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ID2 (Inhibitor of DNA Binding 2) Is a Rhythmically Expressed Transcriptional Repressor Required for Circadian Clock Output in Mouse Liver*[S]

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Id2 **is a helix-loop-helix transcription factor gene expressed in a circadian manner in multiple tissues with a phase-locked relationship with canonical clock genes (1). Our previous studies have identified circadian phenotypes in *Id2* null mice, including enhanced photo-entrainment and disruption of activity rhythms, and have demonstrated a potent inhibitory effect of ID proteins upon CLOCK-BMAL1 transactivation of clock gene and clock-controlled gene activity (1). We have now begun to explore the potential role that ID2 may play in specifically regulating clock output. Here we show that ID2 protein is rhythmically expressed in mouse liver. Time-of-day-specific liver gene expression in $Id2^{+/+}$ and $Id2^{-/-}$ mice under circadian conditions was studied using DNA microarray analysis, identifying 651 differentially expressed genes, including a subset of 318 genes deemed rhythmically expressed in other studies. Examination of individual time courses reveals that these genes are dysregulated in a highly time-specific manner. A cohort of different functional groups were identified, including genes associated with glucose and lipid metabolism, *e.g.* serum protein *Igfbp1* and lipoprotein lipase. We also reveal that the $Id2^{-/-}$ mice show a reduction in lipid storage in the liver and white adipose tissue, suggesting that disruption of normal circadian activity of components of lipid metabolism can result in overt physiological alterations. These data reveal a role for the transcriptional repressor ID2 as a circadian output regulator in the periphery.**

Circadian rhythms are endogenous biochemical, physiological, and behavioral 24-h oscillations that persist under constant conditions and are generated by a series of intracellular transcriptional-translational feedback loops, composed of positive and negative components (2, 3). The positive loop consists of CLOCK (circadian locomotor output cycle kaput) and BMAL1 (brain-and-muscle-ARNT-like 1; also called MOP3), both basic helix-loop-helix (bHLH)[3] PER-ARNT-SIM domain proteins. CLOCK and BMAL1 form a heterodimer that can bind to the transcriptional E-box element (CACGTG). The heterodimer transactivates gene expression of clock genes: *cryptochrome* genes (*cry1* and *cry2*) and *period* genes (*mPer1*, *mPer2*, and *mPer3*). The additional components REV-ERBα and RORα close the interlocking loops. Complexes of mPER and CRY proteins act to repress the transactivational activity of the CLOCK-BMAL1 heterodimer. A significant circadian output is derived from the positive limb of the circadian clock: CLOCK-BMAL1 heterodimers can bind to E-box elements in the promoter regions of clock-controlled genes (CCGs), thereby rhythmically controlling cellular processes (2).

The master oscillator in the mammal is located in the hypothalamic suprachiasmatic nucleus (SCN). The SCN receives photic signals from the retina via the retinohypothalamic tract. The SCN synchronizes the phase of oscillators in peripheral tissues such as the heart, retina, and liver to coordinate biochemical and physiological processes in a temporal manner (2). These peripheral tissues have their own self-sustaining oscillators that are able to persist without input from the master pacemaker (4).

There are additional proteins that can interact with CLOCK and/or BMAL1, such as the bHLH orange transcriptional repressors Dec1 (Stra13/SHARP2/Bhlhb2) and Dec2 (5, 6). These factors may provide additional interlocking feedback loops that in turn provide stability and precision for the circadian clock molecular machinery (7–9). The addition of interlocking feedback loops may also provide regulatory elements in cellular output pathways; an example is defined by Dec1, which can regulate several downstream CCGs (5).

Clock output is a critical aspect of the circadian system, connecting the core molecular oscillator with the rhythmic regulation of cellular effectors, such as neurotransmitters, ion channels, and enzymes. These, in turn, regulate rhythmic physiology at the cell, tissue, and organism levels and finally regulate circadian aspects of metabolism and behavior (2, 3, 10). The core clock machinery orchestrates circadian rhythms of physiology and behavior through regulation of CCGs. These are defined as

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[3] The abbreviations used are: bHLH, basic helix-loop-helix; CT, circadian time; FFA, free fatty acid; qRT-PCR, quantitative reverse transcription-PCR; SCN, suprachiasmatic nucleus; WT, wild type; ANOVA, analysis of variance; CCG, clock-controlled gene.

genes driven by the core oscillator components, but these genes do not contribute to the generation of global rhythmicity in the cell, and it is through CCGs that specific aspects of cell biology are clock-controlled. Approximately 2–10% of the mRNAs in a given tissue are under circadian regulation (11).

ID2 (inhibitor of DNA binding 2) is an important transcriptional repressor that has been studied in the context of cell proliferation and differentiation (12, 13). Each ID protein contains a helix-loop-hexlix domain through which it can interact with bHLH proteins. Dissimilar to BMAL1 and CLOCK, ID2 lacks the basic domain that allows binding to the E-box element. This leads to the possibility that ID2 is capable of modifying the transactivation of clock genes and CCGs by interfering with CLOCK-BMAL1 heterodimers, the bHLH orange factors, and other bHLH factors.

Our previous studies have revealed a conserved circadian rhythm of *Id2* expression across multiple tissues and cell lines (SCN, heart, and fibroblasts) with a phase-locked relationship with the canonical clock genes (1, 14). *Id1*, *Id3*, and *Id4* genes are also rhythmically expressed in various tissues and phase coincident specifically in the SCN. We have also identified *Id2* null mice circadian phenotypes, including larger phase shifts produced under parametric entrainment conditions, and disruption of activity rhythms in constant dark conditions. Furthermore, we have demonstrated a potent inhibitory effect of ID2, ID1, and ID3 upon CLOCK-BMAL1 transactivation of the clock gene *mPer1* and the CCG *AVP* (arginine vasopressin) (1).

Based on these observations, it is plausible that rhythmic ID2 activity could modulate all three hierarchical components of the circadian clock, namely input, pacemaker, and output. This action might be through ID2 interfering with CLOCK-BMAL1 heterodimer formation and in turn transactivation activity, and additionally through the modulation of the activity of other bHLH factors. We used DNA microarray and qRT-PCR analysis of time-of-day-specific collections of RNA from wild type (WT) and $Id2^{-/-}$ mouse liver to examine canonical clock components ("clock genes"), CCGs, and circadian clock output processes that might be affected by the absence of ID2.

EXPERIMENTAL PROCEDURES

Mouse Tissue Collection—Animal husbandry protocols were approved by the University of Notre Dame and Dartmouth Medical School Institutional Animal Care and Use Committees. Generation of *Id2* mutant mice and determination of genotypes were as described previously (1). $Id2^{+/+}$ and $Id2^{-/-}$ mice were on a mixed background (129sv/C57BL6J/FBVN, $n \geq 3$ for each time point, aged 3–5 months at time of sacrifice). Standard diet food and antibiotic water were provided *ad libitum*. The mice received sterile water treated with antibiotic (sulfamethoxalzole and trimthoprim oral suspension (Alpharma, Fort Lee, NJ) at a final dilution of 400 and 80 mg/liter, respectively) and sterile food. The mice were maintained on a 12:12 light-dark cycle for at least 4 weeks and transferred to constant darkness one full day prior to tissue collection. WT and $Id2^{-/-}$ mice were sacrificed at 4-h intervals at circadian times (CT) 0, 4, 8, 12, 16, and 20. CT12 is the time when the lights went off during the prior light-dark cycle. The liver samples were collected and immediately frozen in dry ice and subsequently stored at −80 °C.

RNA Extraction—RNA extraction was performed as described previously (1, 14, 15). The tissues were homogenized with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA from CT8, CT12, and CT20 was used for microarray analysis, and samples from all time points were used for real time qRT-PCR ($n = 3–6$ for each genotype and circadian time). The samples were visualized following separation on an agarose gel to test for RNA quality, and the samples showing degradation of ribosomal RNA were eliminated from further studies.

