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Report

Circadian Orchestration of the Hepatic Proteome

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Summary

Circadian rhythms are essential to health. Their disruption is associated with metabolic diseases in experimental animals and man [1–3]. Local metabolic rhythms represent an output of tissue-based circadian clocks [4]. Attempts to define how local metabolism is temporally coordinated have focused on gene expression by defining extensive and divergent "circadian transcriptomes" involving 5%–10% of genes assayed [5–8]. These analyses are inevitably incomplete, not least because metabolic coordination depends ultimately upon temporal regulation of proteins [9, 10]. We therefore conducted a systematic analysis of a mammalian "circadian proteome." Our analysis revealed that up to 20% of soluble proteins assayed in mouse liver are subject to circadian control. Many of these circadian proteins are novel and cluster into discrete phase groups so that the liver's enzymatic profile contrasts dramatically between day and night. Unexpectedly, almost half of the cycling proteins lack a corresponding cycling transcript, as determined by quantitative PCR, microarray, or both and revealing for the first time the extent of posttranscriptional mechanisms as circadian control points. The circadian proteome includes rate-limiting factors in vital pathways, including urea formation and sugar metabolism. These findings provide a new perspective on the extensive contribution of circadian programming to hepatic physiology.

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Results

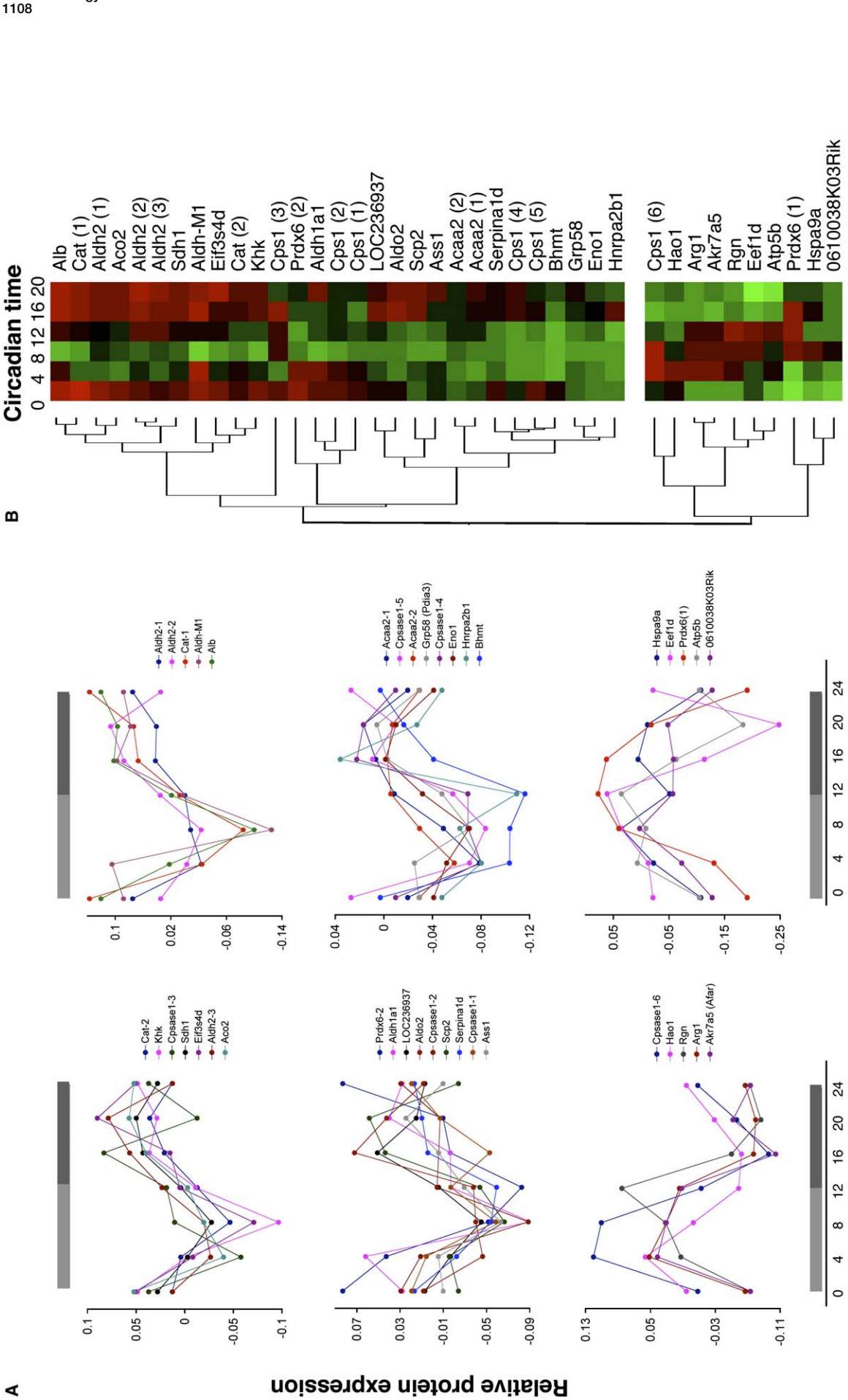
Analysis of the Circadian Proteome of the Liver

