Supplementary Materials for

Population level rhythms in human skin: implications for circadian medicine

Gang Wu[1], Marc D. Ruben[1], Robert E. Schmidt[1], Lauren J. Francey[1], David F. Smith[2,3], Ron C. Anafi[4], Jacob J. Hughey[5], Ryan Tasseff[6], Joseph D. Sherrill[6], John E. Oblong[6], Kevin J. Mills[6], John B. Hogenesch[1*]

*To whom correspondence should be addressed: john.hogenesch@cchmc.org

This file includes:

Other Supplementary Material for this manuscript includes the following:

Data file S1. List of genes cycling in human skin at population level.

Materials and Methods

LCM protocol

Samples were frozen in optimum cutting temperature compound (OTC) (Sakura Finetek, Torrance, CA). Embedded tissue blocks were cut into 14 μm sections and mounted on polyethylene naphthalate slides (Life Technologies, Grand Island, NY) and kept at -80°C until use. Sections were immediately fixed in 95% ethanol for 1 min then rinsed in deionized water. After water rinse, the sections were stained (30 seconds) with cresyl violet (Sigma Aldrich, St. Louis, MO) and eosin Y (Sigma Aldrich, St. Louis, MO) and subsequently rinsed with deionized water. The sections were then dehydrated in 95% and 100% ethanol, cleared in xylene and allowed to air dry at room temperature. After drying, epidermal skin cells were isolated from the sections by LCM with the PALM Microbeam system (Carl Zeiss MicroImaging, Munchen, Germany) for transcriptome analysis.

Salivary Melatonin and Cortisol

Melatonin (N-acetyl-5-hydroxytryptamine) is a hormone produced by the pineal gland that helps orchestrate the sleep-wake cycle. Light input to the retina inhibits melatonin synthesis while darkness promotes it, therefore, melatonin production has a pronounced circadian rhythm. In general, in people with normal sleep patterns, melatonin secretion begins to increase in the evening and reach a peak during hours that most people tend to sleep, which is the pattern we observed in this study (fig. S8).

Cortisol, also known as hydrocortisone, is a glucocorticoid that is produced in the adrenal cortex and is released in response to stress, as well as, a low level of circulating glucocorticoids. Its

primary functions are to increase blood sugar through gluconeogenesis, suppress the immune system, and aid in fat, protein and carbohydrate metabolism. In most healthy individuals, cortisol shows a pronounced circadian rhythm, with circulating levels beginning to increase in the morning and then tapering off later in the day (fig. S9). However, there are many stressors and disease states that can cause chronically elevated cortisol levels and this can have detrimental effects on a number of tissues, including the skin. In this cohort we identified a cortisol rhythm that aligns with the published literature, albeit with large interindividual variability. The rhythms of melatonin and cortisol add a robust, internal control to this study allowing for meaningful detection of circadian transcriptional rhythms.

Extracting expression profiles and running MetaCycle on time series datasets

We used the RMA algorithm in the affy R package *(41)* to extract expression profiles from the raw CEL files of time-series human skin samples. Raw CEL files of mouse anagen and telogen skin were downloaded from the GEO database. The RMA algorithm in the oligo R package *(42)* was applied to each tissue separately to get the expression profile. The RMA normalized expression profiles of 12 mouse tissues *(19)* were downloaded from the GEO database. The time-series human skin samples were analyzed with meta3d using the default settings in addition to cycMethodOne = "ARS" and period length being set to 24 for both ‘minper’ and ‘maxper’. Transcripts with meta3d_Base less than 16 were filtered out. The probe sets were annotated with gene symbols and one representative transcript was selected for each gene based on the following criteria: the lowest *P* value or the largest rAMP (multiple transcripts have equal *P* values). MetaCycle::meta2d was used to detect circadian transcripts with the default settings and period length set to 24 for both ‘minper’ and ‘maxper’ for mouse skin tissues *(7)* and 12 other tissues *(19)*. The cycMethod=c ("JTK", "LS") was used for analyzing those 12 mouse tissues.

The annotation files for the human and mouse array were downloaded from the Affymetrix (annotation date is Mar 2016 and October 2014 for human and mouse respectively; http://www.affymetrix.com/support/technical/byproduct.affx?product). The probe sets were annotated with gene symbols, and one representative probe set was selected for each gene with the similar procedure as mentioned above, with the meta2d_Base cut-off at 64 for mouse telogen and anagen, and 63.1 for other 12 mouse tissues. The circadian genes identified from two mouse anagen and telogen skin were linked to human homolog genes using the NCBI homologene file (Build 68) for comparison analysis.

Batch effect adjustment

All 298 human skin samples listed in table S1 were combined together for the hybrid design. The RMA algorithm was used to extract expression profiles of raw CEL files from these 298 human skin samples. Through PCA analysis using the ggfortify package *(43)*, an obvious batch effect was observed. The ComBat function in the sva package *(44, 45)* was used to adjust these batch effects. The ComBat adjusted expression profiles were annotated with gene symbols. Probe sets with no annotated gene symbols or stable expression in all samples were filtered out. The remaining probe sets were grouped by gene symbols, and one probe set with the maximum median absolute deviation value was selected for each gene.

Testing the new CYCLOPS pipeline with mouse time series skin samples

To improve the ordering of human samples with CYCLOPS *(17)*, we changed the original pipeline. Three key steps were added to the new pipeline: i) assess the robustness of the clock in the data set using an expression correlation matrix (ECM) of core clock and strong output genes, ii) order samples with selected eigengenes from Oscope *(40)*, and iii) validate the best ordering result using samples with time information. The ECM of clock genes in mouse anagen and

telogen (fig. S10, A and C) are similar with the ECM obtained from mouse tissues with known robust clocks (Fig. 4A). This indicates that there is a functional clock in these samples. If the dataset is noisy or there is a weak clock (like human blood samples; Fig. 4A and fig. S6), the ECM will be much weaker than the ECM obtained from mouse. Expression profiles of a set of seed genes (those circadian genes identified from at least two time-series skin dataset mentioned above) were inputs to CYCLOPS to obtain a set of eigengenes. Oscope was used to select the best eigengenes using the pair-sine model, termed the eigengene cluster. In addition to those eigengene clusters automatically selected by Oscope, we also included eigengene pairs with dissimilarity values ≤ the 20th percentile of the dissimilarity matrix given by OscopeSine function. Only the first two eigengenes extracted from mouse anagen and telogen data sets show periodic expression patterns (fig. S10, B and D), which were also selected by Oscope. Using the first two eigengenes, CYCLOPS gave the order for mouse anagen and telogen samples. Lastly, we evaluated the sample order from CYCLOPS. The sample order given by CYCLOPS is in a linear relationship with the real sampling time (fig. S10E), which indicates the CYCLOPS order is correct.

