**Supplemental Data**
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# Ablation of ARNT/HIF1β in Liver Alters Gluconeogenesis, Lipogenic Gene Expression, and Serum Ketones

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## Experimental Procedures

### Animals, Adenoviruses, and Plasma/Serum Biochemical Measurements

ARNT flox/flox mice were generated using the Cre-*loxP* system. Exon 6, encoding the conserved basic-helix-loop-helix domain of the protein, was flanked by *loxP* sites and introduced into the *Arnt* gene by standard gene disruption techniques using embryonic stem cells as described previously (Tomita et al., 2000). All animals were housed on a 12 hr light/12 hr dark cycle. All subsequent animal studies were carried out in accordance with National Institutes of Health guidelines on animal care regulations and were approved by the Animal Care and Use Committee of the Joslin Diabetes Center and Harvard Medical School. An adenoviral vector encoding a CRE protein (Ad-CRE) and its control vector (Ad-vector) were purchased from Gene Transfer Vector Core (University of Iowa).

Three groups of mice were studied: male ARNT flox/flox mice (9-11 months of age) on normal chow diet (MNC), female ARNT flox/flox mice (9-11 months of age) on normal chow diet (FNC), and female ARNT flox/flox mice (9 months of age) that had been on a 60% high fat diet for 3 months (FHF). In each group, half of the mice were injected the adenoviral-Cre construct and half with a control adenovirus via the tail vein. The dosage of adenovirus vectors used was $1 \times 10^8$ pfu/g suspended in 200 μl of normal

saline. Experiments were performed 4-6 days after adenovirus injection. Blood glucose was measured with Elite Glucometer (Bayer). Plasma insulin and serum biochemical measurements were assayed by specialized assay core (Joslin Diabetes Center, Boston, MA).

**Microarray Analysis**

Microarray analysis was performed using U133A Affymetrix to assess expression of ARNT in liver specimens from normal lean individuals, obese individuals, and obese type 2 diabetic individuals with both well-controlled and poorly controlled blood glucose levels. Gene expression data were normalized with Affymetrix internal gene expression parameters using the Bioconductor program.

**Glucose Tolerance (GTT) and Pyruvate Challenge Tests**

For GTT, ARNT flox/flox mice (Control) and L-ARNT KO mice deprived of food for 16 hr were injected intraperitoneally with glucose (2 g/kg body weight). Blood glucose was measured at 0, 15, 30, 60, 90, and 120 min time points by glucometer from tail vein blood. For the pyruvate challenge test, mice deprived of food for 16 hr were injected intraperitoneally with pyruvate dissolved in saline (2 g/kg). Glucose was measured at 0, 15, 30, 60 and 120 min as above.

**Hyperinsulinemic-Euglycemic Clamp Experiments**

The jugular veins of ARNT flox-flox and control mice were cannulated. After a 2 day recovery period, Cre adenovirus was injected as described above. After an

additional 5 day recovery period and a 4 hr fast, a hyperinsulinemic-euglycemic clamp was performed with D-[3-$^{3}$H]glucose as described by Norris *et al.* (2003), except that a continuous insulin infusion dose of 3.5 mU/kg/min was used during the clamp. Adenoviral injection and the clamp technique were carried out by two different people so that the clamp was performed without knowledge, i.e. blinded, of the type of genotype of the group until all calculations were completed. Hepatic glucose production was assessed by subtraction of the glucose infusion rate from whole-body glucose turnover as measured with D-[3-$^{3}$H]-glucose.

**Two Step Real-Time RT-PCR**

cDNA was synthesized by reverse transcription from total RNA that had been extracted from the liver of ARNT flox/flox and L-ARNT KO mice. Real-time PCR reactions were performed using primers specific for the genes and SYBR Green PCR master mix (Applied Biosystems). The primer sequences used for the genes studied are listed in Table S1. Real-time PCR data were analyzed using the SDS version 1.7a software package.

