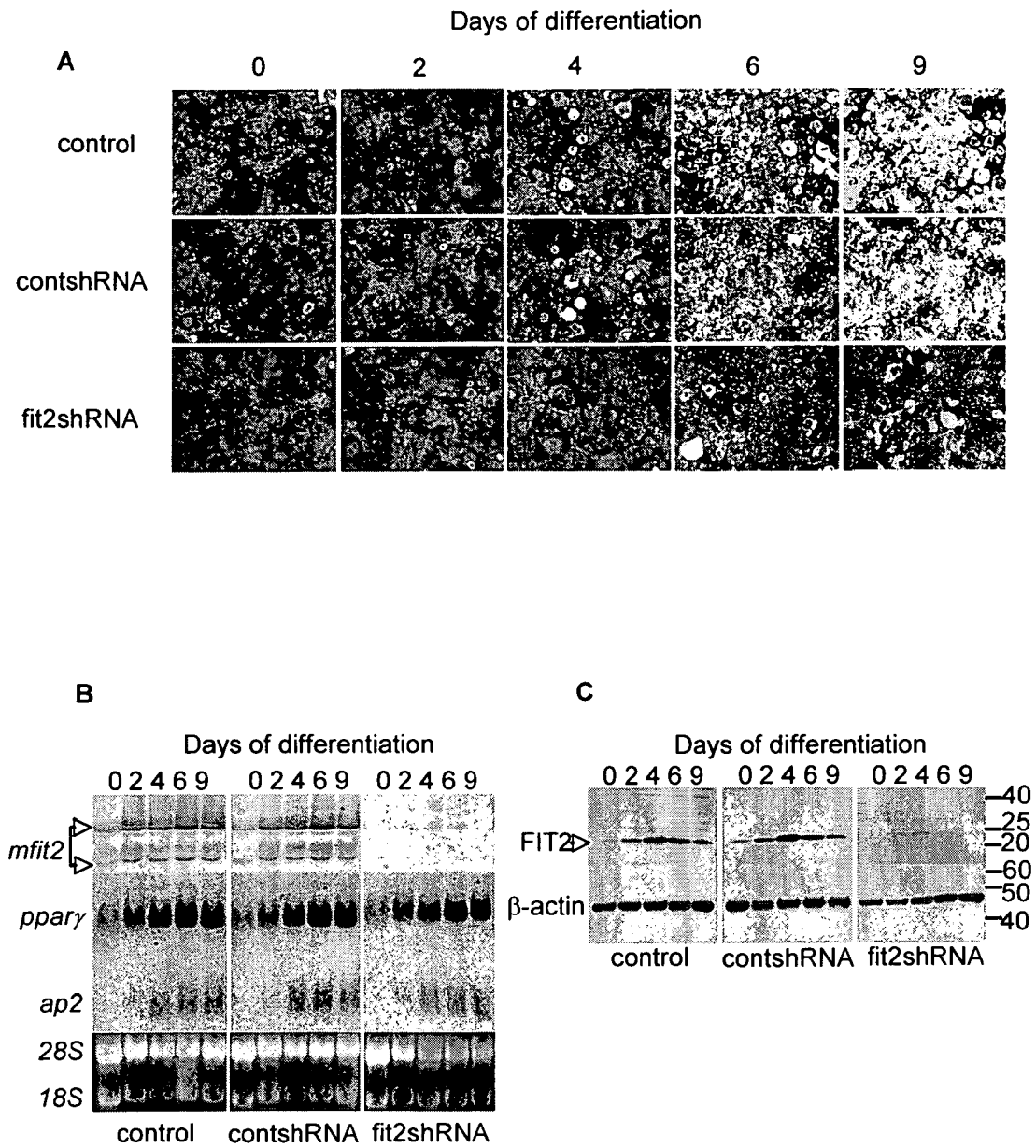

A
Days of differentiation
0
2
4
6
9
control
contshRNA
fit2shRNA
B
Days of differentiation
0 2 4 6 9 0 2 4 6 9 0 2 4 6 9
mfit2
pparγ
ap2
28S
18S
control
contshRNA
fit2shRNA
C
Days of differentiation
0 2 4 6 9 0 2 4 6 9 0 2 4 6 9
FIT2
β-actin
40
25
20
60
50
40
control
contshRNA
fit2shRNA

Figure 4A-4C

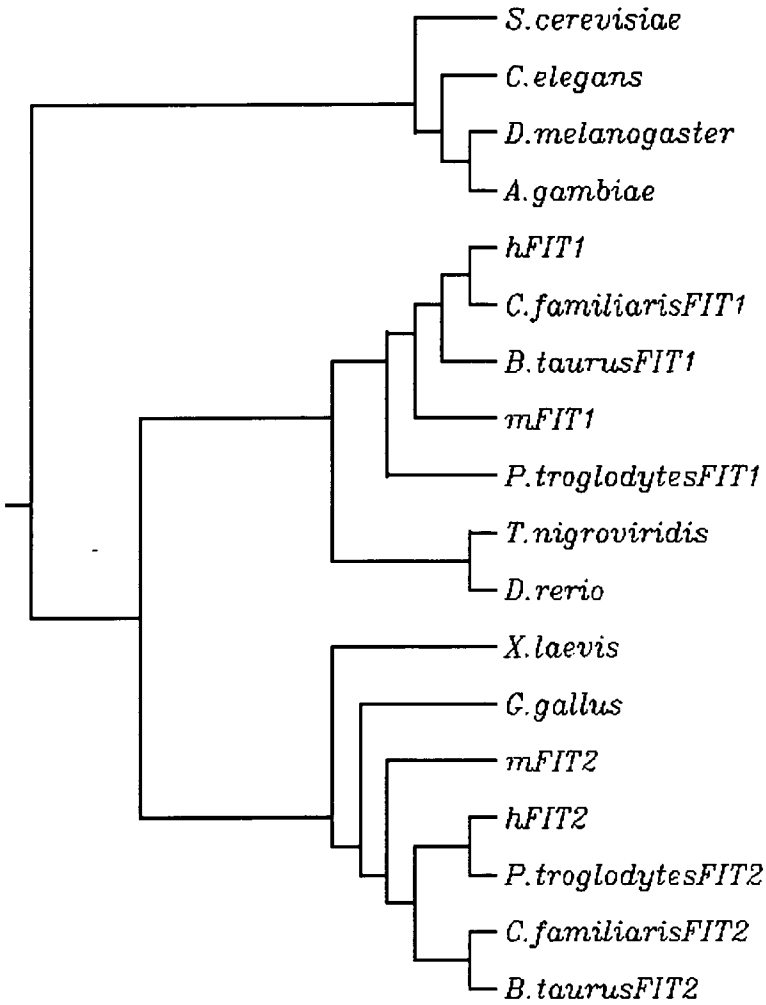

S.cerevisiae
C.elegans
D.melanogaster
A.gambiae
hFIT1
C.familiarisFIT1
B.taurusFIT1
mFIT1
P.troglodytesFIT1
T.nigroviridis
D.rerio
X.laevis
G.gallus
mFIT2
hFIT2
P.troglodytesFIT2
C.familiarisFIT2
B.taurusFIT2