Ordering human skin samples with the new CYCLOPS pipeline

The improved CYCLOPS pipeline was used to order all 298 human skin samples using circadian genes identified from at least two time-series skin datasets as seed list genes. The default settings were used to run both CYCLOPS and Oscope, except 'Frac_Var', 'DFrac_Var' and 'Seed_MinMean' were set at 0.99, 0.01 and 16, respectively for CYCLOPS, and 'quan' and 'maxK' were set at 0.5 and 10 for OscopeKM function. In addition to the default output parameters, the false discovery rate (FDR) was calculated using the p.adjust function in R

(3.3.2), and the relative amplitude (rAMP) was calculated by dividing the amplitude (amp) with fitted expression baseline (fitmean).

Evaluation of CYCLOPS output

A total of 10 eigengene clusters were selected by Oscope and were used for CYCLOPS ordering. From these results, we selected the best sample phase ordering based on two evaluation criteria. First, the sample phase given by CYCLOPS was compared to sampling time, using samples with time information, as mentioned above. The sample phase orders that reflected external sampling time were considered valid. Second, the phase order of clock genes were evaluated. The phase order of clock genes are evolutionarily conserved and have fixed relationships. If no clock genes are rhythmic and/or the phase order of these clock genes have a low correlation with the mouse clock genes, the sample order is likely wrong. The top sample phase order was selected based on the above two evaluation criteria.

Comparison of circadian genes identified from human skin and mouse telogen

135 circadian genes identified from modified cosinor regression analysis on the best CYCLOPS ordered samples were separated into five groups based on the following comparison with circadian genes identified from mouse telogen: i) The NoProbe group includes 10 genes without mouse homolog genes or with mouse homolog genes not represented on the mouse affymetrix array platform. Based on the rAMP and *P* value from the MetaCycle analysis of mouse telogen, the remaining 125 circadian genes were separated into additional other four groups: ii) rAMP $\leq$ 0.1 and P value $\geq$ 0.05, iii) rAMP > 0.1 and P value $\geq$ 0.05, iv) rAMP $\leq$ 0.1 and P value < 0.05, and v) rAMP > 0.1 and P value < 0.05. The 39 circadian genes with mouse homolog genes and robustly cycle in mouse telogen (rAMP > 0.1, *P* value < 0.05) were selected for phase

comparison. Circadian gene phases were adjusted to *ARNTL*/*Arntl* phase and converted to a positive value between 0 and 2π.

Phase set enrichment analysis of circadian genes identified from mammalian skin

PSEA *(18)* was used to identify biologically related gene sets showing temporally coordinated expression of circadian genes identified from time-series human skin ($P < 0.1$), mouse telogen samples ($P < 0.05$), and CYCLOPS ordered human population skin samples (FDR < 0.4). Before running PSEA, mouse gene symbols were aligned to human homologous genes, and MetaCycle gene phases were adjusted with *ARNTL*/*Arntl* phase and converted to a positive number between 0 and 2π. The gene set file (c2.cp.v5.0.symbols.gmt) was downloaded from MSigDB. The following parameters were used to run PSEA on circadian genes identified from time series human skin samples and CYCLOPS: Min items/set is 5, Max sims/test is 100,000, Domain is from 0 to 2π, q value is smaller than 0.05. Similar parameters were used for running PSEA on circadian genes identified from mouse telogen, except Min items/set is 15. Output results in the vsUniform folder were used in this study.

Comparison of the expression relationship of core clock genes in time-series samples collected from multiple mouse tissues, human blood and skin samples

We compared the ECM of core clock genes obtained from human skin with human blood samples and the ECM from multiple mouse tissues to assess the strength of the circadian clock between these two tissues *(19)*. Ten core clock genes which have validated locomotor activity rhythm changes in knockout mice were selected *(24)*. The pair expression correlation of these ten core clock genes in a human tissue was expected to be similar as seen from mouse tissues if this tissue contains a functional running clock, as these relationships are evolutionarily conserved. The human blood samples were from a well-designed research study investigating the effects of

insufficient sleep on circadian rhythmicity *(25)*. Briefly, 26 young and healthy subjects were recruited to take part in a 6 h sleep-restriction condition and a 10 h control condition for one week. Following the last day of sleep-restriction or control condition, blood samples were collected at 3 h intervals for 27 hours. The quantile normalized signal intensities of all blood samples and associated annotation files were downloaded from GEO database (GSE39445), and 201 samples collected from 21 subjects (each subject has at least seven samples) under sleep control condition were used for determining the ECM of core clock genes in human blood. Samples taken from human skin with known sampling time information (n = 79) from this study were used to determine the ECM of core clock genes in human skin. In order to have an equal comparison, samples collected from each mouse tissue were restricted to a time-window between ZT24 and ZT46. Spearman's rank correlation was used to calculate the ECM of core clock genes. Mantel test ('mantel.test' function implemented in the ape R package) was used to test the correlation between ECMs.

Functional clock evaluation in hundreds of human blood samples

To evaluate the functional clock at the population level from human blood samples, quantile normalized expression profiles of peripheral $CD14^+$ monocytes (GSE56045) and $CD4^+$ T cells (GSE56580) isolated from human blood samples *(46)* and their associated annotation files were downloaded from the GEO database. Only those samples collected from subjects younger than 60 years old (593 monocytes samples and 121 T-cell samples) were used for calculating ECM of core clock and strong output genes.

