ONLINE APPENDIX

SUPPLEMENTARY METHODS

Luciferase assay. The rat PEPCK gene promoter (nucleotides –998 to +106 relative to the transcription start site) was cloned by PCR and ligated into the pGL3-basic vector (Promega, Madison, WI), yielding pGL3-WT. For generation of a mutant PEPCK gene promoter, a region containing a consensus binding motif for KLF15 (AATCACCCCTC, nucleotides –587 to –577) was replaced by TTTTTTTTTTT with the use of PCR, and the resulting construct was ligated into pGL3-basic to give pGL3-Mut. HepG2 human hepatoma cells cultured in 12-well plates were transfected with 200 ng of pGL3-WT or pGL3-Mut, 30 ng of pCMV encoding β-galactosidase, and 100 ng of pcDNA3.1 encoding KLF15 or the empty vector with the use of the FuGENE6 reagent (Roche, Basel, Switzerland). Forty-eight hours after transfection, total cell lysates were prepared and assayed for luciferase and β-galactosidase activities, with the former being normalized by the latter.

ChIP analysis. The ChIP assay was performed with the use of a Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA). HL1c cells that had been infected with an adenoviral vector encoding Flag-KLF15 were incubated with 1% formaldehyde for 15 min at 37°C, lysed, and subjected to immunoprecipitation with protein A–agarose beads coated either with antibodies to Flag or with control IgG. DNA was extracted from the immunoprecipitates, and a fragment of the rat PEPCK gene (nucleotides –617 to –383 relative to the transcription start site) was detected by PCR with the primers 5′-CAGGCATTGCCTCCTCGGAATG-3′ and 5′-TTGACAACCAGCAGCCACTGGC-3′.

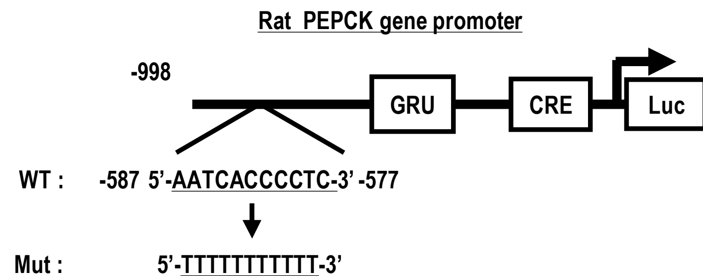
Ubiquitination analysis. For detection of KLF15 ubiquitination, HepG2 cells were transfected with a mammalian expression vector encoding hemagglutinin epitope (HA)–tagged ubiquitin (kindly provided by A. Kikuchi) with the use of the FuGENE6 reagent, 24 h after which the cells were infected with an adenoviral vector encoding Flag-KLF15. Twenty-four hours after infection, the cells were examined for the effect of metformin on KLF15 ubiquitination.

Primers for RT and real-time PCR analysis. Primers (forward and reverse, respectively) for RT and real-time PCR analysis were as follows: mouse KLF15, 5'-CCCAATGCCGCCAAACCTAT-3' and 5'-GAGGTGGCTGCTCTTGGTGTACATC-3'; rat KLF15, 5'-CTCACCCTCCTCCAGACGCCAACCT-3' and 5'-TCTAAGTCAGACTGGGAGGCGCTAG-3'; rat HPD, 5'-GTCCAGCACATCGCTCTCA-3' and 5'-CTTCACTTGGATCTTGGAGGTCTTG-3'; mouse HPD, 5'-AGCCATGGGTGGAGCAAGAC-3' and 5'-GGGCCTCGAATCCAGGTAAGA-3'; mouse ALT1, 5′-GGTGCAGGGCGCTATGTATTC-3' and 5'-CACAACGCAGATGCCAGTCTC-3'; mouse ProDH, 5'-TCATCAGTGCCCGCACCTAC-3' and 5'-TGCAGTGAGCTTAATGGCTGAGA-3'; mouse TDO2, 5′-AGGAACATGCTCAAGGTGATAGCTC-3′ and 5′-TCTGGAAGCCTGATGCTGGA-3′; rat CREB, 5′- ACAGTTCAAGCCCAGCCACAG-3′ and 5′-GCACTAAGGTTACAGTGGGAGCAGA-3′; and rat CRTC2, 5′-CCAATGTTAACCAGATTGGCTGTG-3′ and 5′-CTCCGAGATGAATCCAAAGGTGA-3′.