Figure 5A

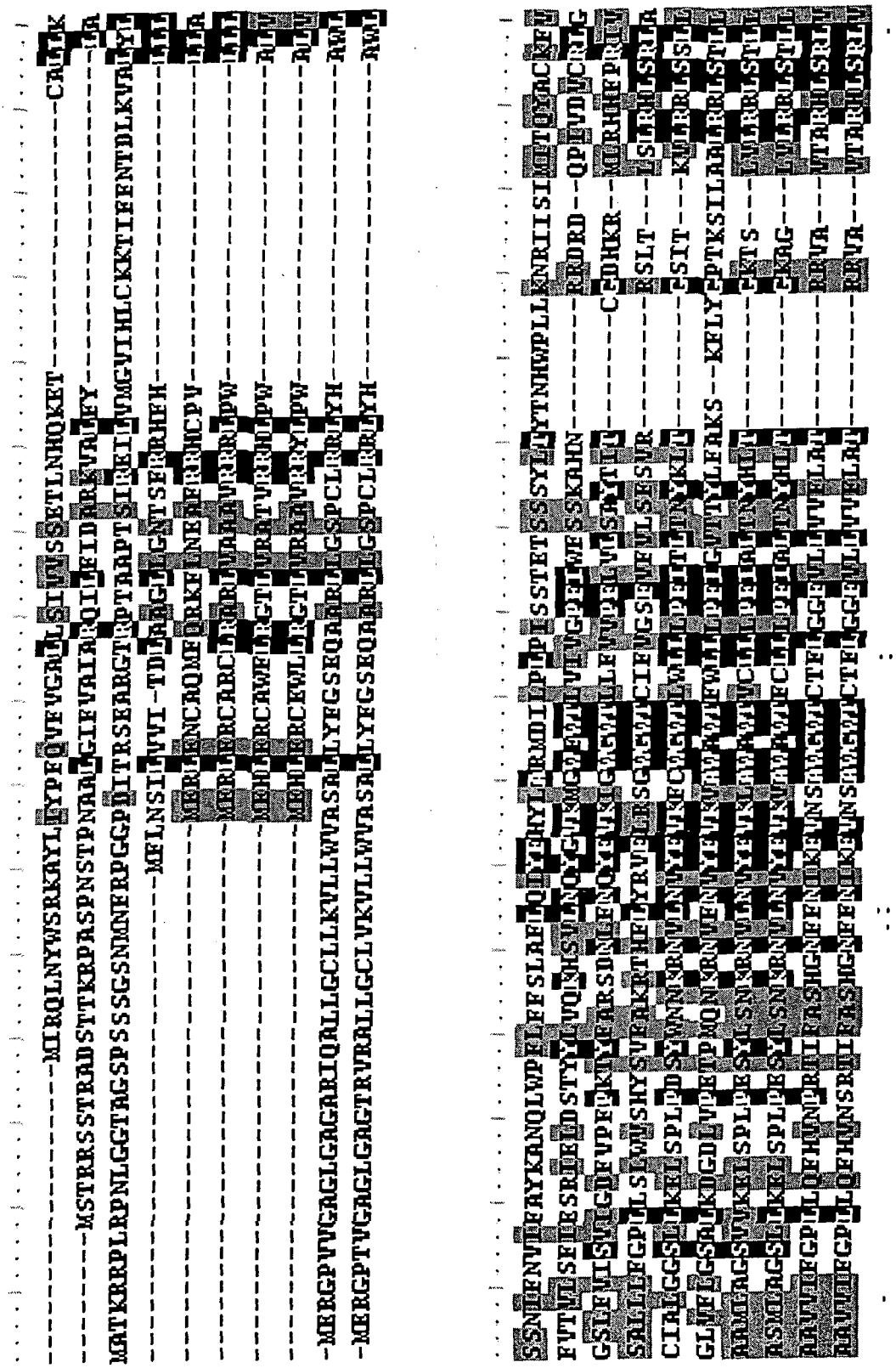

S.cerevisiae
C.elegans
D.melanogaster
D.rerio
X.laevis
G.gallus
mFIT2
hFIT2
hFIT1
mFIT1
Clustal Consensus

