CHAPTER II

MATERIALS AND METHODS

Cell Culture and Plasmids

Cos-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM, BioWhittaker Inc,Walkersville, MD) containing 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (BioWhittaker). NIH 3T3 cells were maintained in DMEM containing 10% bovine serum. The pCMV5-Runx1 Δ379-430 deletion mutant was created by PCR amplification of the 1-379 and 430-480 fragments with the inclusion of a unique Nhe I restriction site for cloning using primer sequences listed in Table 1.

Table 1. Primer Sequences for Generation of Runx1 Δ379-430 Deletion Construct

Name	Sequence (5′→3′)
CMV30	AAT GTC GTA ATA ACC CCG CCC CGT TG
380Nhe	CCG GCT AGC GGT GTG GTA GCG CGT GGC CGA GCC
430Nhe	CGC GCT AGC CCC TGT ACC AAC GCC TCC ACG GGC
3′BglII	GCG AGA TCT CAG TAG GGC CGC CAC ACG GCC TC

In Vitro Transcription and Translation

In vitro purification studies were performed using the bacterially expressed GST-fusion proteins, which were created by subcloning the full length SUV39H1 or the ΔSet and 243-412 fragments from the GAL4 plasmids into pGEX4T. The purified proteins were quantified after SDS-PAGE using Coomassie Brilliant Blue and approximately

equal amounts of protein were used for each reaction. RUNX1 or RUNX1a were transcribed and translated using the quick coupled system (TnT; Promega, Inc., Madison, WI) in the presence of ^{35}S-Methionine. An equal amount of the reticulocyte lysate was diluted into PBS containing 0.5% Triton X-100 and added to the glutathione beads and incubated on ice for 4-12 hr. Samples were washed 3 times with PBS containing 0.5% Triton X-100 and analyzed by SDS-PAGE.

Luciferase Assays

NIH 3T3 cells were transfected using the Superfect or Polyfect reagent (Qiagen) with 300 ng WWP-Luciferase (*p21$^{Waf1/Cip1}$*), 300 ng pCMV5-RUNX1 (unless otherwise noted) or deletion constructs, and 300 ng pCMV5-secreted alkaline phosphatase (SEAP) plasmids. Empty pCMV5 was used as a control and pBluescript KS was added to bring the total amount of DNA in each sample to 1.5 µg. Luciferase activity was measured using the Luciferase Assay System (Promega) and normalized to SEAP activity.

Co-Immunoprecipitation

Cos-7 cells (3 x 10^6 cells in 100 mm dishes) were transfected using lipofectamine or lipofectamine 2000 (Life Technologies) with up to 5 µg of expression plasmids. Cells were extracted with phosphate buffered saline supplemented with 1 mM EDTA, 0.2 mM PMSF, and 0.1 T.I.U/ml aprotinin, and containing 0.5% Triton X-100. Lysates were immunoprecipitated with either anti-Flag M2-Agarose Affinity Gel (Sigma) or GAL4 antibody (Sigma) and precipitated using Protein G Sepharose (Sigma).

Flow Cytometry Analysis

Single cell suspensions of hematopoietic tissues were obtained by either flushing the tibia and femur, or mincing the spleen or the thymus of *Mx:Hdac3* mice. Following lysis of the red blood cells using the Erythrocyte lysis buffer, 1 X 10^6 to 4 X 10^6 cells were aliquoted into individual tubes. The cells were stained with antibodies against: CD3, CD4, CD8, Ter119, Gr-1, Mac-1, B220, Sca-1, and c-Kit. Fluorescence activated cell sorting (FACS) analysis was performed on a Becton Dickinson FACSCalibur, LSRII, or FACSAria flow cytometer.

Methylcellulose Assay (MCA)

Single cell suspensions were obtained by flushing cells from the tibia and femur of *Mx:Hdac3* mice. The red blood cells were lysed with Erythrocyte lysis buffer (Sigma). The cells were mixed with complete methocult M3434 media (Stem Cell Technologies) and plated on 35mm dishes in duplicate. Colonies were grown at 37C, 5% CO_2 for 12 days and colonies were counted.

