Supplemental Data

Mediator MED23 Links Insulin Signaling to the Adipogenesis Transcription Cascade

Wei Wang, Lu Huang, Yan Huang, Jing-wen Yin, Arnold J. Berk, Jeffrey M. Friedman,3 and Gang Wang1,*

Table S1. Med23-Regulated, Insulin-Responsive Genes

Gene symbol	Gene title	fold of insulin induction
Egr2 (Krox20)	early growth response 2	11.95
Fos	FBJ osteosarcoma oncogene	10.76
Egr3	early growth response 3	9.96
Ier2	immediate early response 2	5.13
Egr1	early growth response 1	4.73
Ptgs2	prostaglandin-endoperoxide synthase 2	4.58
Nr4a1	nuclear receptor subfamily 4, group A, member 1	4.06
Junb	Jun-B oncogene	3.34
Btg2	B cell translocation gene 2, anti-proliferative	2.99
Ier3	immediate early response 3	2.88
Errfi1	ERBB receptor feedback inhibitor 1	2.41
Ereg	epiregulin	2.26
Snai1	snail homolog 1 (Drosophila)	2.25
Phlda1	pleckstrin homology-like domain, family A, member 1	2.22
Fosl1	fos-like antigen 1	2.17
Sc4mol	Sterol-C4-methyl oxidase-like	2.02
Jun	Jun oncogene	2.00
Arl4c	ADP-ribosylation factor-like 4C	1.99
Dusp6	dual specificity phosphatase 6	1.96
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	1.91
Srf	serum response factor	1.88
Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	1.87
Trib1	tribbles homolog 1 (Drosophila)	1.83
Dnaja1	DnaJ (Hsp40) homolog, subfamily A, member 1	1.78
F3	coagulation factor III	1.67
Vegfa	vascular endothelial growth factor A	1.66
Nuak2	NUAK family, SNF1-like kinase, 2	1.64
Adm	adrenomedullin	1.60
Klf10	Kruppel-like factor 10	1.59
Slc30a1	solute carrier family 30 (zinc transporter), member 1	1.58
Has2	hyaluronan synthase 2	1.54
D5Ertd579e	DNA segment, Chr 5, ERATO Doi 579, expressed	1.52
C030046G05	hypothetical protein C030046G05	1.51
Socs3	suppressor of cytokine signaling 3	1.51

Table S2.Primers Used in Real-time PCR and ChIP Assays

For real-time PCR analysis	
EF2-F	5′-AAAAGTATGAGTGGGACGTTGC-3′
EF2-R	5′-CCTTGATCTCATTCAGGTACTGC-3′
Krox20-F	5′-AGAAGGTTGTGATAGGAGGTTCTC-3′
Krox20-R	5′-GTTCGGATGTGAGTAGTAAGGTGG-3′
C/EBPβ-F	5′-GCACAAGGTGCTGGAGCTGAC-3′
C/EBPβ-R	5′-CTTGAACAAGTTCCGCAGGGT-3′
αP2-F	5′-GATGCCTTTGTGGGAACCTG-3′
αP2-R	5′-TCCTGTCGTCTGCGGTGATT-3′
PPARγ2-F	5′-TGTCGGTTTCAGAAGTGCCTTG-3′
PPARγ2-R	5′-TTCAGCTGGTCGATATCACTGGAG-3′
Adipsin-F	5′-CTGAACCCTACAAGCGAT-3′
Adipsin-R	5′-GACCCAACGAGGCATTCT-3′
Egr1-F	5′-CCAACATCAGTTCTCCAGCTC-3′
Egr1-R	5′-TTGCTCAGCAGCATCATCTC-3′
Klf5-F	5′-ACCTCCGTCCTATGCCGCTAC-3′
Klf5-R	5′-TCCGGGTTACTCCTTCTGTTGT-3′
For ChIP assay	
Krox20p-F	5′-GGAGAGAGYCAGTGACGGATAGAC-3′
Krox20p-R	5′-GAACTGAGCCTAGGATGGTATCTC-3′
Krox20c-F	5′-AAGGTTGTGATAGGAGGTTCTCAC-3′
Krox20c-R	5′-GGTTCGGATGTGAGTAGTAAGGTG-3′
EF2p-F	5′-TCAAGTGTTAAGTCCCAAAGACC-3′
EF2p-R	5′-CTTTTATAGGTCGACGCCGGTT-3′
IL12bp-F	5′-CCCCCAGAATGTTTTGACAC-3′
IL12bp-R	5′-CTGGACACCCACTGTTCCTT-3′