Figure 5B

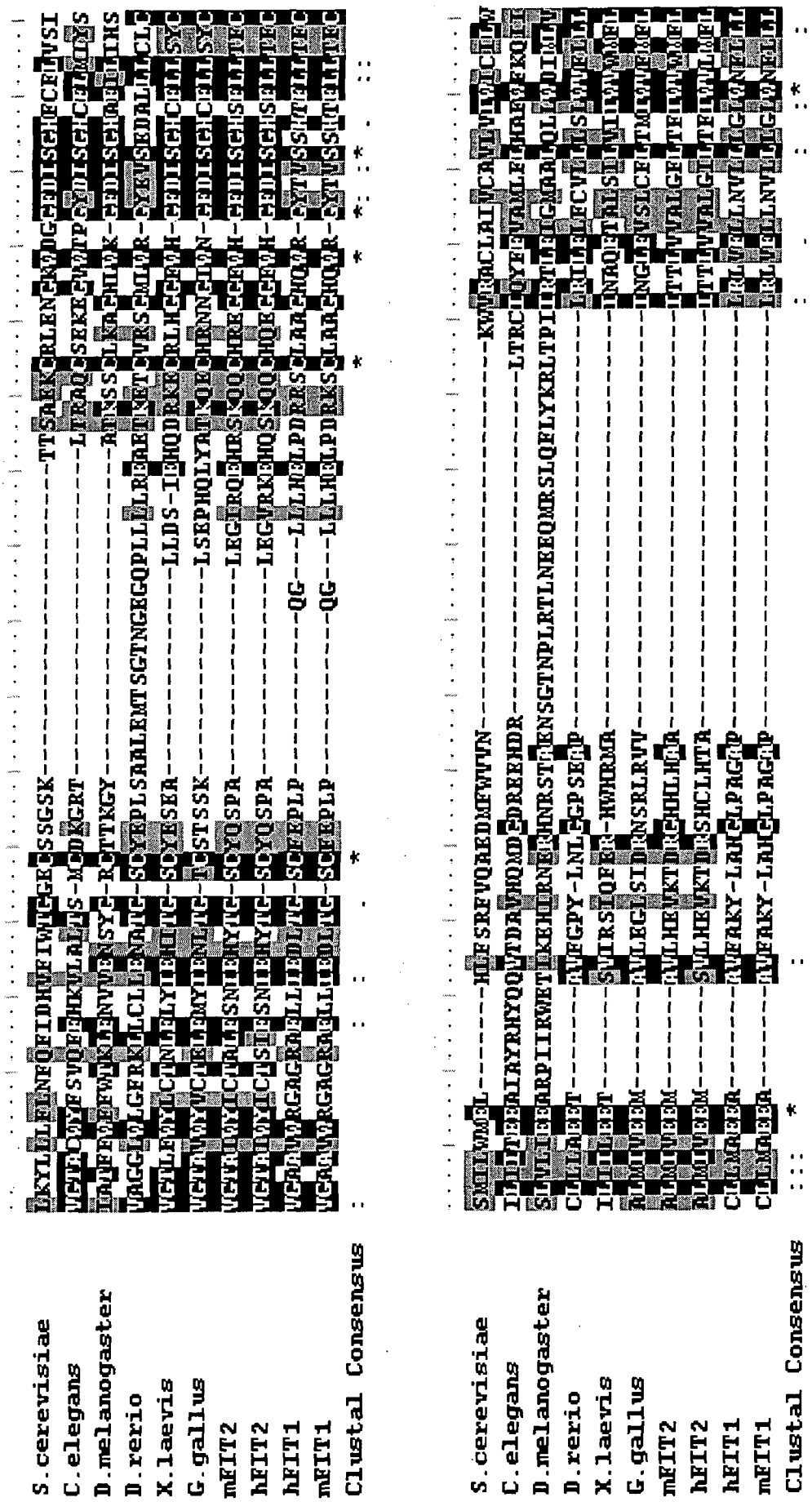


Figure 5B continued

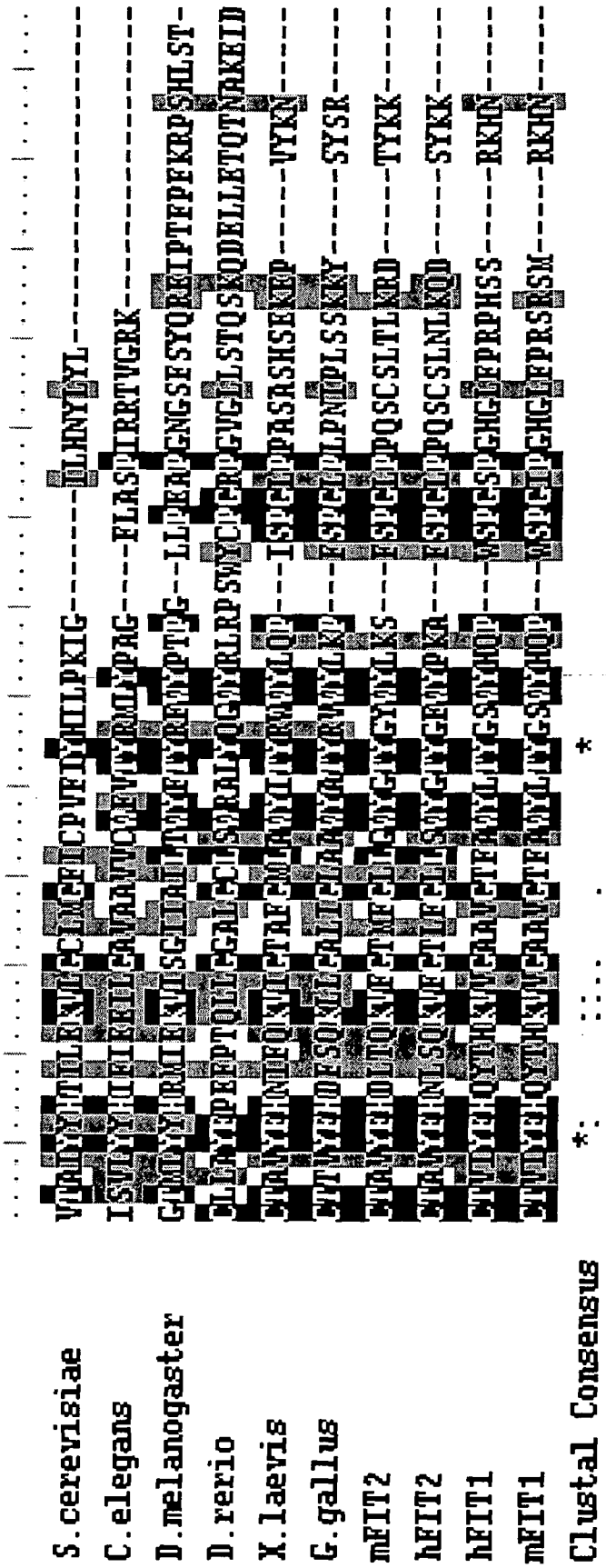


Figure 5B continued

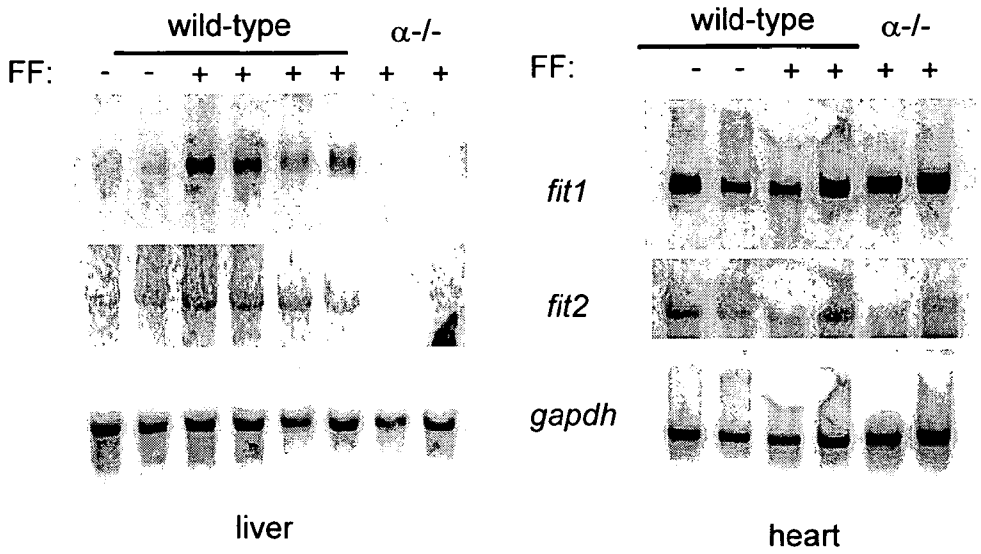