Antibodies

The Runx1 antibody was obtained from EMD Biosciences. The Flag M2 antibody (F-1804) was obtained from Sigma. The following antibodies were obtained from Upstate Cell signaling: Histone H3 (05-928), Histone H4 (07-108), acetyl-Histone H4 (Lys5) (07-327), acetyl-histone H4 (Lys8) (06-760), acetyl-histone H4 (Lys12) (06-761), acetyl-histone H4 (Lys16) (07-329), acetyl-histone H3 (Lys9) (06-942), and phospho-Histone H2A.X (Ser139), clone JBW301 (05-636). The antibodies for Hdac3

(ab-32369-100 and ab2379) and GAPDH (clone 6C5, ab8245) were obtained from Abcam. The antibody for Pparγ (sc-7196) was obtained from Santa Cruz. The p70 S6 Kinase (2708) and phospho-p70 S6 Kinase (Thr398) (9206) antibodies were obtained from Cell Signaling Technology. The cytokeratin wide spectrum antibody (Z0622) was obtained from DakoCytomation. The sources of the secondary antibodies are as follows: anti-mouse IgG (Sigma) and anti-rabbit IgG (Promega, Inc. Madison, WI).

Generation of *Hdac3* Knockout Mice

The targeting construct was designed to flank *Hdac3* exon 7 with LoxP sites and place a floxed *Neo* cassette in the intron between exons 7 and 8. The 5′ arm of the targeting construct was generated containing a LoxP site by PCR using splicing by overlap extension (SOE). Initially, two PCR products making up the 5′ arm were generated using the primers 5′arm fwd, 5′-CGA CTC GAG GTG CAA CTG TAA GCC AGG CAG TGG-3′ and 5′arm mid rev, 5′-GAA TTC ACG CGT GGC TGA AAT GTG AGC AGG CAG CA-3′ and 5′arm rev, 5′-CGA ATC GAT AAG CCA GCC TGG TCT GTA GCA ATA-3′ and 5′arm mid fwd, 5′-CGA CTC GAG CCG CTG CAT GTT CCC TTT CTC CCC-3′. TL1 ES cell DNA was used as the template and the PCR conditions were 94^{o}C, 8 min followed by 30 cycles of 94^{o}C, 1 min, 58^{o}C, 1 min, 72^{o}C, 3 min with a 72^{o}C, 10 min final extension. These PCR products were used as templates for the SOE. The reaction conditions and the primers, containing a LoxP site (italics) and an internal *Mlu*1 site (underlined; added for diagnostic purposes), were as follows: first set of reactions used primers 5′ arm fwd, and LoxP Rev, 5′-*ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T*AC GCG TGG CTG AAA TGT GAG CAG GCA GCA G-3′;

5′arm rev, and LoxP Fwd, 5′-ACG CGT *ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA T*CC GCT GCA TGT TCC CTT TCT CCC C-3′. The PCR conditions for the first set of reactions were 94°C, 8 min followed by 30 cycles of 94°C, 1 min, 58°C, 1 min, 72°C, 3 min with a 72°C, 10 min final extension.

The full-length 5′ arm was created by adding the two products of the above reactions together diluted 1:20 with the primers 5′arm fwd and 5′arm rev. PCR reaction conditions were 94°C, 3 min followed by 30 cycles of 94°C, 1 min, 60°C, 45 sec, 72°C, 3 min with a 72°C, 10 min final extension. The product of this reaction was sequenced to verify the presence of the LoxP site and *Mlu*I sites. The approx. 1.8 kb product was subcloned into the *Xho*I and *Cla*I sites of pNTK(A)LP2 creating the 5′arm *pNTK* construct. The *pNTK(A)LP2* vector used for making the targeting construct contains a floxed Neo cassette bounded by multiple cloning sites with *HSV TK* and was provided by the Vanderbilt-Ingram Cancer Center Transgenic Mouse/Embryonic Stem Cell Shared Resource.

