Elovl3: a model gene to dissect homeostatic links between the circadian clock and nutritional status[S]

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Abstract The ELOVL3 protein is a very long-chain fatty acid elongase found in liver, skin, and brown adipose tissues. Circadian expression of the *Elovl3* gene in the liver is perturbed in mutant CLOCK mice but persists in mice with severe hepatic dysfunction. A reliance on an intact clock, combined with the refractoriness to liver decompensation and the finding of a robust sexually dimorphic pattern of expression, evince a particularly complex mode of transcriptional control. The *Elovl3* gene upstream region was repressed by RevErbα and activated by sterol-regulatory element binding protein-1 (SREBP1) transcription factors. We propose that the temporal coordination of RevErbα and SREBP1 activities integrates clock and nutrition signals to drive a subset of oscillatory transcripts in the liver. Proteolytic activation of SREBP1 is circadian in the liver, and because the cycle of SREBP1 activation was reversed after restricting meals to the inactive phase of the day, this factor could serve as an acute sensor of nutritional state. SREBP1 regulates many known lipogenic and cholesterogenic circadian genes; hence, our results could explain how feeding can override brain-derived entraining signals in the liver. This mechanism would permit a rapid adjustment in the sequence of key aspects of the absorptive and postabsorptive phases in the liver.—Anzulovich, A., A. Mir, M. Brewer, G. Ferreyra, C. Vinson, and R. Baler. ***Elovl3*****: a model gene to dissect homeostatic links between the circadian clock and nutritional status. *J. Lipid Res.* 2006.** 47: **2690–2700.**

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ELOVL3 is a 30 kDa membrane-bound protein expressed only in liver and brown adipose tissues (significantly lower levels can also be detected in skin) that is strongly induced during perinatal development, consumption of a high-fat diet, or exposure to cold stress (1). *Elovl3* is a member of a recently described mammalian gene family involved in, according to current evidence, the elongation of fatty acids to produce very long-chain fatty acids (VLCFAs) and, secondarily, sphingolipids (2) in a tissue-specific manner (3). The dramatic induction observed in *Elovl3* expression in brown adipose tissue during cold stimulation and the ensuing brown fat recruitment links this protein to the thermogenic process (3).

The regulation of the *Elovl3* gene presents several interesting features. First, steady-state levels of *Elovl3* mRNA follow a robust circadian profile in the liver, according to a recent transcriptome analysis (4). This rhythm, which appears to be perturbed in the CLOCK mutant mouse (4), displays a late-night acrophase, suggesting that if there is a link to the circadian BMAL/CLOCK pathway, it is most likely indirect, because BMAL/CLOCK activity peaks during the second half of the light phase (5). Therefore, analysis of the *Elovl3* promoter could lead to the identification of downstream transcriptional programs linked to the core mechanism of the clock in the liver. Second, thermogenesis in the brown adipose tissue can be acutely modulated by photic signals acting via the suprachiasmatic nucleus (6, 7). Because of its presumed role in thermogenesis, *Elovl3* represents a potentially useful link between clock output from the suprachiasmatic nucleus and the regulation of physiological variables such as circadian changes in core body temperature. Finally, a day/night liver transcriptome analysis of the transgenic "fatless" mouse model (A-ZIP/F-1) showed that despite the known metabolic disruption in the liver, the daily rhythm in *Elovl3* gene expression remained virtually unaffected. In fact,

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Abbreviations: ACC, acetyl-coenzyme A carboxylase; ACS, acyl-CoA synthase; G3PAT, glycerol-3-phosphate acyltransferase; iBAT, interscapular brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol-regulatory element binding protein; STAT, signal transducer and activator of transcription; VLCFA, very long-chain fatty acid; ZT, Zeitgeber time.

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the nocturnal induction of the *Elovl3* gene displayed the largest amplitude of day/night rhythmic expression between the two time points selected, among all the genes present in the microarray. This observation suggested that at least some rhythmic BMAL/CLOCK activity is present in A-ZIP/F-1 mice and that *Elovl3* represents a potential downstream clock-controlled gene.

This hypothesis prompted us to look more closely at the regulation of the *Elovl3* gene in normal mice. In this study, we found that *Elovl3* is a tightly regulated sex-specific gene, with virtually undetectable expression in the female liver. In contrast, a uniform low level of *Elovl3* gene expression was observed in both genders in the interscapular brown adipose tissue (iBAT). In addition, we found that the *Elovl3* gene promoter was not activated by BMAL/CLOCK in transfected cells, in spite of the presence of a perfect E-box. This result is consistent with the notion that its deregulation in the CLOCK mouse reflects an indirect connection to the core clock mechanism. In apparent contrast to a previous report, we found that expression of the *Elovl3* gene was arrhythmic in the liver of CLOCK mutant mice.

Our results suggest the RevErbα and the sterol-regulatory element binding protein-1 (SREBP1) transcription factors as likely mediators of circadian *Elovl3* transcriptional activity in the liver. Upon closer examination, we observed that the rhythmic *Elovl3* transcript level in the liver was modulated by the feeding schedule, which was consistent with changes in the rhythm of proteolytic activation of SREBP1 observed under identical conditions. Therefore, the activity of the SREBP1 factor likely functions either to integrate or to dissociate the central circadian cues and the oscillating rhythms in the liver in response to the availability of food, a time cue significantly less predictable than the photoperiod.

MATERIALS AND METHODS

Animal care and experimental protocols

Animals (C57BL/6 or CLOCK heterozygous and homozygous mice) (8) were entrained to a 12 h/12 h light/dark schedule [lights on at 6 AM; this time is referred to as Zeitgeber time (ZT) 0] for 2 weeks before experiments were carried out. The level of photo entrainment in mice was assessed by monitoring the locomotor activity using the Vital-View system software (Mini-Mitter Co.). Experimental procedures involving the A-ZIP/F-1 fatless strain of transgenic mice and all related supplemental data can be viewed at the following URL: http://neuroscience.nih.gov/Baler/CIG30ms/supplementaldata.pdf. (In accordance with the Mouse Nomenclature Committee, the assigning symbol for the mouse gene *Cig30* is changed to *Elovl3*.) For restricted feeding experiments, C57BL/6 male mice were entrained to a 12 h/12 h photoperiod for 2 weeks. During the third week, half of the animals were allowed to feed only between ZT5 and ZT9. Animals were anesthetized by CO_2 inhalation and immediately decapitated. Liver samples were rapidly removed and quick-frozen in a 1.5 ml microtube in solid CO_2. All experiments were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, and all pertinent Animal Welfare Act regulations, according to experimental protocols that were approved by the Animal Care and Use Committee and met the guidelines of the National Institutes of Health. No procedures performed during the course of this study caused more than slight momentary pain or distress.