Circadian biomarker selection with ZeitZeiger

The default settings were used to run ZeitZeiger on CYCLOPS ordered samples, with two modifications. First, we normalized the expression value of each gene with the average

expression value of three non-cyclers (*GPKOW*, *BMS1* and *NAPG*) in skin. The three skin non-cyclers were selected by their average expression value (median-high expression genes with intensity value between 60 and 120) and standard deviation (top 10 median-high expression genes ranked by their standard deviation) among all skin samples. Second, we filtered out those genes with an rsq value ≤ 0.1. This reduced the number of genes used to search for candidate biomarkers. We ran ZeitZeiger using the training data set with the sumabsv = 3. We then filtered those genes in the first two SPCs with an absolute coefficient value ≥ 0.05 to obtain circadian marker genes. Then expression profiles of these selected circadian marker genes in the same training set were used for a second run of ZeitZeiger with sumabsv = 3, and used the first two SPCs to predict the sample phase in the testing data set.

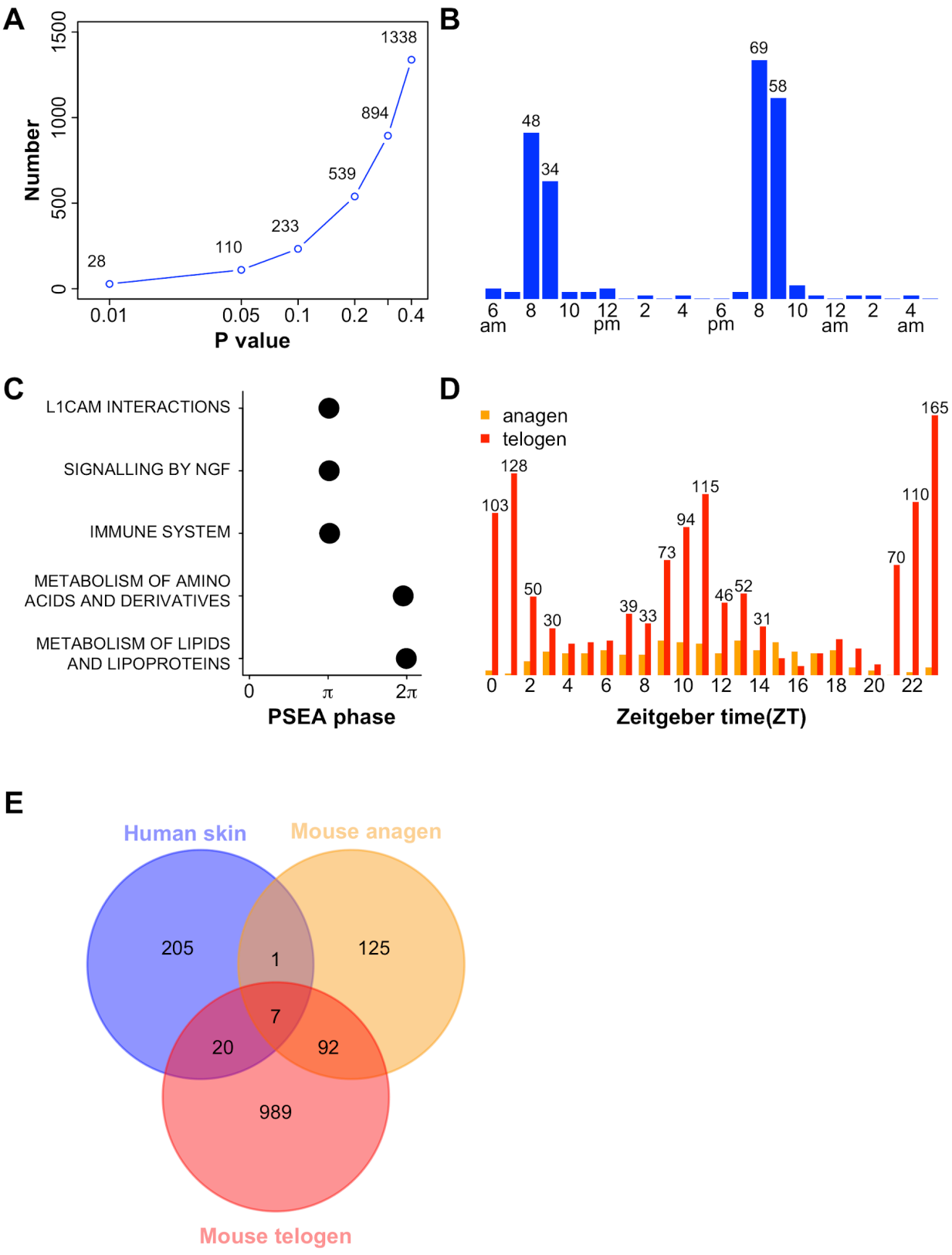
A
Number
P value
28
110
233
539
894
1338
B
48
34
69
58
C
L1CAM INTERACTIONS
SIGNALLING BY NGF
IMMUNE SYSTEM
METABOLISM OF AMINO ACIDS AND DERIVATIVES
METABOLISM OF LIPIDS AND LIPOPROTEINS
PSEA phase
D
anagen
telogen
Zeitgeber time(ZT)
E
Human skin
Mouse anagen
Mouse telogen
205
1
125
7
20
92
989

Fig. S1. Comparison of clock-regulated genes identified from time-series analyses of human and mouse skin. Gene expression data were analyzed using meta3d (human) and meta2d (mouse) using the MetaCycle package. (A) Number of circadian genes identified from time-series analysis of human skin at a series of *P* value cut-offs. Histograms of the phase distribution of circadian genes found in human skin (B) is depicted. (C) Enriched time-dependent pathways (q-value < 0.05) from PSEA analysis using 233 circadian genes ($P < 0.1$) identified from human skin. The x-axis depicts the circular average phase of circadian genes mapped in the same pathway, which is *ARNTL* adjusted. (D) Histograms of the phase distribution of circadian genes found in mouse anagen and telogen skin. (E) Venn diagram showing overlap in circadian genes between human (blue) and mouse homologs from anagen (orange) and telogen (red).

