Supplemental Information

Circadian Amplitude Regulation via FBXW7-targeted REV-ERBα Degradation

Supplemental Experimental Procedures

DNA expression constructs

Mouse Rev-erbα and Rev-erbβ expression constructs were generated by inserting cDNA fragments into HindIII/NotI sites of pcDNA 3 vector. Rev-erbα T275A mutant expression vector was generated using Quikchange II site directed mutagenesis kit (Stratagene). pCMV-neo-HA-CDK1 and its dominant negative mutant (D146N) were provided by Dr. Ed Harlow (Harvard Medical School) and purchased from Addgene (Cambridge, MA). CMV-cyclin B2 expression vectors were provided by Dr. Robert Weinberg (Whitehead Institute). The shRNA-producing plasmid targeting human CDK1 was generated by inserting CDK1-specific RNAi sequence GGGGTTCCTAGTACTGCAA into pSUPER vector. Myc-tagged human FBXW7α expression plasmid was generated by inserting FBXW7α cDNA fragment from pCMV-flag2-FBXW7α (provided by Dr. Steven Reed at Scripps Research Institute, La Jolla) into pcDNA6-Myc-His vector. Expression vector of dominant negative FBXW7α with F-box domain deletion was created using Quikchange II site-directed mutagenesis kit (Stratagene). 6xHis-ubiquitin expression construct was provided by Dr. Geoffrey Wahl (Salk Institute). pBabe-puro-REV-ERBα WT and T275A mutant constructs were created by inserting REV-ERBα cDNA fragments into BamHI/SnaBI sites on pBabe-puro vector. Human FBXW7 and CDK1 siRNAs were purchased from Dharmacon.

Chemicals and antibodies

MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI). Cdk inhibitors Roscovitine, Olomoucine, CDK1 inhibitor (CGP74514A), CDK1/5 inhibitor, CDK2/5 inhibitor (PNU112455A) and a Cdk4 inhibitor (NSC625987) were purchased from EMD Bioscience. Nocodazole, cycloheximide and MG132 were purchased from Sigma. Antibodies were purchased as following: anti-Flag M2 antibody (Sigma), Anti-phospho-CDK substrate antibody (Cell Signaling, 2321S), anti-HA tag antibody (F-7, Santa Cruz Biotechnology), anti-CDK1 antibody (Cell Signaling), anti-REV-ERBα antibody (Cell Signaling), anti-Myc (9E10) (Santa Cruz Biotechnology), anti-GST antibody (Cell Signaling).

Cell culture and transfection

AD293, HEK293T, U2OS cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. NIH3T3 cells and MEFs stably expressing WT REV-ERBα and T275A mutant were maintained in DMEM supplemented with 10% calf serum. All cells were grown at 37°C in a humidified atmosphere of 5% CO2. REV-ERBα null fibroblasts were described (Liu et al., 2008) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.292 mg/ml glutamine and 1% penicillin/streptomycin. Serum shock to induce circadian clock resetting on NIH3T3 cells was performed as described (Yin et al., 2006). Transient transfections were carried out using Fugene 6 transfection reagent (Roche) following manufacturer's instructions, and cells were lysed in NETN buffer (20mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT) supplemented with protease inhibitors and phosphatase inhibitors (Pierce).

Retrovirus production and infection

Retroviruses were produced by transfecting pBabe-puro expression constructs into Phoenix cells. REV-ERBα single null and REV-ERBα/β double null fibroblasts were transduced by incubating in retrovirus-containing media containing polybrene (8 μg/ml) overnight. 36 hours later, cells were selected by 1.5 μg/ml puromycin for 6 days.

