Cite as: S. Ray *et al.*, *Science* 10.1126/science.abf1941 (2021).

Response to Comment on "Circadian rhythms in the absence of the clock gene *Bmal1*"

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Abruzzi *et al.* argue that transcriptome oscillations found in our study in the absence of *Bmal1* are of low amplitude, statistical significance, and consistency. However, their conclusions rely solely on a different statistical algorithm than we used. We provide statistical measures and additional analyses showing that our original analyses and observations are accurate. Further, we highlight independent lines of evidence indicating *Bmal1*-independent 24-hour molecular oscillations.

In their Comment, Abruzzi *et al.* (*1*) challenge our observations on circadian molecular oscillations in the absence of *Bmal1*. We were notified by the authors, and found independently, that the genotypes of processed liver RNA-seq data were swapped by mistake during analysis (corrected in Erratum). This, however, does not affect the major conclusions of the paper. There are still oscillations in *Bmal1*$^{-/-}$. Inverting those data does not change this. All other data, including fibroblast RNA-seq, proteomics, and phosphoproteomics, demonstrate circadian rhythms in *Bmal1* knockouts. Correcting the genotypes does not affect our inferences about E26 transformation-specific (ETS) transcription factors. ETS factors are found in both genotypes, and only the specific factors involved are changed [figure S6A of (*2*)].

Abruzzi *et al.* comment on *Bmal1* transcript oscillations in *Bmal1*$^{-/-}$ tissue. We examined the *Bmal1* transcript in both genotypes. This shows that exon 5 (the targeted region) is missing in the knockout (*3*), whereas other exons (e.g., 7 and 8) of the transcript are transcribed, along with introns (Fig. 1A). Thus, the transcript oscillates when quantified by the RNA-seq pipeline we used, which performed whole-transcript quantification. The mature transcript in *Bmal1*$^{-/-}$ would, however, produce a truncated, nonfunctional protein, which is not detected. This was described originally, and we found the same by immunoblotting [figure S4A of (*2*)] (*2*, *3*). We find that *Bmal1* is also rhythmic in *Bmal1*$^{+/+}$ tissue, as is *Cry2* (Fig. 1B).

Abruzzi *et al.* showed JTK_cycle results for all of their analyses and did not compare their findings to RAIN, the principal algorithm used in (*2*). As stated in (*2*), we used RAIN because of its ability to detect asymmetric circadian profiles (e.g., nonsinusoidal patterns) and its superior sensitivity relative to JTK (*4*). JTK is not a "gold standard" in the field. According to the "Guidelines for Genome-Scale Analysis of Biological Rhythms" (*5*), no gold standard exists (*4*). Most studies use a range of methods to define circadian patterning, as we did [figure S1C of (*2*)] (*5*). There is no justification to regard an exclusively JTK analysis as superior to ours.

Given Abruzzi *et al.*'s claims, we compared raw *P* values and FDR-adjusted *P* values from RAIN with those generated by JTK, and also by harmonic regression (HR), another valid method for circadian detection (*6*) (Fig. 1C). As we had found [figure S1C of (*2*)], JTK gives lower numbers of transcripts than RAIN for any statistical threshold, and HR gives comparable numbers to RAIN (Fig. 1C). From examining their code, we note that Abruzzi *et al.* performed analyses with HR and JTK simultaneously but did not show HR results in their Comment (reason undisclosed), and focused only on JTK. Furthermore, FDRs in JTK are more conservative than the true FDR (*7*); that is, JTK underestimates rhythmic transcripts.

Many recent circadian transcriptome studies, including those published by Abruzzi and Rosbash, use low statistical stringency (i.e., *P* values without FDR adjustment) to obtain deeper coverage of the rhythmic transcriptome (*8*, *9*). In Abruzzi *et al.* (2017), for example, they state "JTK_cycle

identified transcripts as cycling that had … a *p*-value cutoff of less than 0.05" (*8*). Another recent paper by these authors used a $P < 0.05$ cutoff (*9*). It is not clear why the authors of this Comment use far less stringent statistical cutoffs for their own published work but insist that much stricter criteria are required for our work. Regardless, whichever algorithm is chosen, there are hundreds to thousands of transcripts that show circadian oscillations at FDR < 0.05 or 0.1 in *Bmal1*$^{-/-}$.