We analyzed the mouse liver proteome across circadian time by two-dimensional difference gel electrophoresis (2D-DIGE) by using tissue harvested from groups of mice on the second cycle after transfer from synchronized (12L:12DR, i.e., 12 hr light:12 hr dim red light) to free-running conditions (DR:DR, i.e., constant dim red light). Proteins were extracted from each liver individually, and equal amounts were pooled from six mice per 4 hr time point. Across the matrix of 2D-DIGE analyses, 642 protein "spots" were reliably detected. To count in the detection, the spots had to be present in more than 75% of the gels and have a "spot volume" $\geq 5 \times 10^4$ as determined by the analysis software (see the Supplemental Experimental Procedures available with this article online). Of these consistently detected proteins, 60 (i.e., 9.4%) exhibited highly significant ($p < 0.01$) circadian variation, as shown by analysis of variance (ANOVA). With ANOVA set at $p < 0.05$, 135 (i.e., 21%) of these spots exhibited a significant temporal change [11]. Thus, we estimate that up to 20% of robustly expressed soluble liver proteins are under circadian regulation, a notable advance on previous estimates that the circadian transcriptome incorporates between 5% and 10% of genes assayed [5–8].

We selected the 60 rhythmic spots identified with highest stringency ($p < 0.01$) for collection and identification either by using peptide mass fingerprinting (MALDI-TOF mass spectrometry) or by generating uninterpreted peptide fragmentation data by using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) and using the Mascot search engine (see Supplemental Experimental Procedures). Of these 60, 39 spots could be identified and were shown to correspond to the products of 29 different genes because in some cases several distinct spots represented multiple isoforms of the same protein (see Table S2 in the Supplemental Data for an annotated list). A second analysis of an independent series of liver samples obtained at CT11, 23, and 35 allowed us to identify a further ten rhythmic proteins from ten genes. When multiple isoforms were taken into consideration, we established a final list of 39 unique genes for which the corresponding 49 protein products were rhythmic. Expression of these novel circadian proteins fell into a range of phase groupings, and overall ten peaked during the subjective day, whereas 39 were distributed in two clusters that were most abundant during the subjective night (Figures 1A and 1B).

The Molecular Clock Controls Circadian Protein Expression

As a technical and biological validation of our novel circadian proteins identified with 2D-DIGE and MS, we assayed expression of a subset of them by using immunoblotting of liver tissue from further, independent sets of


A
Relative protein expression
Circadian time
Cat-2
Khk
Cpsase1-3
Sdh1
Eif3s4d
Aldh2-3
Aco2
Aldh2-1
Aldh2-2
Cat-1
Aldh-M1
Alb
Prdx6-2
Aldh1a1
LOC236937
Aldo2
Cpsase1-2
Scp2
Serpina1d
Cpsase1-1
Ass1
Acaa2-1
Cpsase1-5
Acaa2-2
Grp58 (Pdia3)
Cpsase1-4
Eno1
Hnrpa2b1
Bhmt
Cpsase1-6
Hao1
Rgn
Arg1
Akr7a5 (Afar)
Hspa9a
Eef1d
Prdx6(1)
Atp5b
0610038K03Rik
B
Circadian time
Alb
Cat (1)
Aldh2 (1)
Aco2
Aldh2 (2)
Aldh2 (3)
Sdh1
Aldh-M1
Eif3s4d
Cat (2)
Khk
Cps1 (3)
Prdx6 (2)
Aldh1a1
Cps1 (2)
Cps1 (1)
LOC236937
Aldo2
Scp2
Ass1
Acaa2 (2)
Acaa2 (1)
Serpina1d
Cps1 (4)
Cps1 (5)
Bhmt
Grp58
Eno1
Hnrpa2b1
Cps1 (6)
Hao1
Arg1
Akr7a5
Rgn
Eef1d
Atp5b
Prdx6 (1)
Hspa9a
0610038K03Rik
Low
High

wild-type and circadian mutant tissues. Our choice of targets was dictated by the availability of reliable, prevalidated antisera for candidate proteins. Bmal1 acted as a positive control for circadian protein expression and exhibited a clear 24 hr cycle in liver from wild-type animals (n = 6 per time point), with protein levels peaking in the subjective night (Figure 2A). Aldolase and Catalase were also expressed rhythmically and, consistent with the 2D-DIGE analyses, levels were highest during circadian night. In contrast, Arginase, which was identified as a daytime protein by 2D-DIGE, was also rhythmic but had an earlier phase than the other proteins. Thus, Western blot analyses revealed clear rhythmicity for three candidates identified as rhythmic by 2D-DIGE analyses, independently confirming their circadian expression and validating the procedure.