*F: forward primer, R: reverse primer

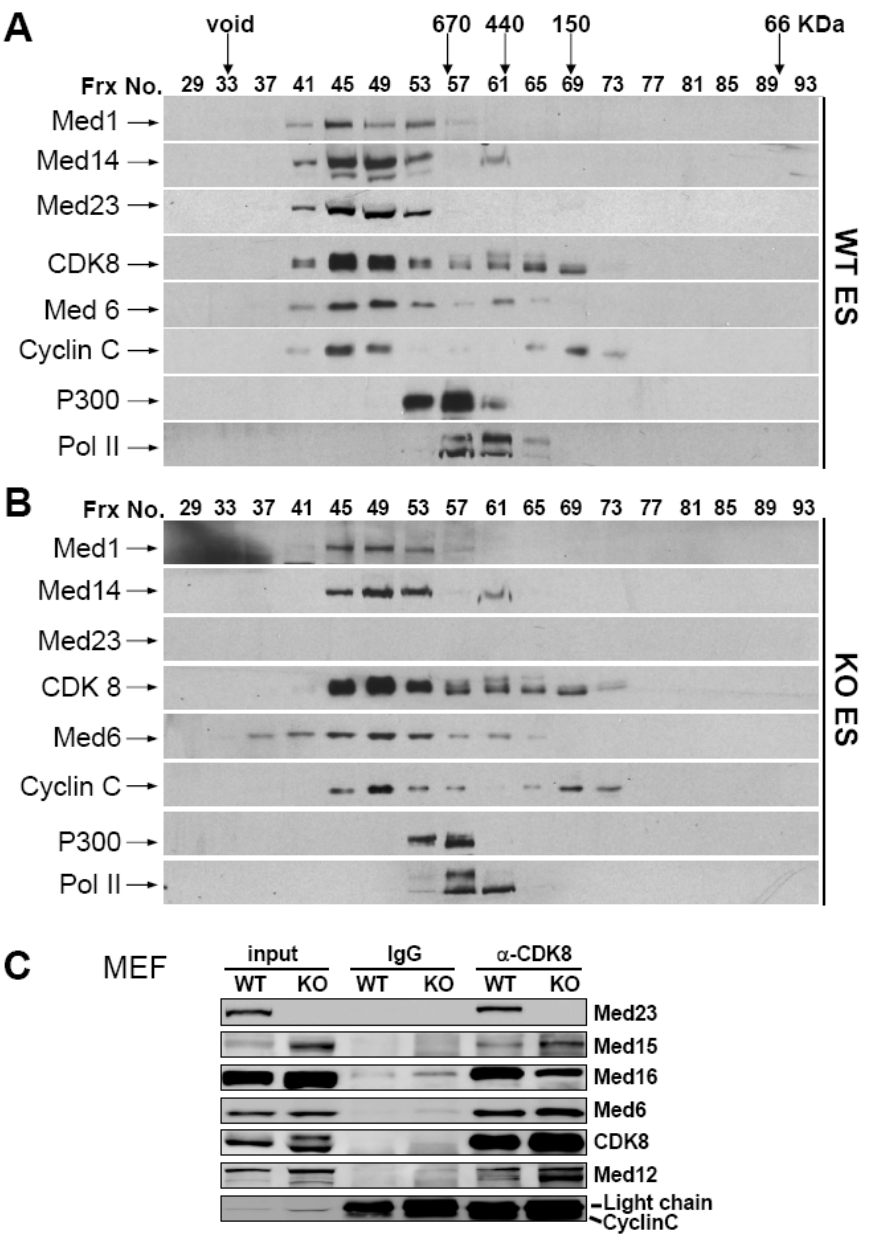

Figure S1. Gel Filtration Chromatography and Immunoprecipitation Analysis of the Mediator Complex in Wt And Ko Cells

(A and B) Gel filtration chromatography of nuclear extracts from wt (A) and ko (B) ES cell nuclear extracts demonstrates that Mediator complex from ko cells is similar in size to the complex from wt cells. A Superose 6 column was run in 0.3 M KCl in buffer D. Every fourth column fraction was analyzed by western blot with antibodies against Med1, Med14, Med23, CDK8, Med6, cyclin C, P300, and the RPB1 subunit of Pol II as indicated. The column fractions where the peaks of protein standards (670, 440, 150, and 66 kDa) eluted and the void volume are indicated.

(C) Cell lysates from wt and ko MEFs were immunoprecipitated with antibodies against CDK8 or IgG and analyzed by western blot with the indicated antibodies.