Microarray Experiment—5 μg of RNA from CT8, CT12, and CT20 ($n = 3$ for each genotype) was further purified using the RNeasy kit (Qiagen), including DNaseI treatment on the column. RNA quality was verified by using an Agilent BioAnalyzer (RNA integrity no. ≥ 8.0). Subsequent steps including reverse transcription, labeled cDNA synthesis, hybridization onto Mouse 430 2.0 microarray chips (Affymetrix, Santa Clara, CA), and scanning of microarrays were conducted at the Dartmouth Medical School Genomics and Microarray Laboratory according to the Affymetrix manual directions.

Microarray Data Analysis—Microarray analysis was performed using Genespring GX7.1 software (Agilent). Both the CEL and CHP files (MAS5 condensation algorithm) were analyzed to create the most robust list possible (16). CEL files were normalized using the Guanine Cytosine Robust Multi-Array Analysis (GC-RMA) method available through the Genespring software. The data were normalized using default options (data transformation set measurements from < 0.01 to 0.01; all of the measurements on each chip were divided by the median value per chip so as to adjust variations in intensity between chips, and each measurement per gene probe was divided by the median intensity of every measurement taken for that specific gene throughout the other chips; per gene was used to adjust for differences in efficiency of detection betweens spots). Genes were deemed to be expressed in the liver where at least two of six samples (CT8, CT12, and CT20 comparisons) or five of twelve samples (CT8/CT12 combined comparison) were considered present in the CHP files according to the MAS5 algorithm, *i.e.* "flagged Present." This level of filtering was considered appropriate such that if a gene was not expressed in one genotype but expressed in the other, it would still be included in the analysis. Mean fold changes of ≥1.3 were determined to be differentially expressed between WT and $Id2^{-/-}$ samples. This level of filtering was based on the findings of other microarray studies that a large proportion of CCG profiles exhibit low amplitude rhythms in this range (1.3–2.5-fold) (5, 11, 14, 17, 18). To be consistent with some other studies (19, 20), we also highlighted genes that exhibited a ≥2.0-fold change (highlighted in bold within supplemental Tables S1–S4). A two-tail Student's *t* test was performed to compare between WT and $Id2^{-/-}$ samples for each time-specific cluster, and genes with a *p* value < 0.01 were included in further analysis. *p* values < 0.01 were determined to be significant because no multiple testing corrections were conducted. Four lists, corresponding to CT8/CT12, CT8, CT12, and CT20 time-specific analyses, were generated. To determine whether a gene was a rhythmically

expressed CCG, published studies and online microarray data bases were analyzed (17, 20–23) (supplemental Tables S1–S4). In some of the data bases (21, 22), genes were found with COSOPT multiple measures corrected β (MMC-β) values $<$ 0.20 (19) (in some cases genes exhibiting pulsatile rhythms, that would not score well with the COSOPT cosine wave fitting algorithm, were also noted as rhythmic based on visual inspection of the gene profile in the data base). Genes that have previously been ascribed a role in lipid metabolism were identified (supplemental Tables S1–S4).

Annotations for the genes were obtained by using NetAffyx or through the National Center for Biotechnology Information websites. Venn diagram analysis was conducted to identify genes common to the various time point analyses using the Pangloss protocols website.

qRT-PCR Analysis—Total RNA extracted was treated with DNaseI (Invitrogen) and used for cDNA synthesis using a TaqMan reverse transcriptase kit (Applied Biosystems, Foster City, CA) primed with random hexamers. PCR thermocycling and qRT-PCR were performed as previously described (1, 15), using SYBR green reagents, an ABI PRISM 7700 sequence detection system, and quantification based on the generation of standard curves. Dissociation curves to test for primer dimers were generated using dissociation curve software (ABI). Normalization of genes was calculated relative to *ARP* (acidic ribosomal phosphoprotein) (1, 15). Two-tailed Student's t-tests were performed at individual time points to determine the statistical significance between WT and $Id2^{-/-}$ samples.

Western Blot Analysis—Liver tissue was harvested from the right anatomical lobe ($n = 3$ mice/time point). Liver was homogenized in radioimmune precipitation assay lysis buffer plus 1% protease/phosphatase inhibitor mixture (Sigma-Aldrich) passed through a 25-gauge 1.5 syringe and incubated at 4 °C for 30 min. The samples were boiled in Laemmli sample buffer for 5 min (Bio-Rad) and loaded at 40 or 80 μg of protein/lane onto 12.5% SDS-polyacrylamide gels. Following separation at 125 V for ~90 min, the proteins were transferred onto nitrocellulose membranes, which were then blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20. The membranes were incubated with rabbit antisera against ID2 (C-20, sc-489, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or actin (1:1000; Cytoskeleton Inc., Denver, CO) overnight at 4 °C. Immunoreactive bands were visualized using anti-rabbit secondary antiserum and ECL detection (Thermo Scientific, Waltham, MA). Quantification of the bands was conducted using National Institutes of Health ImageJ software. Cell lysates from Cos7 cells transfected, using Lipofectamine PLUS reagent, with human *Id2* tagged with FLAG (hId2-F) in pET12c vector were used as a positive control.

Gonadal Fat Pad Analysis—Weights were determined gravimetrically. 15–40-week-old male and female $Id2^{+/+}$, $Id2^{-/-}$, and $Id2^{+/-}$ mice ($n \geq 6$) were weighed and subsequently sacrificed to collect the gonadal fat pads proximal to the testis/uterus. Fat pad weight was determined, and the mass of gonadal fat pads/body mass/age (weeks) was calculated. One-factor ANOVA followed by *post-hoc* Dunnett's t-tests were conducted to determine statistical significance between WT and $Id2^{-/-}$ samples.

Oil Red O Staining—WT and $Id2^{-/-}$ mice ($n = 3$ for each genotype) were intracardially perfused with phosphate-buffered saline 4% paraformaldehyde. Liver was post-fixed overnight, cryopreserved in sucrose, and frozen at −80 °C. 8-μm frozen sections were cut using a cryostat. The slides were air dried, fixed in ice-cold 10% formalin (Fisher) for 5 min, rinsed in two changes of distilled water, placed in two changes of propylene glycol (Fisher) for 5 min, stained in 0.5% oil red O solution for 7 min while agitating, rinsed in 85% propylene glycol for 3 min in distilled water, washed in tap water for 5 min, and finally rinsed in two changes of distilled water. The slides were mounted with fresh glycerol jelly (0.5 g of gelatin powder, 3 ml of water, 3.5 ml of glycerol). The liver sections were visualized using an Olympus AX70 microscope at 40× magnification, and digital colored images were captured with a Spot 2.3.0 camera (Diagnostic Instruments Inc., Sterling Heights, MI; $n = 10$ for each animal). The images were analyzed using ImageJ software, and measurements were determined in pixels for the total area that the red fat droplets occupied. Two-tailed Student's t test was performed to determine the statistical significance between WT and $Id2^{-/-}$ samples.

RESULTS

Circadian Expression of ID2 in the Liver—Previous studies in our laboratory have shown *Id2* to oscillate with a 24-h rhythm in fibroblasts, SCN, and the heart (1, 14). Using qRT-PCR, we have confirmed earlier reports that show *Id2* to oscillate in the liver, with a peak phase at CT4 (Fig. 1*A*) (20, 23). Because there are examples of rhythmically expressed transcripts being constitutively expressed at the protein level (24), it was important that we analyzed levels of ID2 protein from WT mice. When 40 μg of liver protein was subjected to SDS-PAGE and probed with an anti-ID2 antibody, ID2 was only detected at CT8, CT12, and CT16, with a peak intensity at CT12 (Fig. 1*B*). When 80 μg was examined under the same conditions, ID2 was detected at all time points and in a clearly rhythmic profile (Fig. 1*C*; one-factor ANOVA, $F_{5,12} = 3.6$, $p < 0.05$). The peak to trough amplitude of the ID2 protein rhythm was 4.5-fold when measured at 80 μg of protein concentration and considerably larger when examined using the 40-μg blots. The peak and nadir phases occurred consistently at CT12 and CT20–CT0, respectively. The presence of a 24-h rhythm at both the transcript and protein level reveals that ID2 is under pronounced circadian regulation in the liver. ID2 protein peak level phase lags that of the transcript rhythm by ~6–8 h. These results highlight the possibility of downstream rhythmic regulation of genes through ID2-bHLH factor interaction.