Northern blot analysis

Total cellular RNA was extracted from frozen pieces of tissue (in duplicate) using the Trizol reagent (Invitrogen-Life Technologies, Carlsbad, CA), fractionated (8–20 μg/lane) on a 1% formaldehyde-agarose gel, and examined by Northern blot analysis. Probes were labeled by random-priming with [α-^{32}P] dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) using the MegaPrime kit (Amersham Pharmacia Biotech). A rat *Elovl3* probe was obtained through a RT-PCR amplification procedure. Briefly, total rat liver RNA (1 μg) was reverse-transcribed using oligo-dT primers (Invitrogen-Life Technologies), and an 857 bp *Elovl3* cDNA product was then amplified by PCR using the following primer pair (bracketing the fragment between positions 8,384 and 10,208 in GenBank AF054504) (1): forward (in exon 5), 5′-GGTAAGCTCATTTCTCATAGTGGTCG-3′; and reverse (in the 3′ untranslated region), 5′-GGGTGACATTTCTTGGTTGTCA-TAGCTTCC-3′. Northern blot membranes were stripped (boiling in 0.1% SDS and 0.1% sodium chloride, sodium citrate-trisodium salt stock solution, twice for 15 min) and reprobed with a glyceraldehyde-3-phosphate dehydrogenase probe. Densitometric analysis of mRNA levels was performed using ImageQuant™ software (version 3.0; Amersham Pharmacia Biotech).

Cell lines, plasmid vectors, and recombinant DNA protocols

The mouse hepatocyte line AML12 (9) (American Tissue Culture Collection; CRL-2254) was cultured in a 1:1 mixture of DMEM and Ham's F12 medium containing insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 μg/ml), dexamethasone (80 ng/ml), and 10% fetal bovine serum. NIH-3T3 fibroblasts (CRL 1658) were cultured in DMEM (4 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 4.5 g/l glucose) and 10% bovine calf serum (HyClone, Logan, UT). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. A mouse *Elovl3* promoter reporter vector was constructed by splicing a 1.16 kb genomic region (located upstream and immediately adjacent to the mouse *Elovl3* translation start site) between the *Nhe*I and *Bgl*II sites in the pGL3Basic firefly luciferase vector (Promega, Madison, WI). To generate a cytomegalovirus-driven RevErbα expression vector, a fragment derived from the human RevErbα sequence (10) was generated by RT-PCR using the following primers (5′ to 3′): TGAAGACATGACGACCCTGGACTCCAA-CAACAACACAGGT and GGTCACTGGGCGTCCACCCGGAAG-GACAGCAGCTTC. The resulting 1.8 kb coding sequence was subcloned into a *Not*I/*Xba*I-linearized pCDNA3.1 (Invitrogen) and fully sequenced. The CS10a, CS10c, and CS2 expression vectors for the N-terminal truncation derivatives of SREBP1a, -1c, and -2, respectively (11), were kindly provided by Dr. Tim F. Osborne (University of California). Human BMAL and mouse CLOCK expression vectors were a gift from Drs. Nick Gekakis and Charles Weitz (Harvard University). The *vasopressin*/LUC reporter used in this study has been described previously (12).

Transient transfection assays

Transfections were performed using Lipofectamine/Plus (Invitrogen)-DNA lipoplexes as described previously (12). *Elovl3* transactivation patterns were analyzed in the nontransformed mouse hepatocyte cell line AML12 and in the NIH-3T3 mouse embryo fibroblast line. Results are representative of at

least five independent experiments performed in triplicate. Equivalent transfection efficiency was assessed by cotransfection with a Renilla luciferase reporter gene driven by a cytomegalovirus promoter. Statistical analysis was performed using Student's *t*-test for unpaired samples. The intragroup variability remained consistently at or below the 10% level.

Western blot analysis

Duplicate liver samples were harvested from groups of four mice every 3 h during a 12 h/12 h light/dark cycle and placed immediately in dry ice. For whole cell protein extraction 10 μl of ice-cold buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium fluoride, 5 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 25% glycerol) was added for every 1 mg of liver tissue. The tissue was then homogenized on ice with 20 strokes of a tight-fitting, 1.5 ml microtube pestle, vortexed, and refrozen on dry ice for 10 min. Lysates were then incubated in ice water for 15 min and centrifuged for 15 min at 12,000 rpm (4°C). For Western blot analysis, ~40 μg of soluble protein from whole liver lysates was electrophoresed through a 4–12% Tris-Glycine-SDS Nu-Page cassette (Invitrogen) and transferred onto an Immobilon P (Millipore, Bedford, MA) polyvinylidene difluoride membrane according to the manufacturer's recommendations. Membrane-bound proteins were investigated with anti-human $SREBP1a_{301–407}$ (13) (BD Biosciences, San Diego, CA) and finally with a mouse monoclonal antibody against the rat 14.3.3 ϵ protein (14), which was kindly provided by Dr. David Klein (National Institute of Child Health and Human Development). After incubation with a HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), luminescence was quantified using an Image Station 440 CF (Kodak, Rochester, NY).

RESULTS

Rhythmicity of *Elovl3* gene expression in the mouse liver persists in transgenic fatless mice

In a recent transcriptome analysis comparing day/night rhythms in gene expression between wild-type and A-ZIP/F-1 mice (http://neuroscience.nih.gov/Baler/CIG30ms/supplementaldata.pdf), the *Elovl3* gene displayed the most robust amplitude among only 11 transcripts whose circadian oscillations remained largely unaffected by the severe liver decompensation characteristic of this transgenic model (**Table 1**) (15, 16). The level of *Elovl3* expression had been reported previously to increase by ~4.8-fold in the CLOCK mutant mouse at ZT8 (4). This is interesting because the late-night peak in *Elovl3* mRNA develops in antiphase with the diurnal peak in BMAL/CLOCK activity in the liver (5). Together with the refractory nature of the *Elovl3* rhythm to the perturbation in hepatic physiology in A-ZIP/F-1 mice, these observations suggest that the rhythm in *Elovl3* transcription could provide an interesting model for the study of the homeostatic integration between the core clock machinery and downstream gene oscillations in the liver.