Figure 6

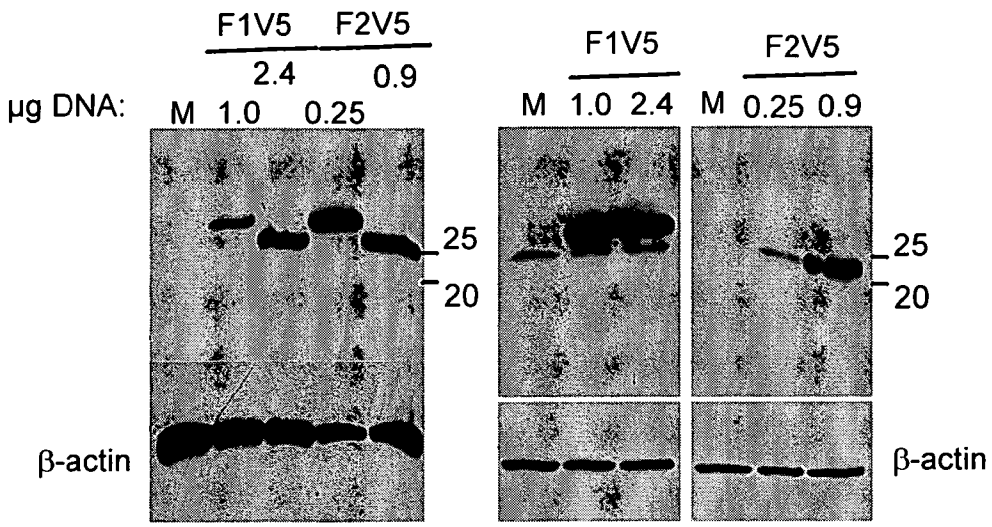

F1V5
F2V5
2.4
0.9
µg DNA:
M 1.0 0.25
25
20
β-actin
F1V5
M 1.0 2.4
F2V5
M 0.25 0.9
25
20
β-actin

