Circadian control of oscillations in mitochondrial rate-limiting enzymes and nutrient utilization by PERIOD proteins

Adi Neufeld-Cohen, Maria S. Robles, Rona Aviram, Gal Manella, Yaarit Adamovich, Benjamin Ladeix, Dana Nir, Liat Rousso-Noori, Yael Kuperman, Marina Golik, Matthias Mann, and Gad Asher

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 7610001, Israel; Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Martinsried 82152, Germany; and Department of Veterinary Resources, Weizmann Institute of Science, Rehovot 7610001, Israel

Edited by Joseph S. Takahashi, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, and approved January 15, 2016 (received for review October 3, 2015)

Mitochondria are major suppliers of cellular energy through nutrients oxidation. Little is known about the mechanisms that enable mitochondria to cope with changes in nutrient supply and energy demand that naturally occur throughout the day. To address this question, we applied MS-based quantitative proteomics on isolated mitochondria from mice killed throughout the day and identified extensive oscillations in the mitochondrial proteome. Remarkably, the majority of cycling mitochondrial proteins peaked during the early light phase. We found that rate-limiting mitochondrial enzymes that process lipids and carbohydrates accumulate in a diurnal manner and are dependent on the clock proteins PER1/2. Concurrently, we uncovered daily oscillations in mitochondrial respiration that peak during different times of the day in response to different nutrients. Notably, the diurnal regulation of mitochondrial respiration was blunted in mice lacking PER1/2 or on a high-fat diet. We propose that PERIOD proteins optimize mitochondrial metabolism to daily changes in energy supply/demand and thereby, serve as a rheostat for mitochondrial nutrient utilization.

Significance

Mitochondria are major cellular energy suppliers and have to cope with changes in nutrient supply and energy demand that naturally occur throughout the day. We obtained the first, to our knowledge, comprehensive mitochondrial proteome around the clock and identified extensive oscillations in mitochondrial protein abundance that predominantly peak during the early light phase. Remarkably, several rate-limiting mitochondrial enzymes that process different nutrients accumulate in a diurnal manner and are dependent on the clock proteins PER1/2. Concurrently, we uncovered daily oscillations in mitochondrial respiration that are substrate-specific and peak during different times of the day. We propose that PERIOD proteins regulate the diurnal utilization of nutrients.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The MS proteomics data have been deposited in the ProteomeXchange Consortium (proteomecentral.proteomexchange.org) through the Proteomics Identifications (PRIDE) partner repository (dataset identifier PXD001732).

See Commentary on page 3127.

1A.N.-C. and M.S.R. contributed equally to this work.
2To whom correspondence may be addressed. Email: robles@biochem.mpg.de or gad.asher@weizmann.ac.il.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1519650113/-/DCSupplemental.
of different nutrients by the mitochondria and thus, optimize mitochondrial function to daily changes in energy supply/demand.

Results

Quantitative Proteomics Identify a Predominant Daily Phase for Mitochondrial Protein Accumulation. To examine the daily regulation of mitochondrial function, we commenced with a MS-based quantitative proteomics approach. Mice were killed at 4-h intervals over 2 d, livers were harvested, and mitochondria were isolated by biochemical fractionation. For each time point, mitochondria from four individual animals were independently analyzed. Proteomic analysis identified 1,537 proteins with label-free intensities (22) in at least three of four biological replicates for each measured time point. As expected, the quantified proteome was statistically enriched with mitochondrial-annotated proteins (Fig. S1A and Dataset S1). In addition, we identified some endoplasmic reticulum- and peroxisome-associated proteins that were likely to be copurified with mitochondria. Cycling analysis using a 24-h periodicity revealed that, of 1,537 proteins, 452 proteins (29%) exhibited statistically significant daily oscillations \( q \) value (permutation-based false discovery rate) < 0.15 (Fig. S1B and Dataset S2A), with median amplitude of ∼1.4-fold (Fig. S1C). Oscillating proteins exhibited two prominent phases 12 h apart: Zeitgeber time (ZT) ∼4 and ∼ZT16 (Fig. S1D and E). Interestingly, when the analysis was limited to exclusively mitochondrial-annotated proteins (Uniprot annotation), a larger fraction oscillated (223 of 590; 38%; \( q \) value < 0.15) (Fig. 1A and Dataset S2B), with median amplitude of 1.3-fold (Fig. 1B). Remarkably, the majority of cycling proteins peaked at ∼ZT4 (Fig. 1C and D). Phase enrichment analysis (using a less stringent cutoff \( q \) value < 0.5) showed that proteins from key mitochondrial functions cycle with phases statistically enriched (false discovery rate < 0.1) during the light phase (Fig. S2).