**Immunoblot Analysis**

ARNT flox/flox and L-ARNT KO mice were sacrificed 6 days after adenovirus injection. The livers from these mice were removed, and a 30 mg aliquot of total homogenates was prepared and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and detected with antibodies against ARNT (BD Transduction Laboratories), C/EBPα (Santa Cruz), ACC1 (Cell Signaling Technology), FAS (Cell

Signaling Technology), α-actin (Santa Cruz), p-AMPK, and AMPK (Cell Signaling Technology). The proteins were visualized using ECL (Pierce) and quantified by densitometry. NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) was used to extract nuclear and cytoplasmic extracts from human Huh7 cells. α-actin protein level was used as a loading control.

**Immunofluorescent Staining**

Huh7 cells cultured on coverslips were incubated with or without insulin for 5 min. The cells were then fixed with 4% paraformadehyde for 20 min and permealized with 0.2% Triton X-100 in 1% bovine serum albumin (BSA)/in phosphate buffered saline (PBS). After incubation for 30 min in a blocking solution which consists of 1% BSA/PBS, the coverslips were incubated with an antibody against ARNT (BD Transduction Laboratories) at 4°C for 24 hr. After several washes in PBS, cells were incubated with GFP-conjugated IgG (Jackson) for 1 hr at room temperature in the dark. The cells on the coverslips were washed in PBS three times and mounted with mounting medium (Vecta-shield, Vector Labs). The cells were evaluated using a Leica confocal fluorescent microscope.

**Hepatic De Novo Lipogenesis**

L-ARNT KO and Control mice were injected with 0.5 mCi of tritiated water (0.4 ml of 1.25 mCi/ml) for 1 hr. The rate of hepatic de novo lipogenesis was determined by measuring the amount of newly synthesized FA and TG present in the liver 1 hr after intraperitoneal injection of 0.5 mCi of $^{3}H_2O$. $^{3}H$-labeled fatty acids were isolated by

saponification of liver samples in KOH. After extraction of nonsaponifiable lipids and acidification with $H_2SO_4$, the $^3H$-labeled fatty acids were extracted with chloroform and separated by thin layer chromatography. The plate was stained with primulin, the FA and TG "spots" were scraped off the plate, and the isolated spots were added to scintillation fluid (CytoScint, MP Biomedicles; Solon, OH) and counted in a liquid scintillation counter (LS6500 Beckman Coulter; Fullerton, CA). The specific activity of body water was determined and used to calculate de novo lipogenesis as micromoles of $^3H_2O$ incorporated into FA/hr/g and TG/hr/g of liver tissue.

**Liver Histology**

Liver tissues were dissected from ARNT flox/flox and L-ARNT KO mice, fixed with 10% buffered formalin, and sectioned. Fixed slides were rinsed in distill water and placed in propylene glycol for 5 min. These slides were stained with oil red O, rinsed with 85% propylene glycol and distilled water, and stained in hematoxylin solution sequentially. The stained slides were rinsed thoroughly in distilled water, mounted with aqueous mounting medium, and examined under a light microscope (Nikon; Tokyo, Japan). Each image represents the liver samples from 5-6 mice, and each sample was representative of three randomly selected fields/section (40× magnification).

**Quantification of Hepatic Malonyl-CoA and Triglyceride Contents**

Liver tissues were removed from ARNT flox/flox and L-ARNT KO mice and immediately frozen in liquid nitrogen. Malonyl-CoA was measured by radioactive assays. Liver was homogenized and deproteinized with 10% perchloric acid, and

the filtrate neutralized as described previously (Saha et al., 1997). Malonyl CoA was determined radioenzymatically in the neutralized filtrate by a modification of the method described previously (McGarry et al., 1978). For the measurement of hepatic triglyceride contents, 0.1-0.2 g of liver tissue from each mouse was homogenized in 500 μl buffer containing 150 mM NaCl and 10 mM Tris (pH 7.5) and subsequently extracted by 400 μl methanol and 800 μl chloroform. The chloroform layer, containing triglyceride, was extracted, evaporated overnight, and resuspended in 70% ethanol. Hepatic triglyceride content was measured by enzymatic method (Serum Triglyceride Determination Kit; Sigma). Triglyceride content was expressed relative to total cellular protein content measured by protein concentration solution (Bio-Rad).