The amplitudes shown in figure S3A of (*2*) are expressed as a peak-to-trough ratio. This is stated in the figure legend. "Amplitude" and "fold change" are used interchangeably in the field (*5*). We used peak-to-trough ratio because RAIN does not calculate amplitude as an output. The calculation used by JTK determines deviation from median expression, which will be at least half the peak-to-trough value. Thus, their amplitudes will not accord with ours unless rescaled. We found similar amplitude distributions for rhythmic transcripts in *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ (Fig. 1E). Furthermore, the amplitude distributions are comparable to earlier liver circadian transcriptome studies by the Hogenesch group (*10*) (Fig. 1F).

We disagree with Abruzzi *et al.*'s results concerning the numbers of transcripts that are rhythmic among different 37°C datasets. We used an alternative metric requiring that a transcript is rhythmic in at least two datasets to identify consistently rhythmic ones (Fig. 2, A and B). It may not be expected that high numbers of transcripts intersect among all the datasets; circadian meta-analyses of the same tissue show little concordance (*11*). Thus, we do not think that intersection of all datasets is a fair comparison, given the known variability among such datasets. Also, clones of seemingly identical fibroblasts do not show the same circadian characteristics from generation to generation. This introduces further variability in passaged cells that could explain some variability seen among our transcriptomic datasets (*12*).

Abruzzi *et al.* speculate that transcriptomic oscillations in *Bmal1*$^{-/-}$ cells and tissues are externally driven by "unintended daily interventions." We went to great lengths to ensure constant conditions. The temperature was held constant during synchronization and sampling phases [figure S1B of (*2*)]. No medium changes occurred after dexamethasone synchronization. Plates were kept in constant darkness in light-tight incubators. Incubators were situated on anti-vibration benches to minimize vibration. Sample collection was done by trained personnel in a randomized fashion. Furthermore, the phase distribution is very similar to that seen in in vivo liver RNA-seq data (*13*), falling into two predominant phases (Fig. 2C). This is expected, rather than unusual (as claimed).

Finally, regarding differential entrainment of fibroblasts, Abruzzi *et al.* show some genes that do not respond to opposite entrainment. Such transcripts are not surprising, given that not all transcripts will display this behavior. However, a large number of transcripts do show the expected behavior ($n = 83$ show a 9- or 12-hour phase difference between AM and PM datasets), and we provide further examples here (Fig. 2, D and E). Thus, their assertion that particular transcripts (defined by their filters) are indicative of the overall results is mistaken.

Is there other evidence for oscillations in *Bmal1* knockouts? Welz *et al.* found circadian oscillations in skin using a different *Bmal1* knockout model (*14*). A comparison of those data with ours demonstrates that 882 transcripts overlap between our skin fibroblast data and in vivo epidermis data (Fig. 2, F and G). Furthermore, *Bmal1*$^{-/-}$ skin fibroblasts show circadian metabolic flux oscillations under constant conditions (*15*). These data provide further evidence of self-sustained circadian oscillations in *Bmal1*$^{-/-}$ cells and tissues under constant conditions.