To examine the relation between transcript and protein levels, we compared our cycling mitochondrial-annotated proteome with a widely used mouse liver transcriptome dataset (23). We
applied the same statistical algorithm that was used for the proteome analysis to identify oscillations in the corresponding transcripts. Of 223 cycling mitochondrial proteins (216 had matched transcript data), 185 showed rhythms at the mRNA level (86%); however, the phase distribution of cycling proteins and transcripts differed (compare Fig. 1C with Fig. 1E). The majority of cycling transcripts encoding for oscillating mitochondrial proteins peaked at \( \sim ZT17 \). The phase correlation between transcript and their corresponding protein was very low (Pearson’s \( r = 0.01 \)) (Fig. 1F). Hence, we observed a wide distribution in the phase delay between the peak in protein accumulation and the peak in its respective transcript (Fig. 1G). Some proteins followed their transcript levels within a reasonable timeframe, whereas others were significantly delayed. About two-thirds of proteins accumulated in mitochondria more than 6 h later than the peak in their transcript levels, suggesting that posttranscriptional mechanisms shape the phase of protein accumulation in mitochondria.

Our comprehensive quantitative proteomics analysis thus revealed daily oscillations in a large fraction of the mitochondrial proteome. Cycling mitochondrial proteins mostly reached their zenith levels during the early light phase. Although the transcript levels of the majority of oscillating proteins cycled, the transcript to protein phases differed, pointing toward the involvement of posttranscriptional control. Conceivably, the diurnal oscillations in mitochondrial proteins are expected to affect mitochondrial function throughout the day.

Key Mitochondrial Metabolic Enzymes Accumulate in a Daily Manner.

To pinpoint mitochondrial processes that might be under circadian control, we generated flow charts of several central mitochondrial metabolic pathways (i.e., carbohydrate metabolism, fatty acid uptake and FAO, the Krebs cycle, and electron transport chain). In each pathway, we marked the enzymes that accumulate in a daily manner together with their peak abundance time according to our proteomics analysis, and we highlighted the rate-limiting steps (Figs. 2 and 3). The rate-limiting step in mitochondrial carbohydrate metabolism is carried out by the PDC, a multiprotein complex that catalyzes the oxidative decarboxylation of pyruvate (24). We found that several components of the PDC, namely the catalytic pyruvate dehydrogenase Pdh-E1\( \beta \) (Pdhb), PDH-E2 (Dlat), and the regulatory subunit PDHX (Pdhx), accumulate in a daily manner with maximum levels at \( \sim ZT3 \) (Fig. 2A and Dataset S3). The mRNA levels of Pdh-E1\( \beta \) and Pdhx also cycled but reached their zenith levels at \( \sim ZT16 \), whereas Pdh-E2 (Dlat) was relatively constant throughout the day (Fig. S3A). The rate-limiting step for the entry of long-chain fatty acids into the mitochondrial matrix is the synthesis of acetylcarbamoyl from acyl CoA and carnitine, which is mediated by carnitine palmitoyl-transferase 1 (CPT1) (25). We identified oscillations in CPT1 (Cpt1a) protein levels with zenith levels at \( \sim ZT17 \) (Fig. 2B and Dataset S4). The mRNA levels of Cpt1cycled throughout the day, with peak levels at \( \sim ZT12 \) (Fig. S3B). After fatty acids enter the mitochondria, they are oxidized. Several enzymes within the FAO pathway cycled in a