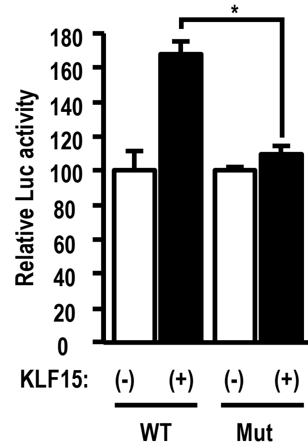
©2010 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1679/DC1

Supplementary FIG. 1. .KLF15 directly binds to and regulates the gene for PEPCK. (*A*) Schematic representation of reporter constructs for the rat PEPCK gene promoter. The WT and mutant (Mut) sequences of the putative KLF15 binding motif are shown. GRU, glucocorticoid-responsive unit; CRE, cAMP-responsive element; Luc, luciferase gene. (*B*) Activity of WT and mutant PEPCK gene promoters assayed in HepG2 cells in the absence or presence of exogenous KLF15. Data are means ± SEM from three independent experiments. *$P < 0.05$. (*C*) HL1c cells infected with an adenovirus encoding Flag-tagged KLF15 were subjected to ChIP analysis with protein A–agarose beads coated either with antibodies to Flag or with control IgG. The immunoprecipitates (IP) or total cell lysates were subjected to PCR analysis for detection of a fragment of the PEPCK gene promoter containing the putative KLF15 binding sequence (arrow). Data are representative of 3 independent experiments.