The 3′ arm was also generated using SOE. The first set of products were generated by the following sets of primers: 3′arm fwd 5′-CGA CTC GAG CTG CTT CTT GAG TGC TGG GAT TAA-3′ and 3′arm mid rev 5′-GTG CTG ACA TCT GGA TGG AGT GTG-3′. The second product was generated by 3′arm rev 5′-ACA GGA TCC TGG TTC ACT GCT CAG GAC AGA GTA-3′ and 3′arm mid fwd 5′-GAT TCA CTC TGG TGT CTC AGC CAT-3′. Reaction conditions for the first round of PCR were 94°C, 3 min, 30 cycles of 94°C, 1 min, 63°C, 45 sec, 72°C, 6 min, with a final extension of 72°C, 10 min. The full-length 3′arm was generated by adding the above two products together after diluting each 3-fold and 8-fold, respectively, and performing PCR with

3′arm fwd and 3′arm rev with the following reaction conditions: 94°C, 3 min, 30 cycles of 94°C, 1 min, 65°C, 45 sec, 72°C, 7 min, with a final extension of 72°C, 10 min. The approx. 3.7 kb product was subcloned into PCR-Script for sequencing. The product was released *by XhoI* and *BamH*I digest and subcloned into the *Sal*I and *BamH*I digested 5′arm *pNTK*.

The targeting construct was restricted with *Not*I for electroporation into TL1 ES cells. Primers for the 3′ probe included: 3′probe fwd 5′-CTG AGC AGT GAA CCA GTA GAC CAC-3′ and 3′ probe rev 5′-TCA CAG CAA CTC GAG TGG TCT CAG-3′ with reaction conditions 94° C, 3 min, 30 cycles of 94° C, 1 min, 65° C, 45 sec, 72° C, 7 min, with a final extension of 72° C, 10 min. TL1 DNA was used as a template for the reactions. The 3′ probe was used to hybridize a Southern blot of *Nhe*I digested ES cell DNA to identify clones that had undergone homologous recombination. Potential positives were identified and a PCR screen examining the 5′ end of the targeting construct was performed to ensure proper homologous recombination. This screen used the following primers: 5′ outside, 5′-TTA GCC CCA AGC AGC TGT TGA TCT G-3′ fwd (outside of the arm of the targeting construct) and *Hdac3* 1597 rev, 5′-GGA CAC AGT CAT GAC CCG GTC-3′ (inside the arm of the targeting construct). This PCR product spans the LoxP site and the *Mlu*I site that was incorporated with the SOE reaction for diagnostic purposes. The PCR products were cut with *Mlu*I to identify the positive clones. Two positive clones were expanded and injected into C57BL/6 blastocysts. The resulting chimeric mice were bred to heterozygosity and crossed with transgenic mice expressing *Cre* ubiquitously from the *EIIA* promoter. Mice that had lost the *Neo* sequences but that retained LoxP sites

flanking exon 7 were identified and used for these studies. Mice that had lost both the *Neo* and exon 7 on one allele (heterozygous) were also identified and bred.

Generation of Liver-specific *Hdac3* Knockout Mice

Mice harboring a conditional allele (fl) or a null allele (-) of *Hdac3* were crossed to transgenic mice expressing *Mx1*-Cre recombinase (318) or *Albumin*-Cre recombinase (319, 320). The offspring from these mice were then bred to yield mice with a conditional allele in conjunction with either a wild-type (fl/+) or null allele (fl/-), and *Cre*. To induce Cre expression in the *Mx1-Cre* expressing mice, 5-week old animals received an intraperitoneal (IP) injection of 500 μg polyinosinic-polycytidylic acid (pIpC) in PBS every other day for 13 days, for a total of 7 injections, with the last day of injection being denoted as Day 0. Mice were sacrificed at 1 day, 2 day, and 1, 2, 3, 4 and 5 weeks after the last injection.

In vivo Inhibitor Treatment

Three-week old *Alb:Hdac3* mice were used in both the GW9662 (Cayman Chemicals) and rapamycin (LC Laboratories) studies. Inhibitors were diluted to a working stock in vehicle solution (5% Tween-80, 5% PEG-400). For the GW9662 experiments, mice were injected with either vehicle or 2mg/kg GW9662, daily for 4 weeks. For the rapamycin experiments, mice were injected with vehicle or 10mg/kg rapamycin, daily for 3 weeks.