![Fig. 2. Diurnal oscillations of enzymes in pyruvate metabolism and fatty acid uptake and oxidation. Schematic depiction of the following principal mitochondrial pathways: (A) pyruvate metabolism and (B) fatty acid uptake and FAO. Metabolites are marked in gray, and enzymes are in black; known rate-limiting enzymes are shown as squares. Oscillating enzymes according to the proteomics analysis are marked with a wave sign (\( \Theta \)) together with their peak time indicated by ZT. Metabolites used as substrates for mitochondrial respiration assays in Figs. 4 and 5 and relevant enzymes are underlined.](image-url)
daily manner and peaked at ~ZT4 [e.g., acyl CoA dehydrogenase VL (ACADVL), ACAD11, ACAD9, HADH, and HADHa] (Fig. 2B and Dataset S4). Moreover, our analysis identified daily rhythms in several enzymes of the Krebs cycle and various proteins within the respiratory complexes (Fig. 3 and Datasets S5 and S6). Finally, we found that several members of the mitochondrial protein translocation machinery, namely the TIM/TOM complex, accumulate in a daily manner, reaching their peak levels predominantly during the early light phase (Dataset S7), which might suggest that protein entry to the mitochondria is temporally gated.

We also quantified 2 mitochondrial-encoded proteins of the known 14, namely NADH-ubiquinone oxidoreductase chain 2 (MTND2) and MTND5. MTND2 was found to be rhythmic, with peak levels at ~ZT13 (q value = 0.041), whereas MTND5 did not show statistically significant oscillations (q value > 0.15). In accordance, the transcript levels of Mtd2 were rhythmic with zenith levels at ~ZT12, whereas Mtd5 transcript levels were relatively constant throughout the day (Fig. S3C).

In summary, our quantitative proteomics analysis evinced that key enzymes in principal mitochondrial metabolic pathways oscillate throughout the day.

**Diurnal Oscillations in Mitochondrial Respiration in Response to Different Nutrients.** To corroborate the diurnal accumulation of mitochondrial enzymes identified in our proteomics analysis, mitochondrial protein extracts were analyzed by SDS/PAGE and immunoblots. We centered our analysis on the following key rate-limiting enzymes, each related to a distinct mitochondrial pathway: (i) CPT1 (i.e., CPT1a), the rate-limiting enzyme for the entry of long-chain fatty acids to the inner mitochondrial matrix; and (ii) PDH (i.e., PDH-E1β), which catalyzes the rate-limiting step in mitochondrial carbohydrate metabolism as part of the PDC. Immunoblot analysis showed that the protein levels of CPT1 and PDH oscillate in mitochondrial extracts prepared from WT mice (Fig. 4A and Fig. S4A). In line with our proteomics analysis, CPT1 reached zenith levels at ~ZT20, whereas PDH accumulated at ~ZT4 (Fig. 4A and Fig. S4A).

Because these key enzymes accumulated at specific times of the day, we posited that mitochondria might display a distinct diurnal metabolic response in the presence of their corresponding substrates. To test this hypothesis, we monitored the respiration rate of isolated mitochondria prepared from mice killed throughout the day using the Seahorse Flux Analyzer. CPT1 function was examined in the presence of palmitoyl CoA and carnitine, and PDH activity was tested with pyruvate and malate. Our working premise was that, for any substrate provided in excess to isolated mitochondria, the respiration rate would primarily reflect the activity of the first rate-limiting enzyme (26). On incubation with palmitoyl CoA and carnitine, mitochondrial respiration exhibited a diurnal response, with peak levels at ~ZT20 (Fig. 4B), which corresponded to the oscillations in CPT1 protein levels (Fig. 4A). In the presence of pyruvate and malate, mitochondrial respiration showed diurnal oscillations with maximum levels at ~ZT4 (Fig. 4C), concomitant with the accumulation profile observed for PDH protein (Fig. 4D).