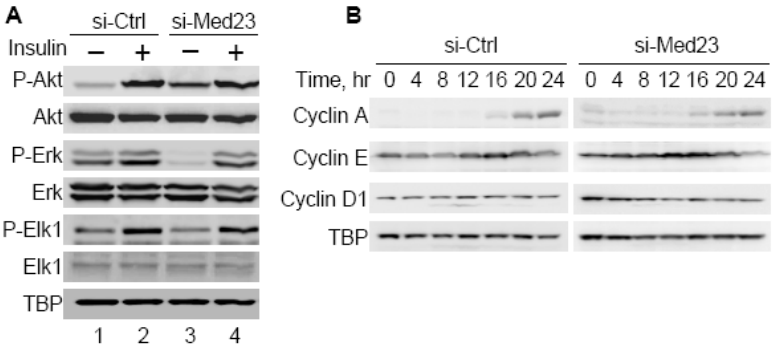

Figure S2. Effect of Med23 Knockdown on Insulin Signaling and Cell Cycle Progression

(A) Confluent si-Ctrl and si-Med23 3T3-L1 cells were treated with 5 g/ml insulin for 30 min. Whole cell lysates were subjected to SDS-PAGE and immunoblotted with indicated antibodies.

(B) Day 0 post-confluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes. At the indicated times, whole cell lysates (50 g of protein) were subjected to SDS-PAGE and immunoblotted with indicated antibodies.

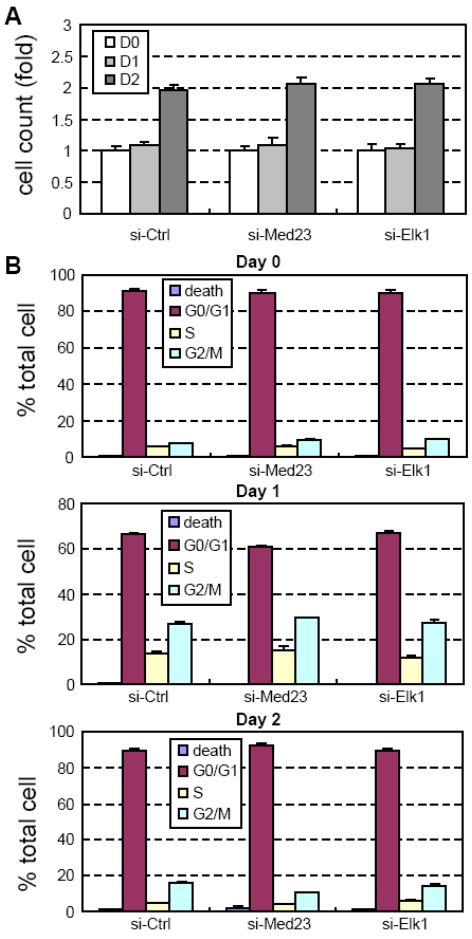

Figure S3. Analysis of mitotic clonal expansion during adipocyte differentiation

(A) si-Ctrl, si-Elk1, and si-Med23 3T3-L1 cells were counted during the early phase of hormone-induced differentiation. The fold change was calculated by normalization to the cell number at day 0. The error bars represent the standard deviation of three independent experiments.

(B) The knockdown of Med23 or Elk1 does not affect the cell cycle. Flow cytometric analysis of si-Ctrl, si-Elk1, and si-Med23 3T3-L1 cells at day 0, day 1, or day 2

experiments.

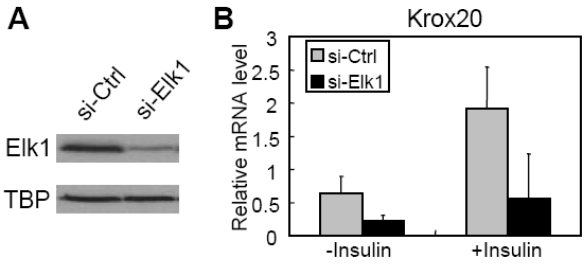

Figure S4. Knockdown of Elk1 attenuates Krox20 expression
MEF cell lines stably expressing an Elk1-specific or control siRNA were generated.
(A) The reduced protein expression of ELK1 in si-Elk1 MEFs was detected by western blot.
(B) The attenuation of both the basal and the insulin-stimulated Krox20 expression following Elk1 knockdown was detected by real-time PCR.