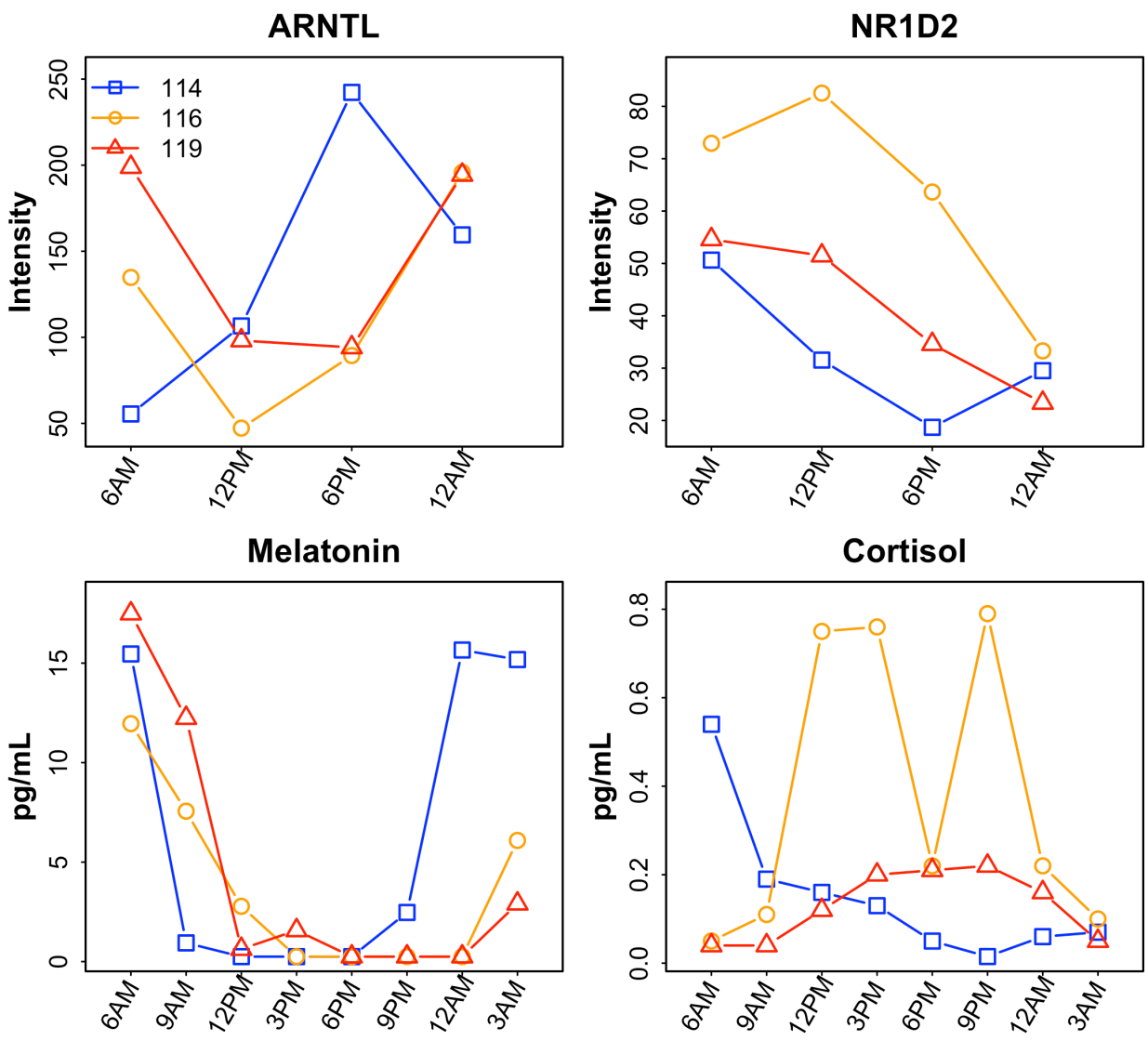

Fig. S2. Salivary melatonin and cortisol levels and clock gene expression profiles in subjects 114, 116, and 119. Subjects indicated with different colors.

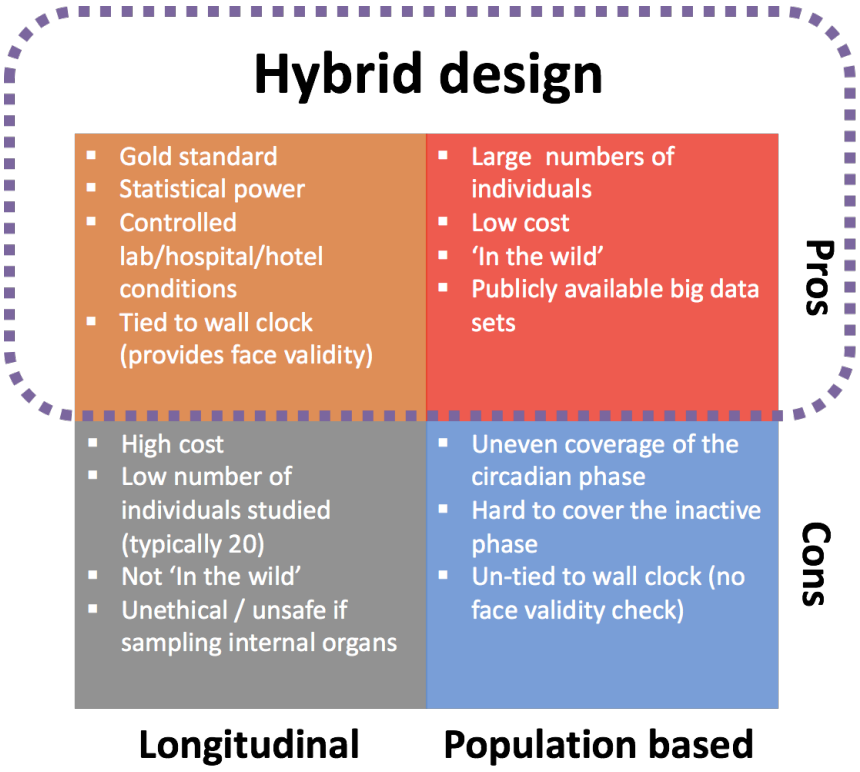

Fig. S3. Pros and cons of experimental designs for human circadian biology. Hybrid design exploits the advantages of both longitudinal and population based experimental designs.

