Shifting the feeding of mice to the rest phase creates metabolic alterations, which, on their own, shift the peripheral circadian clocks by 12 hours

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The molecular mechanisms underlying the events through which alterations in diurnal activities impinge on peripheral circadian clocks (PCCs), and reciprocally how the PCCs affect metabolism, thereby generating pathologies, are still poorly understood. Here, we deciphered how switching the diurnal feeding from the active to the rest phase, i.e., restricted feeding (RF), immediately creates a hypoinsulinemia during the active phase, which initiates a metabolic reprogramming by increasing FFA and glucagon levels. In turn, peroxisome proliferator-activated receptor alpha (PPAR α) activation by free fatty acid (FFA), and cAMP response element-binding protein (CREB) activation by glucagon, lead to further metabolic alterations during the circadian active phase, as well as to aberrant activation of expression of the PCC components nuclear receptor subfamily 1, group D, member 1 (*Nr1d1/RevErb α*), Period (*Per1* and *Per2*). Moreover, hypoinsulinemia leads to an increase in glycogen synthase kinase 3 β (GSK3 β) activity that, through phosphorylation, stabilizes and increases the level of the RevErb α protein during the active phase. This increase then leads to an untimely repression of expression of the genes containing a RORE DNA binding sequence (DBS), including the *Bmal1* gene, thereby initiating in RF mice a 12-h PCC shift to which the CREB-mediated activation of *Per1*, *Per2* by glucagon modestly contributes. We also show that the reported corticosterone extraproduction during the RF active phase reflects an adrenal aberrant activation of CREB signaling, which selectively delays the activation of the PPAR α -RevErb α axis in muscle and heart and accounts for the retarded shift of their PCCs.

shifted eating | shifted peripheral circadian clocks | metabolic alterations | RevErb α | PPAR α

Pioneering studies (1, 2) have established that switching the feeding time in mice from the “active” phase [dark period of the light-dark (L/D) cycle] to the “rest” phase (light period), i.e., restricted feeding (RF), overrides the suprachiasmatic nucleus (SCN) circadian clock (CC)-derived signals and acts as a “zeitgeber” for peripheral CCs (PCCs), leading to a 12-h shift in the time at which components of PCCs are expressed. Numerous studies have shown that under homeostatic conditions, the functions of PCCs and metabolism are tightly linked and that perturbations in their interactions leads to pathologies, e.g., obesity and metabolic syndrome (3–5). The identity of some of the molecular pathways that couple PCCs to metabolism are known (3–5), but it is largely unknown how environmental cues, e.g., altered feeding schedules, may directly perturb the expression of individual CC components (5–7), thereby leading to obesity and a metabolic syndrome-like pathology (5). Assuming that specific metabolic perturbations generated by switching the feeding time could selectively affect the time of expression of some of the CC core components, we looked for both metabolic and PCC alterations at early RF times. We report here a comprehensive temporal analysis, and we found that the shift of PCCs on RF is directly linked, at the molecular level, to metabolic reprogramming that directly impinges on CC component expression.

Results

RF Leads to a Starvation-Like State Characterized by Metabolic and CC Alterations. We initially observed that on RF, gastric emptying is severely delayed (Fig. S1B), thereby creating a “starvation-like state.” This observation led us to investigate the effect of a 24-h starvation on metabolic parameters, which revealed reductions in glucose and insulin (INS) and increases of ketone bodies (β OHB; β -hydroxybutyrate) and free fatty acid (FFA) (Fig. S1A and E), whereas concomitant transcript analyses showed an increase and altered pattern of expression for Period 1 and 2 (*Per1* and *Per2*) and nuclear receptor subfamily 1, group D, member 1 (*Nr1d1/RevErb α*), but not of aryl hydrocarbon receptor nuclear translocator-like protein 1 (*ARNTL1/Bmal1*), cryptochrome 1 (*Cry1*) and a nuclear Factor, interleukin 3 regulated (*NFIL3/E4BP4*) (Fig. S1C and D). We therefore posited that RF could be accompanied by metabolic changes that could affect PCCs.