Rhythmic protein abundance in the liver is likely dependent upon the local molecular clockwork. We therefore tested whether genetic disruption of normal circadian patterning affected rhythmic expression of the three novel circadian proteins. As a control, in independent liver samples taken from wild-type mice at CT0, 6, 12, and 18, there was again clear circadian variation in the levels of Bmal1, Aldolase, and Catalase (Figures 2B and 2C), and consistent with 2D-DiGE, Arginase expression was also rhythmic and phase advanced relative to the other proteins. However, in livers from *clock* mutant mice [12, 13], the daily rhythms in expression of both Bmal1 and the novel circadian proteins were disrupted (Figure 2B). Furthermore, in heterozygous *mPer2*^{Ldc} mutant mice, which show normal circadian behavior [14], the target proteins were expressed rhythmically as in wild-type animals, whereas in arrhythmic homozygous mutants rhythmic protein expression was abrogated (Figure 2C). To pursue the impact of this *per2* mutation further, DiGE was used to compare protein expression in livers from heterozygous and homozygous mutants sampled at CT18. Of the five gels analyzed, 625 protein spots were reliably identified; of these, 27 (4.3%) were consistently disregulated by a factor of 1.5 or more in the homozygous mutant. Of these, 20 were picked for identification by mass spectrometry (16 downregulated and four upregulated in null mutants), and three proteins (from six spots) previously identified as being under circadian regulation were found to be downregulated in the *per2* mutant. These three proteins were major urinary protein, selenium binding protein (three isoforms), and peroxiredoxin. In combination, therefore, our results demonstrate that local rhythmic expression of circadian metabolic proteins is dependent on an intact molecular clock.

Posttranscriptional and Translational Control of Circadian Gene Expression

As noted above, transcriptomic analyses will not provide information concerning the contribution of posttranscriptional mechanisms to circadian coordination. The role of such processes is emphasized by our observation that individual genes yielded multiple, rhythmic isoforms of particular proteins. In the cases of Acaa2, Cat, and Serpina1, two isoforms were identified for each protein, whereas for Aldh2, the rhythmic transcript generated three isoforms (Figure 2D; Table S2). Interestingly, six different rhythmic Cps1 isoforms were represented on our 2D gels. Moreover, they were differentially phased: one held a daytime peak, whereas the other five peaked at night, in the corresponding antiphase. Quantitative PCR confirmed that the transcripts encoding Aldh2 and Cps1 were expressed rhythmically in mouse liver (Figure 2D), with both peaking at the start of circadian night and holding a variety of phase relationships to their respective protein isoforms. These findings demonstrate that posttranscriptional control is a significant component in generating differentially phased, and patterned, protein species. Moreover, this temporal complexity of metabolic coordination is not evident from transcriptomic profiling.

One likely form of posttranslational modification that might also be expected to play a role in regulating tissue-specific clock output is phosphorylation. To examine this further, we combined 2D-DIGE with phosphoprotein staining and observed that of the 36 rhythmic spots for which phosphorylation state could be determined, 24 (67%) represented phospho-proteins. As an example, we isolated concurrently two phosphorylated forms of the same rhythmic protein, Peroxiredoxin 6 (Prdx6) (Figure 2E). This protein is a member of a recently described family of highly conserved anti-oxidant proteins [15, 16]. The isoforms carried different charges and so segregated by iso-electric focusing. Although we cannot exclude the possibility that a modification other than phosphorylation caused the difference in charge, it is plausible that this protein was differentially phosphorylated in a circadian manner. Remarkably, the two protein isoforms cycled in anti-phase, illustrating time-dependent post-translational modification of Prdx6. Because Prdx6 mRNA also expressed a circadian cycle in the liver and peaked around CT10 (Figure 2E), one isoform was in phase with the transcript, and the other was out of phase (as noted above for Cps1 isoforms, Figure 2D). Together, our data reveal that circadian coordination of tissue function is more complex than previously anticipated and occurs at multiple regulatory levels—transcriptional, posttranscriptional, and posttranslational.