Genotyping of Transgenic and Conditional *Hdac3* Deletion Mice

Genomic DNA was extracted by incubating mouse tail piece in Jeffer's mix (0.1M Tris, pH 8.0, 5mM EDTA, 0.2% SDS, 0.2M NaCl) plus 250 μg Proteinase K at 55°C for 3 hours-O/N, followed by centrifugation, decanting solution to a new tube, and precipitating with ice cold isopropanol at -20°C for 30 minutes. DNA was pelleted by centrifugation, washed 1-2 times with 100% ethanol, air dried, and resuspended in nuclease-free water. One-2 microliters of DNA was used for PCR screening to identify mice carrying a floxed exon 7, wild-type exon 7 and a deleted exon 7 of the *Hdac3* allele with primer set 1263T/2158B, or a floxed exon 7 and wild-type exon 7 of the *Hdac3* allele with primer set 1133T/1597B (Table 2). Transgenic mice expressing *Mx1*-Cre recombinase or *Albumin*-Cre recombinase were genotyped using the Cre 685/Cre1054 primer set, with the Mtg16-48/Mtg16-49 primer set (Table 2) as a positive control. The Rosa26 reporter allele was identified using the WildRosa/TransRosa/CommonRosa primer set (Table 2).

Table 2. Transgenic and knockout mouse genotyping primer sequences.

Name	Sequence (5′→3′)
1263T	CCA CTG GCT TCT CCT AAG TTC
2158B	CCC AGG TTA GCT TTG AAC TCT
1133T	CTC TGG CTT CTG CTA TGT CAA TG
1597B	GGA CAC AGT CAT GAC CCG GTC
Cre 645	ACC TGA AGA TGT TCG CGATTA TCT
Cre 1054	ACC GTC AGT ACG TGA GAT ATC TT
Mtg16-48	CAC ACG TGT ATA TTA GGT TGC CTG CCC
Mtg16-49	CAC GTA GCC TGT TCG TAG CAG ACA GCC
WildRosa	GGA GCG GGA GAA ATG GAT ATG
TransRosa	GCG AAG AGT TTG TCC TCA ACC
CommonRosa	AAA GTC GCT CTG AGT TGT TAT

Preparation of Bone Marrow and Liver Lysates

Whole cell lysates (WCL) of total bone marrow were made by homogenizing in PBS plus 0.5% Triton X-100, 0.1% DOC, and 0.1% SDS, sonicated and cleared by centrifugation. To make cellular fractions of liver, fresh or frozen liver tissue was homogenized in Buffer A (PBS plus 1.5 mM $MgCl_2$, 10 mM NaF, 0.1 mM $NaVO_3$, 0.2 mM PMSF, 5μg/mL leupeptin, 1 mM EDTA, 0.5 mM DTT), centrifuged at 850 g, and the supernatent was collected as the cytoplasmic fraction. The remaining pellet was washed twice with Buffer A, resuspsended in Buffer B (Buffer A plus 270 mM NaCl, for a final concentration of 0.4 M NaCl), incubated on ice for 30 minutes with frequent, gentle vortexing, then centrifuged at 14000-15000 g for 15 minutes at 4°. Supernatent was collected as the nuclear fraction. The remaining pellet was washed twice with Buffer B, resuspended in Buffer C (Buffer B plus 0.5% Triton X-100, 0.1% DOC, 0.1% SDS), and sonicated until no longer viscous. All fractions were centrifuged at 14000-15000 g to clear lysates. WCL were made by homogenizing in Buffer A plus 0.5% Triton X-100, 0.1% DOC, and 0.1% SDS, sonicated and cleared by centrifugation.