Analyzing the RF effect on these parameters during the RF first day (RF1; Fig. 1A) indicated INS and triglyceride (TG) decreases, whereas glucose, FFA, and glucagon increased (Fig. 1B). Looking at CC component expression during RF1 showed that *Per1*, *Per2*, and *RevErb α* transcripts increased between “Zeitgeber” (ZT) 20 and ZT4 (food withdrawal at ZT12), whereas *Bmal1*, *Cry1*, and *E4BP4* were unaltered (Fig. 1C and Fig. S1F–I). Of note, using luciferase reporter mice, Saini et al. (8) observed a similar pattern of expression for *Per2*, *RevErb α* , and *Bmal1* in RF livers. That early RF metabolic changes were similar to those generated under starvation and that RF and starvation altered the same CC components prompted us to investigate how metabolic alterations may impair PCC during RF.

Significance

Under homeostasis, peripheral circadian clocks (CCs) and metabolism are intimately linked, as pathologies occur on perturbation of their coupling. In mice, shifting the feeding time from the phase of activity (night) to the phase of rest (day) is known to act as a time cue for peripheral CCs, leading to a 12-hour shift of the time at which their CC components are expressed. However, the molecular mechanisms that underlie this shift are largely unknown. Here, we reveal both the origin and the identity of the metabolic signals that are generated on shifting eating to the rest phase and how these signals directly alter the expression of CC components to generate the shift that ultimately leads to metabolic syndrome-like pathology.

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Fig. 1. RF initiation induces metabolic and CC alterations. (A) Schematic representation of the RF1 experiment. (B) Levels of blood components in control and RF1 mice. (C) RNA transcript levels of CC components in RF1 liver. (D) Levels of blood components in control, RF1, and RF1 intraperitoneally glucose administered (RF1+Glucose) mice. (E–G) RNA transcript levels of genes as indicated in liver (E), IEC (F), and pancreas (G) of control, RF1, and RF1+Glucose mice. All values are mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

Glucose Administration Prevents Metabolic and PCC Alterations During the RF Initial Phase. A starvation-induced decrease in blood glucose reduces INS and increases glucagon and FFA levels, thereby activating gluconeogenesis and β -oxidation (9, 10). We therefore posited that maintaining blood glucose may prevent metabolic and PCC alterations during early RF. Indeed, glucose administration to RF mice (RF glucose mice) prevented INS decrease and a rise in FFA, corticosterone, β OHB, and fibroblast growth factor 21 (FGF21) (Fig. 1D). Notably, there was no increase of *Per1*, *Per2*, and *RevErba* in the liver, intestinal epithelial cells (IECs), and pancreas of RF glucose mice (Fig. 1E–G), supporting our hypothesis that the initial RF CC alterations originate from an altered metabolism.

An Early Insulin Decrease Triggers Increased Lipolysis, FFA Release, and Aberrant PPAR α Signaling in RF Mice. A decrease in blood INS triggers adipose tissue lipolysis and release of FFA, which acts as a ligand for peroxisome proliferator-activated receptor alpha (PPAR α) (11). Importantly, FFA-liganded PPAR α (see Fig. 3A) is known to activate transcription of a number of target genes containing a direct repeat 1 (DR1) DNA binding sequence (DBS) (11), and to activate its own transcription by binding to its own DR1 DBS (11). Accordingly, expression of PPAR α and of its target genes was increased in RF liver and IECs between ZT16 and ZT0 (Fig. 2B and Fig. S2B, C, and F), and ChIP assays demonstrated a concomitant increase in PPAR α binding to its own DR1-DBS and to those of its target genes (Fig. 2C). We previously demonstrated that, in absence of *Bmal1*, PPAR α could induce *RevErba* transcription through binding to the DR2 DBS of the *RevErba* promoter (12). As *RevErba* transcripts increased in RF mice (when no *Bmal1* activity could be detected; see above), we speculated that PPAR α

could also contribute to this RF increase in *RevErba* expression. Indeed, PPAR α was recruited to *RevErba* DR2 DBS during early RF (ZT20–ZT0), but not in control animals (Fig. 2D). Moreover, selective PPAR α mutations in liver (PPAR $\alpha^{\text{hep-/-}}$) and IECs (PPAR $\alpha^{\text{iec-/-}}$) demonstrated that the early RF increases in *RevErba*, *HMGCS2*, *FGF21*, and *Flaf* transcripts were dependent on PPAR α (Fig. S2D and E and Fig. 3A). Importantly, glucose administration to RF mice inhibited PPAR α binding to its own promoter and to the DR1 DBS in its target genes, as well as to the *RevErba* DR2 DBS (Fig. S2J and K), leading to a reduction in their expression during RF (Fig. 1E–G and Fig. S2I). Of note, these early RF-induced expressions of PPAR α and of its target genes were not affected in *Bmal1*^{hep-/-} and *Bmal1*^{iec-/-} mutants (Fig. S2G and H).