Id2 Is Not Required for Normal Circadian Pacemaker Activity in the Liver—CLOCK-BMAL1 transactivate their target genes by forming a heterodimer that binds to DNA via their basic domains. ID2, however, lacks the basic domain; thus we hypothesize that ID2 negatively regulates the circadian system through the interference in CLOCK-BMAL1 heterodimerization. Recent studies from our lab have shown that the presence of ID2, ID1, or ID3 has a potent inhibitory effect on CLOCK-BMAL1 transactivation (1). Thus, we analyzed the canonical clock genes *mPer1*, *mPer2*, and *Bmal1*, known to be under both direct and indirect CLOCK-BMAL1 regulation (Fig. 2). All

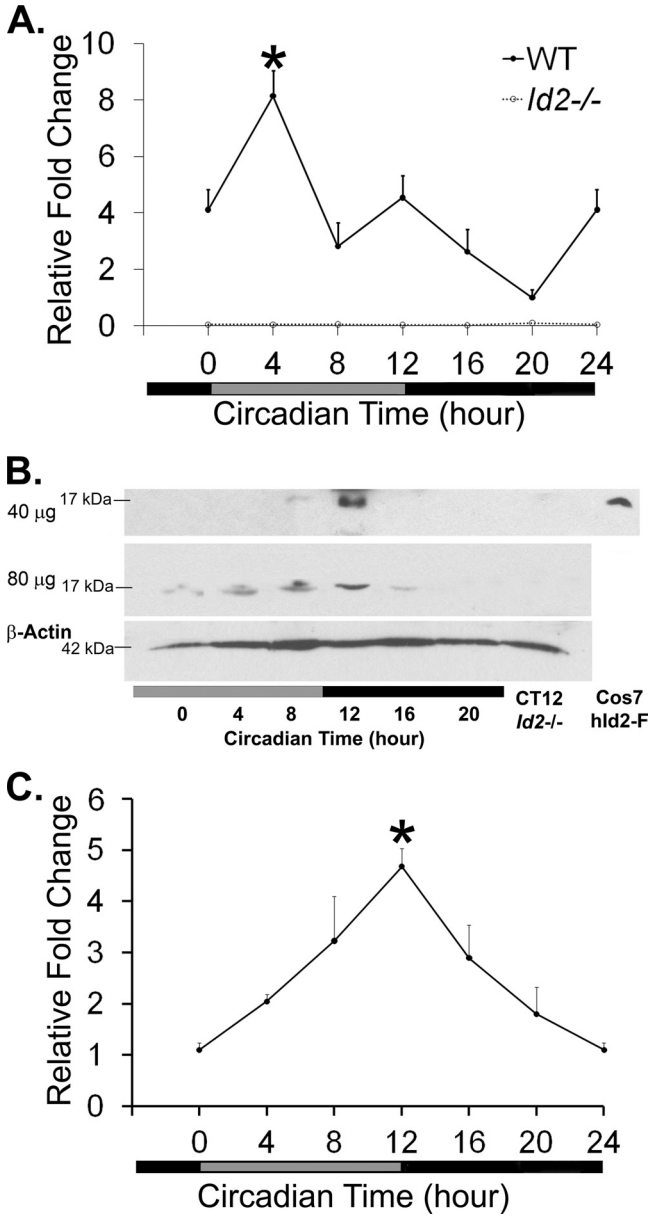

FIGURE 1. *A*, real time qRT-PCR of *Id2* mouse liver ($n \geq 3$) showing rhythmic profiles of *Id2*. The values are the means ± S.E. fold change of expression relative to the lowest expression value (*Id2* and *ARP* using SYBR green, ABI 7700, normalized to *ARP*). One-way ANOVA was performed to determine the significance of the *Id2* transcript rhythm with post-hoc Dunnett's *t*-tests (*, $p < 0.05$). *B*, representative blots for determining ID2 protein rhythm ($n = 3$–5) using 40 and 80 μg of total protein extracts. Visualization of β-actin was used to ensure equal protein loading. A positive control was Cos7 cells transfected with human Id2-FLAG. *C*, quantification of ID2 protein rhythm. Levels of ID2 were determined by normalizing ID2 blots to their respective β-actin signal. Internal normalization was conducted by making the nadir of the rhythm equal to 1.0. One-way ANOVA was performed to determine the significance of the ID2 protein rhythm with post-hoc Dunnett's *t*-tests (*, $p < 0.05$).

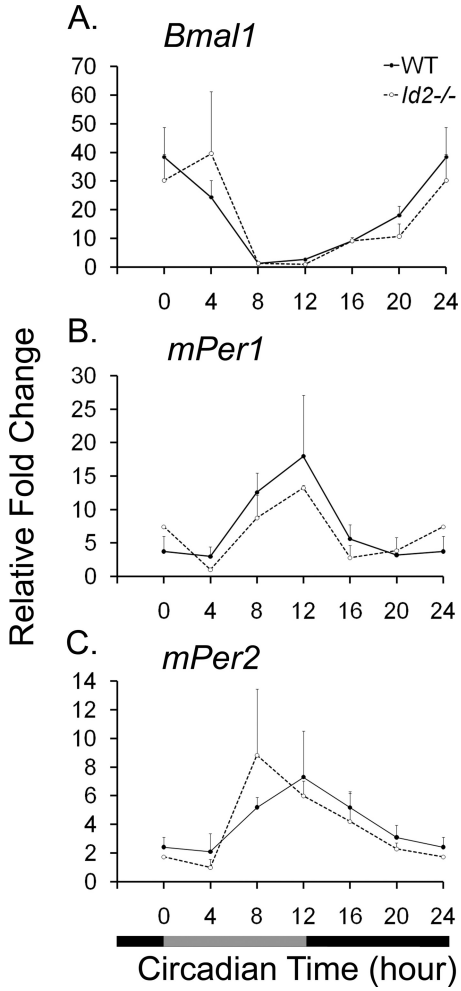

FIGURE 2. **qRT-PCR of *Bmal1* (*A*), *mPer1* (*B*), and *mPer2* (*C*) in mouse liver ($n \geq 3$).** The values are the means ± S.E. fold change of expression relative to the lowest expression value (SYBR green, ABI 7700, normalized to *ARP*). Two-tailed Student's *t* test was performed at individual time points, and it was determined that there was no statistical difference between WT and $Id2^{-/-}$ samples. The data from circadian time 0/24 is double plotted.

three genes oscillated with distinct circadian rhythms in the WT liver, with peak phases of CT0 (*Bmal1*) and CT12 (*mPer1*, *mPer2*) and amplitudes of 40-, 18-, and 9-fold, respectively. These expression profiles are consistent with published data with respect to peak phase and amplitude (15). Profiles derived from $Id2^{-/-}$ liver were not statistically different from WT controls, suggesting that the core molecular oscillator in the liver is intact in the *Id2* null mouse (Student's *t* tests at each circadian time; values not significant).