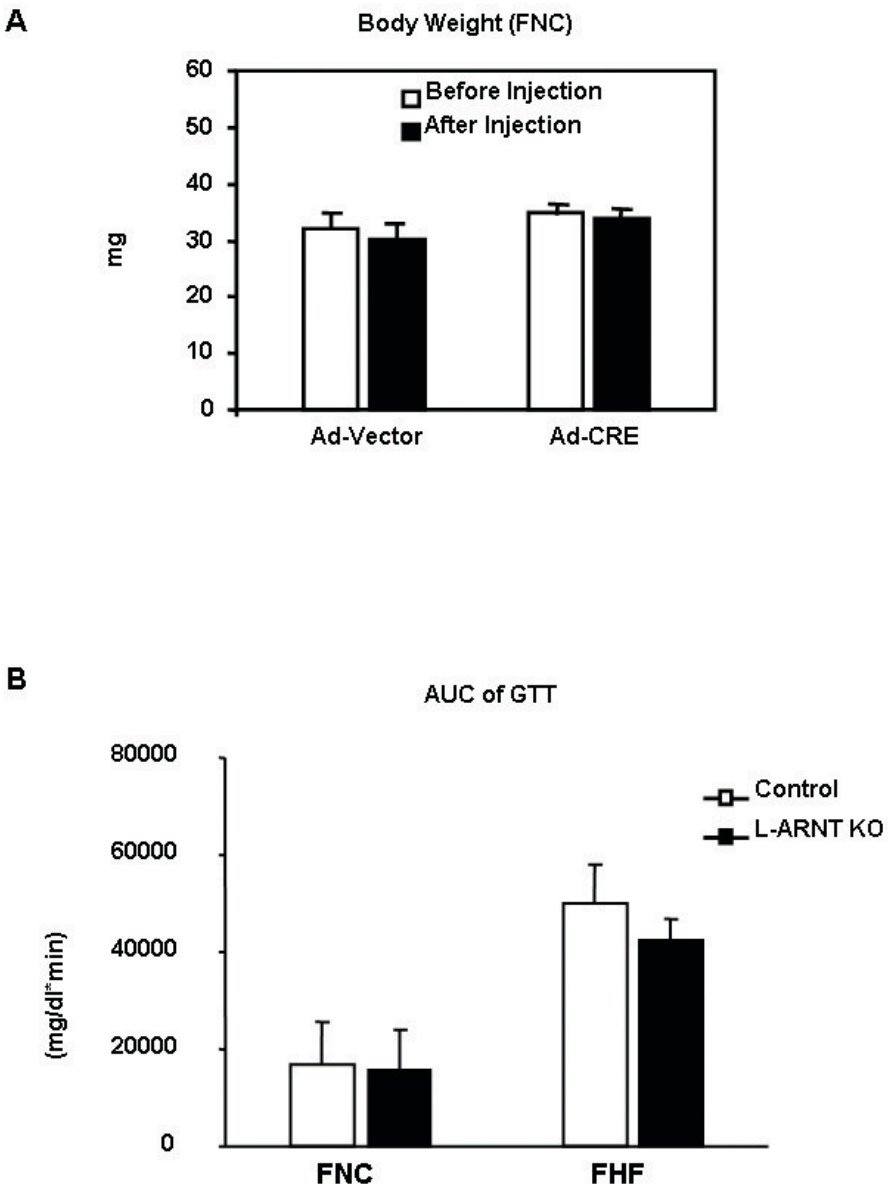
**Statistics**

The results are expressed as means ± SEMs. Significance was established using the Student's t test and ANOVA when appropriate. Differences were considered significant at $p < 0.05$. Two-way ANOVA with repeat measurements was performed to analyze measurements obtained by time course.