We also examined whether mitochondrial respiration is rhythmic in isolated mitochondria from cultured cells that exhibit circadian rhythmicity (i.e., NIH 3T3) (27) (Fig. S5A). In contrast to mitochondria prepared from mouse liver, respiration of isolated mitochondria from NIH 3T3 cells in the presence of palmitoyl CoA and carnitine or pyruvate and malate was relatively even throughout the circadian cycle (Fig. S5B). NIH 3T3 exhibits rhythmic gene expression of core clock and several output genes; however, the amplitude is often shallow compared with liver, likely because of absence of systemic timing cues. Thus, we cannot exclude the possibility that we failed to detect rhythmic respiration in isolated mitochondria from NIH 3T3 because of their low amplitude.

To address the potential dependency of CPT1 and PDH daily accumulation and their respective mitochondrial function on circadian clocks and more specifically, the clock proteins PER1/2, we isolated mitochondria from PER1/2−/− mice throughout the day. The circadian expression of core clock and clock-controlled genes is largely abolished in mice lacking both PER1 and PER2 (28). Immunoblot analysis revealed that mitochondria isolated from PER1/2−/− mice exhibit relatively constant CPT1 and PDH protein levels throughout the day (Fig. 4D and Fig. S4B). Likewise, we did not observe significant diurnal oscillations in mitochondrial respiration in response to their respective substrates (Fig. 4E and F). Thus, the equal daily levels of CPT1 and PDH and the constant mitochondrial respiration in Per1/2−/− mice suggested that the oscillations in these pathways are dependent on the circadian clock PERIOD proteins.

Comparison of the overall daily levels of CPT1 and PDH between WT and Per1/2−/− mice fed ad libitum revealed similar
Fig. 4. Daily oscillations in accumulation of rate-limiting mitochondrial enzymes and mitochondrial respiration are PER1/2-dependent. (A) Mitochondrial protein extracts of WT mice fed ad libitum were analyzed by SDS/PAGE and immunoblot (IB) with indicated antibodies. Oxygen consumption rates (OCRs) of isolated mitochondria prepared from WT mice fed ad libitum were quantified using the Seahorse Flux Analyzer in the presence of the indicated substrates: (B) palmitoyl CoA and carnitine and (C) pyruvate and malate. (D) Mitochondrial protein extracts of PER1/2 null mice fed ad libitum were analyzed by SDS/PAGE and IB. OCRs of isolated mitochondria prepared from PER1/2 null mice fed ad libitum were quantified in the presence of the indicated substrates: (E) palmitoyl CoA and carnitine and (F) pyruvate and malate. (G) Mitochondrial protein extracts of night-fed PER1/2 null mice were analyzed by SDS/PAGE and IB. OCRs of isolated mitochondria prepared from night-fed PER1/2 null mice were quantified in the presence of the indicated substrates: (H) palmitoyl CoA and carnitine and (I) pyruvate and malate. (J) Mitochondrial protein extracts of WT mice fed with a high-fat diet for 3 d were analyzed by SDS/PAGE and IB. OCRs of isolated mitochondria prepared from WT mice fed with a high-fat diet for 3 d were quantified in the presence of the indicated substrates: (K) palmitoyl CoA and carnitine and (L) pyruvate and malate. Carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was specifically added in the case of pyruvate and malate according to standard protocols as detailed in Materials and Methods. For SDS/PAGE and IB, porin levels were used as a loading control, and each time point consists of a mix of mitochondria isolated from three to four individual mice (Fig. S4 shows quantification of the different IBs). OCR measurements are presented in picomoles per minute as means ± SEMs, with individual measurements of three to five animals per time point. Gray shading represents the dark phase. Molecular mass (M.W.) is indicated in kilodaltons.
CPT1 levels but lower PDH levels in \( \text{Per1/2}\sim \) mitochondria (Fig. S6A). The daily mean oxygen consumption rates in the presence of palmitoyl CoA and carnitine or pyruvate and malate were significantly lower in \( \text{Per1/2}\sim \) compared with WT mitochondria (Fig. S6B). Thus, the lower PDH levels in \( \text{Per1/2}\sim \) mitochondria corresponded to the overall reduction in their oxygen consumption rate with pyruvate and malate. By contrast, the decline in mean oxygen consumption rates in the presence of palmitoyl CoA and carnitine could not be attributed to overall changes in CPT1 levels in \( \text{Per1/2}\sim \) compared with WT mitochondria. It is possible that overall CPT levels are similar but that its enzymatic activity in general is reduced in \( \text{Per1/2}\sim \) mice. In this respect, leptin was shown to increase liver and K and PDH). These oscillations were abolished in PER1/2 null mice with the oscillating levels of their rate-limiting enzyme (i.e., CPT1 and PDH). These findings incited us to examine the daily changes in FAO by monitoring the oxygen consumption of isolated mitochondria in the presence of palmitoyl carnitine, the product of CPT1 enzymatic activity (33). To examine whether feeding rhythms might play a role in the diurnal accumulation of CPT1 and PDH and consequently, mitochondrial respiration, we applied a nighttime-restricted feeding regimen on PER1/2 null mice. Immunoblot analysis of mitochondria isolated from night-fed PER1/2\sim mice showed that CPT1 and PDH protein levels are relatively constant throughout the day (Fig. 4G and Fig. S4C), similar to those in \( \text{Per1/2}\sim \) mice fed ad libitum (Fig. 4D). Likewise, the daily profile of mitochondrial respiration in response to their respective substrates was relatively constant (Fig. 4H and I).