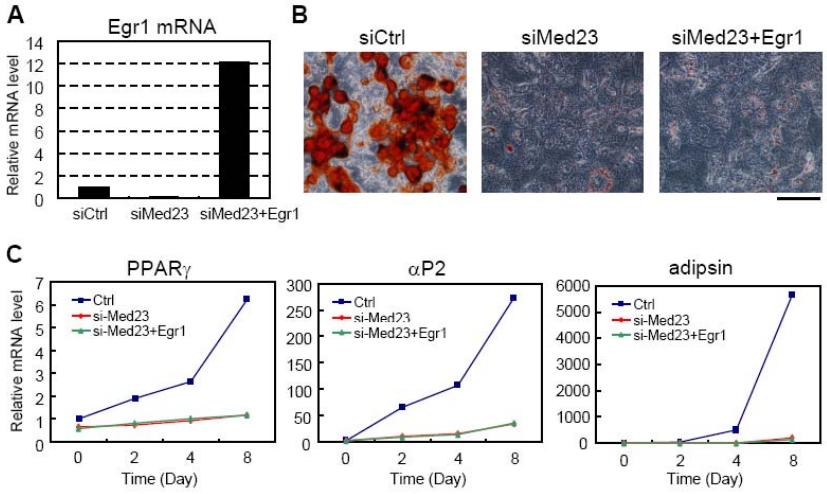

Figure S5 | Effect of Egr1 overexpression on the differentiation of Med23-depleted 3T3-L1 cells
(A) Quantitative real-time PCR analysis showing the Egr1 mRNA level in each cell line as indicated. All values are normalized to EF2 mRNA expression.
(B) Hormone-induced adipogenesis of Ctrl, si-Med23, and si-Med23+Egr1 3T3-L1 cells. At day 8 post-induction, cells were stained for lipid droplets with ORO. Scale bar 100 m.
(C) Real-time PCR analysis of the expression of adipocyte markers in 3T3-L1 cells at different time points post-induction. The expression is normalized to EF2 mRNA expression.

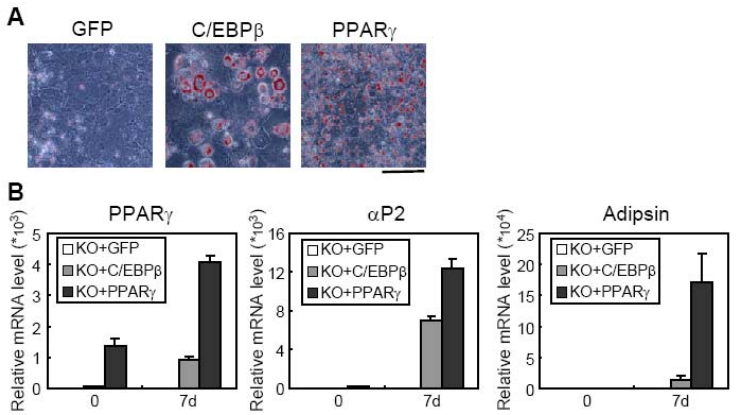

Figure S6. Ectopic Expression of C/EBPβ Or PPARγ in Ko Mefs Rescues Adipogenesis

ko MEFs were infected with a recombinant retrovirus encoding GFP, C/EBPβ, or PPARγ. After puromycin selection, the cells were cultured to confluence and induced for differentiation with the three hormones plus troglitazone.

(A) Cells at day 8 post-induction were stained with ORO staining. Scale bar 100 m.

(B) Real-time PCR analysis for the expression of adipocyte markers. The expression is normalized to EF2 mRNA expression and then further normalized to that of the GFP-transduced cells at day 0. The standard deviation from three replicate experiments are indicated.

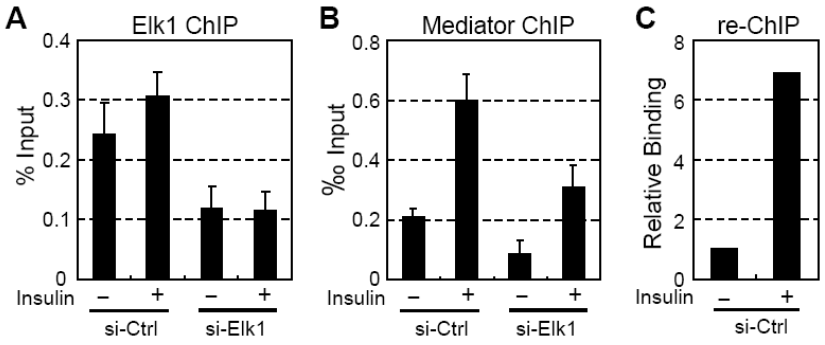

Figure S7. Elk1 Regulates the Binding of the Mediator Complex to the *Krox20* Promoter

3T3-L1 cells were grown to confluence and treated with insulin at a final concentration of 5 g/ml for 30 min. ChIPs were then performed with antibodies to ELK1 (A) or the Mediator complex components, Med1 and Med17 (B).

(C) Re-ChIP assays were performed to determine the co-occupancy of ELK1 and the Mediator complex at the *Krox20* promoter with or without insulin stimulation. The relative binding level was calculated, and the binding level under no insulin induction is 1.