REFERENCES

1. K. C. Abruzzi, C. Gobet, F. Naef, M. Rosbash, Comment on "Circadian rhythms in the absence of the clock gene *Bmal1*". *Science* **372**, eabf0922 (2021).
2. S. Ray, U. K. Valekunja, A. Stangherlin, S. A. Howell, A. P. Snijders, G. Damodaran, A. B. Reddy, Circadian rhythms in the absence of the clock gene *Bmal1*. *Science* **367**, 800–806 (2020). doi:10.1126/science.aaw7365 Medline
3. M. K. Bunger, L. D. Wilsbacher, S. M. Moran, C. Clendenin, L. A. Radcliffe, J. B. Hogenesch, M. C. Simon, J. S. Takahashi, C. A. Bradfield, Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103**, 1009–1017 (2000). doi:10.1016/S0092-8674(00)00205-1 Medline
4. P. F. Thaben, P. O. Westermark, Detecting rhythms in time series with RAIN. *J. Biol. Rhythms* **29**, 391–400 (2014). doi:10.1177/0748730414553029 Medline
5. M. E. Hughes, K. C. Abruzzi, R. Allada, R. Anafi, A. B. Arpat, G. Asher, P. Baldi, C. de Bekker, D. Bell-Pedersen, J. Blau, S. Brown, M. F. Ceriani, Z. Chen, J. C. Chiu, J. Cox, A. M. Crowell, J. P. DeBruyne, D.-J. Dijk, L. DiTacchio, F. J. Doyle, G. E. Duffield, J. C. Dunlap, K. Eckel-Mahan, K. A. Esser, G. A. FitzGerald, D. B. Forger, L. J. Francey, Y.-H. Fu, F. Gachon, D. Gatfield, P. de Goede, S. S. Golden, C. Green, J. Harer, S. Harmer, J. Haspel, M. H. Hastings, H. Herzel, E. D. Herzog, C. Hoffmann, C. Hong, J. J. Hughey, J. M. Hurley, H. O. de la Iglesia, C. Johnson, S. A. Kay, N. Koike, K. Kornacker, A. Kramer, K. Lamia, T. Leise, S. A. Lewis, J. Li, X. Li, A. C. Liu, J. J. Loros, T. A. Martino, J. S. Menet, M. Merrow, A. J. Millar, T. Mockler, F. Naef, E. Nagoshi, M. N. Nitabach, M. Olmedo, D. A. Nusinow, L. J. Ptáček, D. Rand, A. B. Reddy, M. S. Robles, T. Roenneberg, M. Rosbash, M. D. Ruben, S. S. C. Rund, A. Sancar, P. Sassone-Corsi, A. Sehgal, S. Sherrill-Mix, D. J. Skene, K.-F. Storch, J. S. Takahashi, H. R. Ueda, H. Wang, C. Weitz, P. O. Westermark, H. Wijnen, Y. Xu, G. Wu, S.-H. Yoo, M. Young, E. E. Zhang, T. Zielinski, J. B. Hogenesch, Guidelines for Genome-Scale Analysis of Biological Rhythms. *J. Biol. Rhythms* **32**, 380–393 (2017). doi:10.1177/0748730417728663
6. J. Yeung, J. Mermet, C. Jouffe, J. Marquis, A. Charpagne, F. Gachon, F. Naef, Transcription factor activity rhythms and tissue-specific chromatin interactions explain circadian gene expression across organs. *Genome Res.* **28**, 182–191 (2018). doi:10.1101/gr.222430.117 Medline
7. A. L. Hutchison, M. Maienschein-Cline, A. H. Chiang, S. M. A. Tabei, H. Gudjonson, N. Bahroos, R. Allada, A. R. Dinner, Improved statistical methods enable greater sensitivity in rhythm detection for genome-wide data. *PLOS Comput. Biol.* **11**, e1004094 (2015). doi:10.1371/journal.pcbi.1004094 Medline

8. K. C. Abruzzi, A. Zadina, W. Luo, E. Wiyanto, R. Rahman, F. Guo, O. Shafer, M. Rosbash, RNA-seq analysis of Drosophila clock and non-clock neurons reveals neuron-specific cycling and novel candidate neuropeptides. *PLOS Genet.* **13**, e1006613 (2017). doi:10.1371/journal.pgen.1006613 Medline

9. Q. Wang, K. C. Abruzzi, M. Rosbash, D. C. Rio, Striking circadian neuron diversity and cycling of *Drosophila* alternative splicing. *eLife* **7**, e35618 (2018). doi:10.7554/eLife.35618 Medline

10. M. E. Hughes, L. DiTacchio, K. R. Hayes, C. Vollmers, S. Pulivarthy, J. E. Baggs, S. Panda, J. B. Hogenesch, Harmonics of circadian gene transcription in mammals. *PLOS Genet.* **5**, e1000442 (2009). doi:10.1371/journal.pgen.1000442 Medline

11. K. P. Keegan, S. Pradhan, J.-P. Wang, R. Allada, Meta-analysis of Drosophila circadian microarray studies identifies a novel set of rhythmically expressed genes. *PLOS Comput. Biol.* **3**, e208 (2007). doi:10.1371/journal.pcbi.0030208 Medline

12. Y. Li, Y. Shan, G. K. Kilaru, S. Berto, G.-Z. Wang, K. H. Cox, S.-H. Yoo, S. Yang, G. Konopka, J. S. Takahashi, Epigenetic inheritance of circadian period in clonal cells. *eLife* **9**, e54186 (2020). doi:10.7554/eLife.54186 Medline