Protein extraction, immunoprecipitation and western blotting

For detecting phosphorylation on REV-ERBα using pT-P antibody, AD293 cells were lysed in RIPA buffer (Sigma) supplemented with protease inhibitors and phosphatase inhibitors (Pierce). For immunoprecipitation with anti-Flag antibody, equal amounts of lysate containing 300-500 μg of total cellular protein were incubated overnight with 1 μg of anti-Flag antibody (M2, Sigma) and protein G-sepharose (Roche). The immunoprecipitated complex was washed with lysis buffer for four times and heated with LDS sample loading buffer (Invitrogen) before loading on SDS-PAGE for western blotting. For co-immunoprecipitations to detect protein-protein interactions, cells were lysed in NETN buffer supplemented with protease inhibitors and phosphatase inhibitors (Pierce). The cell lysates were incubated overnight with antibodies as described at 4°C. For western blotting, 20–50 μg of total cell extracts or nuclear extracts were mixed with LDS sample loading buffer and heated before resolving by SDS-PAGE. Proteins were detected by SuperSignal chemiluminescent ECL kit (Pierce). Liver whole cell lysates were prepared as described (Lamia et al., 2009).

Nanoflow HPLC-MS/MS analysis
The precipitated protein was resuspended and subjected to digestion with trypsin. The resulted peptide mixtures were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Agilent 1100 quaternary HPLC system (Agilent, Palo Alto, CA) connected to an LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA) through an in-house built nanoelectrospray ion source. The peptide mixture was pressure-loaded onto a 15 cm long capillary column (100-μm i.d.) packed with 3μm Aqua C18 resins (Phenomenex, Ventura, CA). They were separated with a 4-hr gradient from 5% to 60% acetonitrile in 0.1% formic acid and a flow rate of 300 nL/min (through split). As peptides were eluted from the analytical column, they were electrosprayed (distal 2.5 kV spray voltage) into the mass spectrometer. MS instrument method consisted of a full-scan MS analysis (400-1800 m/z) followed by data-dependent MS/MS scans of the 5 most intense precursors at a 35% normalized collision energy with dynamic exclusion for 60 s. MS/MS spectra were processed and searched with the SEQUEST algorithm against the EBI human IPI database (ftp://ftp.ebi.ac.uk/pub/databases/IPI/, version 3.30), that was concatenated to a decoy database in which the sequence for each entry in the original database was reversed. SEQUEST search results were assembled and filtered using the DTASelect (version 2.0) program (Cociorva et al., 2007; Tabb et al., 2002).

Quantitative PCR
qRT-PCR was performed on cDNA (synthesized of TRIzol extracted total RNA using iScript reagent (Bio-Rad)) using SsoAdvanced SYBR Green reagent on the CFX384 detection system (Bio-Rad). Relative expression values were determined using the standard curve method, and abundance was normalized to 36B4. Values represent averages from three biological samples. Primer sequences were described previously (Cho et al., 2012).

Animal studies
Mice were housed under 12 hour light:dark cycle. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at The Salk Institute for Biological Studies. Homozygous FBXW7 floxed mice (stock number: 017563) and Albumin-Cre animals (B6.Cg-Tg(Alb-cre)21Mgn/J, stock number: 003574) were obtained from Jackson Laboratory. Homozygous FBXW7 floxed and hemizygous Cre transgenic males were bred with homozygous Fbxw7floxed females to generate Cre-positive and Cre-negative littermates. Mice entrained to 12-hour light-dark cycle were sacrificed using CO2 at indicated ZT and the livers flash-frozen. GTTs and PTTs were conducted after overnight fast. Mice were injected intraperitoneally with 2 mg of glucose or 1.5mg of pyruvate per gram body weight, and blood glucose was monitored at 0, 15, 30, 60, 90 and 120 min using an OneTouch Ultra glucometer (Lifescan Inc.). ITTs were conducted after overnight fasting. Mice were injected intraperitoneally with insulin (0.75 U/kg body weight, Humulin R; Eli Lilly) and blood glucose monitored at 0, 15, 30, 60, 90 and 120 min using an OneTouch Ultra glucometer. Real-time metabolic analyses were conducted in a Comprehensive Lab Animal Monitoring System (Columbus Instruments). CO2 production, O2 consumption, RQ (relative rates of carbohydrate versus fat oxidation) and ambulatory counts were determined for 6 consecutive days and nights, with at least 24 h for adaptation before data recording. Analysis of total body composition was performed with an EchoMRI-100 (Echo Medical Systems, LLC). To observe *in vivo* REV-ERBα phosphorylation in mouse liver, animals were injected intraperitoneally with Roscovitine (100mg/kg) at ZT6. 4 hours later, animals were sacrificed and liver sample were harvested for western blotting using anti-REV-ERBα and pT275 antibodies.