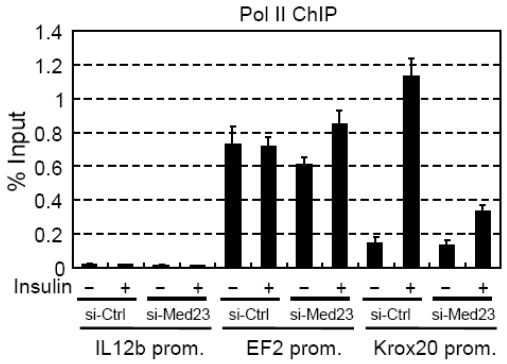

Figure S8. Pol II Binding at the *IL12b*, *EF2* and *Krox20* Promoters
3T3-L1 cells were grown to confluence and treated with insulin at a final concentration of 5 g/ml for 30 min. A ChIP assay was then performed with an antibody to Pol II. Pol II binding at the different gene promoters was analyzed by quantitative real-time PCR.

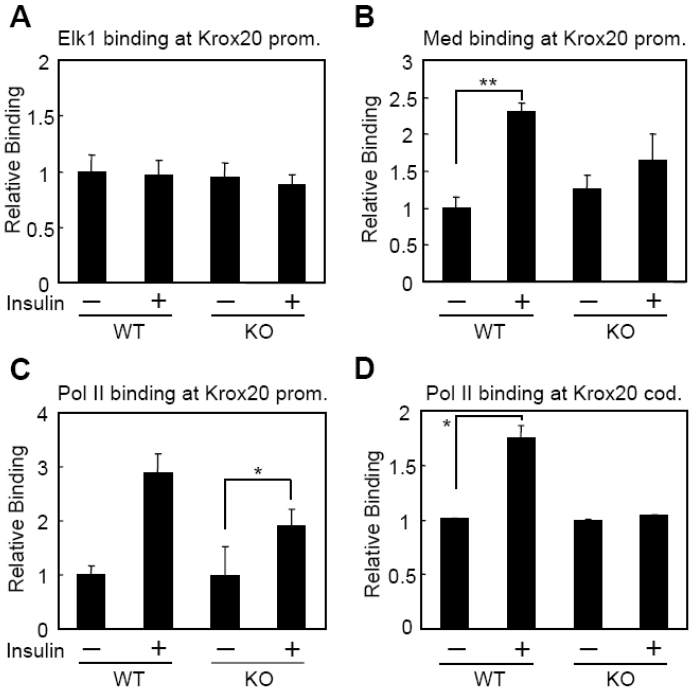

Figure S9. Effect of insulin on the binding of Elk1, Mediator, and Pol II to the *Krox20* locus
wt and ko MEFs were grown to confluence and then treated with insulin at a final concentration of 5 g/ml for 30 min. ChIP assays were performed, and the immunoprecipitated DNA was analyzed using real-time PCR. Experiments were performed with antibodies to Elk1 **(A)**, the Mediator complex (Med1 and Med17; **B**) and Pol II (**C, D**).
The precipitated DNA was analyzed by real-time PCR with primers targeting the *Krox20* promoter region (Krox20p; **A, B, C**) or the coding region (Krox20c; **D**). The primer sequences are provided in Supplementary Table 2. The relative binding of each factor was calculated by normalizing the binding to the PCR value of the *EF2* promoter (EF2p) from the same immunoprecipitated DNA sample, which was further normalized to that in wt cells without insulin treatment. The mean of three separate experiments is shown, with the standard deviation indicated. Student's t-test, *p<0.05, **p<0.01.

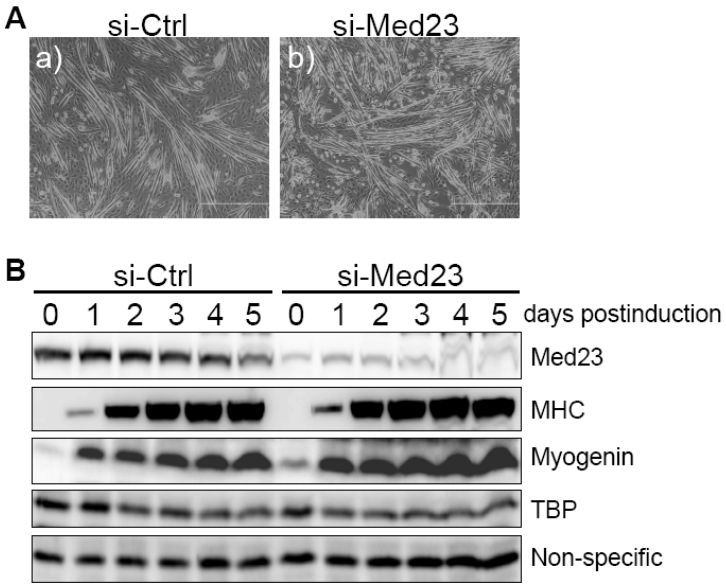

Figure S10. Effect of Med23 Depletion on Skeletal Muscle Differentiation

(A) C2C12 cells were infected with the si-Ctrl (panel a) or si-Med23 (panel b) retroviruses, selected, and induced to differentiate in conventional differentiation medium (DMEM supplemented with 2% horse serum, 30 mg/ml penicillin, and 100 mg/ml streptomycin). The differentiation medium was changed every 24 h until day 5, when the photos were taken. Scale bar 500 m.