Next, we asked whether the diet composition might play a role in the diurnal accumulation of CPT1 and PDH and consequently, mitochondrial respiration. Thus, WT mice were fed with a high-fat diet for 3 days. Immunoblot analysis of mitochondria isolated from high-fat diet-fed mice showed that CPT1 oscillated throughout the day, with trough levels at \( \sim\text{ZT0} \) (Fig. 4F and Fig. S4D), similar to mice fed with regular chow (Fig. 4A). Mitochondrial respiration in the presence of palmitoyl CoA and carnitine, cycled and exhibited a similar pattern under both diets (Fig. 4B and K). By contrast, mitochondria isolated from high-fat diet-fed mice showed relatively constant levels of PDH protein (Fig. 4J and Fig. S4D), and their respiration in the presence of pyruvate and malate was fairly even throughout the day (Fig. 4L).

Taken together, we detected daily oscillations in key mitochondrial enzymes and observed concomitant diurnal changes in mitochondrial respiration in the presence of their respective substrates. Thus, the response to palmitoyl CoA and carnitine as well as pyruvate and malate elicited daily cycles in mitochondrial respiration with different phases (\( \sim\text{ZT20} \) and \( \sim\text{ZT4} \), respectively), concurrent with the oscillating levels of their rate-limiting enzyme (i.e., CPT1 and PDH). These oscillations were abolished in PER1/2 null mice irrespective of the feeding schedule, suggesting that they are dependent on the clock PERIOD proteins independent of feeding time. Remarkably, a high-fat diet specifically affected the daily accumulation of PDH and related mitochondrial respiration but did not alter the diurnal oscillations in CPT1 levels and its respective mitochondrial function.