Adenovirus preparation and administration
Full length FBXW7 and its enzymatic deficient mutant were cloned into pAd-CMV expressing vector. Adenovirus expression Cre recombinase or GFP were purchase from University of Iowa Viral Vector Core Facility, and were

amplified using 293a cells (Life Technology, Carlsbad, CA). Adenovirus were purified using Virapur purification system. 2x109 plaque-forming unit adenovirus was delivered using retro-orbital injection to C57BL6 or FBXW7 flox mice anaesthetized with isoflurane. Animals were sacrificed 4-7 days after injection and liver samples were harvested for gene expression analysis and western blotting.

RNA-Seq library preparation and sequencing

Total mouse liver RNA was isolated using Trizol (Invitrogen) and the RNeasy mini kit with on-column DNase digestion (QIAGEN). RNA purity was assessed by Agilent 2100 Bioanalyzer. Sequencing libraries were prepared from 100 ng of total RNA using the TruSeq RNA sample preparation kit v2 (Illumina) according to the manufacturer's protocol. Briefly, mRNA was purified, fragmented and used for first- and second-strand cDNA synthesis followed by adenylation of 3′ ends. Samples were ligated to unique adaptors and subjected to PCR amplification. Libraries were then validated using the 2100 BioAnalyzer (Agilent), normalized and pooled for sequencing. RNA-Seq libraries prepared from three biological replicates for each experimental timepoint were sequenced on the Illumina HiSeq 2500 using barcoded multiplexing and a 100-bp read length. Image analysis and base calling were done with Illumina CASAVA-1.8.2. Short read sequences were mapped to a UCSC mm9 reference sequence using the RNA-Seq aligner STAR (Dobin et al., 2013). Known splice junctions from mm9 were supplied to the aligner and de novo junction discovery was also permitted. Differential gene expression analysis, statistical testing and annotation were performed using Cuffdiff 2 (Trapnell et al., 2013). Transcript expression was calculated as gene-level relative abundance in fragments per kilobase of exon model per million mapped fragments and employed correction for transcript abundance bias (Roberts et al., 2011) . Results for genes of interest were also explored visually using the UCSC Genome Browser. Heatmaps were generated by Cluster with Javatree view software.

Statistics

All data are shown as means ± s.d. or means ± s.e.m. as indicated. Statistical analysis was performed using a two-tailed Student’s t-test. For all tests, P-values lower than 0.05 were considered statistically significant.

Supplemental tables

Table S1. Phenotypic comparison between FBXW7 Liver KO animals and REV-ERBα KO animals shows opposite metabolic parameters, Related to Figure 5.

	REV-ERBα KO	**FBXW7 LKO**
Hepatic triglyceride	decreased	increased
Plasma LDL cholesterol	increased	decreased
Plasma HDL cholesterol	increased	decreased
Bile acid level	decreased	increased
Fasting blood glucose	increased	decreased

Table S1. A phenotypic comparison between *FBXW7* liver knockout animal and REV-ERBα knockout animals (Le Martelot et al., 2009) shows opposite metabolic parameters between these two strains, suggesting the role of FBXW7 is to down-regulate REV-ERBα levels.

Table S2. Hepatic gene expression comparison between FBXW7 liver KO animals and REV-ERBα Liver transgenic animals at ZT8-12, Related to Figures 3, 4, 5 and S6.

Genes	**REV-ERBα Tg**	**FBXW7 LKO**
Bmal1	not detectable	not detectable
Rev-erbα	decreased	decreased
Cry1	no change	decreased
Per1	decreased	decreased
Dbp	decreased	decreased
Cyp7a1	increased	increased
Insig2a	decreased	decreased
hmgcr	increased	increased
Fas	increased	decreased
Elovl6	no change	no change
Fads	increased	increased
insig1	increased	increased
Aacs	no change	no chnage
ldlr	no change	no change

Table S2. Hepatic gene expression comparison between FBXW7 liver KO animals and REV-ERBα liver transgenic animals (Kornmann et al., 2007; Le Martelot et al., 2009) shows consistent pattern suggesting that REV-ERBα activity is elevated in FBXW7 KO animals.

Supplemental references

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