(B) Samples of C2C12 cells undergoing differentiation were taken daily until day 5 post-induction, and whole cell lysates were prepared. The lysates were subjected to western blot with antibodies against Med23, myosin heavy chain (MHC), myogenin, and TBP. TBP and a non-specific band are shown as loading controls.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture And Adipocyte Differentiation

Med23$^{-/-}$ MEFs were isolated from 9.5-day embryos and self-immortalized using standard procedures. Immortalized MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Hyclone) in 5% CO2. 293T and 3T3-L1 cells were grown in DMEM containing 10% newborn calf serum (NCS) in 5% CO2.

To induce MEFs to differentiate into adipocytes, cells were plated at a density of 5×10^4 per 3.5 cm dish in cell culture medium with or without BMP4 (10 μg/L). Two days after the cells reached confluence, the cells were treated with culture medium containing differentiation cocktail (5 mg/L insulin (Sigma), 0.5 mM IBMX (3-isobutyl-1-methylxanthine) (Sigma), 1 μM dexamethasone (Sigma)), with or without 0.5 mg/L troglitazone (Sigma). After 48 hr, the cells were switched to medium containing 5 mg/L insulin, and the medium was replenished every 2 days.

3T3-L1 cells were induced to differentiate by adding 1 mg/L insulin, 0.5 mM IBMX, and 1 μM dexamethasone for 48 hr and then switched to medium with 1 mg/L insulin. RNA was collected for real-time PCR analysis at the indicated time points. Lipid droplets were stained with Oil Red O (Sigma).

Insulin Stimulation And Signaling Inhibition

Insulin was directly added to the cell culture medium to a final concentration of

5 ng/L. The cells were harvested for total RNA extraction at the indicated time points. For signaling inhibition, the cells were pretreated with various signaling inhibitors 30 min before the addition of insulin. RNA was isolated 30 min after the addition of insulin. The chemical inhibitors used and their final concentrations in the medium are 10 μM U0126 (LC Laboratories; specific to MEK1/2), 30 μM H89 (LC Laboratories; specific to PKA), 30 μM SB203580 (LC Laboratories; specific to p38), 20 μM LY294002 (Calbiochem; specific to PI3K), and 1 μM wortmannin (Calbiochem; specific to PI3K). The effect of wortmannin on Krox20 expression is equivalent to that of LY294002 (data not shown).

Retrovirus Plasmids And Infection

The human Med23 cDNA was amplified by PCR from an expression vector (pCS2-Med23) and cloned into the pMSCV/Puro and pMSCV/Hygro vectors (Clontech). The mouse Egr1 and Elk1 cDNAs were cloned into the pMSCV/Hygro vector (Clontech). The KOD Hot Start DNA Polymerase kit (TOYOBO) was used to mutate Ser-383 and -389 of pMSCV-Elk1 to alanine. The pMSCV-Krox20, pMSCV-PPARγ2, and pMSCV-C/EBPβ plasmids have been described previously(Chen et al., 2005).

For knockdown of Med23 and Elk1 in 3T3-L1 cells, retrovirus-mediated siRNA expression was used. The targeted sequence was determined using the Whitehead Institute siRNA designing tool and verified by BLAST searches to ensure specificity. As recommended by Clontech for the RNAi-Ready pSIREN-RetroQ system, two complementary oligos for each targeted sequence were designed, annealed, and ligated into the BamHI/EcoRI-linearized pSIREN-RetroQ vector. The resulting construct, upon packaging into retroviruses, allows for the stable expression of the siRNA hairpin specific for the target gene. The sequences of the oligonucleotides cloned into pSIREN-RetroQ were si-Med23, gagataagtaagttacatg; si-Elk1, agttggtggatgcagagga; and si-Ctrl, gtgcgctgctggtgccaac. Retroviruses were generated following the cotransfection of recombinant pMSCV or pSiren-RetroQ plasmids with pCL10A1 helper plasmid into 293T cells using Lipofectamine 2000 (Invitrogen). Tissue culture supernatants containing retroviruses were harvested 48 hr later and passed though a 0.45 μm filter. Cells were plated into 6-well plates before retroviral infection. Virus-containing supernatants were supplemented with 20 μg/ml polybrene and added to the cells for a spin infection procedure, which was performed in the 6-well plates by centrifugation at 2500 rpm at 30°C for 1.5 h. MEFs were selected with 50 μg/ml puromycin and 3T3-L1 cells were selected with 300 μg/ml hygromycin or 5 μg/ml puromycin. One representative result from at least two siRNA experiments is shown.