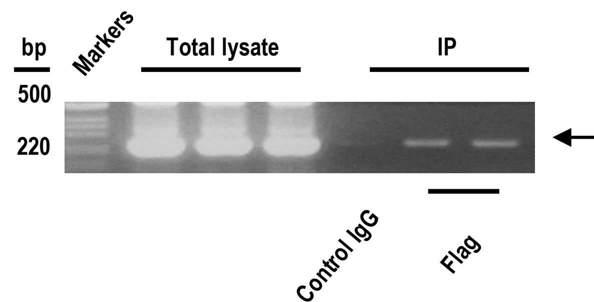
A



B

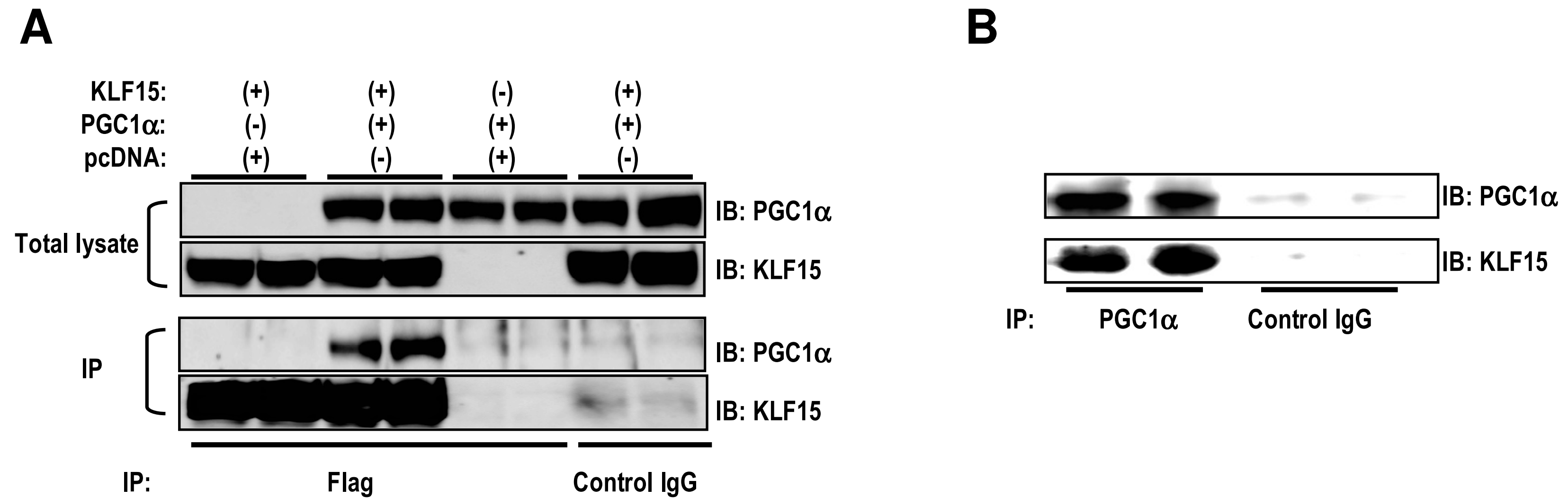


C



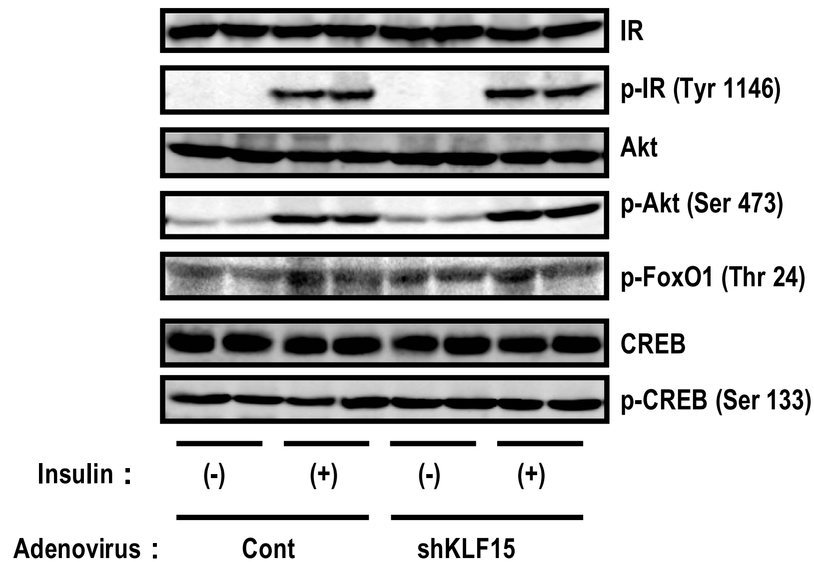
©2010 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1679/DC1