Rhythmic, sex-specific, and tissue-specific expression of the mouse *Elovl3* gene

To analyze the pattern of *Elovl3* gene expression in normal animals, groups of male and female C57BL/6 mice were entrained to a 12 h/12 h light/dark photoperiod for 2 weeks before being euthanized every 4 h for the collection of liver, iBAT, and muscle tissue samples. Northern blot analysis (**Fig. 1A**) confirmed the rhythmic expression in liver that had been observed previously in a liver transcriptome analysis performed on cDNA microarrays (4). Unexpectedly, the circadian expression of *Elovl3* transcription in the liver was sex-specific, because no signal was detected in the livers of female mice (Fig. 1A, compare left and right panels). Interestingly, no such difference was observed in the iBAT, which showed low and virtually constant levels of *Elovl3* mRNA in both sexes (see supplementary data). As expected, no *Elovl3* expression was detected in skeletal muscle of either gender (data not shown). The rhythmic, tissue-specific, and sexually dimorphic pattern of *Elovl3* gene expression suggests interesting links between the complex regulation of this gene and the well-established differences in the control of thermogenesis (17, 18) and liver gene expression (19) between genders in the rodent species.

TABLE 1. Genes found to display similarly robust night/day (N/D) rhythms in the livers of both wild-type and A-ZIP/F-1 mice

Gene	IMAGE Clone Identifier	Unigene Cluster Identifier	Ref. Seq. or Representative mRNA Accession Number	A-ZIP/F-1 Ratio, N/D	Wild-Type Ratio, N/D
Elovl3 (or *Cig30*), Elongation of very long-chain fatty acids-like 3	1280273	Mm.21806	NM_007703	8.31	15.35
Ppp1r5, Protein phosphatase 1 binding protein PTG	870856	Mm.24724	U89924	2.34	3.52
EST	618155	Mm.80570	BI078026	2.02	2.78
Similar to Galectin (RIKEN cDNA 1110067D22 gene)	1478745	Mm.76694	BC019131	2.02	3.34
Ak4, Adenylate kinase	403946	Mm.42040	NM_009647	0.44	0.50
Alas1, Aminolevulinic acid synthase 1	583638	Mm.19143	NM_020559	0.41	0.43
Cish, Cytokine-inducible SH2-containing protein	335058	Mm.4592	NM_009895	0.36	0.38
Pbef, Pre-B-cell colony-enhancing factor	541305	Mm.28830	NM_021524	0.36	0.48
Tdag, Pleckstrin homology-like domain, family A, member 1	374607	Mm.3117	NM_009344	0.30	0.27
Thra, Thyroid hormone receptor α	1040517	Mm.26587	NM_011584	0.30	0.23
Similar to uridine phosphorylase (RIKEN cDNA 1700124F02 gene)	987456	Mm.200370	BC027189	0.20	0.25

Genes are listed from strongly nocturnal to strongly diurnal. This list of 11 genes represents the small group of overlapping genes with rhythmic expression that remained unchanged between the livers of wild-type and fatless (A-ZIP/F) mice (see Materials and Methods and supplementary data for more details).

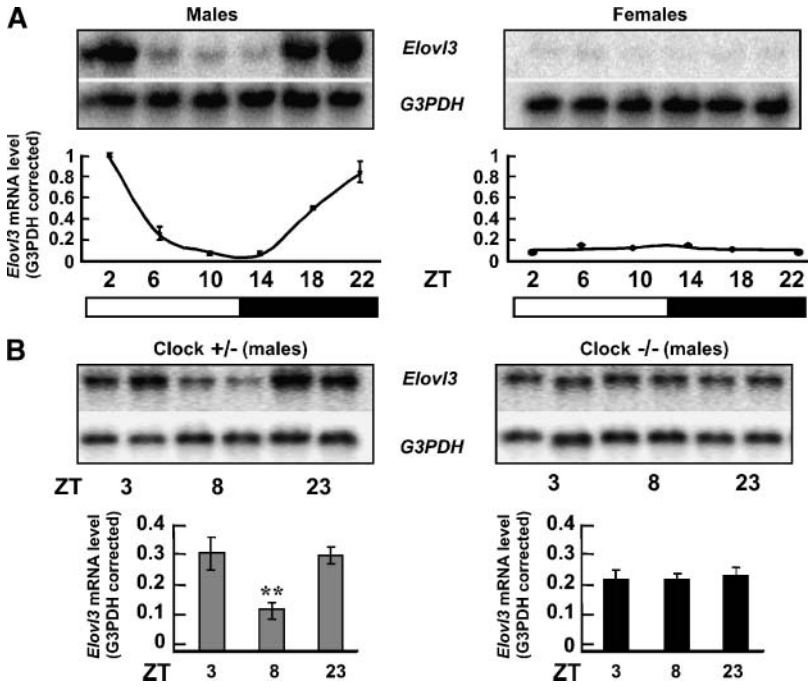

Fig. 1. Northern blot analysis of the daily rhythm in steady-state *Elovl3* mRNA level in the livers of wild-type and heterozygous and homozygous Clock^{-} mutant mice. *Elovl3* mRNA levels were measured in total liver RNA extracted at 4 h intervals from either two male or two female wild-type C57BL mice (A) or at Zeitgeber time (ZT) 3, ZT8, and ZT23 from the livers of two heterozygote (gray bars) or homozygote (black bars) CLOCK mutant male mice (B). Blots were probed sequentially with mouse *Elovl3* and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) probes as described in Materials and Methods. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24 h photoperiod.

Effect of the CLOCK mutation on rhythmic *Elovl3* gene expression in the liver

It is reasonable to propose that daily oscillations in the level of *Elovl3* mRNA in the liver are somehow linked to the circadian transcriptional activity of the BMAL/CLOCK heterodimeric complex, because *Elovl3* mRNA levels have been reported to increase by ~4.8-fold in the livers of CLOCK mutant mice at ZT8 (4, 8). To independently corroborate that result at time points that are more relevant to *Elovl3* gene rhythmicity, heterozygote and homozygote CLOCK mutant male mice were photo-entrained for 2 weeks and euthanized at ZT3, ZT8, and ZT23 to assess the level of liver *Elovl3* mRNA by Northern blot analysis (Fig. 1B). We found that *Elovl3* mRNA was marginally higher in the livers of Clock$^{-/-}$ mice at ZT8; more importantly, the daily rhythm in *Elovl3* gene expression was essentially abolished in Clock$^{-/-}$ mice (Fig. 1B, right panel). This result represents a significant refinement of previously available data (4). The present study suggests a more complex effect of the CLOCK mutation upon rhythmic *Elovl3* gene expression in the rodent liver, which should be reinterpreted in light of the promoter and functional studies described below.