Figure 7

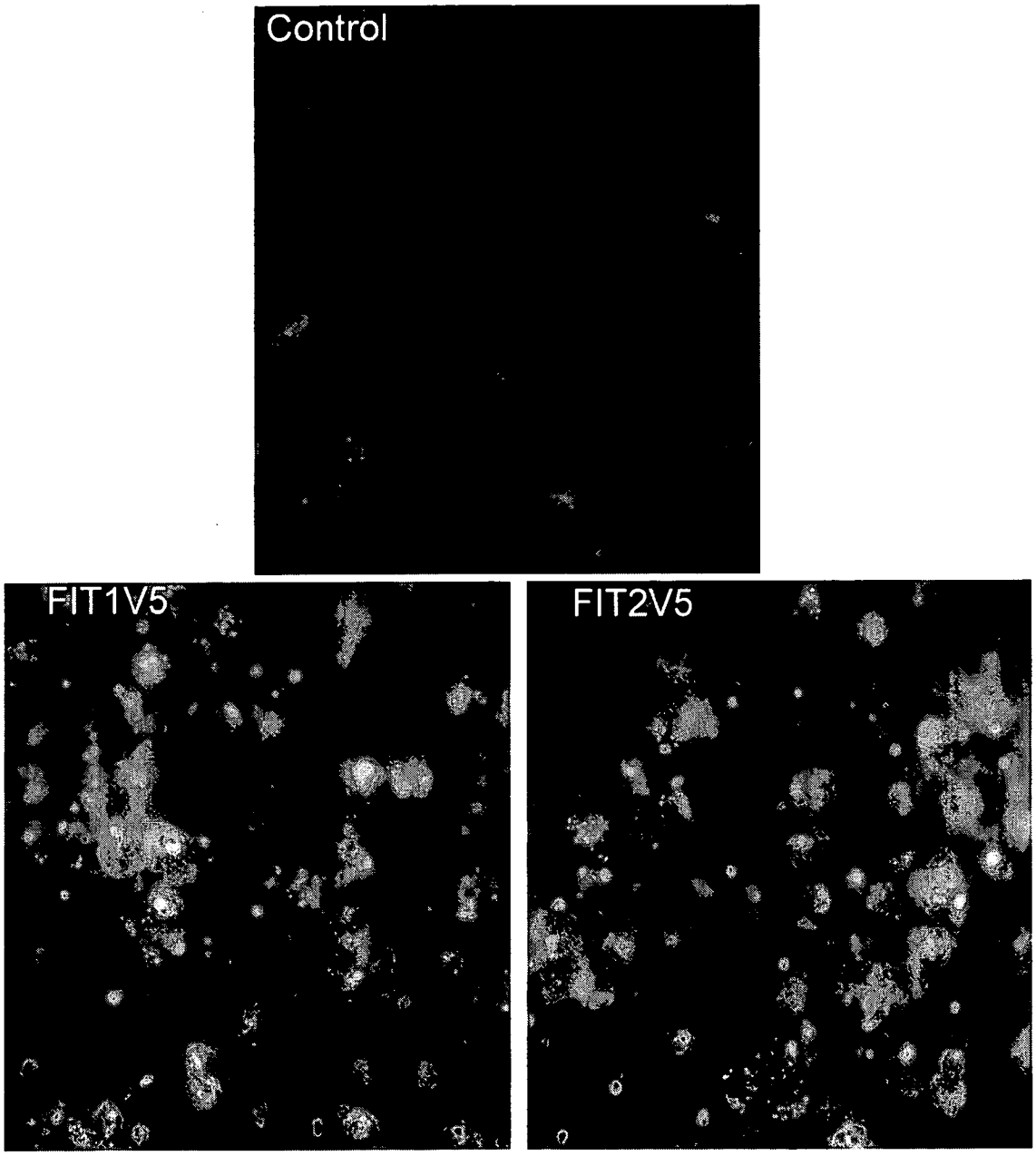


Figure 8

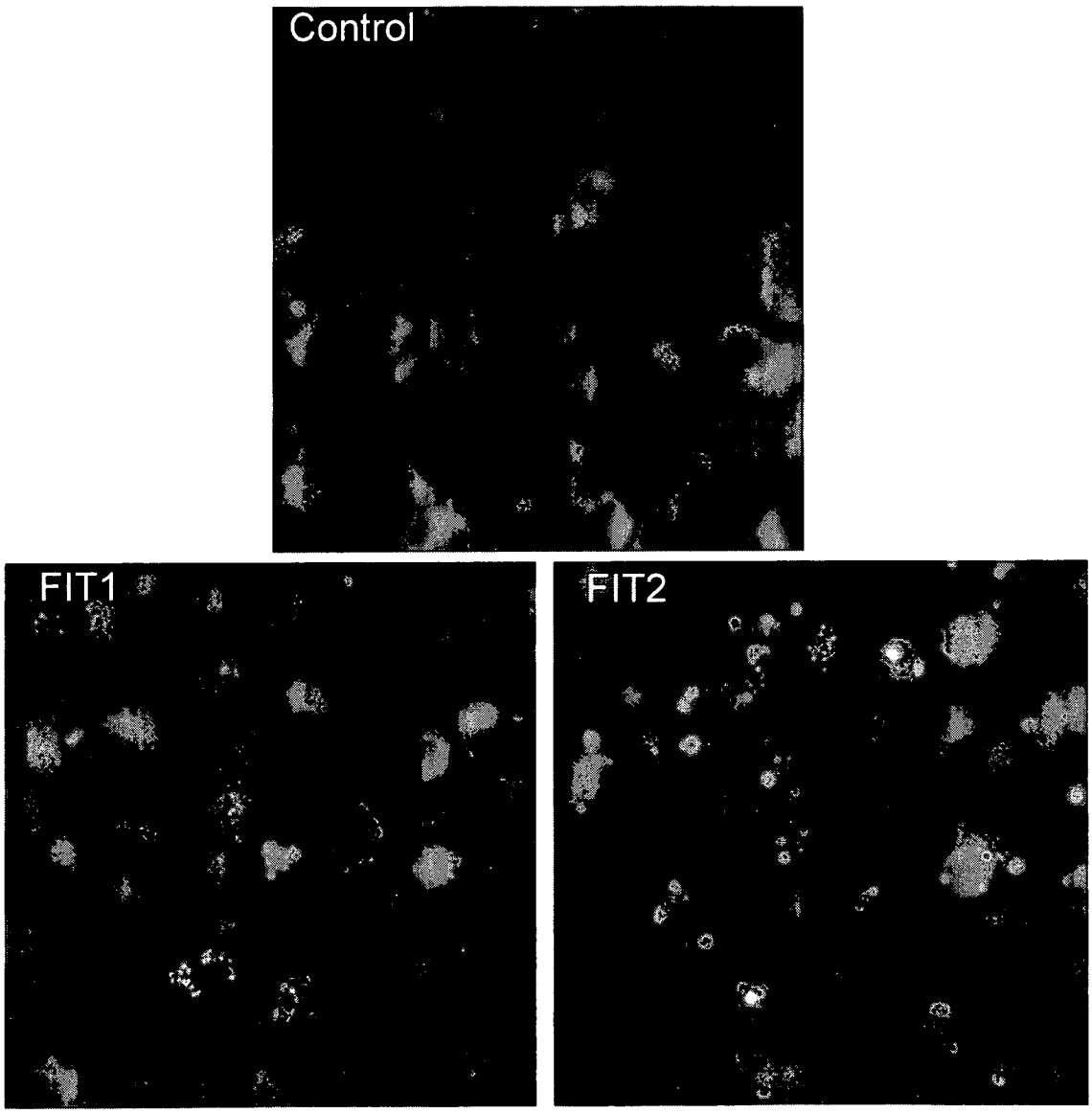


Figure 9