Id2 Is Involved in Regulating Clock-controlled Outputs in the Liver—The peak of ID2 protein occurs at CT12, with the second highest value at CT8 and has a nadir at approximately CT20. Thus, we conducted our microarray experiment on tissue harvested at these specific phases. The microarray data for CT8 and CT12 were analyzed independently and in combination to generate gene lists corresponding to the predicted highest inhibitory effect of ID2. CT20 was analyzed separately because it corresponds to the predicted minimal effect of ID2 on its targets. For the CT8/CT12 gene list, 79 genes are found to be differentially expressed between WT and $Id2^{-/-}$ mice (Fig. 3*A* and supplemental Table S1). Published microarray analyses were then consulted to determine whether the differentially expressed genes in the $Id2^{-/-}$ liver were known rhythmically expressed CCGs. Using this approach, 70% of the CT8/CT12 differentially expressed genes were previously identified CCGs. For the CT20 gene list, 131 genes are found to be differentially expressed between WT and $Id2^{-/-}$ mice, and 47% of those

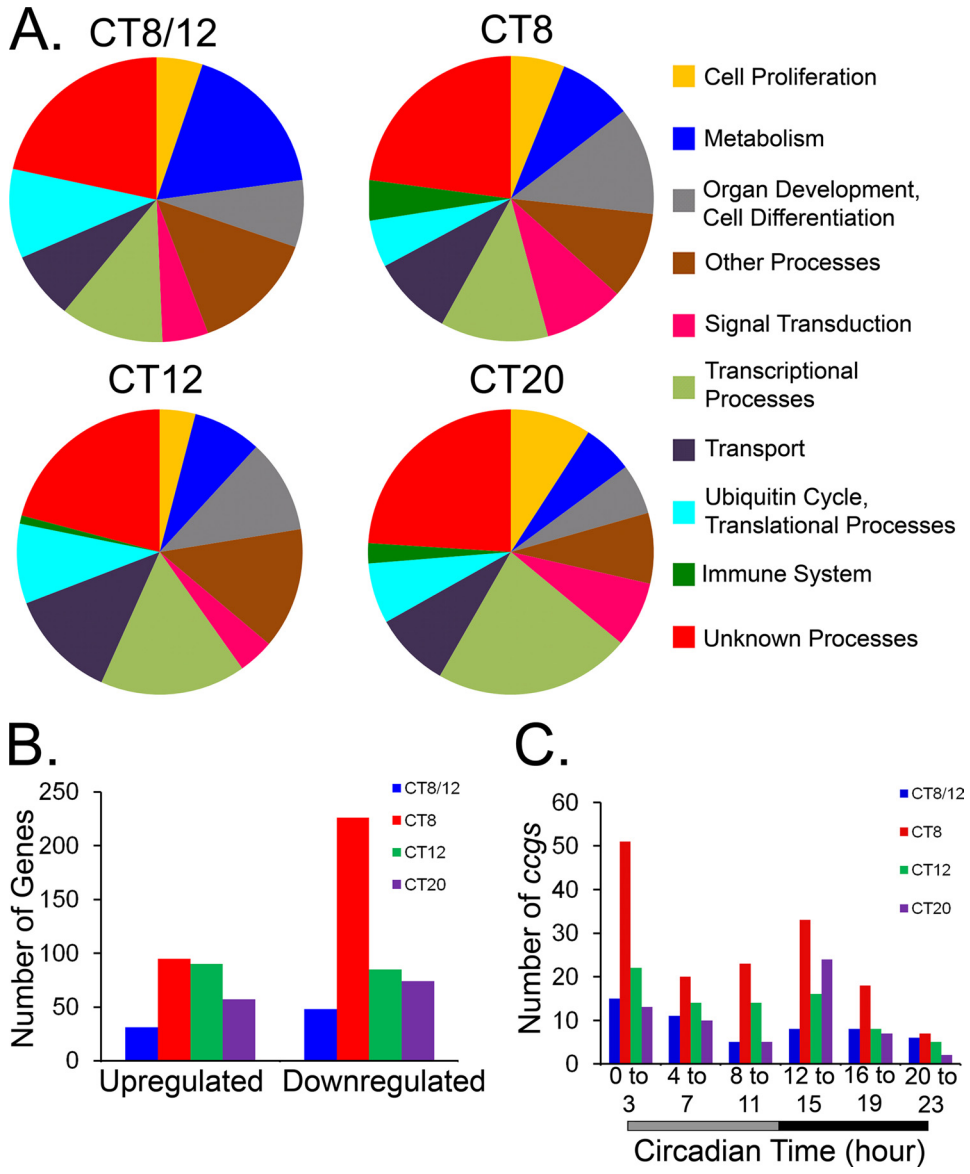

FIGURE 3. *A*, genes involved in various cellular processes were identified by microarray experiment as differentially expressed in the absence of *Id2* at CT8/12, CT20, CT8, and CT12. Microarray experiments ($n = 3$/genotype) at CT8, CT12, and CT20 identified genes involved in various cellular processes that are differentially expressed ≥1.3-fold change in the absence of *Id2*. *B*, 38 and 62% of genes identified as differentially expressed were up- and down-regulated, respectively, in the absence of *Id2*. *C*, distribution of $Id2^{-/-}$ differentially expressed CCGs clustered according to phase of peak expression; 60% of these genes had a peak phase in subjective morning, whereas 40% of genes had a peak phase in the subjective night; CCGs were identified from published reports and microarray data bases (17, 20–23).

genes are known CCGs (Fig. 3*A* and supplemental Table S2). For the CT8 and CT12 gene lists, respectively, 321 and 175 genes were differentially expressed (Fig. 3*A* and supplemental Tables S3 and S4), 47 and 45% of which are known CCGs. These numbers are reduced if we use the more stringent criteria of the COSOPT algorithm of MMC-β values < 0.15 (306 genes) and < 0.10 (295 genes) rather than ≤ 0.20 (318 genes) in our examination of the published data bases (19, 21, 25). Using a 2-fold expression change as a filter, we identified 15, 41, 77, and 31 genes at CT8/CT12, CT20, CT8, and CT12, respectively (genes noted in bold type in supplemental Tables S1–S4).

39 ± 5% of genes determined to be differentially expressed were shown to be up-regulated, whereas 61 ± 5% were down-regulated (Fig. 3*B*). These distributions showed limited change when CT8/12, CT8, CT12, and CT20 were analyzed separately (39, 30, 51, and 44% up-regulated at CT8/12, CT8, CT12, and CT20, respectively). Because ID2 acts as a transcriptional repressor, this finding is surprising because one would expect most genes to be up-regulated in the absence of ID2. Rhythmically expressed CCGs with various peak phases, as identified from published papers and data bases (17, 20–23), were differentially expressed in the absence of ID2. Their distribution of peak phases fell almost equally between subjective day (59 ± 4% of CCGs) and night (41 ± 3%; Fig. 3*C*).

For CT8/CT12, 13 genes (18%) of differentially expressed genes are involved in metabolic processes and represent the largest proportion of genes in a functional group (Fig. 3*A*). For CT8, CT12, and CT20, 25 (8%), 10 (6%), and 11 (8%) genes are components of metabolic pathways, respectively. One observation to note is that no core clock components (clock genes) were identified as differentially expressed in the absence of *Id2*. This is consistent with our qRT-PCR analysis of *mPer1*, *mPer2*, and *Bmal1*. Venn diagram analysis revealed an overlap of genes between the specific time points (supplemental Table S5) and cross-study comparison revealed 23 differentially expressed genes common to $Id2^{-/-}$ and *Clock* gene homozygous (*Clk/Clk*) mutant mice and to $Id2^{-/-}$ and $Dec1^{-/-}$ mice (supplemental Table S6 and supplemental Results and Discussion).