PPAR α and RevErba Are Instrumental in RF-Induced Breaking of Existing PCCs. As RF early metabolic alterations did affect the expression of *Per1*, *Per2*, and *RevErba*, we analyzed the transcript of CC components after RF for 4, 8, 15, 30, and 90 d. In liver and IECs, all of them were shifted after RF4, but it took 8 d to achieve the same shifts in muscle and heart (see below). Importantly, once established these shifts were stable as long as RF was not interrupted, whereas the “original circadian active and rest phases” remained unchanged throughout the RF period (13).

Analysis of CC shifts in liver and IECs during the first 108 RF hours (Fig. 4A and Fig. S3A) revealed that they occurred in two steps. The first phase (broken clock; compare Fig. 3B and C) was triggered by metabolic alterations occurring during the active phase (ZT12–ZT0) through (i) an increase of FFA-liganded PPAR α that activated aberrant *RevErba* expression (Figs. 3C and 4A and Fig. S3A) mediated by its binding to the *RevErba* DR2 DBS (Fig. 4D). This *RevErba* increased expression then repressed during the ac-

Fig. 2. RF-induced aberrant activations of PPAR α and CREB signaling play a critical role in metabolic and CC alterations. (A) Schematic representation of the RF1 experiment. (B) RNA transcript levels of genes, as indicated, in RF1 liver. (C) ChIP-qPCR assays to analyze PPAR α recruitment to DR1 DBS-containing genes in liver of control and RF1 mice. (D) ChIP-qPCR assays to analyze PPAR α recruitment to the RevErba DR2 DBS in liver and IEC of control and RF1 mice. (E) ChIP-qPCR assays to analyze CREB binding to CRE DBSs present in the indicated genes in control and RF1 liver. (F) NAD $^{+}$ levels in control and RF1 liver. (G) Immunoblot analyses of control and RF1 liver with indicated antibodies. (H) RNA transcript levels of genes as indicated in liver of control and RF1 mice, with or without selective mutation of *Bmal1* (*Bmal1*^{hep-/-}). All values are mean $\pm$ SEM. ** $P < 0.01$.

tive phase the expression of ROR α /RevErba response element (RORE)-containing genes (Fig. 4A and B and Fig. S3A and B), and (ii) a simultaneous pCREB increase that activated *Per1*, *Per2* expression (Fig. 4A and Figs. S3A, S4C, and S5A). Importantly, besides these *Per1*, *Per2*, and *RevErba* transcript alterations during RF1 night, there was no changes in the profile of other CC components (*Bmal1*, *Cry1*, and *E4BP4*; Fig. 4A and Fig. S3A). In contrast, the expression of all CC components was altered during RF2 (compare RF1 and RF2 in Fig. 4A and Fig. S3A), because (i) during the active phase, the levels of *Bmal1*, *Cry1*, and *E4BP4* transcripts were diminished, (ii) during the rest phase (ZT0–ZT12), there was a decrease in *RevErba* transcripts that were abnormally expressed during the active phase, and (iii) the physiological ZT8–ZT12 expression of *Per2* transcripts disappeared, whereas both *Per1* and *Per2* transcripts were modestly expressed during the ZT20–ZT4 period (Fig. 4A and Fig. S3A).