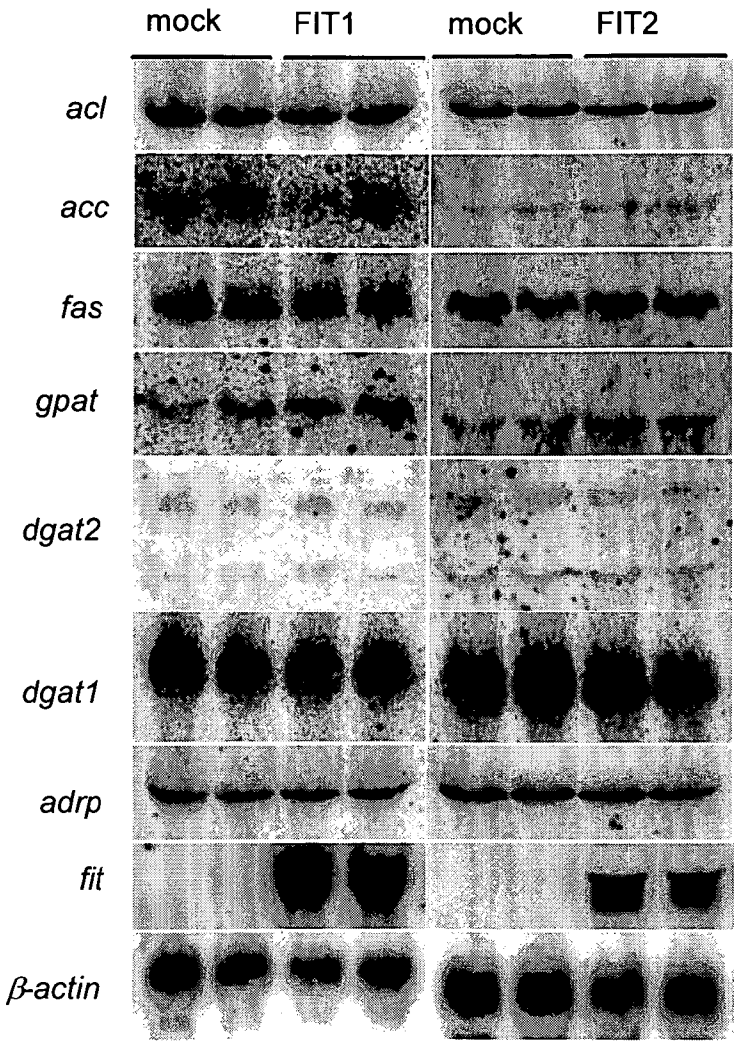

mock
FIT1
mock
FIT2
acl
acc
fas
gpat
dgat2
dgat1
adrp
fit
β-actin

Figure 10