Histology and Immunohistochemistry

Tissue was fixed in 4% paraformaldehyde at 4°, dehydrated and embedded in paraffin. 5μm sections were stained with hemotoxylin and eosin (H&E) and periodic acid Schiff (PAS). Apoptotic cells were detected by TUNEL using the ApopTag Plus Kit (Chemicon International). Cycling cells were detected by injecting the mice IP with 100μg/g BrdU in PBS 1 hour prior to sacrifice. BrdU was detected using anti-BrdU for immunohistochemistry and BrdU positive cells were counted per 100 nuclei in control

and *Hdac3*-null liver sections. Frozen sections were prepared by fixing tissue in 4% paraformaldehyde at 4° for 2 hours, then 30% sucrose for 3-12 hrs. Tissue was then embedded in OCT, frozen on dry ice, and 10μm sections were stained with Oil Red O and counterstained with hematoxylin. For immunofluorescence, 5μm sections were cut, permeabilized with 0.5% Triton X-100 for 5 minutes, washed 3 times with PBS, and blocked with 10% normal goat serum in PBS for 30 minutes, followed by 3 washes in PBS. Primary antibody was diluted in 10% normal goat serum in PBS, and tissue sections were incubated for 1 hour at RT, followed by 3 washes in PBS. Tissue was then incubated with secondary antibody diluted in 10% normal goat serum, for 45 minutes at RT, in the dark, followed by 3 washes with PBS. Nuclei were stained with Hoechst dye (B-1155, Sigma) at RT for 10 minutes, washed 3 times with PBS, and sections were mounted with coverslips using Permamount (Fisher).

Liver DNA and RNA Extraction, Q-RT-PCR and Microarray Analysis

Liver DNA was purified using Qiagen DNeasy Tissue Kit. Liver RNA was extracted using Versagene RNA Tissue Kit (Gentra Systems). cDNA was prepared from 2-3 μg RNA using random hexanucleotide primers (Applied Biosystems) and M-MLV Reverse Transcriptase (Promega). For Q-RT-PCR, cDNA was analyzed using iQ SYBR Green Supermix (Biorad). Primer sequences are listed in Table 3.

For the microarray analysis, pools of RNA were prepared from a total of 10 control and 10 *Alb:Hdac3*$^{fl/-}$ mice at P17 and P28. Briefly, RNA was prepared from individual whole livers, tested for quality, and high quality RNA was pooled into 2 groups of 5 to normalize for biological variability between animals prior to being

hybridized to Applied Biosystems 1700 Mouse Expression Array System chips for analysis in the Vanderbilt Microarray Shared Resource. Data was analyzed using GeneSpring (Agilent Technologies), and genes whose expression changed at least 2-fold in 2 of 2 *Alb:Hdac3*$^{fl/-}$ biological replicates (determined using a T-test) were included in the data sets. Ingenuity Pathway Analysis software (Ingenuity Systems, Mountain View, CA) and Panther Classification System software (321, 322) were used to group the regulated genes into ontology groups.

Chromatin Immunoprecipiation (ChIP)

The ChIP assay was performed following the standard protocol provided in the Chromatin Immunoprecipitation Assay Kit from Upstate Cell Signaling, with slight modifications. Antibodies for immunoprecipitation of protein/DNA crosslinks included Histone H3, acetyl-Histone H3K9, acetyl-Histone H4 K5, K8, K12 or K16 (Upstate). Liver tissue was minced using a razor blade for formaldehyde crosslinking. DNA was purified using the Qiagen PCR Purification kit after uncrosslinking for 4 hours. Primers designed to the designated promoter sequences were used for quantification of histone acetylation using Q-PCR and are listed in Table 4. Histone H3 and input sonicated DNA were used as reference controls.