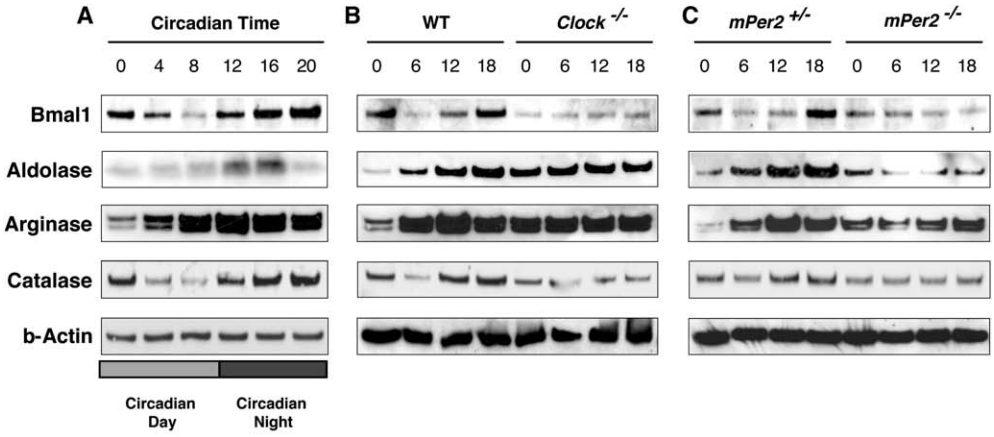
Marked Dissociation between the Circadian Transcriptome and Proteome

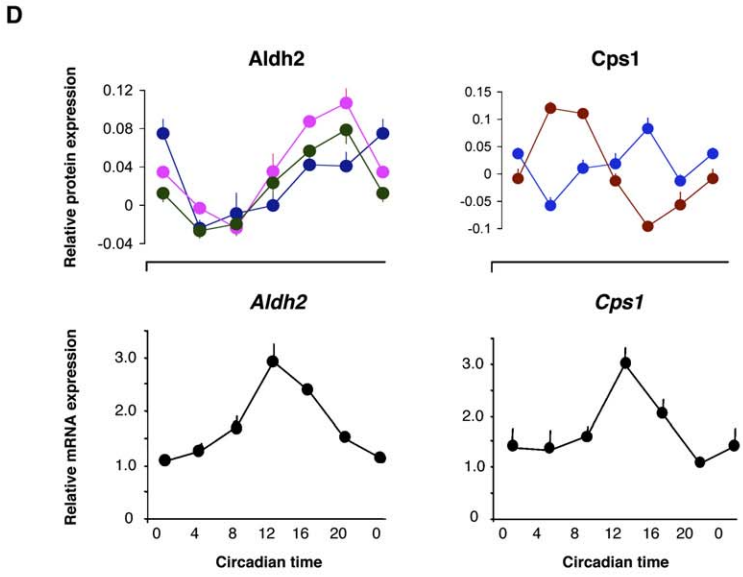
Our implicit assumption was that these protein rhythms originated from rhythmic transcriptional programmes, and this view was supported for the three sets of protein isoforms and their corresponding mRNA profiles

Figure 1. The Circadian Proteome of the Liver

(A) Circadian profiles of representative rhythmic proteins that fell into three broad phase clusters, each cluster represented by two separate plots (left and right), with peaks in circadian night (upper four panels) or circadian daytime (lower two panels). Data are plotted as the mean value, with all plots significant by ANOVA $p < 0.01$. CT0 data are replotted, and SEM is omitted for clarity (n = 3).

(B) Hierarchical clustering (by average distance correlation) of representative novel circadian proteins detected by 2D-DIGE. Green represents low levels of protein expression, black represents intermediate levels, and red represents high levels of expression. Profiles for multiple 2D gel spots representing isoforms of the same protein are indicated by parentheses.





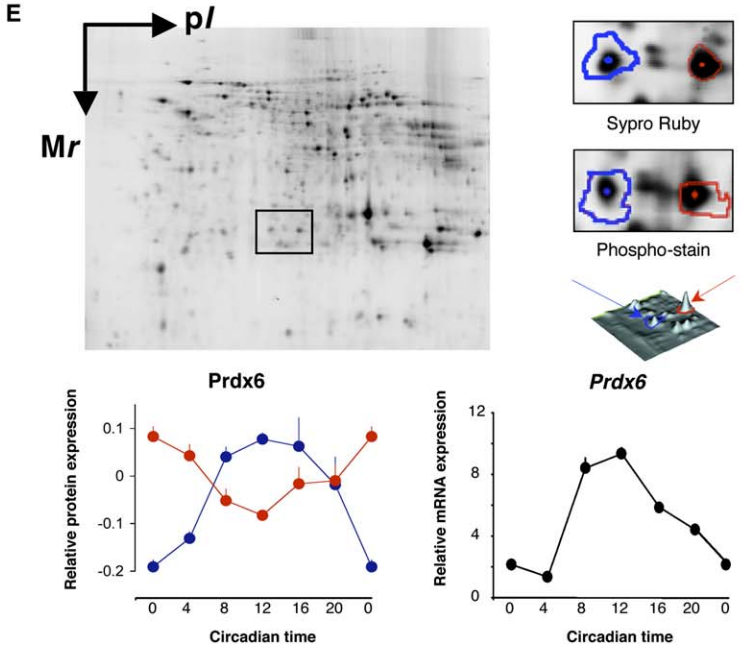


Figure 2. The Circadian Clock Regulates Hepatic Protein Expression via Transcriptional and Posttranscriptional Mechanisms

(A) Circadian profiles for Bmal1, Aldolase, Catalase and Arginase1 assayed by immunoblotting from wild-type livers.