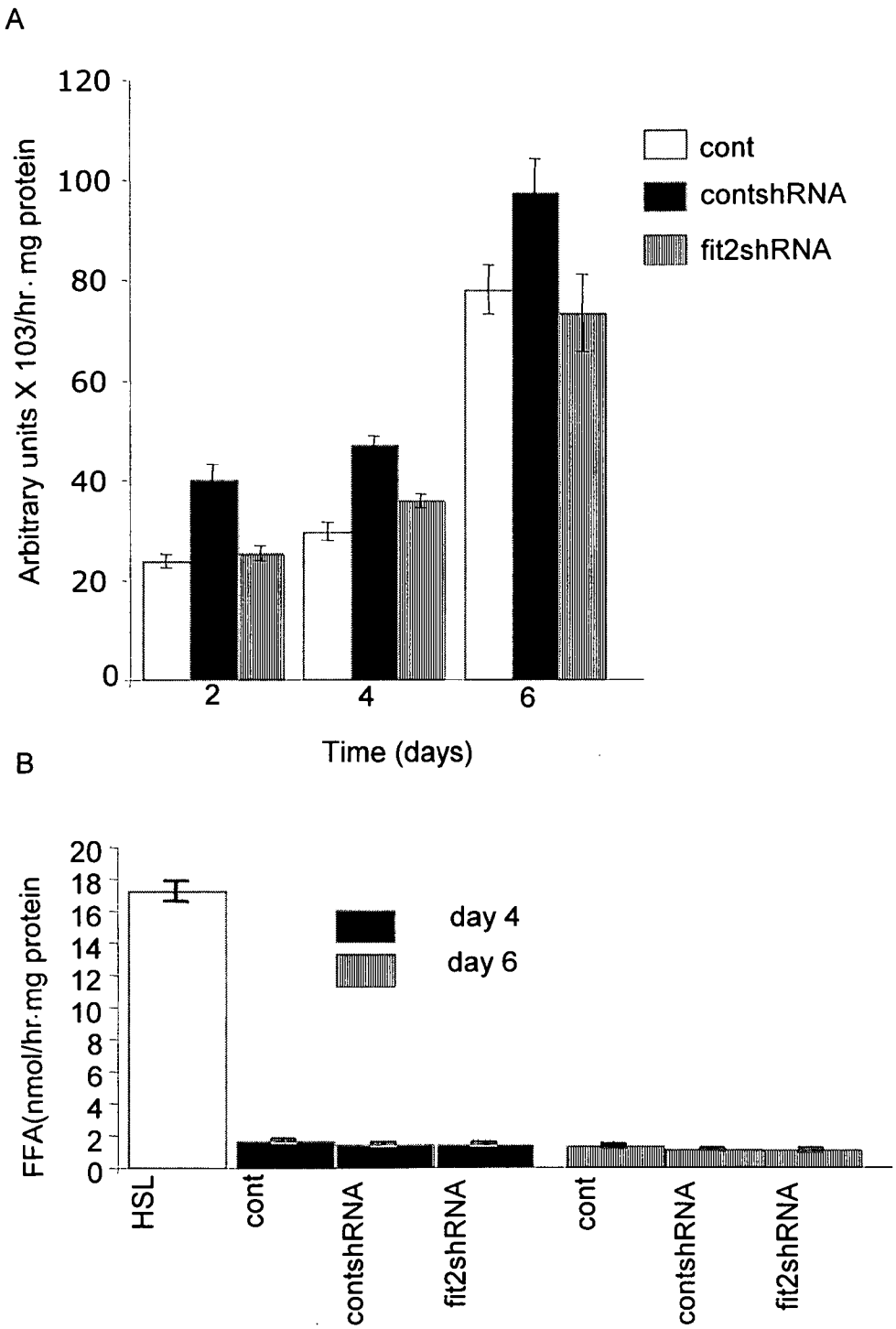


Figure 11A-11B

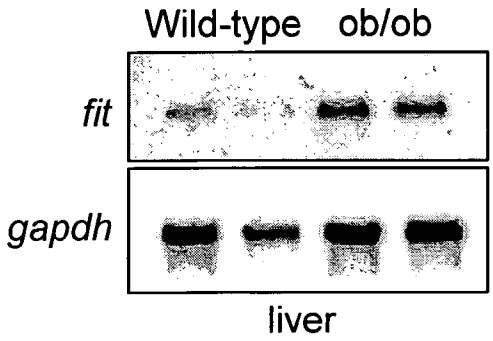
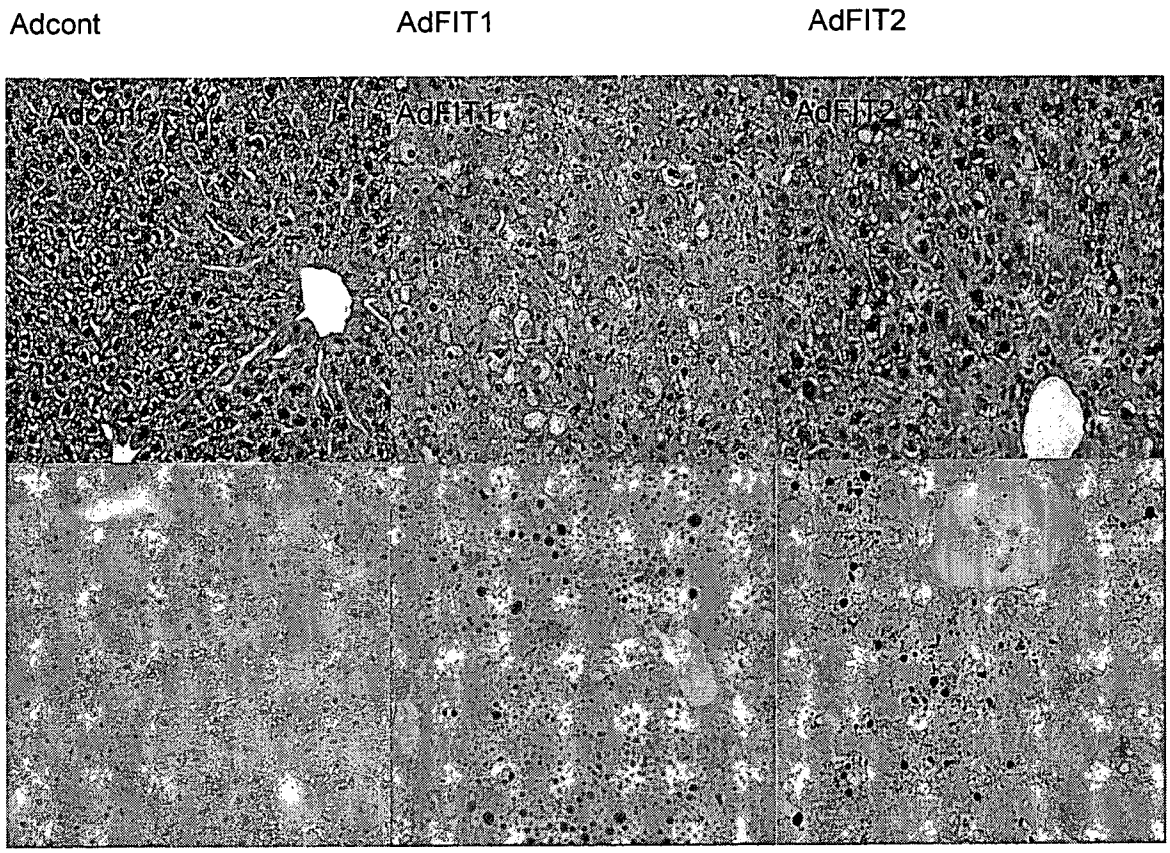