ChIP assays were performed in RF liver and IECs to explore the basis for these aberrant expressions of PCC components. It is known that *RevErba* expression is controlled by *Bmal1* (3) and, in addition, that the *RevErba* promoter contains a DR2 DBS to which RevErba binds to trigger its autorepression, whereas the binding of FFA-liganded PPAR α to the same element enhances *RevErba* expression (12, 14) (Fig. 3C). As *PPAR α* ^{hep-/-} and *PPAR α* ^{iec-/-} mutants (Fig. S2D and E) together with *Bmal1*^{hep-/-} and *Bmal1*^{iec-/-} mutants (Fig. S2G and H) revealed the *Bmal1*-independent critical role of PPAR α in *RevErba* early expression in

RF mice, we analyzed PPAR α binding on the *RevErba* DR2 DBS in RF tissues. Remarkably, this binding was only detected in RF mice during the ZT16–ZT0 period of the first two RF nights (Fig. 4D; see below), thereby leading to aberrant *RevErba* transcription during the active phase (Figs. 3C and 4A and Fig. S3A).

As expected (9), RF hypoinsulinemia also decreased pAKT, thereby activating glycogen synthase kinase 3 β (GSK3 β) which, in turn, through phosphorylation, increases the amount of RevErba (steps 8–11 in Fig. 3A and Fig. S3C) (13). ChIP assays (Fig. 4B and Fig. S3B) performed to investigate the consequence of this active phase RevErba expression revealed a time-restricted (ZT8–ZT12) RevErba binding to *Bmal1*, *Cry1*, and *E4BP4* RORE DBS (3) in both control and RF mice during the RF1 rest phase (Fig. 4B and Fig. S3B). In contrast, during the active phase of RF2, RevErba was recruited on these RORE DBSs between ZT20 and ZT4, which resulted in their repression during the active phase (Fig. 4B, Fig. S3B, and see steps 1–3 in Fig. 3C).

The Shifted Expression of *Bmal1* Generates Shifted PCCs. As *Bmal1*-dependent transcription of *Per1*, *Per2*, and *RevErba* is known to drive CCs (3–5), ChIP assays were performed to analyze its contribution in initiating the RF PCC shift. In control mice, ChIP assays showed a rest phase (ZT4–ZT12) *Bmal1* binding to the E-Box DBS of *Per1*, *Per2*, and *RevErba* (Fig. 4C and Fig. S4A) (15). Strikingly, on RF1 night, no *Bmal1* binding could be detected on the E-box of these genes when they were initially

Fig. 3. A schematic representation of how RF-induced metabolic alterations induce a shift of PCCs. (A) Metabolic alterations induced by RF. The up and down blue arrows correspond, respectively, to increases and decreases of indicated components during RF. The red arrowheads indicate components present in the blood. (B) The two interlocked feedback loops constituting the normal CC oscillator. (C) The early phase of peripheral CC disruption. The up and down blue arrows correspond to increases and decreases of indicated components in RF, respectively. Red crosses represent RF-induced impairments. (D) The late phase of reconstruction of a new shifted PCC.

aberrantly expressed (Fig. 4C and Fig. S4A), due to PPARα binding on the *RevErbα* DR2 DBS and CREB recruitment on the CRE present in *Per1* and *Per2* genes (Fig. 4D and Figs. S4C and S5A). Remarkably, no Bmal1 binding to these E-Boxes could be observed during the ZT4–ZT12 period of RF2, due to the repression on *Bmal1* exerted by *RevErbα* aberrantly expressed during the RF2 night (Fig. 4A and B and Fig. S3A and B). Importantly, refeeding RF mice (at ZT0) reduced the FFA blood level and consequently decreased PPARα binding on the *RevErbα* DR2 DBS (Fig. 4D), thus reducing *RevErbα* expression (see ZT4 of RF3; Fig. 4A and Fig. S3A). This resulted in a derepression of *Bmal1* expression that was delayed to the ZT12–ZT20 period of RF3, thereby initiating the PCC shift (Fig. 4A and Fig. S3A; see Fig. 3C and D).

During the second phase of the RF CC shift (Fig. 4A and Fig. S3A), the now shifted *Bmal1* expression progressively restored the Bmal1-dependent expression of *RevErbα*, *Per1*, and *Per2* during the ZT20–ZT4 period (Fig. 4A and C and Figs. S3A and S4A; see Fig. 3D). Importantly, this Bmal1-dependent *RevErbα* synthesis during the ZT20–ZT4 period (Fig. 4C and Fig. S4A) not only prevented further binding of PPARα on the *RevErbα* DR2 DBS (making its transcription independent of metabolism), but also started to repress itself through binding to its own RORE and DR2 DBS (Fig. 4E and Fig. S3B). This pattern of *RevErbα* expression (ZT20–ZT4) with a zenith at ZT0 instead of ZT8 in WT mice was then repeated on the subsequent RF days, thereby creating an RF permanently shifted temporal window (ZT12–ZT20) for the RORα/γ-dependent expression of RORE

mutation of *RevErba* in either liver (*RevErba*^{hep-/-}) or IECs (*RevErba*^{iec-/-}) prevented the RF CC shift in these tissues, but not the RF-induced metabolic alterations (Fig. S5 C-G).