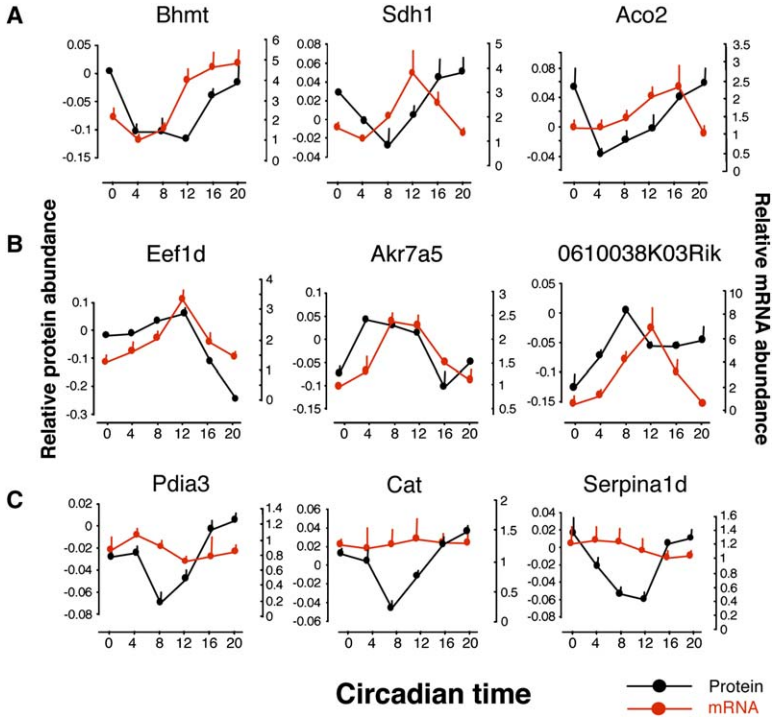


Figure 3. Comparison of Proteomic (2D-DIGE) and Transcriptomic (qPCR) Assays of Circadian Rhythmicity

(A) Comparison of three representative circadian protein (black) and mRNA (red) profiles for genes that are rhythmic at both transcriptional and translational levels, with the mRNA phase leading the protein phase.

(B) As in (A) but with simultaneous or delayed mRNA peaks relative to the protein cycle.

(C) Circadian profiles for three representative proteins whose mRNA is nonrhythmic.

All data are plotted as the mean +SEM, n = 6. For protein profiles, $p < 0.01$; for mRNA cycles in (A) and (B), $p < 0.05$. mRNA profiles shown in (C) were not statistically significant ($p > 0.05$).

discussed above. We therefore tested this assumption systematically, first by performing an in silico analysis of data sets from several Affymetrix-based studies [6–8] and thus comparing our cycling proteome with the corresponding transcriptome in the liver. We were able to obtain transcript data for 30 of our 39 genes that encoded rhythmic proteins. Unexpectedly, our analysis revealed that the corresponding genes for only five rhythmic proteins were also associated with a statistically rhythmic mRNA, as reported by at least one of a number of micro-array studies [5–8] (see Figure S1 and Table S1). We subsequently conducted our own more extensive statistical tests of archive RNA data from these investigations by utilizing spectral analysis and autocorrelation. These identified a number of further genes (*apoa4, hspa9a, and selenbp1*) that were probably rhythmically expressed, as well as others (*akr1a, acaa2, aldh2, arg1, ass1, cps1, eno1, gnpat, gstm1*) that were possibly rhythmic. These genes were potential false negatives from the original transcriptomic screens for circadian genes. Nevertheless, based on our stringent reanalysis of available micro-array data, a possible 13 of 30 genes that encode rhythmic proteins were arrhythmic at the RNA level.

Given that differences between each of the previous micro-array studies (e.g., lighting conditions and mouse genetic backgrounds) may have accounted for discrepancies both between the studies and between the transcriptome and proteome, we used highly sensitive Taqman quantitative real-time PCR (qPCR) on the same samples as our 2D-DIGE analysis in order to make a direct comparison between the proteome and transcriptome in a single sample set. We obtained profiles for 32 of our 39 target genes, and only 16 (50%) of these tested genes that encode rhythmic proteins exhibited statistically significant circadian variation in mRNA levels. This included 8 of 12 "probable" or "possible" rhythmic genes from our initial statistical re-analysis of micro-array data, thereby validating our current ANOVA analysis of qPCR circadian data. In addition, the *aldo2* and *hspd1* transcripts, which were rhythmic in at least one micro-array study, were not rhythmic by qPCR (Figures 3A and 3B; Table S2 and Figure S2A). Thus, at most 18 (16 + 2) of 32 target genes may be rhythmic, i.e., 56%. Three main phase relationships between mRNA and protein profiles were evident: (1) phase-advanced mRNA relative to protein (e.g., *ass1*, *aco2*, *aldh2*, *bhmt*, *sdh1*); (2) synchronous mRNA and protein profiles (e.g., *eef1d*); and (3) phase-delayed mRNA relative to protein (e.g., *0610038K03Rik*, *akr7a5*, *arg1*, *hspa9a*). Clearly, mRNA phase is not a strong predictor of protein phase for circadian products in the liver. Moreover, for