To verify that ID2 regulates a subset of CCGs, we examined five genes found to be differentially expressed by microarray analysis in $Id2^{-/-}$ mouse liver that have been reported as CCGs in other studies (17, 20–23) (Fig. 4). qRT-PCR was used to examine gene expression across the entire circadian day (CT0–CT20), and peak phases of CCG expression profiles in WT liver were consistent with published reports. Significant differences were detected between predicted peak and nadir phases for *Igfbp1* (insulin-like growth factor-binding protein 1), *LpL* (lipoprotein lipase), *Acot1* (acyl-CoA thioesterase 1), and *HexB* (hexosamidase B) (Wilcoxon test or Student's *t* test, $p < 0.05$). *Igfbp1* and *LpL* in both WT and $Id2^{-/-}$ liver showed rhythmic profiles but with changes in

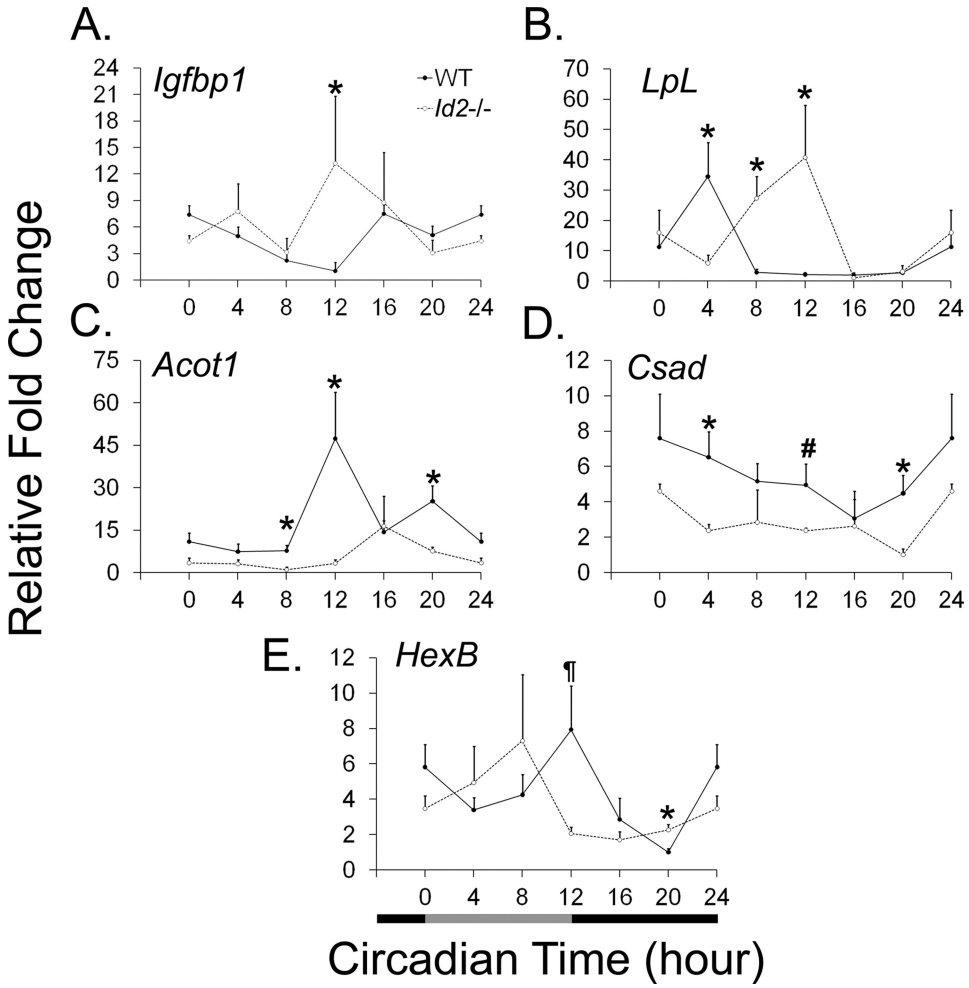

FIGURE 4. **qRT-PCR reveals time-specific disruption of clock output in the absence of *Id2*.** Shown are *Igfbp1*, *LpL*, *Acot1*, *Csad*, and *HexB* in mouse liver ($n \geq 3$ per time point and genotype) at CT0–20. The values are the means ± S.E. fold change of expression relative to the lowest expression value (SYBR green, ABI 7700, normalized to *ARP*). Two-tailed Student's *t* tests were performed at individual time points to determine the statistical significance between WT and $Id2^{-/-}$ samples. *, $p < 0.05$. For *Csad* at CT12, a one-tailed Student's *t* test was performed because it was identified in the microarray experiment as up-regulated in $Id2^{-/-}$ liver. #, *p* value < 0.05. For *HexB* at CT12, a two-tailed Student's *t* test was performed because it was not identified in the microarray experiment as differentially expressed at CT12, and it was determined to be not significant. ¶, $p = 0.065$. The data from circadian time 0/24 is double plotted.

their peak phase: WT peak phases for *Igfbp1* and *LpL* rhythms were at CT0 and CT4, respectively, consistent with published reports. However, their peak phases in $Id2^{-/-}$ occurred at CT12. Examination of *Acot1* and *Csad* (cysteine sulfunic acid decarboxylase) revealed damped levels of expression across most circadian phases. Although *Csad* did not show a change in the peak phase, *Acot1* showed a peak phase change from CT12 in WT to CT16 in $Id2^{-/-}$ liver. *HexB* showed a distinct shift in the peak phase of the rhythm from CT12 to CT8.

Clock Output Regulation of Metabolism Is Modified in $Id2^{-/-}$ Mice—Various genes involved in lipid metabolism were found to be differentially expressed (Table 1 and supplemental Fig. S1). As many as 14 of the 30 genes identified as differentially expressed were previously described CCGs (Table 1) (17, 20–23). The functions of these genes include direct involvement in fatty acid metabolism (*Acot1* and *LpL*), in cholesterol metabolism (*Akr1c20* and *Scarb1*), and as transcription factors regulating lipid metabolism (*G0s2*, *Rarb*, and *Ppargc1a*).

The serum protein lipoprotein is synthesized by the liver and carries lipid molecules throughout the body via the blood circulation. LpL hydrolyzes triglycerides associated with lipoproteins into free fatty acids (FFAs) and glycerol (26). This results in triglycerides shuttling to the liver where very low density lipoprotein and ketone production are increased, allowing glucose to be spared for other tissues, such as brain and muscle (27). FFAs are then activated by the formation of a thioester linkage between FFA and CoA. The enzyme that catalyzes this reaction belongs to a family of acyl-CoA synthases, including acyl-CoA synthase medium chain family member 2 (Acsm2) (28). Acyl-CoA can then be used to generate energy by undergoing β-oxidation or be extended by elongation. *LpL* was found to be up-regulated at both CT8/12 and CT20, and qRT-PCR shows that *LpL* was up-regulated dramatically in $Id2^{-/-}$ mouse liver at CT8 and CT12 (Fig. 4*B*). Conversely, *Acsm2* was down-regulated at CT8/12 and CT20 (supplemental Fig. S1). The altered expressions of these genes may result in altered lipid metabolism.

Enzymes involved in the elongation of acyl-CoA were also found to be differentially expressed (supplemental Fig. S1). For example, the gene for acetyl-CoA carboxylase, which is the committed step in the elongation of acyl-CoA (29), was up-regulated at CT8. Malate dehydrogenase, which is required to generate NADPH for the elongation of acyl-CoA (30), was down-regulated at CT20. Lastly, the hydrolysis of elongated acyl-CoA to yield elongated FFA and CoA is catalyzed by the enzyme Acot1 (31). *Acot1* was down-regulated in $Id2^{-/-}$ mouse liver at all three time points: CT8, CT12, and CT20 (Fig. 4*C* and supplemental Fig. S1).

The elongated FFA can either be stored or be used to activate PPARα, a nuclear steroid hormone receptor involved in regulation of fatty acid metabolism. Signaling through PPARα occurs through the direct interaction between the ligand and PPARα or is mediated through signaling from adiponectin and its receptor (32) (supplemental Fig. S1). *AdipoR2* (adiponectin receptor 2) was down-regulated at CT8 and CT8/12, further suggesting a dysregulation in lipid metabolism in the absence of *Id2*.

Taurine is an amino acid synthesized from cysteine and is important in maintaining glucose homeostasis, osmoregula-

TABLE 1

Genes involved in lipid metabolism identified by microarray experiment as differentially expressed in the absence of *Id2* at CT8/12, CT8, CT12, and CT20

The peak phase of the circadian rhythm of each gene is provided if documented in published reports and/or associated online databases. References refer to the studies linking the genes to their roles in lipid metabolism. NA, not applicable.