Table 3. Q-RT-PCR primer sets

Primer Name	Sequences (5′→3′)
ACoACarb	5′- GAA TCT CAC GCG CCT ACT ATG
	3′- ACG GTG AAA TCT CTG TGC AGG
B-actin	5′- GAC GGC CAG GTC ATC ACT ATT G
	3′- AGG AAG GCT GGA AAA GAG CC
Cby	5′- CGT TTC CTC ACT GAG TTA GG
	3′- TAG TCT GCT AAT CTG ACG GG
Cd36	5′- CCG AGG ACC ACA CTG TGT C
	3′- AAC CCC ACA AGA GTT CTT TCA AA
Cyp51	5′- GGA GCG AAA AGT CCA CCA CAT
	3′- GGT AAG GCT AAA CAC AGG TCC
DopaDC	5′- TAC CCA GCT ATG CTT GCA GAC
	3′- GCG GAT AAC TTT AGT CCG AGC
Ehhadh	5′- GGC TTT AAA TCT CCC ACT GGC
	3′- CAG GAA GAA TTC CCA GCA TCA C
Fasn	5′- AGA GAT CCC GAG ACG CTT CT
	3′- GCC TGG TAG GCA TTC TGT AGT
Gapdh	5′- GCC TTC CGT GTT CCT ACC C
	3′- TGC CTG CTT CAC CAC CTT C
Ggt1	5′- TCC TAC AGG AAG GTG GTT CCG
	3′- CGG GCA TTG ATA ACC TCA ACT T
Glut4	5′- GGA AGG AAA AGG GCT ATG CTG
	3′- TGA GGA ACC GTC CAA GAA TGA
Hdac1RT	5′- TCT ACC GCC CTC ACA AAG C
	3′- ACA GAA CTC AAA CAA GCC ATC A
Hdac2RT	5′- GGA GAT GAG GAT GGA GAA GAC C
	3′- GTC CTT GGA TTT GTC TTC TTC C
Hdac3RT	5′- CGG GTG CTC TAC ATT GAT ATC GAC AT
	3′-TAC CAG GAG AGG GAT ATT GAA ACT CT
Hmgcr	5′- TCG TCA TTC ATT TCC TCG ACA AA
	3′- GAT TGC CAT TCC ACG AGC TAT
Hmgcs	5′- AAC TGG TGC AGA AAT CTC TAG C
	3′- GGT TGA ATA GCT CAG AAC TAG CC
Igf2	5′- GGG ACG TGT CTA CCT CTC AGG
	3′- GAC GAT GAC GTT TGG CCT CTC
Lss	5′- GGA CTG CCC TGA ACT ATG TGG
	3′- GGA CAG CCA GCC AGA ACT T
Mouse 18S	5′- TCG GCG TCC CCC AAC TTC TTA
	3′- GGT AGT AGC GAC GGG CGG TGT
Pcsk9	5′- CCC CAT GTG GAG TAC ATT GAG G
	3′- CAC GCT GTT GAA GTC GGT GA
Pdk4	5′- AGG GAG GTC GAG CTG TTC TC
	3′- GGA GTG TTC ACT AAG CGG TCA

Table 3—continued

Ppara	5′- AAC ATC GAG TGT CGA ATA TGT GG
	3′- AGC CGA ATA GTT CGC CGA AAG
Pparb/d	5′- CCT CCA TCG TCA ACA AAG ACG GG
	3′- GTC CTG GAT GGC TTC TAC CTG G
Pparg	5′- TGT GGG GAT AAA GCA TCA GGC
	3′- CCG GCA GTT AAG ATC ACA CCT AT
Pparg1mRNA	5′- CAG GAG CCT GTG AGA CCA ACA G
Pparg2mRNA	5′- GGT GAA ACT CTG GGA GAT TCT CC
PpargmRNA-Common	3′- GTG TGG AGC AGA AAT GCT GGA G
Sqle	5′- CGC TGC CTT CTC GGA TAT TCT
	3′- ATG TCG TTT CTG AAA GAC CGA TT

Table 4. Primer sets used for amplification of designated promoter regions analyzed in ChIP assay.