Figure 12

A



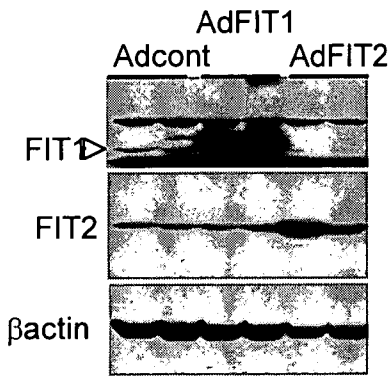


Figure 13A-13B

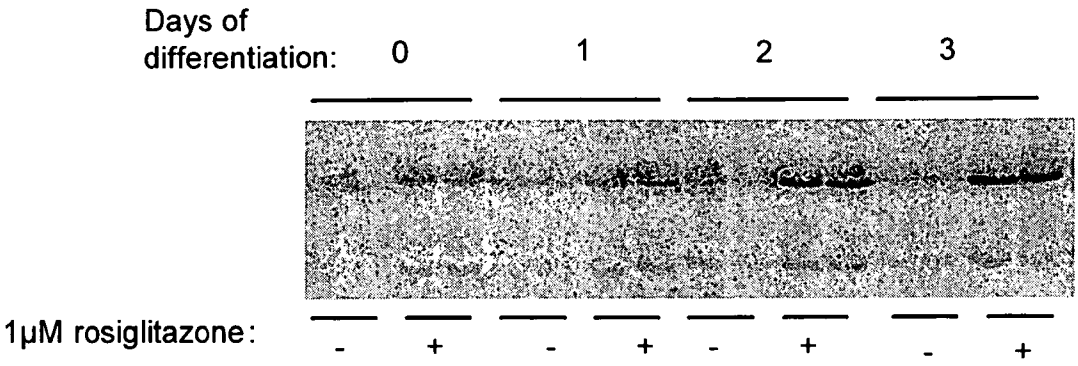


Figure 14

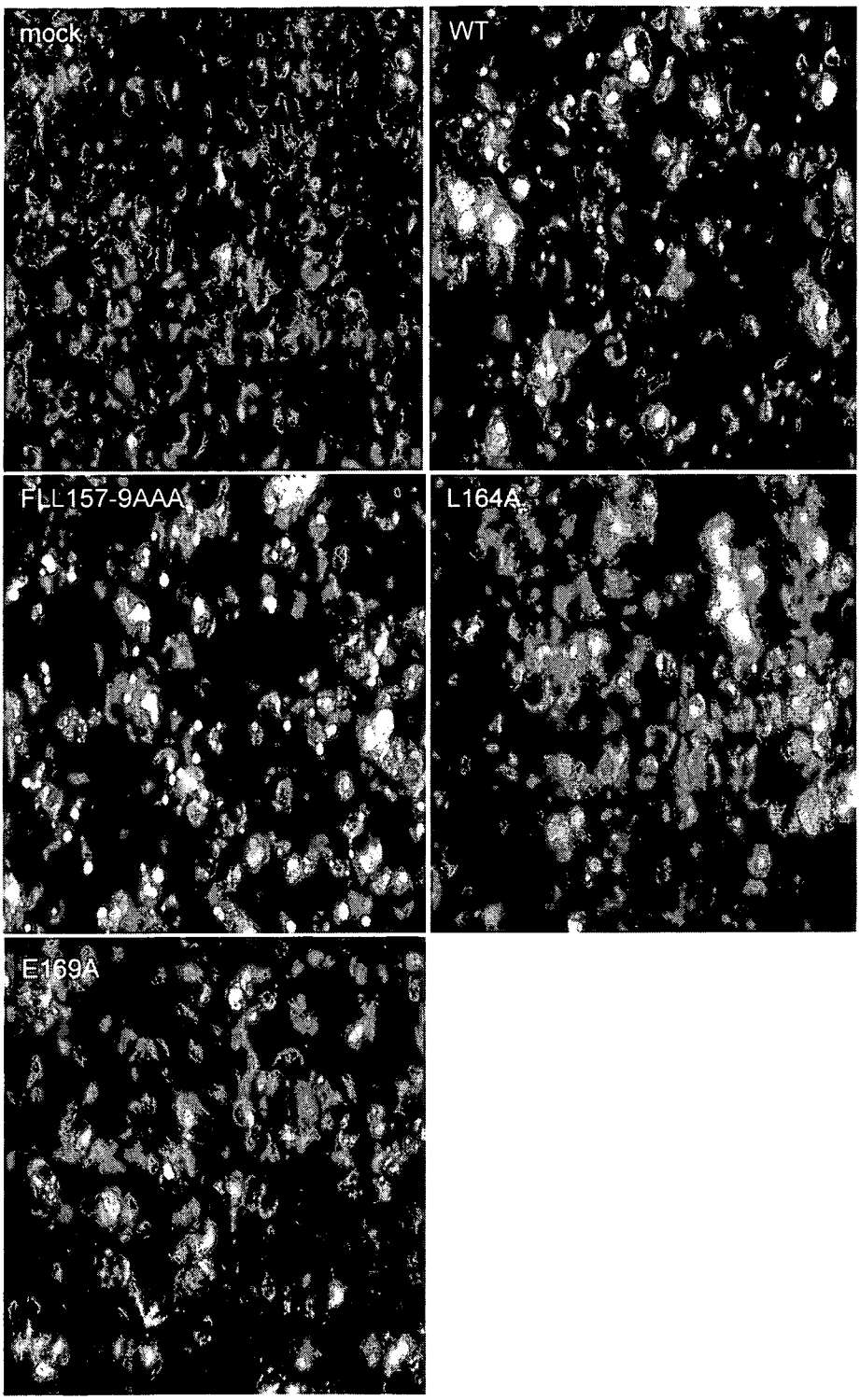


Figure 15

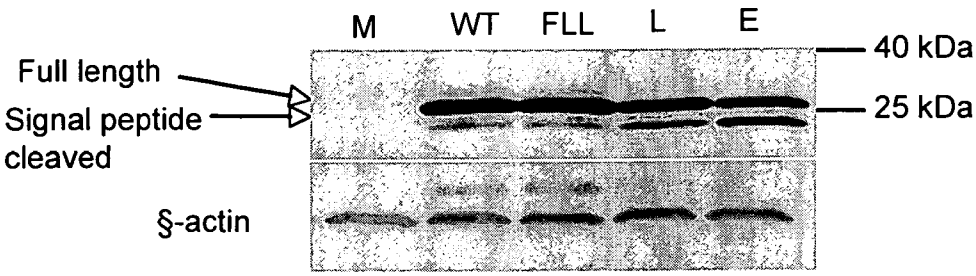

M
WT
FLL
L
E
40 kDa
25 kDa
Full length
Signal peptide cleaved
§-actin

Figure 16

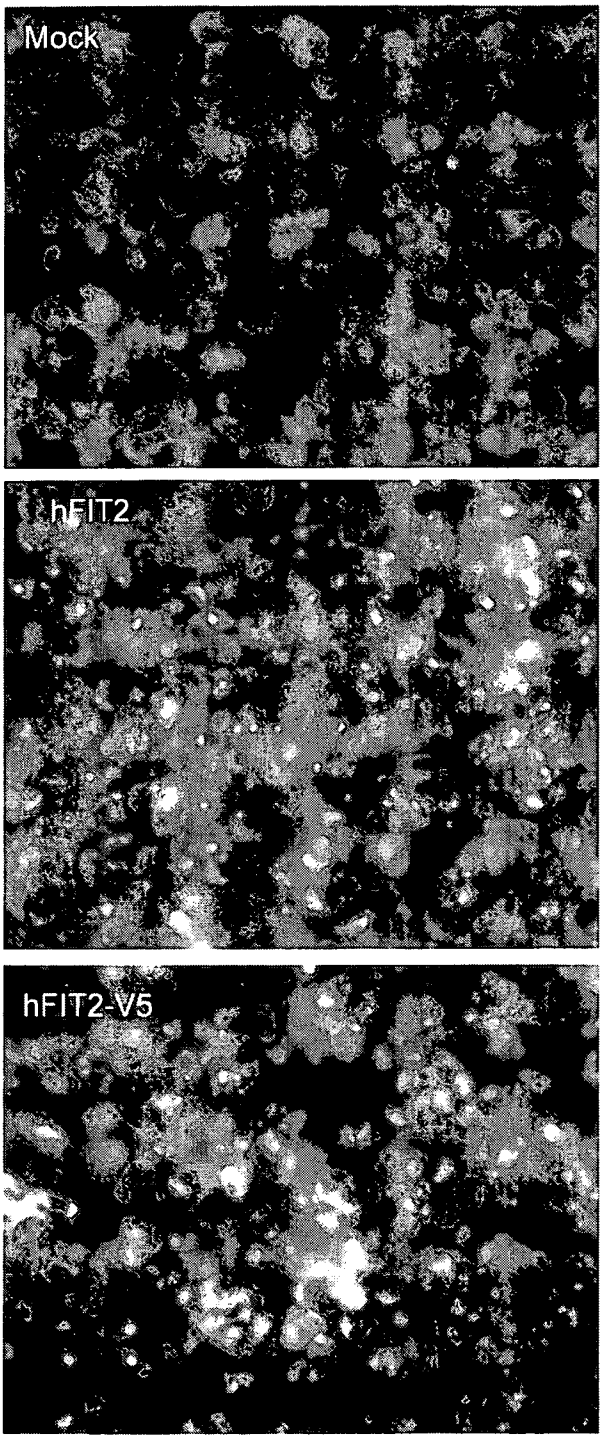


Figure 17