### Daily Rhythms in FAO and Related Enzymes

Long-chain fatty acids are transported into mitochondria through the carnitine shuttle, in which the rate-limiting step is catalyzed by CPT1. After fatty acids enter the mitochondria, they are catabolized through FAO generating acetyl CoA. Our proteomics analysis evinced that several enzymes within the FAO pathway oscillate with zenith levels at \( \sim\text{ZT4} \) (Fig. S2B and Dataset S4), among them several ACADs. ACADs are a class of enzymes that catalyzes the initial step in each cycle of FAO. They differ in their specificity for different chain lengths of fatty acid acyl CoA substrates (34). As a proof of principle, we examined ACAD11, which specifically metabolizes long-chain fatty acid substrates (e.g., palmitoyl CoA) (34).

Analysis of mitochondrial protein levels of ACAD11 throughout the day by SDS/PAGE and immunoblot showed, in accordance with our proteomics analysis, that they oscillate, with peak levels at \( \sim\text{ZT0} \) (Fig. S4A and Fig. S4B). The rhythmical accumulation of ACAD11 was lost in mitochondria purified from PER1/2 null mice fed ad libitum (Fig. S5B and Fig. S4B). However, when we tested mitochondria that were isolated from night-fed PER1/2 null mice, the daily oscillations in ACAD11 accumulation were restored (Fig. S5C and Fig. S4C), albeit with a phase delay compared with that in WT mice (i.e., \( \sim\text{ZT4} \)). Next, we asked whether the food composition might also play a role in the daily accumulation of ACAD11. Remarkably, 3 d of high-fat diet were sufficient to damp the oscillations of ACAD11 accumulation in mitochondria isolated from WT mice (Fig. 5D and Fig. S4D).

The above-described experiments showed that the daily changes in ACAD11 levels correspond to oscillations in FAO as monitored by mitochondrial respiration in the presence of palmitoyl carnitine. Both were rhythmic in WT mice with zenith levels at \( \sim\text{ZT4} \), and the rhythmicity was lost on high-fat diet or in PER1/2 null mice and restored in nighttime-fed PER1/2 null mice.
with WT mice (~45% vs. ~29% during the day and ~55% vs. ~71% during the night in PER1/2 null and WT mice, respectively). Short duration (i.e., 3 d) of high-fat diet in contrast to 6 wk of high-fat diet (38) did not significantly alter the daily feeding habits of WT mice. As expected, mice were mostly active during the dark phase, but PER1/2 null mice were more active during the light phase and less active during the dark phase compared with WT mice (~33% vs. ~24% during the day and ~67% vs. ~76% during the night in PER1/2 null and WT mice, respectively). This effect was maintained under different feeding regimens. A high-fat diet shifted the balance to lipids use throughout the entire day, likely because of their attenuated feeding rhythms. A high-fat diet shifted the balance to lipids use throughout the entire day. These changes matched the oscillations in mitochondrial FAO as reflected by respiration measurements of isolated mitochondria in the presence of palmitoyl carnitine and malate (Fig. 5 E–H).

**Discussion**

In this study, we examined the temporal changes in the mitochondrial proteome by applying MS-based quantitative proteomics on isolated mitochondria from mouse liver. We obtained the first, to our knowledge, comprehensive mitochondrial proteome around the clock and found that ~38% of mitochondrial proteins oscillate throughout the day. Unexpectedly, we discovered that the majority of cycling proteins in mitochondria reach their zenith levels at ~ZT4. Hence, mitochondrial proteins are predominantly gated to accumulate during the early light phase, raising the question of how this temporal coordination is achieved at the molecular level. When we tested the possibility that transcription regulation plays a role in this process, we found that, although the transcript levels of the vast majority of cycling mitochondrial proteins do oscillate, the phase of oscillating mitochondrial proteins poorly correlated with the phase of their cycling transcript levels. It should be noted that our proteomics analysis was done from mice housed under a 12-h light-dark regimen, whereas the transcriptome dataset (23) was obtained under constant darkness. The difference in the experimental setup may account for some differences in peak time of abundance for RNA and protein. Recent proteome studies on whole liver samples also showed that the oscillation of proteins encoded by rhythmically expressed mRNAs greatly differ in their cycling phases (13, 15). Thus, although rhythmic transcription is at the core of circadian regulation, it seems that post-transcriptional mechanisms, such as translational control (39, 40) and temporal regulation of protein synthesis/degradation (41), play a significant role in shaping the circadian proteome landscape. In the case of the mitochondrial proteome, another mechanism that comes to mind is the translocation of protein from the cytoplasm, where the vast majority of mitochondrial proteins are synthesized, to the mitochondria. This process is mediated through the TIM/TOM complex and tightly regulated, for example, by cytosolic kinases, such as casein kinase 2 (42). Interestingly, casein kinases have been previously implicated in regulation of circadian rhythmity (7). Along this line, our proteomics analysis also evinced that several members of the mitochondrial protein translocation machinery, namely the TIM/TOM complexes, accumulate in a daily manner during the early light phase, which might suggest that protein entry to the mitochondria is temporally gated by them. Lastly, it was recently shown that mitochondrial dynamics through fusion, fission, and mitophagy occur in a daily manner under the control of the liver clock (43). Hence, it is plausible that these alterations might also shape the phase of protein accumulation in mitochondria.