Isolation and functional characterization of the mouse *Elovl3* gene promoter

The promoter region of the mouse *Elovl3* gene was isolated via high-fidelity genomic PCR with primers designed using available genomic sequence information (20). To identify putative DNA consensus regulatory sites, we scanned the ~1.1 kb fragment of the *Elovl3* region upstream of the translation start site [including a 171 bp long 5′ untranslated region (boldface in **Fig. 2A, B**)] for significant matches to entries in the TRANSFAC database (21). This procedure revealed, most significantly, a potential RevErbα binding element at position −846 (22, 23), a putative signal transducer and activator of transcription (STAT) (24) recognition site at position −726, two sterol response elements at −776 and −426, a perfect activator protein-1 site at position −326, a likely TATA box ~27 bp upstream of the reported transcription start point, and a perfect E-box, within the 5′ untranslated region at position +21 relative to the transcription start point.

Next, we created a preliminary functional map of possible regulators of *Elovl3* gene expression by testing the performance of an *Elovl3*/LUC promoter reporter construct during the transient coexpression of selected transcription factors in AML12 cells, a murine hepatocyte cell line (9). The factors tested were chosen after the identification of suitable potential cognate sites along the *Elovl3* regulatory region (Fig. 2B) and because of their known involvement in circadian and lipogenesis-related transcriptional regulation.

To ascertain whether the links between the clock and *Elovl3* gene expression operate directly or indirectly, we first evaluated the ability of the *Elovl3* gene upstream

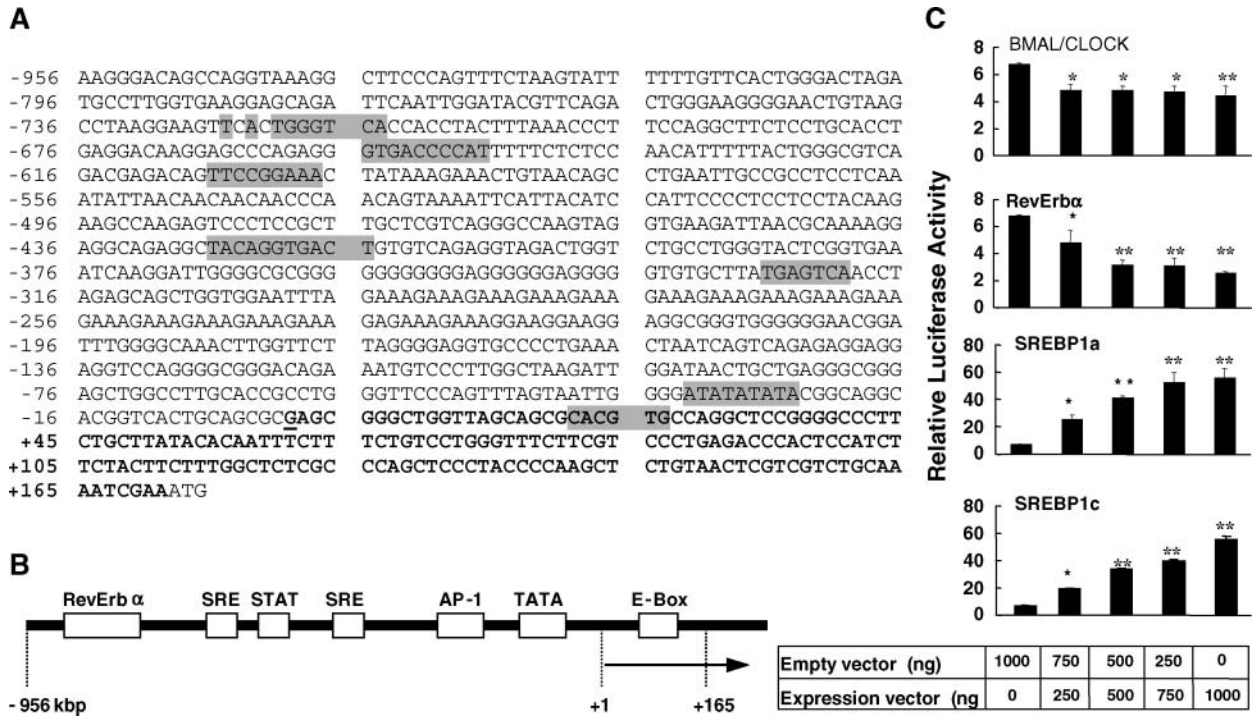

Fig. 2. Proposed architecture and functional analysis of the mouse *Elovl3* proximal promoter region. A, B: An ~1.1 kb mouse genomic region was isolated by high-fidelity PCR as described in Materials and Methods. The sequence was corroborated and scanned for the presence of putative binding sites for relevant transcription factors. The start points of transcription bracket the sequence in boldface. Putative *cis*-acting elements identified are highlighted and boxed (A) and also depicted schematically along the promoter (B); their positions, as indicated in the text, are relative to the transcription start point (+1). AP-1, activator protein-1; SRE, sterol-regulatory element; STAT, signal transducer and activator of transcription. C: The performance of a mouse *Elovl3*/LUC promoter-reporter construct was assessed after its cotransfection into AML12 cells along with the indicated vectors designed to overexpress the BMAL/CLOCK (top), RevErbα (second), and sterol-regulatory element binding protein-1a (SREBP1a; third) and SREBP1c (bottom) transcription factors. Error bars represent ± SD of five independent experiments performed in triplicate. The asterisks indicate statistical significance of transcription factors overexpression versus basal Elovl3 promoter activity at $P < 0.05$ and $P < 0.01$, respectively. *Elovl3* promoter-driven firefly luciferase activity was normalized to the activity generated by a cytomegalovirus promoter-driven Renilla luciferase control reporter. Similar results were observed when the experiments were performed in NIH-3T3 cells.

region to respond to the action of the circadian heterodimer BMAL/CLOCK in transient cotransfection assays. The 1.1 kb *Elovl3* promoter fragment was not transactivated after overexpression of the circadian BMAL/CLOCK factor (Fig. 2C, upper panel), despite the presence of one perfect and three noncanonical E-boxes within the *Elovl3* promoter sequence and the robust transactivation of the control *vasopressin* gene promoter (data not shown), a well-established BMAL/CLOCK-responsive region (12, 25). In fact, we have consistently observed a modest repression of *Elovl3* promoter-driven luciferase basal activity upon forced expression of BMAL/CLOCK. This behavior indicates that the E-box sites found in the mouse *Elovl3* promoter region used are not likely to be direct positive targets of the clock. This result was expected based on the fact that circadian expression of *Elovl3* in the male liver peaks at the end of the dark period, in relative antiphase to the reported peak activity time of BMAL/CLOCK at ZT8–ZT12 in peripheral tissues (5, 26). The *Elovl3* promoter, however, must be strongly connected to the central clock machinery, because its expression is obviously affected by a mutation in the CLOCK gene (4), albeit in a complex manner (Fig. 1B).