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in gluconeogenesis (steps 1–4 in Fig. 3A) (10). In keeping with the RF glucagon surge, there was indeed a pCREB increase (Fig. 2G and Fig. S6D) that led to increased expressions of CRE-containing genes (Fig. 2E and Fig. S6B, C, and E; see Fig. 3A). NAD⁺ is increased in RF liver (Fig. 2F) and plays a critical role in the sirtuin1 (SIRT1)-mediated activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (10). These RF NAD⁺ and NAMPT [nicotinamide phosphoribosyl-transferase; the rate limiting enzyme in the salvage pathway (4, 5)] increases were concomitant (Fig. S6B and C). Note that this RF-induced increase in NAMPT expression persisted in the selective *Bmal1*^{hep-/-} and *Bmal1*^{iec-/-} mutants (Fig. 2H and Fig. S6H), even though *Bmal1* is known to control its circadian expression (4, 5). Instead, we found that these NAMPT increases were related to an increased CREB binding to a CRE DBS (TGACGTCA) located in the NAMPT promoter (Fig. 2E and Fig. S6E; 7 in Fig. 3A). Moreover, ChIP analyses revealed an enhanced CREB binding to the CRE located in the *Per1* and *Per2* gene promoters (Fig. 2E and Fig. S6E) (16), which led to its increased expression during RF1 (see above). As expected, glucose administration to RF mice also prevented the RF-induced CREB activation (Fig. S6G), as well as the increased expressions of its target genes (Fig. 1E–G and Fig. S6I and J). Inhibiting the PKA activity by H-89 administration (17) during RF1 also prevented RF-induced CREB activation (Fig. S6F), as well as the increased expressions of its target genes (Fig. S6K), thus supporting the conclusion that the RF-induced CREB activation plays a critical role in the increased gluconeogenesis and CC alterations that occur on RF.

We next explored the relative contributions of FFA-PPARα-RevErbα and glucagon-CREB-Per1 and Per2 pathways in achieving the complete RF CC shift (Fig. 3C and D). Notably, on RF4, there was no aberrant expression of *RevErbα* in selective *PPARα*^{hep-/-} and *PPARα*^{iec-/-} mutant mice (Fig. S7A and B). However, the CREB-dependent ZT20–ZT4 increase in *Per1* and *Per2* expression persisted in these mutants (Fig. S7A and B), which resulted after 8 RF days in a 4 days-delayed shift of all CC components in PPARα-ablated liver and IECs (Fig. S7C and D), thus demonstrating that CREB activation plays an ancillary role in the RF CC shift, the major role being played by the PPARα-induced aberrant expression of *RevErbα* (Fig. 3C).

An Increased CREB Activity in Adrenal Glands of RF Mice Leads to Extra-Corticosterone Production. RF is known to induce during the ZT20–ZT4 period an extra peak of corticosterone production in an adrenal-dependent (Fig. S8E) (18), but SCN-independent, manner (21). However, the mechanism involved in this RF extra-corticosterone production has not been elucidated. As cAMP-PKA-CREB signaling plays, at ZT12, a critical role in physiological corticosterone production through transactivation of the numerous genes involved in corticosterone synthesis (22, 23), we investigated the role of CREB in extra-corticosterone production. We found, on RF1 night (Fig. S8A) in adrenal glands, increases in pCREB level (Fig. 5C), as well as in transcripts of various CREB target genes (Fig. S8B and C), including those involved in corticosterone synthesis (*Cyp11a*, *Cyp11b*, *Hsd3b*, *Prkar2b*, and *Adcy5*; Fig. S8D). To support the role played by an increased CREB activity in RF extra-corticosterone production, the PKA activity was inhibited by H-89 administration (17), which prevented the RF-induced CREB binding to CREs present in *Cyp11a*, *Cyp11b*, and *Hsd3b* genes (Fig. S8F) and their ZT20–ZT4 transcription (Fig. 5D), thereby reducing the corticosterone blood level (Fig. S8J).