In contrast to previous circadian proteomics that were done on whole-liver samples (13, 15), we performed herein the proteomics analysis on isolated mitochondria from liver. After stringent filtering, we precisely quantified 590 different mitochondrial-annotated proteins of ~1,000 known mitochondrial proteins (44). Comparison of our mitochondrial proteome with formerly reported whole-liver proteome (15) revealed that mitochondrial isolation before the proteomics analysis increases the identification and quantification rate. Of the total of 590 mitochondrial-annotated proteins quantified in this study, only 412 were previously quantified in whole-liver samples. Interestingly, although 157 of 223 cycling mitochondrial proteins (based on this study) were also quantified in the whole-liver proteome, only 6 of them were considered to be cycling. This striking difference further highlights the critical importance of mitochondrial fractionation when examining rhythmic protein accumulation in mitochondria and is likely to reflect the temporal and spatial dynamics in protein accumulation in subcellular compartments.

We detected daily oscillations in key mitochondrial enzymes and observed concomitant diurnal mitochondrial respiration profiles in the presence of their respective substrates. Thus, the response to palmitoyl CoA and carnitine as well as pyruvate and
malate elicited daily cycles in mitochondrial respiration with different phases (\(\sim\)ZT20 and \(\sim\)ZT4) concurrent with the oscillating levels of their rate-limiting enzyme (i.e., CPT1 and PDH). The oscillations in mitochondrial respiration and the abundances of the rate-limiting enzymes were abolished in PER1/2 null mice irrespective of feeding schedule, suggesting that they are dependent on the clock proteins PER1/2 independent of feeding time. Remarkably, high-fat diet specifically affected the daily accumulation of PDH and related mitochondrial respiration but did not alter the diurnal oscillations in CPT1 levels and its respective mitochondrial function. Based on the correlation between the oscillations in the protein levels of the aforementioned rate-limiting enzymes and the diurnal oscillation in mitochondrial respiration in the presence of their respective substrates, we proposed that daily rhythmicity in mitochondrial function is governed, at least in part, by the cycling level of these enzymes. However, it is
Materials and Methods

Animals. All animal experiments and procedures were conducted in conformity with the Weizmann Institute Animal Care and Use Committee guidelines. For mitochondria isolation, we used 3-mo-old WT and Per1/2−/− males as previously described (28). Mice were kept under a 12-h light-dark regimen and fed either ad libitum or exclusively during the dark phase for 2 wk. Animals were fed either regular chow or a high-fat diet (60% of kilocalories as fat; D12492; Open Source Diets). Mice were killed at 4-h intervals throughout the day, ZT0 corresponded to the time that lights were turned on in the animal facility, and ZT12 corresponded to the time that lights were turned off in the animal facility. The temperature in the housing cages and metabolic cages was maintained at 22 °C.