RevErbα, another of the likely candidates to provide clock-dependent information to the pathways controlling *Elovl3* gene expression, is a nuclear hormone receptor-related protein that has a potential binding site along the *Elovl3* promoter (Fig. 2B). Additionally, RevErbα plays a role in the positive limb of the interlocking loops that maintain oscillations in the central core of the mammalian clock (27), it is known to repress the transcription of target genes (28), and its early peak of activity in peripheral oscillators (27, 29) is suitably in phase with the trough of *Elovl3* mRNA expression in the liver. Interestingly, we found that basal *Elovl3* promoter activity was significantly reduced after overexpression of RevErbα (Fig. 2C, second panel from top). This effect is consistent with a possible role for this factor in maintaining the low levels of *Elovl3* mRNA in the male liver during the day and provides the first possible connection to the clock machinery.

In an attempt to identify the factor(s) responsible for the activation of the *Elovl3* gene, we next tested the performance of the *Elovl3* promoter fragment after overexpression of the mature forms of SREBP1a, -1c, and -2. These factors, which also possess putative binding sites along the *Elovl3* promoter (Fig. 2B), have been shown to control the expression of a

large number of lipogenic and cholesterogenic genes (11, 30–32). We found that SREBP1a and -1c had robust and positive effects on *Elovl3* promoter-driven firefly luciferase activity in AML12 cells (Fig. 2C, bottom panels). However, no significant effect was observed after SREBP2 expression (data not shown). The observation of different potencies between the two SREBP proteins is consistent with previous studies showing that the SREBP1 and -2 proteins display differential transactivation properties (33) and effects on gene regulation (31).

SREBPs are unusual basic helix-loop-helix proteins in that they can recognize not only a sterol regulatory element, such as the one found in the low density lipoprotein receptor gene promoter (32, 34) and related sequences (11), but also the palindromic E-box motif (30). Mutation of the *Elovl3* perfect E-box at position +21, however, had no negative effect on SREBP1-mediated stimulation of luciferase activity. On the contrary, E-box mutation resulted in a significantly more robust transactivation (higher stimulation index) of *Elovl3* by SREBP1 (data not shown). Thus, a more detailed analysis of this regulatory region will be needed to identify which of the several potential SREBP binding sites along the *Elovl3* promoter fragment used in this study is responsible for mediating this response.

Together, the responses of the mouse *Elovl3* promoter fragment to BMAL/CLOCK, RevErbα, and SREBP1 suggest that a complex network of factors can potentially cooperate to establish the observed temporal pattern of *Elovl3* gene expression in the rodent liver. It is apparent that SREBP1 may play an important role in driving the robust induction of *Elovl3* transcription in the liver toward the end of the night. The positive action of SREBP1 could reflect a constitutive default mechanism that becomes manifest when RevErbα repression disappears. Alternatively, the SREBP1 rhythmic and antiphasic pathway (35) could have been designed to amplify the response during the dark (active) phase of the cycle.

Restricted feeding during the day shifts the phases of both SREBP1 activation and *Elovl3* gene expression

To test the hypothesis that SREBP1 activation plays a role in the rhythmic activation of the *Elovl3* gene in the liver in vivo, we subjected mice to a restricted feeding paradigm. Providing animals with restricted access to food only during the day (the inactive phase of the cycle for mice) has been found to reverse the phase of many cycling transcripts (36, 37). We found that restricted feeding during the day had two noticeable effects on the pattern of SREBP1 activation. First, it was evident that the relatively shallow rhythm of activation seen in mice fed ad libitum had consolidated around the time of feeding; this was accompanied by a significant increase in the amplitude of the response at peak time. Second, and more importantly, the peak of SREBP1 activation was shifted by ~9 h relative to the peak detected in mice fed ad libitum. An equivalent shift was also observed in the rhythmic expression of *Elovl3* (**Fig. 3**). This result is