13. N. Koike, S.-H. Yoo, H.-C. Huang, V. Kumar, C. Lee, T.-K. Kim, J. S. Takahashi, Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**, 349–354 (2012). doi:10.1126/science.1226339 Medline

14. P.-S. Welz, V. M. Zinna, A. Symeonidi, K. B. Koronowski, K. Kinouchi, J. G. Smith, I. M. Guillén, A. Castellanos, S. Furrow, F. Aragón, G. Crainiciuc, N. Prats, J. M. Caballero, A. Hidalgo, P. Sassone-Corsi, S. A. Benitah, BMAL1-Driven Tissue Clocks Respond Independently to Light to Maintain Homeostasis. *Cell* **177**, 1436–1447.e12 (2019). doi:10.1016/j.cell.2019.05.009 Medline

15. R. Ch, G. Rey, S. Ray, P. K. Jha, P. C. Driscoll, M. S. Dos Santos, D. M. Malik, R. Lach, A. M. Weljie, J. I. MacRae, U. K. Valekunja, A. B. Reddy, Rhythmic glucose metabolism regulates the redox circadian clockwork in human red blood cells. *Nat. Commun.* **12**, 377 (2021). doi:10.1038/s41467-020-20479-4 Medline

ACKNOWLEDGMENTS

Funding: A.B.R. acknowledges funding from the Perelman School of Medicine, University of Pennsylvania, and the Institute for Translational Medicine and Therapeutics (ITMAT), Perelman School of Medicine, University of Pennsylvania.
Author contributions: Conceptualization and writing, A.B.R., S.R., and U.K.V.; all authors agreed on the interpretation of data and approved the final version of the manuscript; analysis and visualization, A.B.R., S.R., and U.K.V.; funding acquisition, A.B.R. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** RNA-seq data are available on the Gene Expression Omnibus (accession number GSE111696 and GSE134333). Analysis scripts are available on GitHub (https://github.com/ReddysLab/Bmal1Paper).