Symbol	Description	Circadian time	Circadian peak	Reference
Abca1	Transporter: lipid removal	12	NA	51
Acac	Enzyme: fatty acid synthesis	8	NA	52
Acot1	Enzyme: hydrolysis of Acyl-CoA to release fatty acid and CoA	20	17 (20)	31
Acsm2	Enzyme: fatty acid ligation with CoA	8/12, 20	NA	28
Adipor2	Receptor: fatty acid oxidation and glucose uptake	8, 8/12	3 (20)	53
Akr1c20	Enzyme: steroid metabolism	8, 8/12	22 (20)	54
Apom	Transporter: lipoprotein processing	8	NA	55
Atrn	Transmembrane protein: energy expenditure	12	NA	56
Bbs4	Putative enzyme: leptin sensitivity	8	NA	57
Cpe	Enzyme: hormone processing, implicated in obesity development	8	10 (20)	58
Crat	Enzyme: fatty acid transport	12	NA	59
Enho	Serum protein: lipid homeostasis	8	3 (20)	60
G0s2	Transcription factor: adipocyte differentiation and adipogenesis	8/12	14 (20)	61
Hdlbp	Transporter: cholesterol excretion	8	NA	62
Hpgd	Enzyme: prostaglandin degradation	8, 12	21 (20)	63
Ifi27	Receptor: adipocytokine receptor	20	NA	64
LpL	Enzyme: hydrolysis of lipoproteins	8/12, 20	2 (20)	26
Lsr	Receptor: lipoprotein processing	8	18 (20)	65
Mgat2	Enzyme: diacylglycerol synthesis	8	NA	66
Mod1	Enzyme: generation of NADPH byproduct for lipid biosynthesis	20	NA	30
Msr1	Receptor: lipoprotein binding	20	NA	67
Osbpl11	Receptor: cholesterol homeostasis	8	NA	68
Pdcd6ip	Adaptor protein: cholesterol homeostasis	8	20 (17)	69
Pex11a	Signaling factor: peroxisome proliferation	20	14 (20)	70
Ppargc1a	Transcription factor: fatty acid oxidation	20	12 (20)	45
Rarb	Receptor: regulation of adipogenesis	20	NA	71
Rps6kb1	Kinase: β-oxidation	20	NA	72
Sc4mol	Enzyme: cholesterol biosynthesis	8/12	NA	73
Scap	Adaptor protein: cholesterol homeostasis	8	3 (20)	74
Scarb1	Receptor: cholesterol uptake	20	2 (20)	75

tion, protein phosphorylation, and calcium modulation (33). Taurine improves insulin sensitivity in Otsuka Long-Evans Tokushima Fatty rat, a model for type 2 diabetes (34). Taurine synthesis begins with the oxidation of cysteine to cysteine sulfinic acid, followed by decarboxylation to hypotaurine, which is catalyzed by the rate-limiting enzyme cysteine sulfinic acid decarboxylase (Csad or Csd) (35). Our microarray and qRT-PCR analyses reveal a dampened rhythm of *Csad* across all circadian phases. Furthermore, the enzyme catalyzing the conversion of cysteamine to hypotaurine, *Ado* (2-aminoethanethiol (cysteamine) dioxygenase) was up-regulated at CT20, opposite to *Csad* that was down-regulated at this circadian phase. Both genes are transcribed in a rhythmic fashion, raising the possibility that the disrupted rhythmic gene expression may result in altered taurine levels.

Gangliosides are sphingolipids containing carbohydrates attached to ceramides and are incorporated into the outer leaflet of the plasma membrane and serve as cell surface markers in cell-to-cell communication (36). Gangliosides are degraded in the lysosome, disruption of which contributes to pathological conditions including atherosclerosis (37, 38). Gm2 ganglioside is degraded by the enzyme *HexB*, and atherosclerotic mice exhibit increased serum Gm2 ganglioside (39). *HexB* was up-regulated at CT20, and we speculate that the disruption of its normal rhythm may contribute to disturbed lipid metabolism.

$Id2^{-/-}$ Mice Exhibit Reduced Gonadal Fat Pad Mass and Fat Deposits in the Liver—While performing surgeries for other studies on mice aged 6 months to 1.5 years, we noticed that *$Id2^{-/-}$* mice had noticeably less white adipose tissue mass throughout the body cavity. To conduct a thorough study, we collected gonadal fat pads from male and female WT, *$Id2^{+/-}$*, and *$Id2^{-/-}$* mice. The analysis was focused on comparing the fat content based on the percentage of gonadal fat/body mass/weeks alive (Fig. 5*A*). One-factor ANOVA determined a significant difference between WT and *$Id2^{-/-}$* gonadal fat pads in both male and female subjects (female $F_{2,38} = 3.2$, $p < 0.05$; *post-hoc t* test, $Id2^{-/-}$ *versus* WT, $p < 0.05$; male $F_{2,33} = 3.3$, $p < 0.01$; *post-hoc t* test, $Id2^{-/-}$ *versus* WT, $p < 0.01$).

Because the liver is a major regulator of lipid metabolism, we investigated whether the level of lipid deposits is uniform throughout the liver. Liver sections were stained with oil red O to visualize the fat deposits (Fig. 5*B*). WT lipid deposits were located uniformly throughout the liver sections, but in contrast, deposits in the *$Id2^{-/-}$* liver were concentrated in specific regions, clustering together to form rings. When the area of droplets was quantified for each field of view, we found a significant reduction in fat deposition between WT and *$Id2^{-/-}$* liver (Fig. 5*C*; two-tailed *t* test $t_{42} = 2.0$, $p < 0.01$).

DISCUSSION

Id2 mRNA and Protein Are Rhythmically Expressed in Adult Liver—Id2 is one of four *Id* genes (*Id1–Id4*) that comprise a family of helix-loop-helix transcriptional repressors that can interact with bHLH transcription factors in a dominant-negative manner (12, 13). Heterodimerization between an ID protein and a bHLH transcription factor results in the suppression of the transcription factor because ID proteins lack the basic domain required for binding to DNA. Consistent with our earlier studies revealing *Id2* transcript rhythms in SCN, heart, and immortalized fibroblasts (1), our current results in the liver indicate that not only does *Id2* oscillate at the transcript level,