(B and C) Circadian expression of Bmal1, Arginase1, Aldolase, and Catalase in (B) wild-type (WT) but not *clock* mutant mice, as well as in (C) circadian-competent heterozygous *mPer2* mutants but not in homozygous mutant mice. Representative blots are shown. β-actin was used as a loading control in all cases.

(D) Three distinct isoforms of Aldehyde dehydrogenase 2 (Aldh2) exhibited robust circadian protein profiles with a common phase, peaking in circadian night. Cps1 was represented by six different forms, five of which peaked together in circadian night (here, these are represented by one isoform [blue curve]: further isoforms are plotted in Figure 4A) and a sixth that peaked in anti-phase, in circadian day (red curve). Both Aldh2 and Cps1 were encoded by highly rhythmic transcripts. All data aree plotted as the mean +SEM (n = 3 for proteins, n = 6 for mRNA).

(E) Two different forms of Prdx6 were identified by 2D-DiGE (boxed area). Staining with the ProQ Diamond phospho-stain showed them both to be phosphorylated. The variants are identified here as red and blue circles on enlarged 2D-gel images and a three-dimensional plot. These two forms of Prdx6 exhibited an anti-phasic oscillation, with one isoform (blue plot) peaking with the rhythmic mRNA and the second peaking 12 hr later. All data are plotted as above.

the remaining 16 genes, qPCR failed to show statistically significant variation over the circadian cycle by ANOVA (Figure 3C; Table S2 and Figure S2B). We cannot of course exclude the possibility that distinct mRNA pools may contribute differentially to translation. For example, more recently synthesized and cyclical mRNA may drive rhythmic protein expression against a background of a larger and older nonrhythmic mRNA pool. Such a mechanism would involve an intriguing and unprecedented complexity in the circadian regulation of gene expression. Nevertheless, our findings show that our initial expectation that rhythmic proteins would have an underlying rhythmic mRNA profile, i.e., rhythmicity begets rhythmicity, was misguided. More importantly, it highlights for the first time that posttranscriptional mechanisms must exert significant control over circadian coordination in vivo.

To determine mechanisms that may account for the differences in the behavior of mRNA and proteins, we first looked for binding sites within the 5′-flanking regions of genes coding rhythmic and nonrhythmic mRNAs and found that although a variety of circadian elements were present (E box, E′ box, D box and RORE), they were equally likely to occur in either group (see Table S2). This suggests that control of promoter activity by known circadian elements is unlikely to explain the observed variance in mRNA rhythmicity [17]. How might nonrhythmic mRNA result in rhythmic protein expression? Translational control of circadian expression has been observed previously for a limited number of circadian proteins in lower organisms, but not in mammals, and not to the extent seen here. For example, in the dinoflagellate *Gonyaulax polyedra*, *luciferin binding protein* (*LBP*) mRNA is not rhythmic, but protein expression (and bioluminescence) exhibits clear circadian oscillation mediated by the rhythmic binding activity of the RNA binding protein CCTR to the *LBP* 3′UTR [18]. Similarly, rhythmic expression of the deadenylase, nocturnin, in the *Xenopus* retina may potentially regulate steady-state downstream target mRNAs to generate protein cycles [19, 20]. Such mechanisms involving RNA binding proteins and their interactions with target UTRs could explain the patterns we observed in mouse liver. Our results thus highlight the role that posttranscriptional mechanisms, which have hitherto received little attention, undoubtedly possess in exerting significant control over the circadian proteome.