A Retarded RF-Induced CC Shift in Muscle and Heart Reflects a Delayed Activation of PPARα. It has been reported that the RF CC shift was delayed in muscles and heart (1) and that this delay could be overcome by adrenalectomy (Adx) (18), but how corticosterone could possibly retard the CC shift in muscle and

heart was unknown. We posited that a retarded activation of PPARα (relative to other tissues such as liver and IECs; see above) in muscle and heart could be at the origin of this delay. Of note, it was reported (19) that a PPARβ-dependent synthesis of phosphatidylcholine 18:0/18:1 in the liver was a prerequisite for PPARα activation in muscle and heart (but not in liver; see below). Most interestingly, we found that the liver expression of *PPARβ*, which was repressed on RF1, was restored by Adx or administration of the glucocorticoid antagonist RU486 (Fig. 5A and B). Moreover, we also found that within the *PPARβ* promoter there is a negative glucocorticoid receptor (GR) response element (IR2 nGRE) (20), which suggested to us that the RF1 extra-corticosterone surge (ZT20–ZT4; Fig. S8G) could account for the RF1 repression of *PPARβ* expression. Accordingly, we found that Adx or RU486 administration, by preventing the RF1 repression of *PPARβ* in the liver, led to an early activation of PPARα in muscle and heart and consequently to a stimulation of *RevErbα* expression in these tissues during RF1, thereby accelerating the pace of the CC shift in these tissues (Fig. 5A and B and Fig. S7I and J; see Fig. 5E and F). Moreover, we found that this “extra-corticosterone”-dependent *PPARβ* repression was relieved by RF4 because the liver *PPARβ* expression shifted on RF4 from ZT20–ZT4 to ZT4–ZT12 (Fig. S7L) and therefore escaped the extra-corticosterone-dependent repression, which led to a gradual increase in *PPARα* level (in muscle and heart), and consequently to a shift in *RevErbα* expression (Fig. S7E–I and Fig. 5E–G).

Discussion

Shifting in the Mouse Eating from the Active Phase to the Rest Phase Induces Alterations of Two Metabolic Pathways That Unequally Generate a 12-Hour Shift of PCCs. Here we demonstrated that on RF, the shift of PCCs involves two distinct phases: (i) breaking the existing PCCs (Fig. 3C) and (ii) generating shifted PCCs (Fig. 3D). The breaking phase originates from the fasting imposed during the active phase, which by reducing INS secretion triggers an untimely increase in glucagon and FFA (Fig. 3A and C). Importantly, in RF tissues (liver, IECs), these alterations impinge on CCs during the active phase through (i) the FFA-PPARα-dependent *RevErbα* expression and (ii) the glucagon-CREB-dependent *Per1* and *Per2* expression (Fig. 3A). Most notably, we found that glucose administration to RF mice by preventing the reduction in INS prevented the metabolic and subsequent PCC alterations (Fig. 1D–G), thus establishing that a fasting state is in fact at the origin of the RF CC shift. Importantly, at the onset of RF, the PPARα-dependent aberrant *RevErbα* synthesis during the active phase breaks the existing PCCs through RORE-mediated repression of *Bmal1* synthesis (see steps 1–3 in Fig. 3C). The postfeeding reduction in FFA-PPARα levels then leads to a decrease in *RevErbα*, an essential event that derepresses *Bmal1* expression but also permanently shifts it to the ZT12–ZT20 period (step 4 in Fig. 3C and D). Subsequently, through controlling the E-Box-mediated expression of *Per1*, *Per2*, and *RevErbα*, this shifted *Bmal1* expression enables the emergence of a shifted repaired CC (step 5 in Fig. 3D). Notably, this whole process progressively restores a separation of 12 hours between the zenith and nadir of expression of all CC components, thus creating an RF peripheral CC oscillator, which is similar to that seen in non-RF mice, but shifted at the RNA level by 8 hours for the *Bmal1*, *Cry1*, *RevErbα*, and *E4BP4* CC components, whereas *Per1* and *Per2* are shifted by 12 hours.