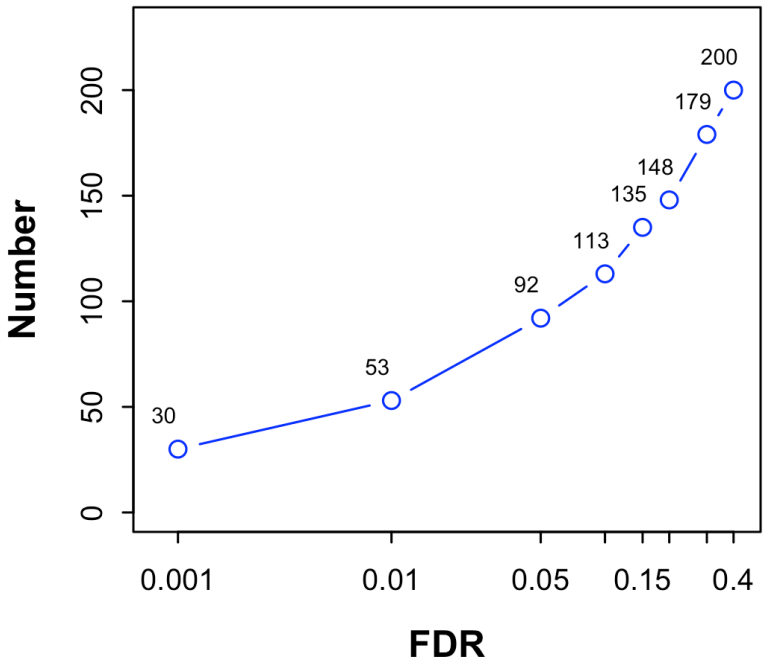

Fig. S4. Number of circadian genes identified in human skin at a series of FDR cut-offs.

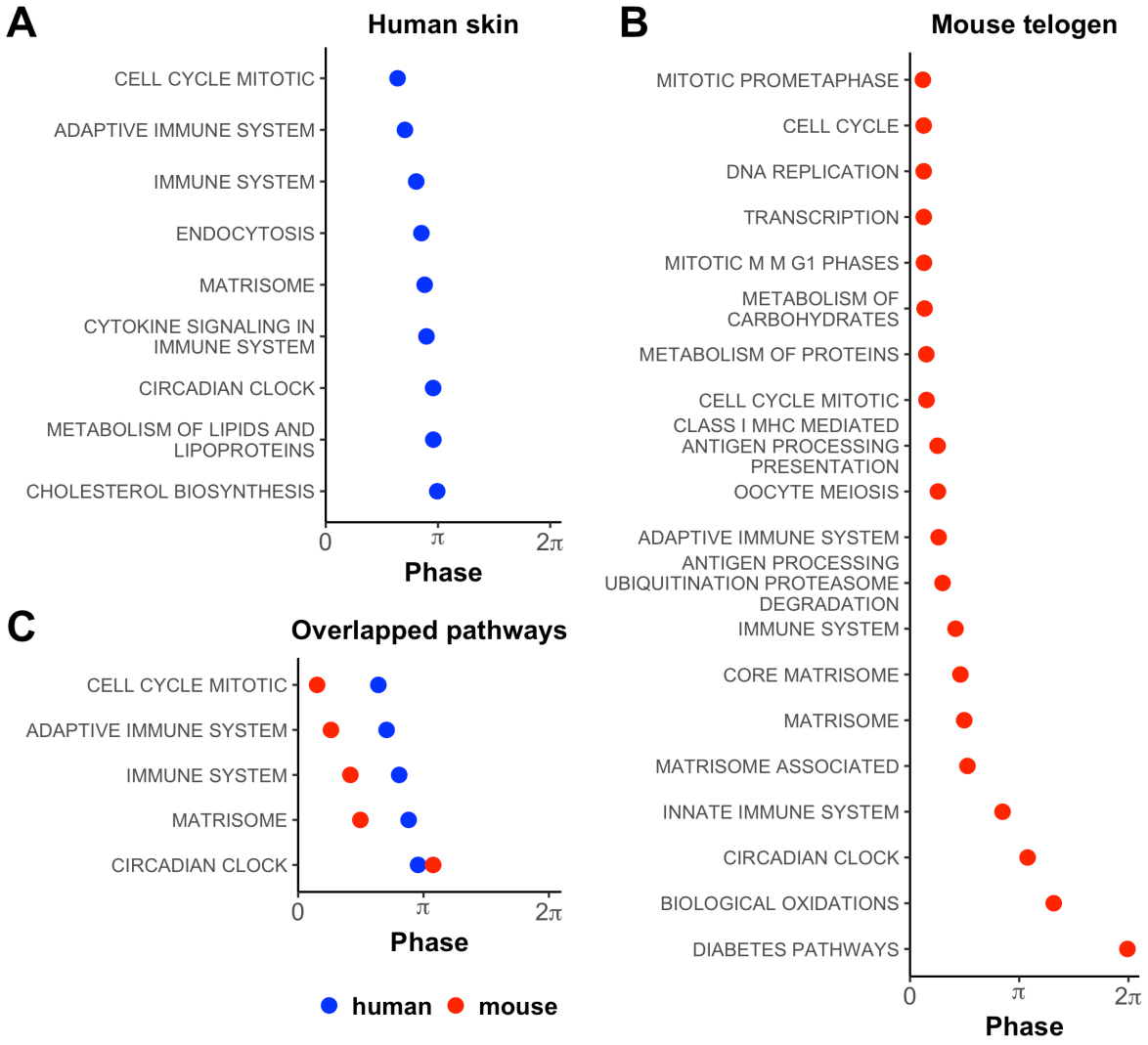

Fig. S5. PSEA analysis of circadian genes identified in human skin (A) and mouse telogen (B). A less significant cut-off (FDR < 0.4) was used to select genes for performing PSEA analysis in human skin. (C) The phase of five overlapping time-dependent pathways in human skin (blue) and mouse telogen (red). The x-axis in (A), (B) and (C) depict the circular average phase of circadian genes mapped in the same pathway, which is *ARNTL*/*Arntl* adjusted.