Circadian Control of Critical Liver Processes: Ureagenesis and Sugar Metabolism

Because we focused on soluble proteins, the circadian proteins we found were predominantly enzymes. Importantly, they are associated with a variety of vital liver functions, and here we focus on two archetypal pathways: urea formation and sugar metabolism. Urea formation is a central function of the liver, and three distinct proteins that control three independent steps in the urea cycle (Carbamoyl phosphate synthetase 1, Cps1; Arginosuccinate synthetase 1, Ass1; and Arginase 1, Arg1) were revealed as robustly rhythmic (Figure 4A). The activity of Arg1 has been shown previously to vary across the circadian cycle in the liver [21], consistent with our findings here, whereas circadian rhythmicity has not been reported previously for the activity or abundance of either Cps1 or Ass1. Importantly, Cps1 acts at the rate-limiting step in ureagenesis by converting ammonia to carbamoyl phosphate; mice genetically deficient in Cps1 die in early development because of an accumulation of excess ammonia [22], and mutations in *cps1* are associated with liver disorders in humans [23]. The relative phasing of these proteins may be significant, insofar as Ass1 and the majority of Cps isoforms peaked during circadian night when nocturnal feeding and digestion would present amino acids to the hepatocytes, whereas Arg1, the final stage before urea production peaked later in circadian day, when digestion would have been complete.

Several enzymes involved in sugar metabolism were also under circadian control. First, Ketohexokinase and Succinate dehydrogenase 1, both involved in the fructose metabolism pathway, upstream of glycolysis, exhibited highly rhythmic protein oscillations (Figure 4B). Second, we found that two key glycolytic enzymes, Aldolase 2 and Enolase 1, showed robust rhythmic expression, as did Aconitase 2, which catalyses the conversion of citrate to isocitrate in the second step of the citric acid (tricarboxylic acid) cycle. All five of these enzymes exhibited synchronous oscillation, with expression levels increasing through the circadian night (Figure 4B). Previous studies have generally shown that transcription of genes encoding metabolic enzymes in this pathway peaks at the onset of circadian night [5, 6], in anticipation of the initiation of nocturnal feeding. The current results demonstrate synchronous activation of a suite of proteins critical to carbohydrate metabolism. Such temporal regulation likely optimises hepatic processing of nocturnal meals and metabolic efficiency in general.

Discussion

Our integration of proteomic and transcriptomic analyses adds a new perspective to the understanding of circadian coordination in the liver and is the first study to assess posttranscriptional regulation in this context. We have revealed extensive and divergent rhythmic control of protein expression, such that the liver proteome is markedly different between circadian day and night. This temporal regulation involves up to 20% of the soluble proteins we were able to detect and highlights circadian control of a variety of archetypal and vital liver-specific processes, including rate-limiting steps in urea, sugar, alcohol and bile acid metabolism. The physiological relevance of circadian changes in the proteome should not, therefore, be underestimated, in that they are as extensive and robust as those described in pathological conditions. For example, using similar methodology, Fella et al. (2005) recently showed that chemically induced liver carcinogenesis is associated with differential expression of approximately 7.5% of liver proteins [24].

This natural and regular daily change in protein composition is likely to be common to many tissues and thus provides a framework for understanding various reports of severe metabolic disturbance both in circadian mutant mice [1, 25] and in humans subject to shift work [2, 26]. In the specific context of hepatic function, circadian variation may have numerous adaptive