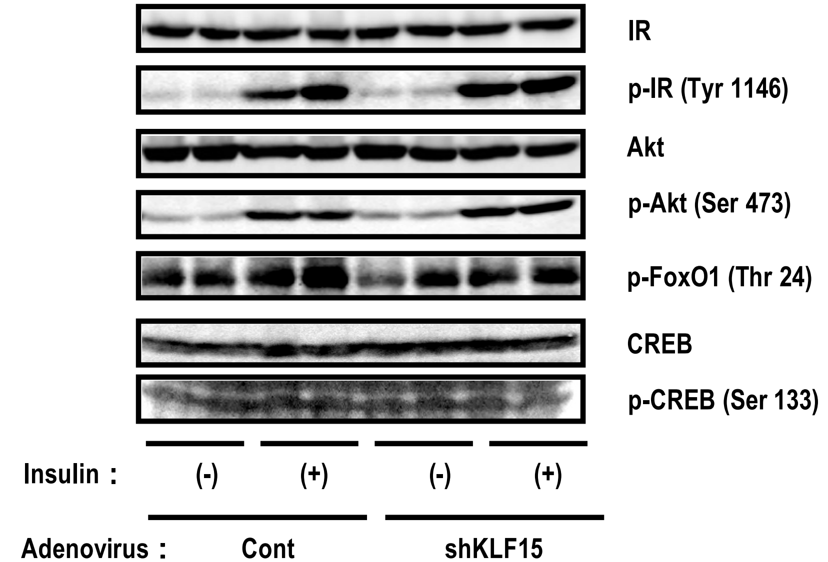
Supplementary FIG. 2. Interaction of PGC1α with KLF15. (*A*) HEK293 cells were transfected with pcDNA3.1 encoding mouse PGC1α or Flag-tagged mouse KLF15 or with the empty vector (pcDNA), as indicated. Total cell lysates were then subjected to immunoprecipitation (IP) with protein G–agarose beads coated either with antibodies to Flag or with control IgG, and the resulting precipitates as well as the original cell lysates were subjected to immunoblot (IB) analysis with antibodies to PGC1α or to KLF15. (*B*) Nuclear extracts of HepG2 cells were subjected to immunoprecipitation with protein G–agarose beads coated either with antibodies to PGC1α or with control IgG, and the resulting precipitates were subjected to immunoblot analysis with antibodies to PGC1α or to KLF15. All data are representative of three experiments.



©2010 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1679/DC1

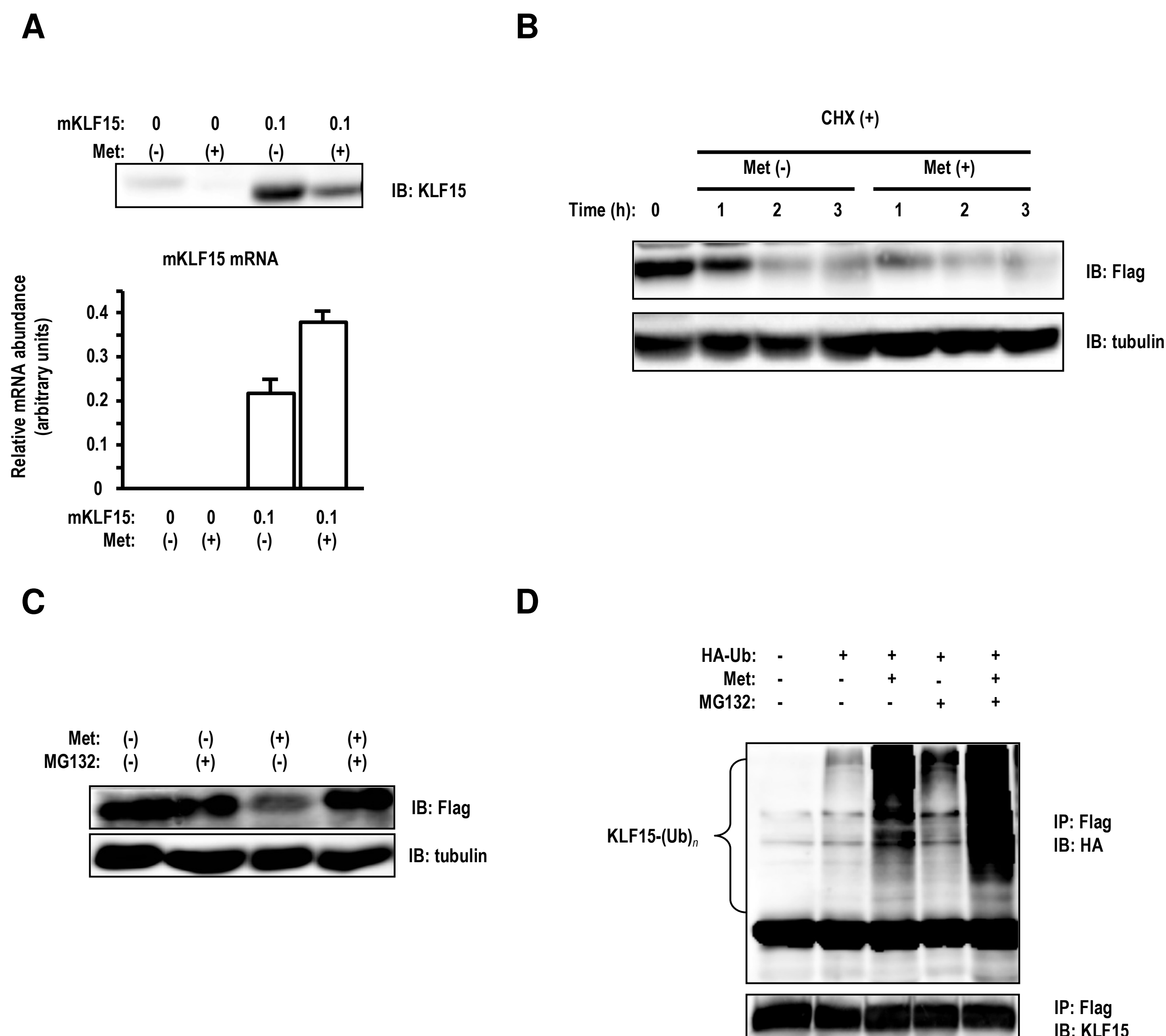
Supplementary FIG. 3. Effects of acute KLF15 depletion on the abundance and phosphorylation of various signaling molecules in mouse liver. C57BL/6 (*A*) or db/db (*B*) mice were injected with an adenovirus encoding KLF15 shRNA (shKLF15) or a control adenovirus containing the U6 promoter alone (Cont). Seven days after virus injection, the mice were deprived of food for 16 h, injected intraperitoneally with human regular insulin (5 U/kg) or vehicle, and killed 7 min later. Total homogenates were prepared from the liver and subjected to immunoblot analysis with antibodies to the indicated proteins. IR, insulin receptor; p-, phosphorylated (on the indicated amino acid residues). All data are representative of at least 3 independent experiments.