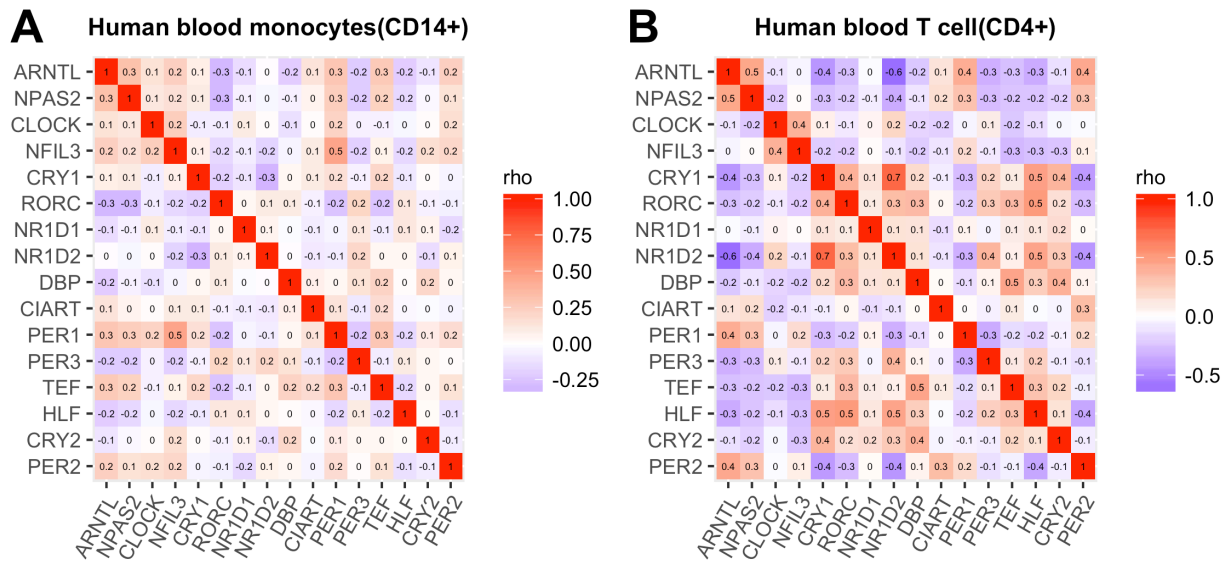

Fig. S6. Weak circadian clock gene correlations in human population-level blood samples. Evaluation of population level circadian clock gene correlations from peripheral $CD14^{+}$ monocytes and $CD4^{+}$ T-cell isolated from blood samples of 593 and 121 human subjects younger than 60 years old *(46)*. Note: the clock in monocytes and T-cells is weaker than in mouse and human skin shown in Figure 4. Red and blue indicate positive and negative Spearman's rho values, respectively.

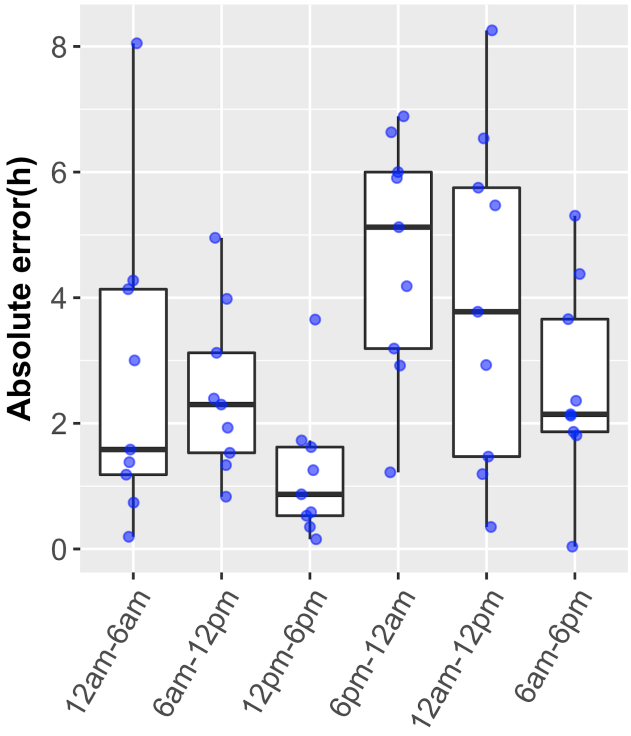

Fig. S7. Validation of skin circadian markers. Absolute errors are plotted for nine subjects at six time windows.