Primer Name	Sequences (5′→3′)
Acacb	5′- GCA CTG GTT GCT CTT GCA GAG
	3′- CTG AAC ACA CTA GCA GCA TCG AC
Apoa4	5′- GTG GAA CTG TAG CTG AGA TGT CC
	3′- GAT TCT GTG AGA TAG TCT GGC TCC
Cyp17a1	5′- GCC CAT TCA AGA GAA TGT ACC TAG
	3′-CTC CCA GAG GCA AAT GCT ATC AG
Cyp7a1Distal	5′-GAG ACT GAC CCA CTA GAG ATG CAC
	3′- GCC TTT CTC TCA CTC TCT GTC AC
Cyp7a1Proximal	5′- GAC AGA CCT TCG GCT TAT CGA C
	3′- GCT TAG CAA AGC AAG ACG GTC
Cyp51 -208	5′- CGA AGG GCT GGT CTC ACA AAG CCC AG
	3′- CTC AGG ACC AGA TTG GTG GCG GAC AGC
Cyp51 -2kb	5′- GGG CTG GCT GTG ATG GCA TAT ATC TTG G
	3′- CAG CTG GTG CAA CCA ACC TTG TCT AC
Cyp51 Ex5	5′- GCA CTG TCT GAG CTC ATA ATT TTG ACA GC
	3′- CTG AAA CTT GGC AGA GGC AGC C
Cyp51 Txn	5′- CGG GCC AGA CTA AGC CAT CAG GCC GG
	3′- CTG CTC CAT CGC CTG TCC GAG CAC C
G0/G1Prom	5′- GTG ATC AGC ACC TTC TGA GTC TAG CAG
	3′- GCT GTG GCT ATC GGT GTA CCT G
GkPPRE	5′- CCA GCC ACT TCA TCA ACC TCT CCA CAG
	3′- GGC TGA GTG TTG CAA AAG ACA GGC AGC C
HexCREB	5′- CTC TAG CCA CGG AAC ACA CGT CCC AAC
	3′- GCA GAA CAA CTC CGG GTA GCC TGT GG
HexPPRE	5′- CTC TCC AGC CTT TGC TGG CTT CAA G
	3′- CTC AAC GAG TCA GCG GAT CCC TG
NCoR	5′- GGA GCA AAG AAC ATG GCT TCA TCG
	3′- GAA GTT TCC ACG CTA GAC CAC GAA TC
Pparg1Promoter	5′- TGT CTA TCA TGT GGG CTT CAG GCT
	3′- TTG TGT GTG TAT GCA CGT GAA CGG
Pparg2Promoter	5′- TGG CCA AAT ACG TTT ATC TGG TGT
	3′- AAT GCT GTC TGC TGC TTT GGC AAG
Saa2	5′- GGA CAC ACA GCA TGA TGT CAC ATC
	3′- GGA TTA GAG GTG TGA GCC ACC ATG
SqleMinus2kb	5′- CTT CCC AAT ACT GTG ACT GTA GCC TG
	3′- GCA CAG AAC ATA GGA TGA GAT CTT GGT GG
SqleSRE	5′- CCA GGA GCC ACC AAG AGT TCC TCC AG
	3′- CGG TAG GCT AAG ATG GCG CTA TAG AGC

Metabolic Analysis

Serum levels of alanine transaminase (ALT) were measured using the ALT (SGPT) Reagent Colorimetric, Endpoint Method (Teco Diagnostics) following the standard protocol with slight modifications to scale down the reactions. Glucose levels were measured in whole blood using the Free Style Flash glucose monitor, courtesy of Dr. William Russell. Insulin and glucagon levels were measured in serum using a double antibody radioimmunoassay, with all primary reagents supplied by Linco Research, Inc. (St. Charles, MO). Hormone levels were determined by the Vanderbilt Diabetes Center Hormone Assay Core. To perform the glucose tolerance test, 10-week old mice were fasted for 6 hours, and injected IP with glucose at 1.5 mg/g of body weight. Blood glucose levels were measured through the tail tip using the Free Style Flash glucose monitor during the indicated time course. Total plasma cholesterol and triglycerides were measured by standard enzymatic assays. Liver tissue extraction and analysis of lipids and cholesterol were performed by the Vanderbilt Mouse Metabolic Phenotyping Center (MMPC) Analytical Resources Core, as previously described (323).

Transmission Electron Microscopy (TEM)

Liver tissue was minced to fine pieces using razor blades and fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer at RT for 1-2 hours, then stored at 4° until processing. Samples were washed in 0.1M cacodylate buffer then postfixed in 1% aqueous osmium tetroxide. After postfixation, the tissues were washed, dehydrated through a graded series of ethanols and embedding in Spurr resin. Thin sections (100 nm) were viewed with an FEI CM-12 transmission electron microscope operated at 80 KeV.