Mitochondrial Preparation. Mice were killed, and livers were isolated and minced in −10 vol Mitochondrial Isolation Buffer (MIB; 70 mM sucrose, 210 mM mannitol, 10 mM Hepes, 1 mM EDTA, pH 7.5) supplemented with 0.2% BSA at 4 °C. All subsequent steps of the preparation were performed on ice. The tissue was then homogenized using a Teflon dounce homogenizer. Homogenate was centrifuged at 600 × g for 10 min at 4 °C. After centrifugation, fat/lipids were carefully aspirated, and the remaining supernatant was centrifuged at 7,000 × g for 15 min. The pellet was resuspended in MIB supplemented with 0.5 mM EGTA and centrifuged for 10 min at 7,000 × g. Subsequently, the pellet was resuspended in MIB and centrifuged for 5 min at 7,000 × g. The final pelleted was collected. Total protein (milligrams per milliliter) was determined using Bradford Assay (Bio-Rad). In the case of mitochondria isolation from NIH 3T3 cells, cells were collected and washed with PBS, homogenized in MIB buffer using a syringe, and isolated by subsequent centrifugations as described above.

Mitochondrial Respiration Assay. Mitochondrial oxygen consumption rate experiments were conducted using the Seahorse Bioscience XF24 Extracellular Flux Analyzer; 5 μg per well mouse liver-isolated mitochondria or 10 μg per well mouse mitochondria purified from NIH 3T3 cells were seeded in Mitochondrial Assay Solution (70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM Hepes, 1 mM EGTA, 0.2% (wt/vol) fatty acid-free BSA, pH 7.2). Mitochondrial Assay Solution was supplemented according to standard protocols (50) with (i) 40 μM palmitoyl CoA and 40 μM carnitine; (ii) 10 mM pyruvate, 2 mM malate, and 5 μM carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP) or (iii) 40 μM palmitoyl-carnitine and 0.5 mM malate. FCCP was added in the case of pyruvate and malate according to established protocols to enable accurate measurements of oxygen consumption rate within the dynamic range in response to these specific substrates.

Metabolic Cages Analysis. The voluntary locomotor activity, food consumption, and RER were monitored using Phenomenex Metabolic Cages (TSE Systems).

RNA Analysis by Real-Time Quantitative PCR. RNA extraction and transcription quantification by real-time PCR technology were performed as previously described (16). Real-time PCR measurements were performed using SYBR green or Taqman probes with a LightCycler II Machine (Roche). Normalization was performed relative to the geometrical mean of five housekeeping genes: Tbp, Hprt, Actn, Rplt, and Gapdh. Primers and probes are listed in SI Materials and Methods.

mtDNA Quantification. Total DNA was isolated from liver using TRI Reagent (SIGMA) according to the manufacturer’s protocol, and quantitative real-time PCR was performed with mitochondrial- (ChrM and Cox1) and genomic-specific (Hprt and Glucagon) primers as listed below. The relative number of mitochondria was determined as the ratio between the average of mtDNA and the average genomic DNA based on the real-time PCR with the different specific primers.

chrM fw 5′-AACAACTCTGCTATGTGGCCAAAAA-3';
chrM rev 5′-CCACGTATACCAAGGCTCGT-3';
ACKNOWLEDGMENTS. We thank K. Mayr, I. Paron, and G. Sowa for their assistance with the MS measurements and D. Wischnewski and M. Elimelech for technical help. We also thank A. Gross and H. Reineke for their valuable comments on the manuscript. A.N.-C. and Y.A. received a postdoctoral fellowship from the Feinberg Graduate School, Weizmann Institute of Science. The work performed in the laboratory of G.A. was supported by Israel Science Foundation Grant ISF 138/12, the Ahsh-Frenkel Foundation, Human Frontier Science Program (HFSP) Career Development Award HFSP CDA00014/2012, and European Research Council Grant ERC-2011 METACYCLES 310320.