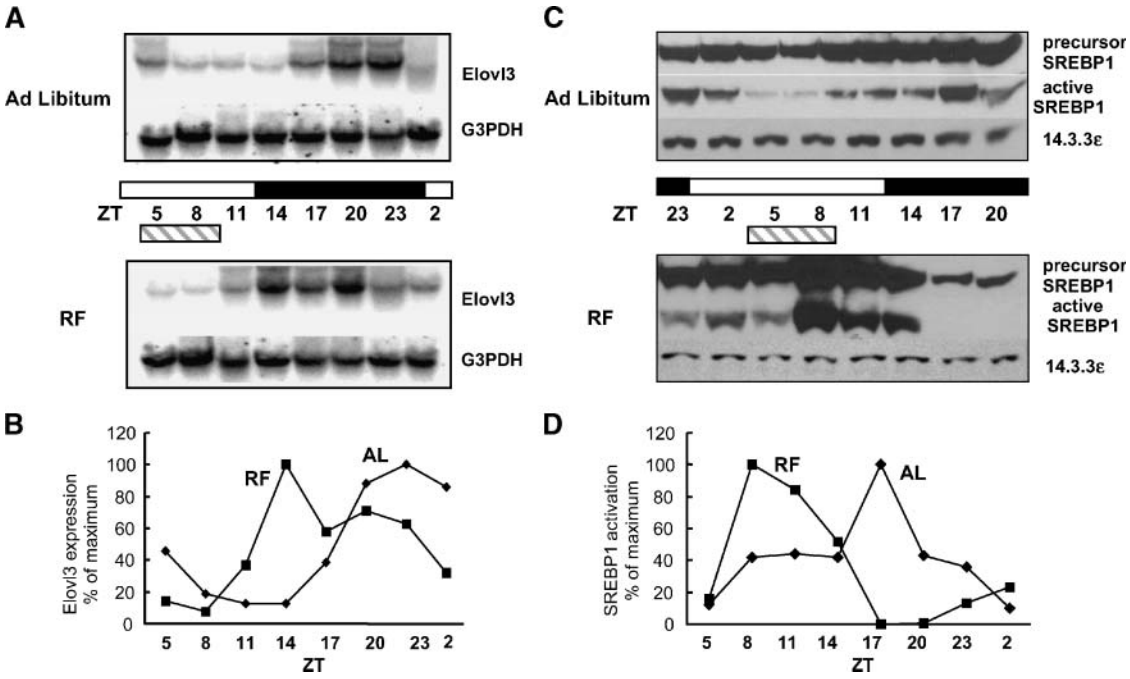

Fig. 3. Effects of restricted feeding upon cyclic activation of SREBP1 and rhythmic expression of *Elovl3*. A, C: Northern (A) and Western (C) blot analysis of total RNA or protein extracted from wild-type male C57Bl/6 mouse livers. *Elovl3* mRNA (A) and precursor and mature forms of SREBP1 (C) were measured after animals were fed either ad libitum (AL) or for a defined 4 h period (ZT5–ZT9) in the middle of the day [restricted feeding (RF)] for 1 week before the end of the experiment. Groups of four mice were euthanized at 3 h intervals to collect individual liver samples for protein and RNA extraction. The experiments were independently repeated three times in replicate liver samples. Open and closed bars represent the distribution of light and dark phases of the 24 h photoperiod. The stippled box marks the period of restricted feeding. B, D: Densitometric quantitation of the Northern (B) and Western (D) representative data.

consistent with a causal relationship between the nutritional activation of the SREBP1 pathway and the setting of the phase of the *Elovl3*, and, likely, many other gene cycles.

DISCUSSION

Elovl3: a model of complex gene regulation

Elovl3 has been identified several times during independent investigations of differentially expressed transcripts. First detected as a gene induced by cold stress in brown adipose tissue and termed cold-inducible glycoprotein of 30 kDa (Cig30) (38), *Elovl3* later reemerged as a result of its strong predawn induction in the liver (4). Finally, our own analysis of the transcriptome in the liver of the fatless mouse (Table 1 and supplementary data) singled out *Elovl3* as a gene under the control of particularly robust and complex transcriptional mechanisms in the rodent liver.

Previous studies have classified the *Elovl3* gene product as a member of the fatty acid chain elongase family, likely involved in thermogenesis and/or brown fat recruitment (i.e., brown adipose tissue hypertrophy) (1). Many biological processes depend on VLCFAs containing >20 carbon atoms. The mouse ELOVL3 protein is very similar to yeast ELO2 and ELO3 gene products. In yeast, disruption of either ELO2 or ELO3 leads to a broad range of defects, encompassing altered expression of the plasma membrane ATPase gene and decreased glucose uptake capacity (39), modified phospholipid composition (40), and resistance to inhibitors of sterol synthesis (41), among others.

This study places the *Elovl3* gene within a large family of sexually dimorphic genes in the rodent liver, a finding that raises many exciting questions that should be explored further. For example, many genes in this set (42, 43) have been shown to be modulated by androgenic signaling working through sex-specific growth hormone secretion profiles (43, 44). This cascade, beyond the growth hormone receptor, appears to recruit STAT5 transcriptional activity, which closely correlates with the sexually dimorphic patterns of growth hormone pulsatile secretion (44). It is possible that the putative STAT5 sites along the *Elovl3* promoter contribute to the dramatic sex differences in *Elovl3* expression we have discovered (Fig. 1A). In addition, and considering current evidence about the effect of a sexually dimorphic growth hormone secretory pattern on SREBP1c and its target genes in liver (45), it will be of interest to investigate the molecular basis for the failure of this activation strategy in the female liver and compare hypophysectomized, as well as ovariectomized, with control animals in future experiments.

In light of its sexually dimorphic pattern of expression and its robust circadian rhythm in the liver, it is tempting to propose the *Elovl3* gene as a fertile model for studying the integration of major physiological systems, such as energy homeostasis, sex, and circadian timing at the level of transcriptional control. The sharp increase in the levels of *Elovl3* mRNA in the livers of male mice at the end of the night already suggested that the transcription factor BMAL/CLOCK was unlikely to be involved directly in this transcriptional rhythm. In fact, such a late-night surge of a circadian gene in the liver, which is a strong peripheral oscillator, was more consistent with a RevErbα-like activity. RevErbα does not possess an Activation Function-2 domain and behaves as a powerful transcriptional repressor that interacts strongly with the Nuclear Receptor Corepressor (46). RevErbα is active during the day, when it represses the activity of target promoters. BMAL itself is driven by a RevErbα-sensitive promoter, and its release from RevErbα repression constitutes the basis for its antiphasic circadian pattern of expression (27). Together with the presence of a putative RevErbα binding site along the *Elovl3* promoter, it was reasonable to hypothesize that a similar RevErbα derepression could be at least partially responsible for the rhythmic increase in *Elovl3* mRNA levels in the liver at night. Thus, the loss of RevErbα rhythmicity in CLOCK null mice (27) could contribute to the observed disruption of *Elovl3* rhythmicity in these mice (Fig. 1B). Our inability to transactivate the *Elovl3* promoter after BMAL/CLOCK overexpression, coupled with the significant reduction in its basal level of expression after RevErbα expression (Fig. 2C, top and middle panels), is consistent with this hypothesis. Furthermore, the slight repression of *Elovl3* promoter-driven expression after BMAL/CLOCK overexpression suggests the induction of a suppressive pathway that might involve the RevErbα protein. Therefore, we propose that *Elovl3* rhythmicity might be at least partially maintained by the negative action of RevErbα during the day, effectively linking the *Elovl3* rhythm to the upstream status of the clock (**Fig. 4**). Moreover, an interruption of the repressive RevErbα cycles in CLOCK mutant mice could explain the transiently higher levels of *Elovl3* mRNA in their livers at ZT8 (4).