Real-Time PCR and Western Blot Analysis

Total RNA was isolated from cells using TRIZOL (Invitrogen). The first-strand cDNA was generated using MMLV transcriptase (Promega), and real-time PCR was performed in triplicate using a SYBR Green PCR master mix in an Eppendorf Mastercycler. All values were normalized to the level of EF2 mRNA, which is constitutively expressed and not changed during the time course of the experiments. Primer sequences used in the experiments are included in the supplemental Table 2. The western blot was performed using an ECL kit (Pierce) based on the manufacturer's recommendations. HRP-conjugated secondary antibodies were purchased from the Jackson Laboratory.

Microarray Analysis

Affymetrix Mouse Genome 430 2.0 expression arrays were probed with cDNA synthesized from total RNA isolated from 3T3-L1 cells treated for 30 min with 5

software. Med23-RNAi and insulin-induction were considered two factors in the two-way ANOVA analysis. Med23-RNAi down-regulated genes were selected by RNAi comparison at p-values <0.01 and >1.5 fold down-regulation, whereas insulin-responsive genes were selected by insulin-induction at p-values <0.01 and >1.5 fold induction. The overlapping set of selected genes is listed in supplemental Table 1.

Soluble Recombinant MED23 Protein Production And Purification

The His-Flag-Med23-expressing baculovirus was generated using the Bac-to-Bac Baculovirus Expression system (Invitrogen), as specified by the manufacturer. Briefly, the PCR-generated hMed23 cDNA from pCS2+hMed23 was subcloned into the pFASTBac-HTc plasmid, and the Flag sequence was added to the N terminus to generate the N-terminal 6xHis-Flag-tagged fusion. This plasmid was introduced into DH10-Bac cells to produce the His-Flag-Med23 baculovirus plasmid (His-Flag-Med23-Bac). His-Flag-Med23-Bac plasmid was then transfected into Tn5 cells to recover recombinant baculovirus Med23-BAC. Tn5 cells (three 10 cm plates) were then infected with Med23-BAC, harvested 72 h post-infection at 2500 rpm, and lysed in 3 ml cytoplasm lysis buffer (20 mM HEPES, pH 7.9, 0.1% Triton X-100, 5 mM β-mercaptoethanol) with 30 µl PMSF (0.1 M) for 30 min at 4 ºC. After centrifugation at 15,000 *g* for 20 min, the supernatant was bound to 200 µl of Ni^{2+} resin (Qiagen) in low flow speed three times at 4 ºC. The resin was washed three times in 1 ml of 20 mM imidazole washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and then twice with 1 ml of 40 mM imidazole washing buffer. The purified Med23 was then eluted from the resin with 3 ml of 0.25 M imidazole elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 10% glycerol). For Ni^{2+} pull down experiment, the Ni-Med23 beads were prepared without elution and stored in PBS at 4 ºC.

Preparation of Recombinant GST-Elk1c and Ni-NTA Pull Down

The pGEX-Elk1c plasmid encodes the C-terminal amino acids 307-428 of human Elk1 fused to glutathione *S*-transferase. Elk1 307-428 was PCR amplified from HeLa cDNA and ligated into pGEX-4t-2 using the BamH1 and NotI sites. The GST-Elk1 fusion protein was purified as described(Guan and Dixon, 1991). Recombinant ERK2 and ATP were purchased from NEB. The purified GST-Elk1 (100 µg) was treated with activated ERK2 and ATP at 30 ºC and then incubated at 65 ºC to inactivate Erk2. The GST-Elk1 (1 ml diluted in D300) was incubated with Ni-Med23 beads (40 µl of 50% PBS slurry) overnight at 4℃, washed twice with 1 ml of D300 (no EDTA, with 0.1% NP40), and finally washed twice with 1 ml of D500 (no EDTA, with 0.1% NP40). SDS loading buffer (50 µl) was added to the beads, and the samples were boiled at 100 ºC for 5 min.