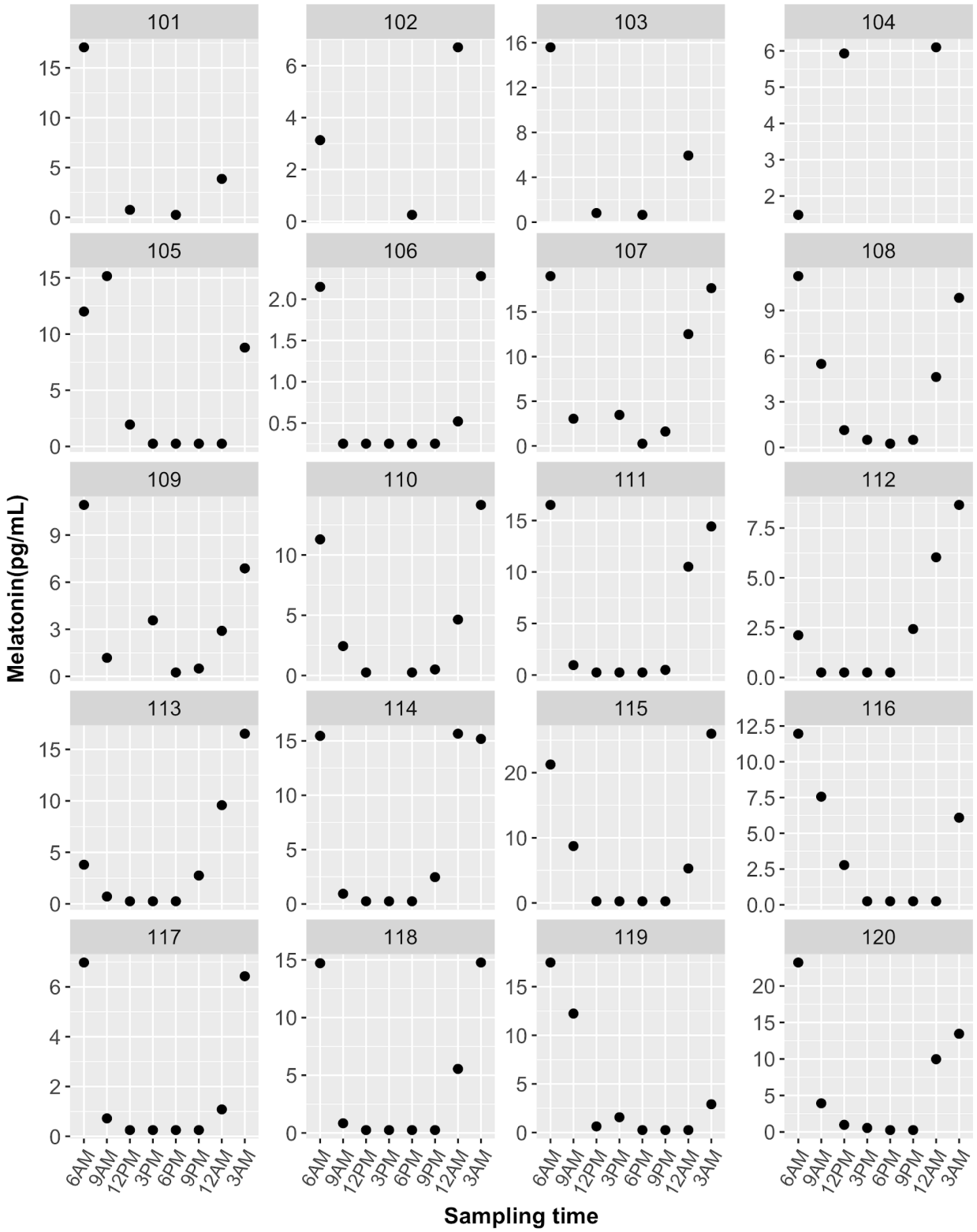

Fig. S8. Daily salivary melatonin levels for 20 subjects. Salivary samples were collected every 3 h for 24 h. Subjects 101, 102, 103, 104, 107, 109 and 110 have some missing values.

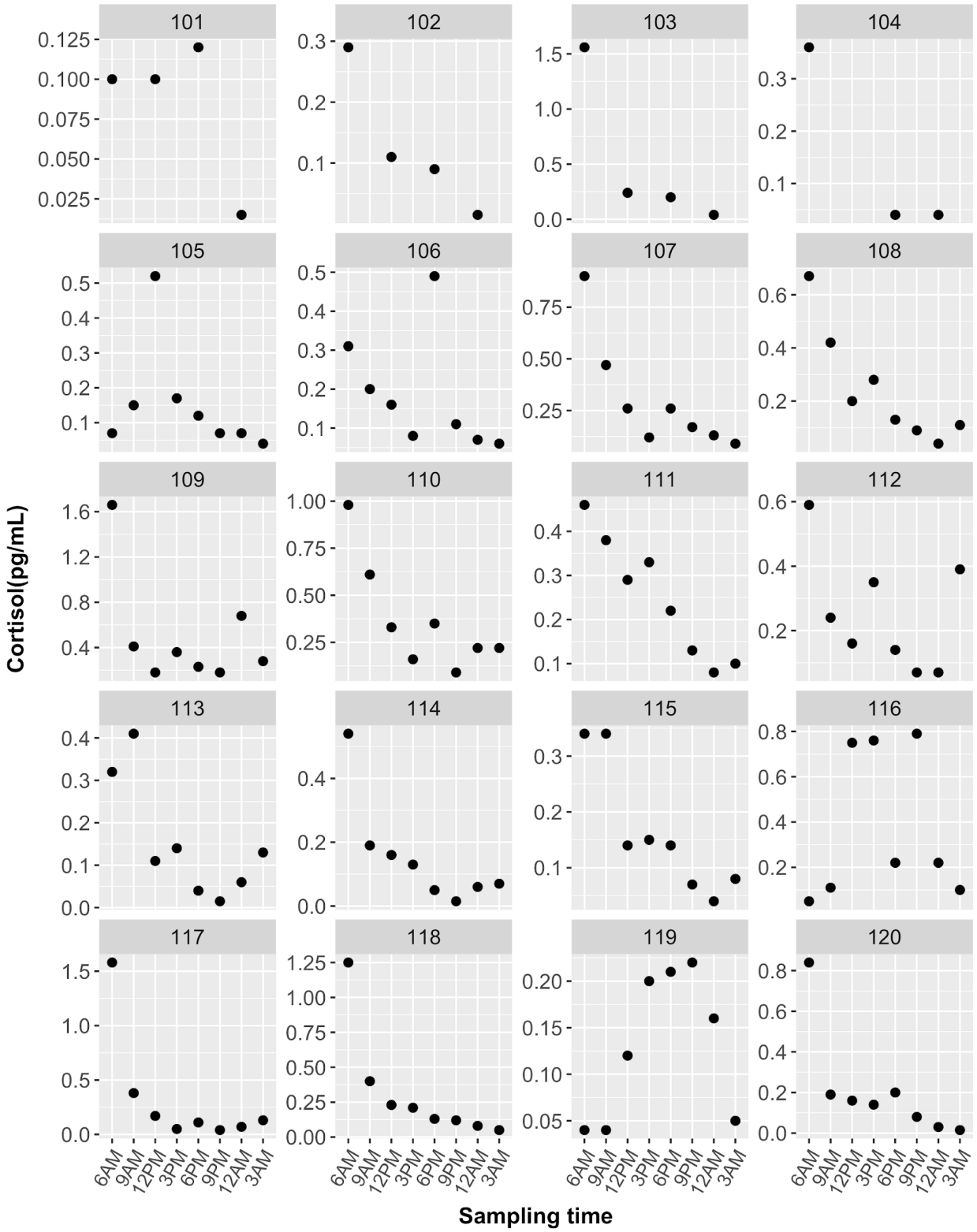
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
Cortisol(pg/mL)
Sampling time
6AM
9AM
12PM
3PM
6PM
9PM
12AM
3AM

Fig. S9. Daily salivary cortisol levels for 20 subjects. Salivary samples were collected every 3 h for 24 h. Subjects 101, 102, 103 and 104 have some missing values.