The responsiveness of the *Elovl3* promoter to the action of SREBP1 is of interest for several reasons. There is overwhelming evidence that SREBPs play an essential role in both cholesterol and fatty acid metabolism (33, 47). SREBPs might be differentially regulated at multiple levels by sterol depletion (48), but they appear to act in concert to maintain the right composition of lipids in cell membranes (31). Our results suggest that *Elovl3* should be added to the growing list of genes targeted by SREBPs (33). This list includes FAS, low density lipoprotein receptor, HMG-CoA reductase, acetyl-coenzyme A carboxylase (ACC), and lipoprotein lipase. Interestingly, many of the genes in this list are also circadian in the liver, and some (e.g., ACC1, FAS, and HMG-CoA reductase) are in phase with the daily *Elovl3* cycle (4, 35). Together, these observations suggest that SREBP1 might play an important role in the orchestration of temporal gene expression, downstream of the circadian clock, in the liver. This hypothesis can now be tested in vivo in suitable genetically modified animals.

It is of interest that a perfect E-box (CACGTG) appears not to be the target of SREBP1 action in *Elovl3* or in other target genes. This site, however, could play a role in preventing SREBP1-mediated activation at inappropriate phases of the cycle, because an E-box mutation resulted in

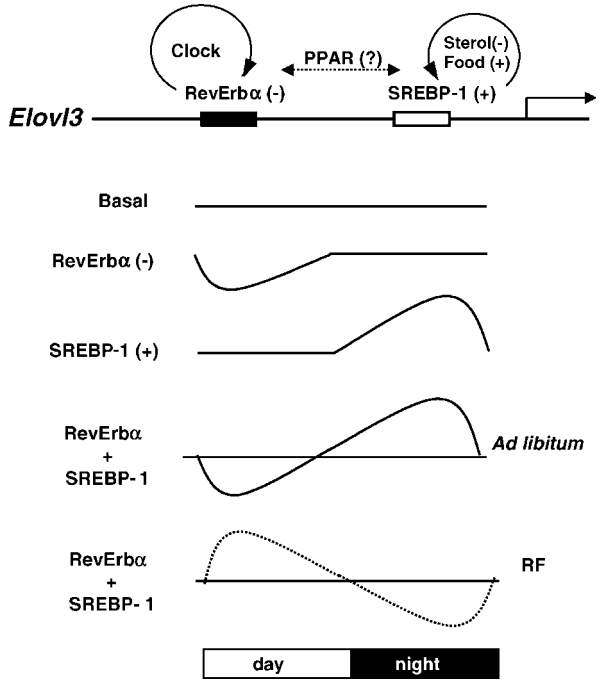

Fig. 4. Proposed biphasic circadian regulation of the *Elovl3* promoter in the male mouse liver. Hypothetical model according to which a high amplitude in *Elovl3* gene expression is accomplished by a combination of out-of-phase negative and positive influences. The circadian clock in the liver can exert negative pressure on the *Elovl3* gene during the day through the action of a RevErbα-dependent pathway. The feeding schedule (clock-interdependent) can positively affect the *Elovl3* promoter at night as a result of the rhythmic activation of SREBP1. Restricted feeding during the day can reverse both RevErbα and SREBP1 rhythms, triggering a quick reversal of the rhythm in *Elovl3* (dashed line) and other similarly regulated genes in the liver. Abolition of rhythmic *Elovl3* gene expression in the liver of CLOCK mutant mice suggests that the two loops at the top of the model are linked. PPAR, peroxisome proliferator-activated receptor.

a significantly more robust transactivation (higher stimulation index) of *Elovl3* by SREBP1 (data not shown).

SREBP-CLOCK connections

The results of this study also give rise to the question of whether or not the BMAL/CLOCK pathway of transcriptional regulation can feed into the SREBP1 activation system in peripheral oscillators. The *SREBP1* gene can be induced by differentiation (49), diets rich in PUFAs (48, 50), or refeeding after fasting (51). *SREBP1* mRNA (UniGene Cluster Mm.214958) also displays a circadian rhythm of expression in the rodent liver (4), as well as SREBP1 active protein, yet SREBP1 precursor remains at a constant level throughout the day (35) (Fig. 3C). The SREBP1 precursor is a cell membrane-anchored protein that becomes activated through proteolytic cleavage of the N terminus upon sterol depletion (52). Thus, the nucleus-bound and transcriptionally active SREBP1 fragments can translate extremely low sterol levels (e.g., cholesterol) into specific patterns of gene expression (53).

It has also been suggested that SREBP1 in the liver could be involved in the transcriptional control of insulin-responsive genes (51) and that the regulation of gene expression by feeding could be exerted in part by insulin acting on the posttranscriptional activation of SREBP proteins (54). To our knowledge, ours is the first report of a detectable overt daily rhythm in the levels of active SREBP1 in the liver during ad libitum feeding (35) (Fig. 3C), which is consistent with an underlying rhythm in food intake-mediated (and perhaps insulin-mediated) SREBP1 activation. Should this interpretation be valid, one would predict that the amplitude of the rhythmic SREBP1 cleavage will vary significantly depending on the extent of consolidation in the feeding behavior. Thus, deviations from our ad libitum results might be observed in other strains or under different conditions.

It is well established that the sterol depletion that ensues after fasting is a strong cue for SREBP activation. The mechanism involves the localized activity of an SREBP cleavage-activating protein (SCAP) whose shuttling back and forth between the endoplasmic reticulum and the Golgi is sterol level-dependent (55). Thus, it is likely that low sterol levels play a role in the consolidation of the phase-shifted pattern of SREBP1 activation we observed during the reverse restricted feeding paradigm (Fig. 3). It will be of interest to investigate whether the SREBP1 regulators, SCAP and CP332 (56), are themselves subject to circadian modulation in this context. More generally, we do not currently know to what extent the rhythmic cleavage of SREBP1 in the liver is also affected by the clock independent of the clear cyclic nature of the feeding behavior. On the other hand, as we and others have shown, key SREBP targets, such as ACC1, FAS, acyl-CoA synthase, glycerol-3-phosphate acyltransferase, ELOVL6, CIG30, and HMG-CoA reductase, display circadian expression patterns in several animal models (4, 35, 57), probably as a result of a dual regulatory mechanism involving nutritional (SREBP) and circadian (RevErbα or BMAL1) signals, as proposed in the *Elovl3* model.

A hypothetical model

We can now attempt to integrate these results into a coherent hypothetical model of *Elovl3* transcriptional control that would incorporate the effect of the CLOCK mutation upon the levels of *Elovl3* gene expression and the likely role of SREBP1 activation as a circadian sensor of nutritional status. We propose the existence of at least two input pathways controlling the *Elovl3* promoter (Fig. 4). The first is clock-dependent, affected by RevErbα, and active during the day. The second is food-dependent, mediated by SREBP1, and operates at the end of the active phase or an unscheduled fasting period (Fig. 3), when sterol levels are at a nadir and glucose levels begin to increase (58). *SREBP1* mRNA has also been found to be under circadian control in the liver (4); this underlying rhythm could serve to replenish the pool of SREBP1 precursor being depleted during the dark phase.