Elk1 Mutagenesis And Luciferase Assay

Multiple serine residues within the Elk1 activation domain of Gal4-Elk1 (307-428) were mutated by site-directed mutagenesis to alanine residues using the KOD Hot Start DNA Polymerase kit (TOYOBO). 293T cells at 90% confluency (in 12-well plates) were transfected with a 5×Gal-E1B-TATA-luciferase reporter construct (250 µg per well) and the Gal4-Elk1 activation domain or Gal4-ELk1 mutants (50 µg per well), in the absence or presence of a plasmid expressing active MEKK (Stratagene, 25 µg per well). Additionally, a plasmid expressing Renilla luciferase was cotransfected (50 µg per well). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. At 48 hours post-transfection, the cells were lysed, and the luciferase expression was

activity was normalized to Renilla luciferase activity.

Coimmunoprecipitation

A PCR-generated Med23 fragment was ligated into pcDNA3-5×myc. The Flag-tagged Elk1 expression vector was a gift from AD Sharrocks. 293T cells (90% confluent) were transfected with 16 μg Myc-Med23 and 4 μg Flag-Elk1 (with or without 600 ng Mekk) using Lipofectamine 2000 (Invitrogen). At 24 hours post-transfection, the cells were washed and lysed in 1 mL lysis buffer (1% NP-40, 10% glycerol, 135 mM NaCl, 20 mM Tris, pH 8.0, 10 mM NaF, 2 mM $NaVO_4$, and freshly added proteinase inhibitors from Roche). After a brief sonication, the lysates were centrifuged at 12,000 rpm for 5 min. The supernatant was then added to 20 μl of equilibrated anti-M2 beads (Sigma) and incubated at 4 ºC. After 3 hours, the beads were washed 3 times with lysis buffer. The Flag peptide (Sigma, 150 ng/μl) was added to the beads and incubated for 30 min. The eluted supernatants were boiled in SDS loading buffer and analyzed by western blot with the indicated antibodies. For the experiment in supplemental Figure S1B, 3×10^7 wt and ko MEFs were harvested, lysed in protein lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and proteinase inhibitors), and centrifuged at 16,000 g for 15 min at 4 ºC. The lysates were incubated with 30 μl of a rabbit anti-CDK8 antibody (Neomarkers) or an equal amount of rabbit IgG (Santa Cruz) for 12 h at 4 ºC. Protein A/G agarose beads (Santa Cruz) were added and incubated at 4 ºC for 3 h. The samples were washed three times with lysis buffer, eluted with SDS loading buffer, and analyzed by western blot with the indicated antibodies.

Nuclear Extract Preparation And Gel Filtration Chromatography

The nuclear extract preparation and chromatography on Superose 6 columns have been described previously (Wang et al., 2001). Briefly, 3 ml of undialyzed nuclear extract was directly loaded onto a 100-ml Superose 6 column (HR 16/50; Pharmacia) preequilibrated with 0.3 M KCl D buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol). Column fractions (1 ml) were collected, and 100 μl of each fraction was precipitated with trichlorolacetic acid and analyzed by western blot using the indicated antibodies.

Cell Count and Flow Cytometry

For the cell counts and cell cycle analysis, 5×10^5 cells were seeded in 10 cm dishes with 10 ml of medium. At the indicated times post-confluence, the cells were trypsinized and counted or washed with ice-cold PBS, fixed in 75% ethanol, and stored at -20 °C. The cells were treated with 0.5 ml RNase A (0.2 mg/ml) for 0.5 h at 37°C and stained with propidium iodide (50 g/ml). The stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Chromatin Immunoprecipitation

ChIP assays were performed as described previously(Wang et al., 2005); however, the immunoprecipitated DNA was quantified using real-time PCR. Two sets of primers were used to amplify DNA from the *Krox20* promoter (Krox20p) or the *Krox20* coding region (Krox20c). All values were normalized to the input.

For supplemental Figure S9, the relative binding values were calculated by the ratio of the amount of PCR product with the Krox20p or Krox20c primers divided by the amount of PCR product with the EF2p primers (specific for the *EF2* promoter region). The ratio for wt cells in the absence of insulin was assigned as 1. The primers for analyzing the ChIP DNA are provided in Supplemental Table 2.

Antibodies

The antibodies to ERα, p-ERα, ERβ, p-ERβ, MED1 (for ChIP), MED24, Med30, Pol II, TBP, TFIIE, p300, Cyclin A, Cyclin D1, Cyclin E, and normal rabbit or goat IgG were purchased from Santa Cruz Biotechnology. The antibodies to Akt and p-Akt (Ser473) were purchased from Cell Signaling. The mouse monoclonal antibody to Med23 was from BD Biosciences. The anti-Med12 antibody was from Bethyl. The anti-CDK8 antibody was from NeoMarkers. The antibodies to Flag and Myc were from Sigma. The antibodies to MED1 (for western), Med16, Cyclin C, and Med6 were gifts from Len Freeman, Robert Roeder, Emma Lees, and Danny Reinberg, respectively.

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