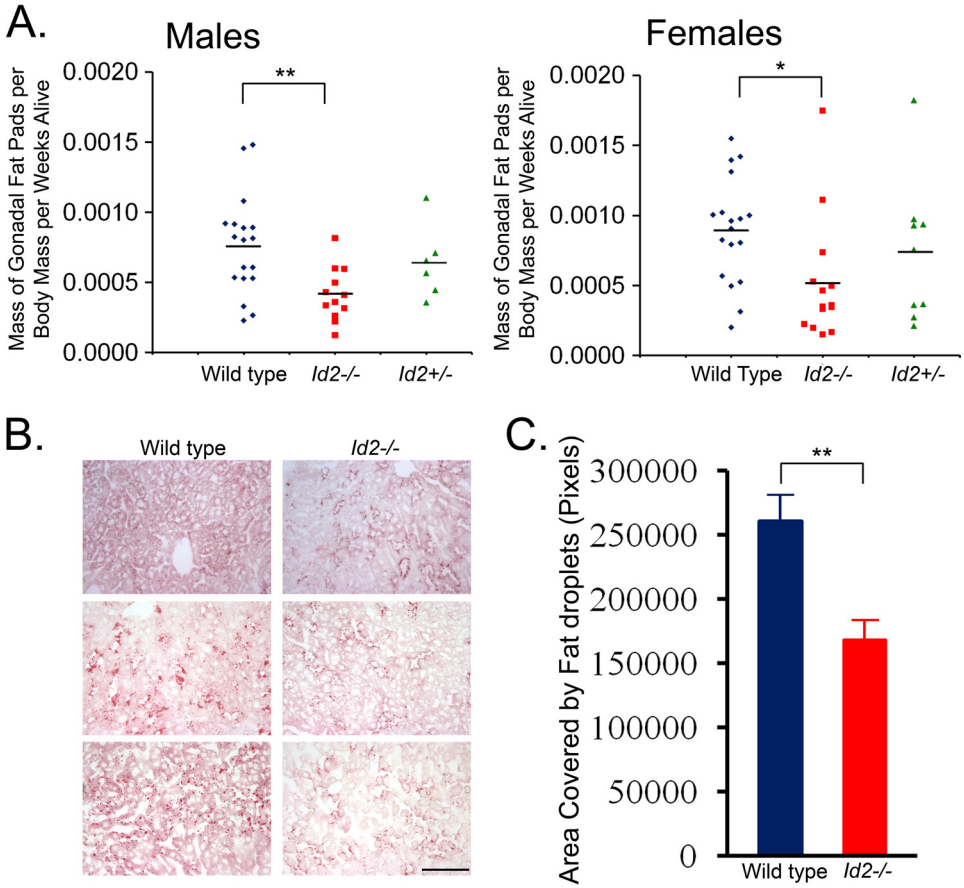

FIGURE 5. *A*, reduced white adipose tissue density in absence of *Id2*. Gonadal fat pads from WT, $Id2^{+/-}$, and $Id2^{-/-}$ male and female mice were weighed and compared based on the percentage gonadal fat per body mass per age (weeks). One-factor ANOVA followed by *post-hoc* Dunnett's t-tests. *, p value < 0.05; **, p value < 0.01. *B*, $Id2^{-/-}$ mice have significantly lower quantities of fat deposits in the liver. Oil red O staining of WT and $Id2^{-/-}$ mouse liver ($n = 3$ mice/genotype). The image shows sections from representative sections from each of three WT (*left*) and three $Id2^{-/-}$ (*right*) mice. *Scale bar*, 100 μm. *C*, quantification of fat deposits in WT and $Id2^{-/-}$ mouse liver sections ($n = 10$) from three different mice/genotype. The values are the means ± S.E. total area of oil O red droplets occupied in square pixels. Two-tailed Student's t test was performed to determine statistical significance between WT and $Id2^{-/-}$ samples. $t_{42} = 2.0$. **, $p < 0.01$.

the protein also oscillates. The amplitude of the protein rhythm is ~4.5-fold, with the peak and nadir phases occurring at CT12 and CT20–CT0, respectively. The presence and phasing of the *Id2* mRNA rhythm is consistent with that reported earlier (20, 23), with a peak at CT4 and nadir at CT16–CT20. Coincidently, such a large phase lag between the mRNA and protein rhythms has been observed for the canonical clock components. For example, the temporal lag between the peak phases of rhythmic transcript and protein for *mPer1* and *mPer2* in mouse liver is 6–9 h (40). Similarly, with the *Id2* transcript level in the mouse liver peaking at CT4 and the peak phase of the protein level delayed to CT12, this represents a phase lag of up to 8 h. Additionally, the peak activity of the CLOCK-BMAL1 transactivation is hypothesized to be at CT8 (20, 40), suggesting that the peak phase of ID2 at CT12 might contribute to stabilizing the positive loop driven by CLOCK-BMAL1 transactivation.

Normal Core Clock Function in the Absence of Id2—Our microarray and qRT-PCR analyses suggest that ID2 is not a critical component of the core oscillator required for the hepatic clock, but rather ID2 is involved primarily in clock output through the regulation of CCG expression. The fact that the rhythmic expression profiles of *Bmal1*, *mPer1*, and *mPer2*, which represent key components of the positive and negative transcriptional loops of the core oscillator, are not modified in the absence of *Id2*, is evidence of a functional circadian pacemaker in the $Id2^{-/-}$ liver. Our previous studies have determined that ID2 is involved in clock input, namely through photic entrainment of the SCN clock (1). A significant proportion of *Id2* null mice also exhibit disturbed or arrhythmic locomotor activity in constant darkness (1). This raises the possibility that ID2 might contribute to core pacemaker function or that this phenotype reflects a disturbance in the output pathways mediating rhythmic cycles of activity and rest. The current results suggest the latter as the most likely case, although care must be taken when comparing such diverse systems as the liver and SCN circadian clocks (15). There may also be redundancy in the functions of *Id1*, *Id2*, and *Id3*, such that the absence of *Id2* results in compensatory actions by *Id1* and *Id3*. Indeed, rhythmic expression of all *Id* genes, *Id1–Id4*, in the SCN all peak at similar times of the day and with similar amplitudes (1).

Gene Regulation in Liver by ID2—Our microarray analysis revealed that 70% of genes identified as differentially expressed between WT and $Id2^{-/-}$ mice at CT8/12 are known CCGs. Similarly, 47% of genes that are identified as differentially expressed at CT20 have been described as CCGs. These proportions are well beyond what might be predicted by chance, because only 3–9% of the genes screened in previous microarray studies of WT mice appear to be under clock control in the liver (11, 18–20, 23). The lack of overlap between genes differentially expressed in the $Id2^{-/-}$ liver during the subjective night (CT20) as compared with subjective day (CT8 and CT12) and the discovery that 49% of these genes are known CCGs provide compelling evidence for the role of ID2 in modulating pathways of clock output.

Genes differentially expressed did not cluster into one specific liver process, suggesting that ID2 contributes to a broad aspect of transcriptional regulation; this is consistent with the fact that the circadian clock controls various cellular and physiological functions (11). Interestingly, only 39% of genes determined to be differentially expressed in the absence of *Id2* were up-regulated (Fig. 3*B*). One might predict that the absence of a transcription factor repressor would result primarily in up-regulation of gene expression. This suggests the existence of

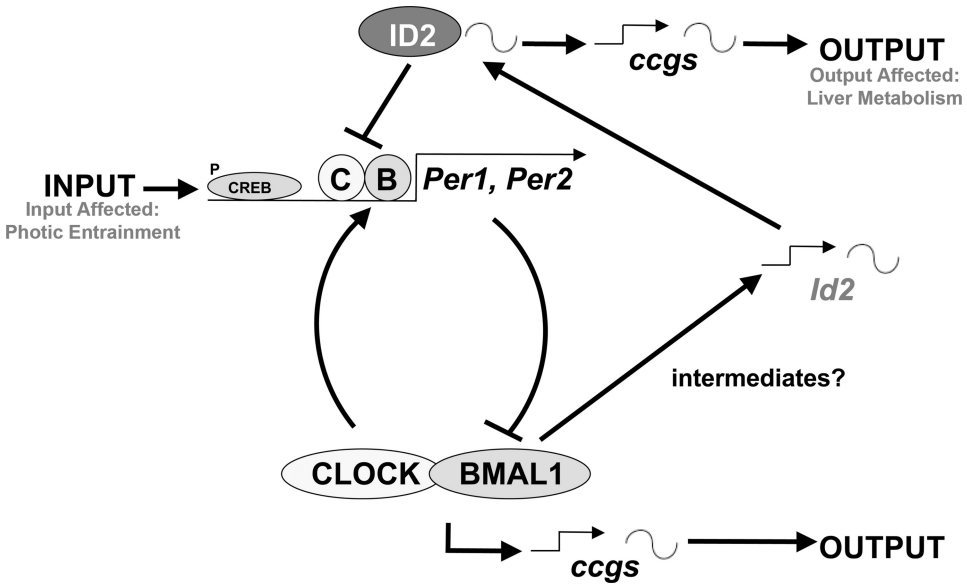

FIGURE 6. **A model for the action of ID2 in the circadian clock.** Expression of *Id2* is controlled by the clock, and ID2 in turn feeds back to regulate the expression of input and output genes yet is not required for circadian rhythmicity. Its action describes an autoregulatory feedback loop that closes outside the core oscillator but that affects aspects of circadian timing, including photic entrainment through the regulation of light responses (1) and regulation of lipid metabolism. Its actions may include interference with CLOCK-BMAL1 transactivation activity in both input and output regulation (1) and/or act through other bHLH transcription factor targets. *C*, CLOCK; *B*, BMAL1; *pCREB*, Ca^{2+}/cAMP response element-binding protein phosphorylated on Ser^{133}.

several regulatory mechanisms in play that are not mutually exclusive, including inhibition of both transcriptional activators and repressors (see supplemental Results and Discussion). Interestingly, our results are comparable with those found for the *Clk*/*Clk* mutant mouse liver, in which 21–42% of differentially expressed genes were up-regulated and 58–79% were down-regulated, and a proportion of these genes were not identified as CCGs (5, 19, 20).