**Table S1. Effect of ARNT on the Expression of Genes Involved in Fatty Acid β-Oxidation and Ketogenesis**

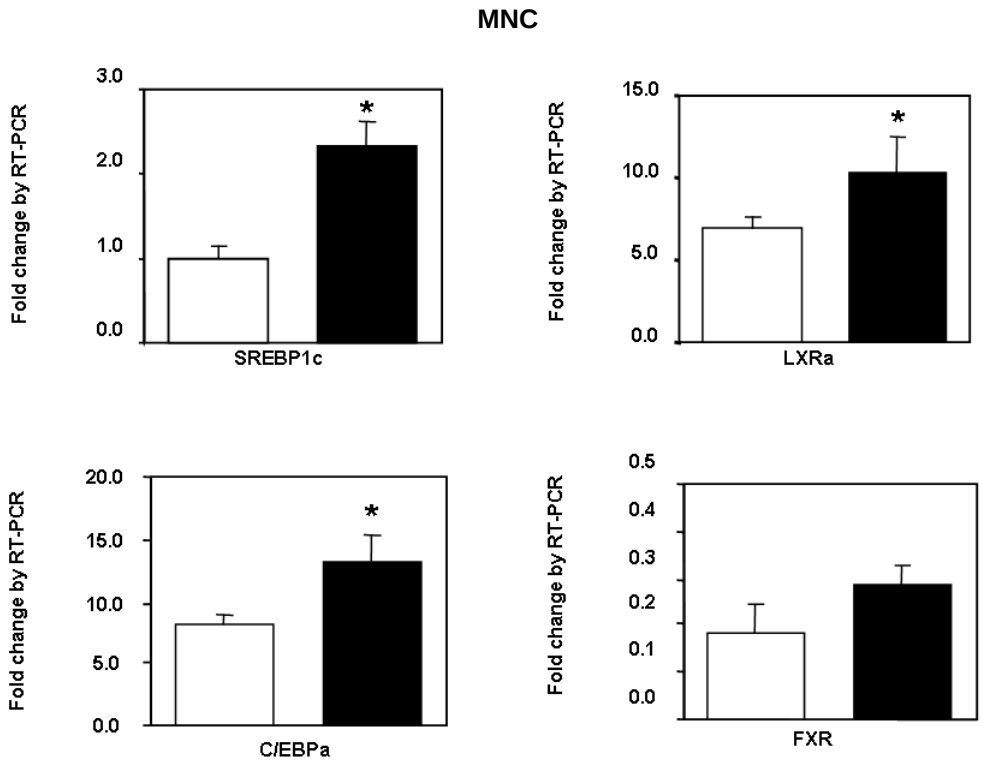
| Enzyme Abbreviation | Changes in mRNA levels (Control vs. L-ARNT KO ) |
|---|---|
| CPT-I | 1.00: 0.90 (p=0.75) |
| CPT-II | 1.00: 0.95 (p=0.84) |
| ACC1 | 1.00: 1.10 (p=0.94) |
| ACC2 | 1.00: 1.65 (p=0.17) |
| Malonyl CoA decarboxylase (MCD) | 1.00: 1.21 (p=0.59) |
| AMPK | 1.00: 1.21 (p=0.72) |
| HMG-CoA Synthase | 1.00: 1.07 (p=0.81) |
| HMG-CoA Lyase | 1.00: 1.42 (p=0.37) |
| 3-hydroxybutyrate dehydrogenase (Bdh1) | 1.00: 0.98 (p=0.94) |