2 November 2020; accepted 11 March 2021
Published online 16 April 2021
10.1126/science.abf1941

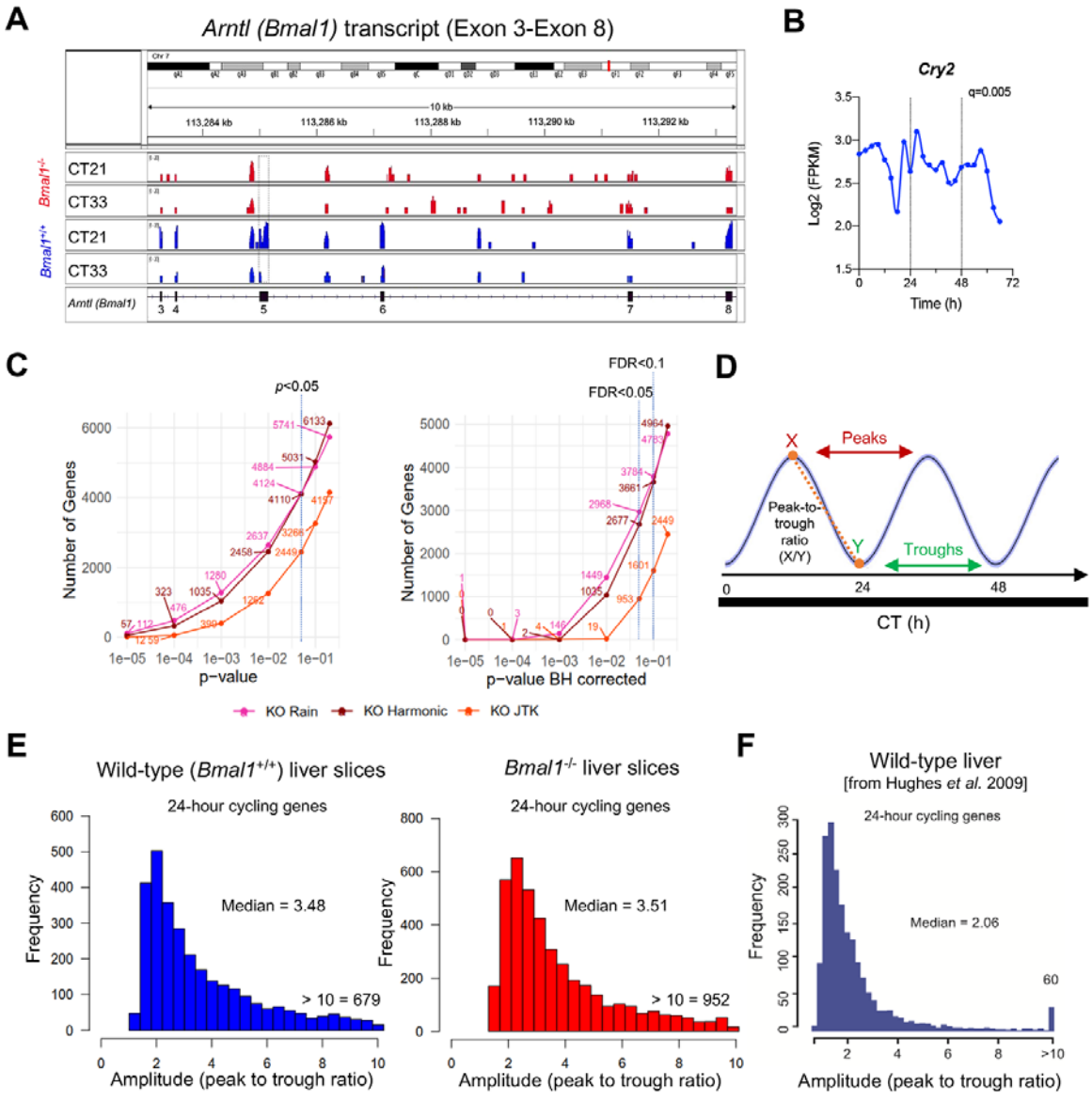

Fig. 1. Substantial numbers of rhythmic transcripts detected in *Bmal1*$^{-/-}$ liver tissue using different algorithms and statistical thresholds. (**A**) Genome browser view of *Arntl* (*Bmal1*) transcript in wild-type and *Bmal1*$^{-/-}$ liver tissues. Sequence reads for exon 3 to exon 8 are shown for both genotypes at the peak (CT21) and at the trough (CT33). Exon 5 is disrupted in the knockout. (**B**) Rhythmic expression (Log_2 transformed FPKM) of *Cry2* in wild-type liver tissue (FDR < 0.05). (**C**) Thousands of 24-hour oscillating transcripts identified at different statistical stringency levels [using three different algorithms: RAIN, harmonic regression (HR), and JTK_cycle] in *Bmal1*$^{-/-}$ liver tissues (after correcting the genotype switching). (**D**) Schematic showing how the amplitudes were calculated as peak-to-trough ratio (fold-change) in our study. (**E**) Amplitude [peak-to-trough ratio (fold-change)] for rhythmic genes in wild-type and *Bmal1*$^{-/-}$ liver tissues (RAIN FDR < 0.1, 24-hour period). (**F**) Comparative analysis of our data with the amplitude of cycling transcripts (profiled using microarray analysis) in mouse liver as reported in (*10*).