Importantly, we found that selective mutation of *PPARα* in liver and IECs delayed by 4 days the completion of their RF CC shift (Fig. S7A–D). In these PPARα-mutant tissues, these shifts were achieved by CREB-dependent *Per1* and *Per2* activation. Therefore, it is the PPARα-*RevErbα* axis, which by perturbing the second loop of the CC oscillator (Fig. 3B and C), efficiently drives the RF CC shift, whereas the CREB-*Per1* and *Per2* axis plays an ancillary role.

The RF-Generated Extra-Corticosterone Production Delays PPAR α Activation in Muscle and Heart, Thereby Retarding the CC Shift in These Tissues. Our study unveiled that the underlying basis for the RF extra-corticosterone production (18, 21) (Fig. S8G) can be ascribed to an aberrant increase in adrenal CREB activity, which induces the adrenal expression of corticosterone synthesizing genes

(Fig. S8D). Importantly, this RF-induced increase in adrenal CREB activity is accompanied by a surge in adrenocorticotrophic hormone (ACTH) secretion (Fig. S8H), which is known to increase the cAMP-PKA-dependent CREB activity (22). Interestingly, this RF extra-ACTH-corticosterone surge temporally coincides with an RF blood increase in the liver-derived growth factor FGF21 (Fig. 1D

Fig. 5. RF-induced PPAR α activation is delayed in muscle and heart. (A) RNA transcript levels of genes, as indicated, in control and RF1 mice on adrenalectomy (Adx). (B) RNA transcript levels of genes, as indicated, in control and RF1 mice with or without RU486 (RU) administration. (C) Immunoblot analyses of adrenal glands in control and RF1 mice with indicated antibodies. (D) RNA transcript levels of genes as indicated in the adrenal glands of control and RF1 mice treated with or without the PKA inhibitor (H-89). (E) Physiological corticosterone production has no effect on PPAR β expression in liver, which controls the activity of PPAR α in muscle and heart. (F) A schematic representation of how RF extra-corticosterone production delays the CC shift in muscle and heart. (G) A schematic representation of how adrenalectomy (Adx) or inhibition of corticosterone signaling by RU486 leads to an RF-induced early CC shift in muscle and heart. All values are mean $\pm$ SEM.

and Fig. S2F), which was recently found to increase the hypothalamic corticotropin-releasing hormone (CRH) and pituitary ACTH secretion and, subsequently, corticosterone production (24). Thus, the RF-dependent increase in the FGF21 level provides a rationale for the previously reported suprachiasmatic nucleus (SCN)-independent corticosterone production during RF (21).

The delayed CC shift in tissues of RF *PPARα* mutants (see above) suggested to us that the delayed CC shift in muscle and heart, previously reported to be lagging behind that of liver (1), could reflect a delayed activation of *PPARα*. As this delay disappeared in ADX mice, it was ascribed to the RF extra-corticosterone production (18). Interestingly, *PPARα* activation in muscle and heart is dependent on the expression of *PPARβ* in liver (19), which reaches its zenith at ZT20–ZT4 (Fig. 5E). Notably, we found that, during early RF, the *PPARβ* promoter that contains an IR2 nGRE DBS (20) is repressed in liver by extra-corticosterone-liganded GR, thus accounting for the delayed activation of *PPARα* in muscle and heart (Fig. S7 E–H and Fig. 5 F and G) and also providing a rationale for the glucocorticoid-dependent inertia (18) of their RF CC shift.

Methods

Mice and Treatments. Eight- to 12-wk-old C57BL/6J male WT and adrenal-ectomized mice (Charles River Laboratories) were used. Control mice were provided food and water ad libitum, under 12-h light (6:00 AM–6:00 PM) and 12-h dark (6:00 PM–6:00 AM) conditions. RF mice were provided food during the entire light period (1). Breeding, maintenance, and experimental manipulations were approved by the Animal Care and Use Committee of IGBMC/ICS.

Statistics. Data are represented as mean $\pm$ SEM of at least three independent experiments and were analyzed by SyStat and Microsoft Excel statistics software using the Student *t* test (RNA transcripts) and one-way ANOVA (blood metabolic analysis). *P* < 0.05 was considered significant.

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