**Figure S1. Physiological Measurements in Control and L-ARNT KO Mice**



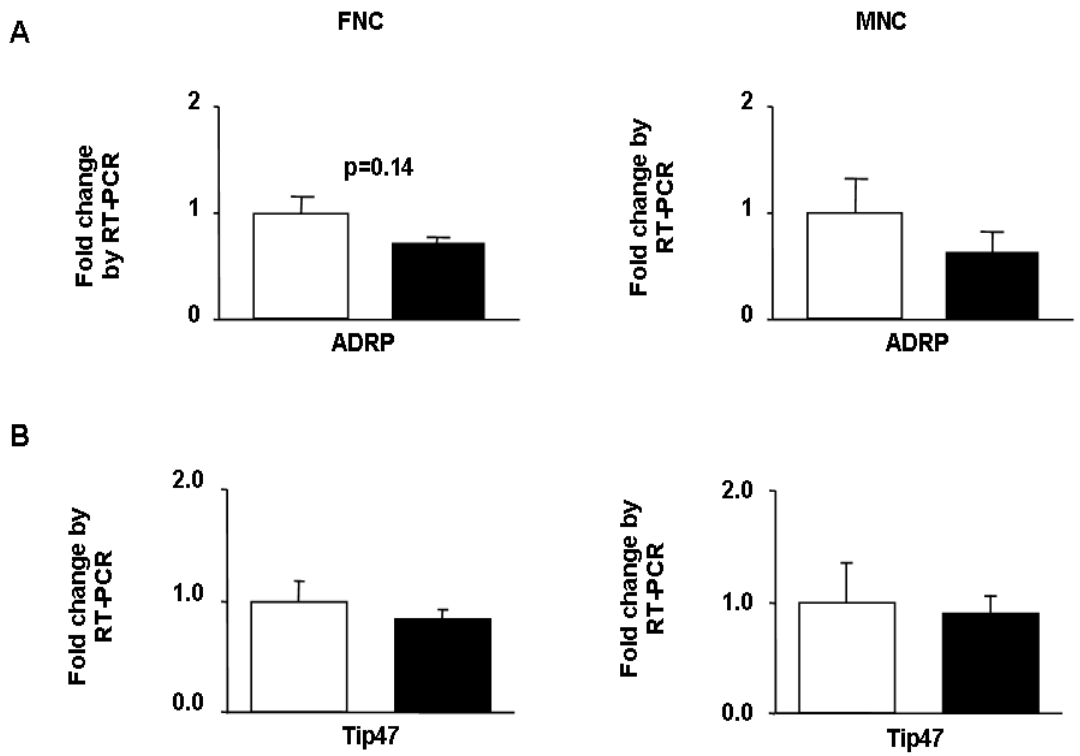

(A) Body weight before and on day 6 after the adenovirus injection in both L-ARNT KO and Control mice from FNC group.
(B) Area under the curve (AUC) of GTT from FHF and FNC groups, respectively.

**Figure S2. Effect of ARNT on the Expression of SREBP1c, LXRa, C/EBPa, and FXR Genes in the Livers of ARNT KO and Control Mice from MNC Group**



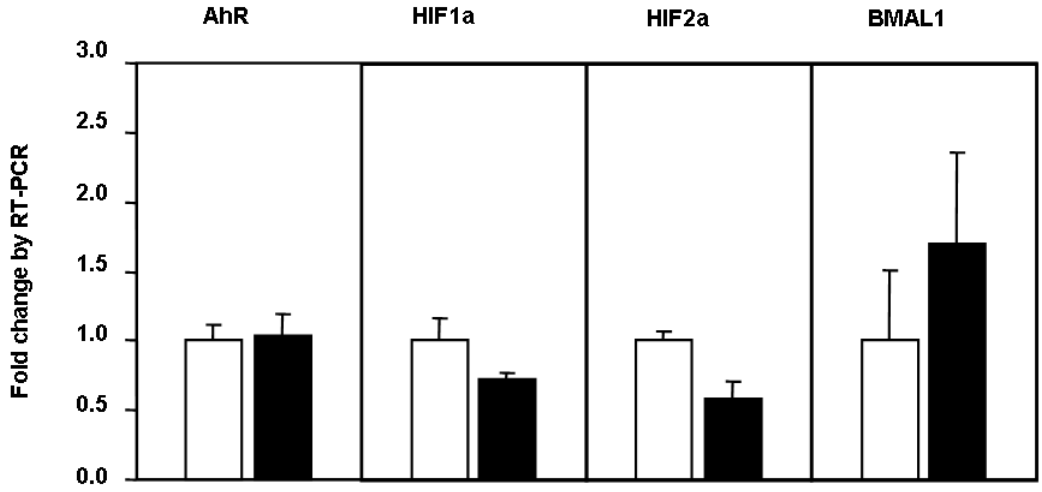

The livers of mice from each group were obtained in the fed state at day 6 after injection. Data are representative of results obtained from 5-6 mice from MNC group.

**Figure S3. Effect of ARNT on the Expression of ADRP and Tip 47 Genes**




(A) mRNA levels of ADRP in the livers of L-ARNT KO mice from both FNC and MNC groups were measured by real-time RT-PCR.
(B) mRNA levels of Tip47 from the livers of FNC and MNC groups. The livers of mice from each group were obtained in the fed state at day 6 after injection. Data are representative of results obtained from 5-6 mice in each group.

**Figure S4. Effects of ARNT on the Expression of Genes Encoding Its Potential Partners**




mRNA levels of AhR, HIF1a, HIF2a and BMAL1 in the livers of L-ARNT KO mice from FNC group were measured by real-time RT-PCR. Data are representative of results obtained from 5-6 mice in FNC group.