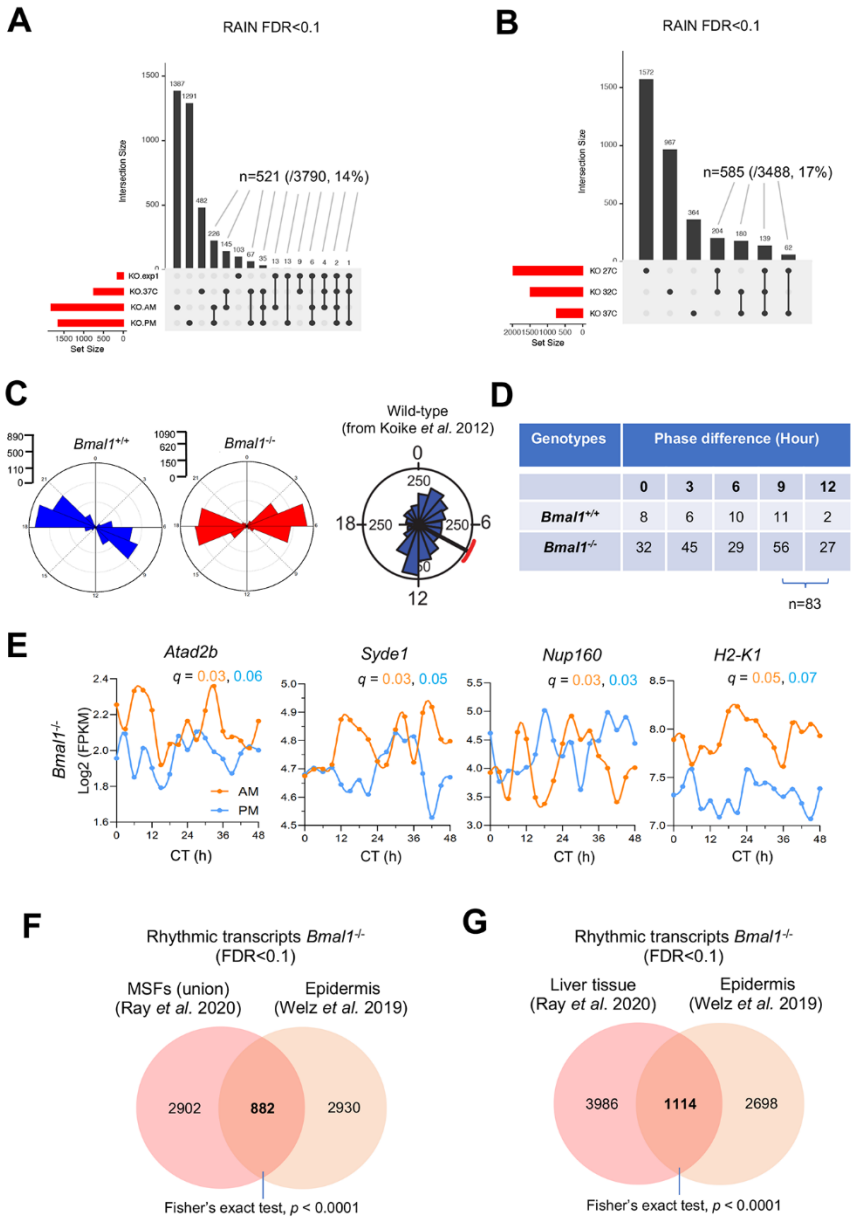

Fig. 2. Overlap among rhythmic transcripts identified in different *Bmal1*$^{-/-}$ mouse skin fibroblast (MSF) experiments. (**A**) Rhythmic transcripts (RAIN, FDR < 0.1) identified in at least two of four *Bmal1*$^{-/-}$ MSFs experiments performed at 37°C. Results represent a significant overlap (521/3790, 14% of all identified rhythmic transcripts) among the datasets. (**B**) Overlap among rhythmic transcripts (RAIN, FDR < 0.1) identified in 27°, 32°, and 37°C *Bmal1*$^{-/-}$ MSF datasets (585/3488 in at least two of three datasets, 17% of all identified rhythmic transcripts). (**C**) Rose plots representing the frequency distribution of the phases of cycling transcripts identified in *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ liver tissues in our study (*2*). We compared our data with the phase distribution of cycling genes (exon) in wild-type mouse liver as demonstrated in (*13*) (whole-transcriptome RNA-seq analysis). (**D**) Phase difference distribution for the rhythmic transcripts (RAIN FDR < 0.1) in AM and PM datasets (*Bmal1*$^{-/-}$ fibroblasts synchronized 12 hours apart). (**E**) Many of the identified rhythmic transcripts in AM and PM datasets exhibited antiphase rhythms in *Bmal1*$^{-/-}$ MSFs (phase difference 9 or 12 hours). Oppositely phased abundance profiles (log_2 transformed FPKM) of a few representative rhythmic genes (RAIN, FDR < 0.1, in addition to those shown in the original manuscript). Samples from three biological replicates were pooled together for RNA-seq analysis at each time point. (**F** and **G**) Venn diagrams showing overlap between rhythmic transcripts (RAIN FDR < 0.1) identified in *Bmal1*$^{-/-}$ MSFs (if rhythmic in any of the 37°C datasets) (F) and *Bmal1*$^{-/-}$ liver tissues (G) in our study (*2*) and in *Bmal1*$^{-/-}$ epidermis (KO-*Arntl*$^{stopFL/stopFL}$) under constant conditions (DD), as identified in (*14*) (GEO accession: GSE114943; FPKM values reanalyzed using the RAIN algorithm).

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Science **372** (6539), eabf1941.
DOI: 10.1126/science.abf1941