The combination of the RevErbα and SREBP1 antiphasic and opposing pathways could account for the high

amplitude rhythm observed in the levels of *Elovl3* mRNA. Restricted feeding during the day has already been shown to reverse the RevErbα mRNA cycle (36). Because we have shown in this study, for the first time, that a similar paradigm shifts the SREBP1 activation cycle and *Elovl3* mRNA acrophase by ~9 h (Fig. 3), these observations are consistent with the hypothesis that the coordinated actions of RevErbα and SREBP1 are ideally positioned to drive the observed rhythm in *Elovl3* expression, and likely many other late-night-peaking genes, in the liver. We would have predicted that once the impact of the RevErbα (clock) loop is abrogated, the influence of the second control mechanism, driven by the SREBP1 (nutrition) factor, would become the sole force driving transcription. Our finding that liver *Elovl3* expression settles for a tonic level in CLOCK mutant mice, however, suggests that the two pathways might be interdependent. One possibility is that the expression of *SREBP1* mRNA is dampened in the CLOCK mutant, which, in turn, would deplete the pool of activatable SREBP1 protein. We are currently analyzing the effect of clock mutations upon the rhythm of *SREBP1* mRNA and SREBP1 proteolytic processing in the liver. Alternatively, the products of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors could play a role in connecting the loops shown in Fig. 4. PPARγ, for example, is a circadian (4) and SREBP1-driven (59) gene and has been reported to affect RevErbα gene transcription. Similarly, mice that do not express the PPARα gene [a strongly circadian gene in the liver (4)] display a severe disruption of SREBP-dependent transcriptional pathways (60).

Potential physiological implications of *Elovl3* circadian expression

In terms of how circadian regulation of *Elovl3* could fit with the needs of the liver to synthesize long-chain fatty acids, we should mention that the main fraction of intracellular VLCFAs in the liver is esterified in various lipids, predominantly in sphingolipids. The bulk of sphingolipids are located in the plasma membrane, and a large body of evidence has been accumulated that implicates sphingolipids and their degradation products in signal transduction, cell proliferation, differentiation, and apoptosis (61) as well as in the formation of functional lipid microdomains (62). Given its circadian expression, as well as its role in synthesizing VLCFAs, it would be reasonable to propose that *ELOVL3*, along with other lipogenic enzymes, could contribute to establishing and/or sustaining a circadian pattern of membrane fluidity and, consequently, regulating the putative circadian rhythmicity of membrane-linked processes in the liver, such as signal transduction involving sphingolipids, apoptosis, metabolite uptake, and turnover of phospholipids.

Regarding daily variation in lipid metabolism, studies using hamsters showed that the diurnal phase is characterized by inactivity and fuel storage (maximal enzymatic activity in fatty acid and triglyceride synthesis), whereas the night phase is one of exercise and fuel mobilization (fatty acid oxidation and sterol synthesis) (63). A circadian rhythmicity has also been reported in the expression level of lipogenic ACC1, FAS, ACS4, and G3PAT genes in mice (4, 35). In this work, the mRNA level of *Elovl3* shows the same pattern as those of FAS and G3PAT, peaking at the end of the night/beginning of the day. *Elovl3* would, in this way, participate in the anabolic activity during the inactivity phase.

Additionally, the fact that *Elovl3* was very sensitive to the acute reversal in the schedule of food consumption in mice is consistent with the significant phase shift in the circadian expression of *Fas* and *g3pat* genes observed under an identical feeding schedule (35). Such a mechanism could contribute to the increased energy storage at the end of the night/beginning of the day, preparing the body for or anticipating a period of food deprivation. The significant reversal in the expression of *Elovl3* under diurnal feeding conditions is also consistent with the reported rapid switch of hepatic fatty acid metabolism from oxidation to synthesis and the concomitant hyperactivation of lipogenesis upon refeeding of diurnally fed rats (64).

Conclusions

The influence of the suprachiasmatic nucleus upon rhythmic gene expression in the liver is likely exerted through direct and indirect connections. It is well accepted that the autonomic nervous system and the establishment of activity/feeding schedules are likely the ultimate drivers of hepatic rhythmic gene expression, as pointed out in several previous studies (36, 37, 65).

The possible communication between the RevErbα and SREBP1 pathways that emerges from our study of the *Elovl3* gene promoter (Fig. 4) introduces a potentially attractive interphase for the homeostatic integration of clock and nutritional signals. The reversal of liver rhythms during restricted feeding occurs even if the photoperiod remains unchanged, and it does not affect the endogenous rhythms in the suprachiasmatic nucleus (36, 37). Therefore, it has been concluded that restricted feeding can effectively uncouple the clock in the liver from the circadian humoral cues derived from the central oscillator in the brain (36). This phenomenon can be interpreted as evidence for the existence of a molecular sensor of nutritional status able to override other circadian entraining signals (37). A possible scenario for how this might be implemented emerges from the restricted feeding experiment (Fig. 3) involving the rapid modulation of the daily rhythm in SREBP1 precursor cleavage we have seen in the liver, which is predominantly linked to nutritional status.

In summary, we found that the expression of *Elovl3* is sexually dimorphic: its circadian expression in the liver is detectable only in the male, whereas both genders express it in iBAT. We also propose for the first time a preliminary functional map of the *Elovl3* upstream regulatory region and report that RevErbα likely plays a connecting role between the core clock machinery and *Elovl3* expression. In addition, our studies revealed that SREBP1 could play an important role in the regulation of the *Elovl3* gene, activating it during the dark phase to consolidate and/or amplify the temporal pattern established by the diurnal action of the RevErbα transcriptional repressor. Finally,

we propose that the daily cycles of SREBP1 activation in the liver are loosely coupled to the clock machinery. We predict that this coupling is disengaged during diurnal restricted feeding, when a reversed pattern of SREBP1 activation overrides central circadian cues to reset the rhythms of many targets in the cholesterogenic and lipogenic pathways. Such a mechanism would be essential to accomplish an acute reversal in the sequence of absorptive and postabsorptive phases in the liver while avoiding a protracted clock-dependent reentrainment that would require several days to be completed.

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