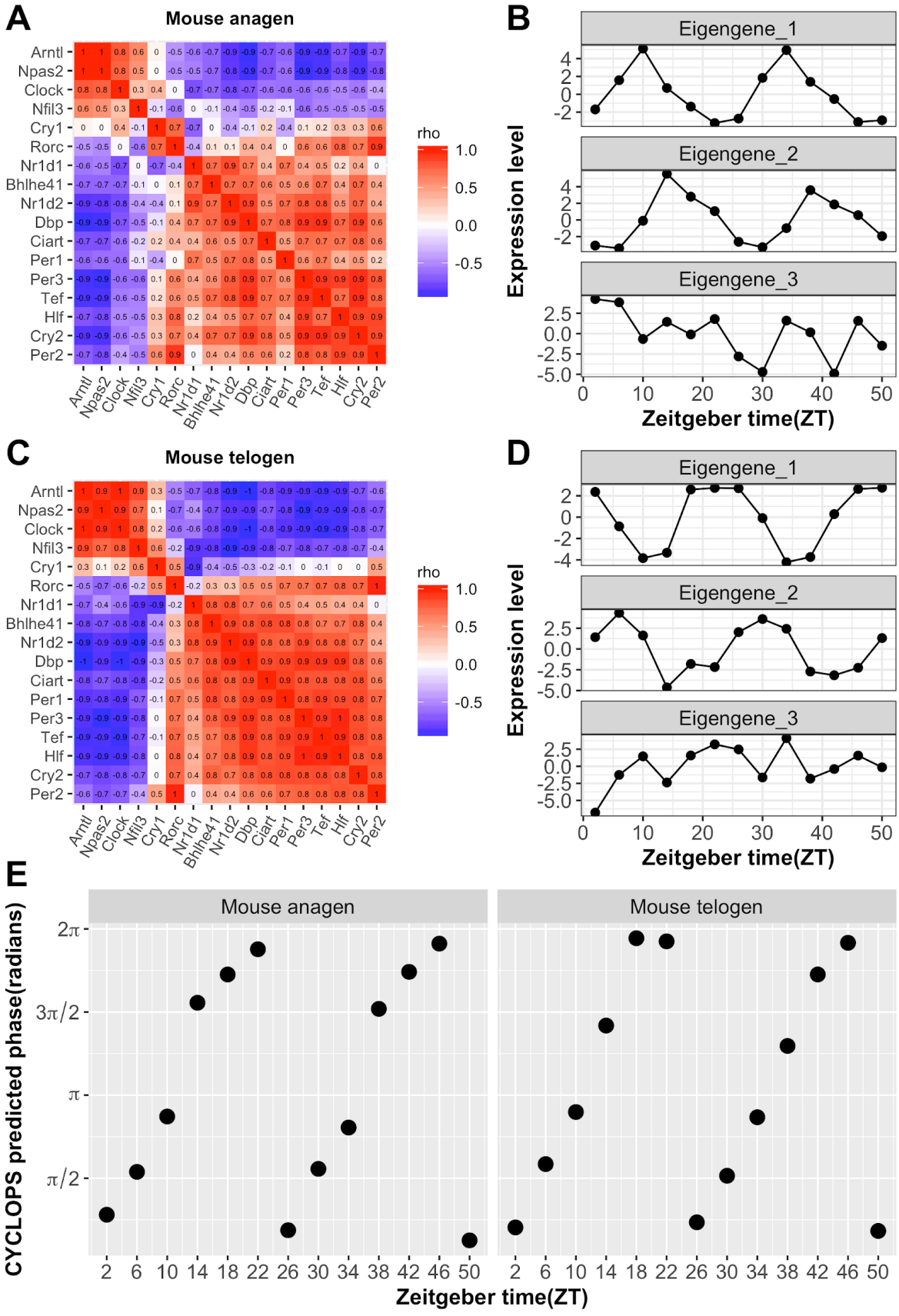
A
Mouse anagen
Arntl
Npas2
Clock
Nfil3
Cry1
Rorc
Nr1d1
Bhlhe41
Nr1d2
Dbp
Ciart
Per1
Per3
Tef
Hlf
Cry2
Per2
rho
1.0
0.5
0.0
-0.5
B
Eigengene_1
Eigengene_2
Eigengene_3
Expression level
Zeitgeber time(ZT)
C
Mouse telogen
D
E
Mouse anagen
Mouse telogen
CYCLOPS predicted phase(radians)
2π
3π/2
π
π/2
Zeitgeber time(ZT)

Fig. S10. CYCLOPS recovers sample orders from mouse anagen and telogen datasets. A heat map of Spearman's rho for core clock and output genes from ordered mouse anagen (A) and telogen data (C) shows conserved correlation of clock genes. The expression profiles of the top 3 eigengenes from mouse anagen and telogen skin data were shown in (B) and (D), respectively. Only Eigengene 1 and Eigengene 2 in mouse anagen and telogen skin data show periodic profiles, which were selected by Oscope and used for ordering. (E) CYCLOPS accurately re-ordered the mouse anagen and telogen samples. The sampling time and the CYCLOPS predicted sample phase are shown on the x and y axes, respectively.

Table S1. List of datasets used in this study

Datasets	**Reference**	**Platform**	**Site**	**#Samples**	**Experimental design**
Mouse anagen	Geyfman M., et al., 2012, PNAS. GSE38623	MoGene-1_0-st	anagen	13	C57BL/6CR mice were housed under 12 h:12 h LD cycles with food and water ad libitum. Whole skin was collected at 4-h intervals for 48 h. Anagen samples were collected from P30 mice. Equal amounts of RNA from the three mice for each time point were pooled.
Mouse telogen	Geyfman M., et al., 2012, PNAS. GSE38622	MoGene-1_0-st	telogen	13	C57BL/6CR mice were housed under 12 h:12 h LD cycles with food and water ad libitum. Whole skin was collected at 4-h intervals for 48 h. Telogen samples were collected from P46 mice. Equal amounts of RNA from the three mice for each time point were pooled.
Caucasian epidermal skin (ordered)	This study. GSE112660	HG-219 array	forearm	79	Four forearm skin samples were collected at 4 time points (12 AM, 6 AM, 6 PM, 12 PM) from each of 20 Caucasian male subjects, except 3 samples from subject 115. The ages of these 20 subjects are between 20 and 50 years old. LCM was performed to separate dermis from epidermis.

Caucasian epidermal skin (unordered)	Kimball A. B., et al., 2018, J Am Acad Dermatol. GSE112660	HG-219 array	forearm	152	One forearm skin sample was taken from each of 152 subjects in a Caucasian female population, aged between 20 and 75 years old. Samples were designed to collect during the working hours, between 9 AM to 5 PM. LCM was performed to separate dermis from epidermis.
African-Americans epidermal skin (unordered)	This study. GSE112660	HG-219 array	forearm	67	One forearm skin sample was taken from each of 67 subjects in a African-Americans female population, aged between 20 and 65 years old. Samples were designed to collect during the working hours, between 9 AM to 5 PM. LCM was performed to separate dermis from epidermis.