©2010 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1679/DC1

Supplementary FIG. 4. Metformin-induced ubiquitination and degradation of KLF15. (*A*) HL1c cells that had been infected (or not) with an adenovirus encoding Flag-tagged mouse KLF15 (mKLF15) at an MOI of 0.1 pfu/cell were incubated in the absence or presence of 5 mM metformin (Met) for 6 h. The cells were then subjected to immunoblot analysis with antibodies to KLF15 (upper panel) or to RT and real-time PCR analysis of mouse KLF15 mRNA (lower panel). Quantitative data are means ± SEM from three independent experiments. (*B*) HL1c cells that had been infected with an adenovirus encoding Flag-tagged mouse KLF15 at an MOI of 10 pfu/cell were incubated in the absence or presence of cycloheximide (CHX, 25 μg/ml) or 5 mM metformin for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to Flag and to α-tubulin. (*C*) HL1c cells infected as in (*B*) were incubated in the absence or presence of 30 μM MG132 or 5 mM metformin for 6 h, after which cell lysates were subjected to immunoblot analysis with antibodies to Flag and to α-tubulin. (*D*) HepG2 cells that had been transfected (or not) with a plasmid encoding hemagglutinin epitope–tagged ubiquitin (HA-Ub) were infected with an adenovirus encoding Flag-tagged KLF15. The cells were incubated in the absence or presence of 5 mM metformin or 15 μM MG132 for 6 h, lysed, and subjected to immunoprecipitation with antibodies to Flag, and the resulting precipitates were subjected to immunoblot analysis with antibodies to HA (upper panel) or to KLF15 (lower panel). The region of the blot containing ubiquitinated KLF15 [KLF15-$(Ub)_n$] is indicated. All immunoblot data are representative of at least 3 independent experiments.



©2010 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1679/DC1