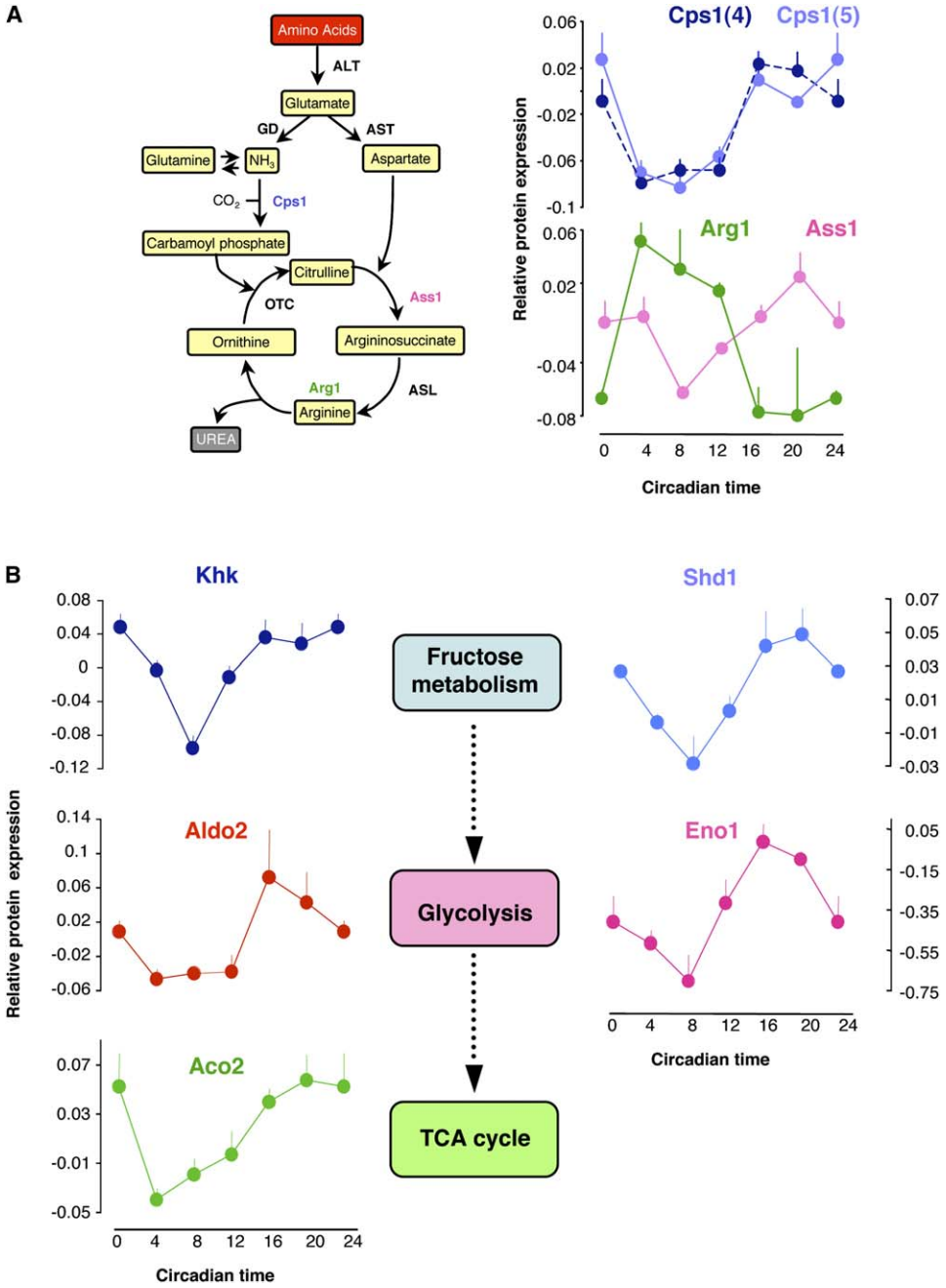


Figure 4. Circadian Coordination of Rate-Limiting Proteins in Vital Hepatic Pathways

(A) A schematic view of the urea cycle illustrates roles of circadian proteins and accompanying representative circadian profiles of Carbamoyl-phosphate synthetase 1 (Cps1, blue), Arginosuccinate synthetase 1 (Ass1, pink) and Arginase 1 (Arg1, green). Data are plotted as mean + SEM, n = 3. Two different isoforms of Cps1 are plotted; Cps1 (4) (dark blue, dotted line) and Cps1 (5) (pale blue). Further isoforms of Cps1 are plotted in Figure 2.

(B) Circadian profiles of proteins involved in carbohydrate metabolism at levels of fructose metabolism (Ketohexokinase [Khk], Succinate dehydrogenase 1 [Sdh1]), glycolysis (Aldolase 2 [Aldo2], Enolase 1 [Eno1]), and the TCA cycle (Aconitase 2 [Aco2]). All five proteins underwent synchronous oscillation, with troughs of expression in the middle of the circadian day, and peaked to coincide with the feeding phase at night. Data are plotted as above.

physiological roles. For example, changes in expression of circadian-regulated liver genes underlie the ability to switch metabolic fuels during environmental stress [27], and its pathological relevance is evident from the observation that an inability to metabolise nutrients as a result of inappropriate meal-times may be a major cause of coronary heart disease in shift workers [28]. Our findings also provide a mechanistic framework within which one can understand temporal aspects of toxicology and drug metabolism, for example, in the circadian pattern of vulnerability to cytotoxic agents [29]. Further characterization of the circadian proteome will

thus highlight potential therapeutic targets [30] and could facilitate more effective chrono-therapeutic strategies.

An unprecedented finding is that for almost one half of the rhythmic proteins, their corresponding mRNA was not rhythmic. Analogous dissociations between expression changes in the transcriptome and proteome have recently been observed in models of cancer and other diseases [9, 10], further illustrating the utility of taking an integrated view of circadian gene expression. A variety of kinases are known to influence the period of the core circadian clockwork by altering the stability of Period and associated proteins in rodents and humans [4, 31] and thus targeting their degradation by the proteosome. Such kinases may play a more general role in sculpting the circadian proteome reported here. Equally, temporally specific metabolism of RNA may underlie some of these daily patterns, and the deadenylase Nocturnin is a clock-regulated factor [19] that offers a precedent for such a mechanism [20]. Elucidating the posttranscriptional and translational control points that regulate circadian protein oscillations therefore remains a critical step in characterizing the molecular programmes that drive circadian physiology and disease. The combined proteomic and transcriptomic analyses developed here offer an effective framework for understanding how local and central clock mechanisms generate appropriately timed daily metabolic programmes.

Supplemental Data

Supplemental data include Experimental Procedures, two figures, and two tables and are available with this article online at http://www.current-biology.com/cgi/content/full/16/11/1107/DC1/.

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