A combination of the nadir and peak phases of the ID2 rhythm imparting time-of-day-specific activity and action through a secondary set of transcriptional activators/repressors would allow the ID2 rhythm to influence the expression of CCGs at almost all circadian phases of the 24-h day. In fact, this pattern of regulation across the entire circadian day is reflected in the microarray analysis, in which there is an almost even distribution of CCGs with peak phases at each circadian time interval (Fig. 3*C*). Overall, these results support the notion that there are multiple direct and indirect cascades of transcriptional control downstream of ID2 that can regulate rhythmic patterns of gene expression.

Surprisingly there were few genes identified as differentially expressed across multiple time points (supplemental Table S5). The highest level of overlap (15 genes) was found between the CT8/CT12 and CT8 groups, and the lowest overlap was between CT20–CT8, CT8–CT12, and CT20–CT12: 9, 8, and 5 genes, respectively. Apart from *Id2*, no other gene was found common between all three time points. The transcriptional regulator *Nudt16* (*Nudix* (nucleoside diphosphate-linked moiety X)-type motif 16) was common to CT8/CT12, CT12, and CT20 analyses. *Nudt16* is a nuclear localized RNA-binding and RNA-decapping hydrolase enzyme implicated in reproductive function and under steroid hormone control (41). Although the low level of overlap between the subjective night and day time points may be expected, it is somewhat surprising that there was little overlap between CT8 and CT12, given that the combined analysis at CT8/CT12 identified 79 differentially expressed genes.

We specifically examined five of the CCGs that were differentially expressed in the $Id2^{-/-}$ liver across the entire circadian day and discovered phase-specific alterations in their expression profiles. The qRT-PCR results showed a diverse response to the absence of ID2, ranging from an exaggerated amplitude (*Igfbp1*), damped expression (*Acot1* and *Csad*), and shifts in peak phases (*Igfbp1*, *LpL*, *Acot1*, and *HexB*). These results clearly reveal that ID2 can regulate gene expression in the liver in a highly temporal manner.

It is plausible that certain patterns of gene expression may be dysregulated in the $Id2^{-/-}$ liver in response to factors operating outside of the liver organ system or result from a developmental manifestation of the *Id2* gene deletion. However, clearly the high proportion of differentially expressed genes being known CCGs, the qRT-PCR examination of specific genes revealing highly temporal-specific regulation of gene expression, and the presence of a robust rhythm of *Id2* mRNA and protein expression in the adult animal provide compelling evidence for gene expression regulation to be under ID2 control both locally within the liver and post-development.

ID2 Regulation of Lipid Metabolism—The study has shown that genes involved in lipid metabolism are affected in a time-specific fashion and that $Id2^{-/-}$ mice have altered physiological characteristics. Microarray analysis identified genes involved in lipid biosynthesis including *LpL*, *Acsm2*, *Acot1*, *Mod1*, *Adipor2*, *Acac*, and *Cart* (28, 30–32, 42, 43) (Table 1 and supplemental Fig. S1). We speculate that because mice feed primarily during the night (44), an abnormal nocturnal rise in *LpL* in the $Id2^{-/-}$ mouse liver would result in reduced lipid storage. Furthermore, a decrease in *Acsm2*, *Acot1*, and *Mod1* transcript levels in the $Id2^{-/-}$ mice would likely reduce lipid storage biosynthesis. When the animal is feeding during the night phase, the lipids released from food are less likely to be stored in the $Id2^{-/-}$ animal. Thus, the desynchronization in expression of lipid catabolic enzymes and biosynthetic enzymes such as *Acac* may be correlated to the decrease in gonadal fat storage and liver fat deposition in $Id2^{-/-}$ mice. Our results are consistent with data from the *Clk* mutant mouse (10, 44), which show that a dysfunctional core clock results in an obese phenotype. Based on our previous studies showing potent ID2 inhibition of CLOCK-BMAL1 transactivation (1), the absence of ID2 might be predicted to result in an increase in CLOCK-BMAL1 transactivation of CCGs, and the expected phenotype should be opposite to that observed in the *Clk* mutant mouse. One interesting gene

to note is *Ppargc1a/PGC-1a* (peroxisome proliferative-activated receptor gamma coactivator 1a), which has been described to create a link between energy metabolism and the circadian clock by acting as a transcriptional activator for the expression of *Bmal1* and *Rev-erbα* and as a transcriptional coactivator regulating energy metabolism (45). *Ppargc1a* was 1.8-fold up-regulated in $Id2^{-/-}$ liver, possibly resulting in disturbance of downstream circadian and metabolic pathways.

ID2 Contributes to the Clock Output Complex—The microarray analysis also points to other cellular processes that are affected by the absence of *Id2*. For example, *Igfbp1*, whose protein product is secreted by the liver, contributes to the regulation of glucose metabolism and cell growth. Interestingly, *Dec1*, which is another rhythmically expressed bHLH gene that regulates circadian output in the liver, also regulates the expression of *Igfbp1* (5). It was speculated that the peak phase of *Igfbp1* at CT0-CT4 is regulated in a Dec1-dependent manner. ID2 may provide an additional loop that controls the down-regulation of *Igfbp1* at CT12.

We speculate that ID2 provides an additional circadian loop that regulates a subset of liver clock output in coordination with other rhythmic and nonrhythmic transcription factors. Rhythmic expression controlled by multiple transcription factors has been documented. For example, the rhythmic gene expression of *Cyp7a* (cholesterol 7 alpha hydroxylase) is controlled by a series of rhythmically expressed transcription factors: DBP (D-box-binding protein), Dec2 and REV-ERBα (46), presumably acting through E-box-, REV-ERBα/ROR-, and DBP/E4BP4-binding elements. This concerted effort allows for the amplitude, phase, and wave form of the rhythmic profile to be finely controlled (47). Similarly, ID2 may regulate gene expression through interaction with bHLH transcription factors that act through E-box elements so as to fine-tune the profiles of downstream CCGs.

Id2 appears to play a role in both the input and output pathways of the circadian clock (Fig. 6). In the input component, *Id2* is involved in modulating the magnitude of photic entrainment in animals (1); the absence of *Id2* results in animals entraining to a new light-dark cycle twice as fast as WT individuals and with correspondingly larger phase shifts. In the output component, *Id2* appears to contribute to the regulation of a variety of liver clock-controlled processes, probably through interference with bHLH transcriptional activators/inhibitors whose actions result in a cascade of transcriptional regulation. One such physiological output is highlighted by lipid metabolism, a system well documented to be under circadian regulation (10, 44, 48). Disruption of CLOCK-BMAL1 transactivation in the *Clk* mutant mouse results in an obese phenotype (44). In our study, we discovered a disruption in the rhythmic expression of genes involved in lipid metabolism that may contribute to a disrupted lipid storage phenotype, as observed in a decrease in $Id2^{-/-}$ mice of lipid deposits in the liver and the mass of white adipose tissue. ID2 appears to function in a comparable manner as the bHLH transcription repressor Dec1 and *Neurospora* PER-ARNT-SIM protein VIVID. Both proteins act as regulators of clock output and input, whereas they are not required for sustained oscillatory functions in the core clock (3, 5, 6, 49, 50). Collectively, our experiments demonstrate a potential regulatory loop closing outside of the core clock that may play a role in both clock input (photic entrainment) (1) and clock output.

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