Supporting Information

Kervezee et al. 10.1073/pnas.1720719115

SI Discussion

Group-Level and Individual-Level Cosinor Analysis. The proportion of significantly rhythmic transcripts as determined by the individual-based analysis is higher than the proportion determined by group-level analysis at baseline and during the night shift condition. This is understandable given that the individual analysis considers each individual time series separately, hence taking into account that the phase of a transcript might be different per individual. Nevertheless, both methods consistently show a robust reduction in the number of significantly rhythmic transcripts during the night shift condition compared with baseline.

Comparison with Published Human Circadian Transcriptomic Datasets. Two recent studies have investigated the circadian regulation of the human transcriptome (1, 2). Archer et al. (1) used a forced desynchrony protocol, in which the sleep/wake cycle is scheduled to a 28-h day. Möller-Levet et al. (2) studied the effect of sleep restriction. In that study, the control condition included a constant routine measurement period during which blood samples were taken for transcriptomic analysis. Since the baseline condition of the study presented by Archer et al. and the control condition described by Möller-Levet et al. are similar to the baseline condition of our study, the results warrant comparison. One important difference between our study and those by Archer et al. and Möller-Levet et al. is that in those studies a custom-designed microarray chip was used that included many duplicate probes targeting circadian clock-related transcripts (n = 495), while we used a commercially available chip that is not enriched for circadian-related probes. This difference should be kept in mind when comparing the results of the three studies. In addition, we performed microarray analysis on PBMCs, whereas the other two studies used whole-blood samples for this purpose, presenting another potential source of differences between the results obtained from the three studies.

A Venn diagram showing the number of overlapping transcripts identified as rhythmic during the control or baseline conditions in the studies by Archer et al. and Moller-Level et al. and our study is shown in Fig. S3*A*. Clock genes that were classified as rhythmic in all three datasets include *PER3* and *NR1D2*, which consistently oscillate with the same phase at baseline across the three datasets (Fig. S3*B*), indicating the reproducibility of rhythms in clock gene expression across studies.

The night shift condition in our study, in which the sleep period of participants was acutely delayed by 10 h and maintained for 4 d, may to some degree be comparable to the out-of-phase condition used by Archer et al., because, despite the different protocols, the sleep in both studies occurs out of phase with the melatonin rhythm of the subjects. Our study and that of Archer et al. uncovered transcripts that are up- or down-regulated following the shifted sleep schedules. While we observed a slightly higher number of up-regulated genes than down-regulated genes during the night shift condition (154 vs. 177), Archer et al. found many down-regulated transcripts and relatively less up-regulated transcripts (913 vs. 206). The reason for this discrepancy could be related to the different study designs and/or statistical analysis methods. Nevertheless, several transcripts were found to be down-regulated (n = 5; *RANBP9*, *SMC3*, *MAP4K5*, *GADD45B*, and *C5AR1*) or up-regulated (n = 2; *OGG1* and *CAMP*) in both studies.

In addition, using grouped or individual cosinor analysis, we observed a marked reduction of significantly rhythmic transcripts, which is similar to the decrease reported by Archer et al. (from 6.4% at baseline to 1.0% in the out-of-phase condition). However, our model selection approach reveals a different picture: the majority of transcripts that are rhythmic at baseline remain cycling during the night shift condition, but with reduced amplitudes. This highlights the strength of our model selection approach: while the mixed-model ANOVA approach used by Archer et al. allows for the detection of significant interactions between conditions and time points, it is not possible to distinguish between different scenarios (e.g., shifted rhythm vs. loss of rhythm). With the model selection approach used in our study, derived from Atger et al. (3), we can distinguish between these different scenarios and classify transcripts into 10 different categories based on a change in mesor and altered rhythmic expression profiles. We believe this method to be useful in the context of recently published guidelines advocating the use of approaches to directly compare rhythmic parameters in two conditions (4).

SI Methods

Participants. Ten healthy subjects (one woman) were enrolled in a simulated night shift work protocol. Eligibility was determined based on results of a medical screening as described previously (5). One male subject only completed the day-oriented session and his results were excluded from all analyses. Another male subject was excluded from further analysis due to technical difficulties involving his samples before microarray analysis. The woman was naturally ovulating, had regular menstrual cycles, and was studied in her follicular phase.

Study Protocol. Subjects maintained a self-selected stable sleep/wake schedule with a sleep period of 8 h for at least 1 wk before admission to the laboratory. Compliance was assessed using a sleep diary and actigraphy. The laboratory protocol (Fig. 1) was composed of a 24-h measurement period consisting of an 8-h sleep period during which the subjects slept according to their habitual sleep times and a 16-h constant posture procedure ("baseline"), involving a semirecumbent posture, minimal activity levels, hourly isocaloric snacks, and exposure to dim light. On the subsequent day, the sleep period was delayed by 10 h relative to the habitual sleep period. This night shift condition was maintained for four consecutive 24-h cycles until the end of the study. During wake period of the first simulated night shift, subjects were given hourly isocaloric snacks. On the days of the second and third simulated night shift, subjects received three meals (45 min, 4 h, and 10 h after lights on) and a snack (2 h before lights off). Outside the two measurements periods, subjects were free to move within their time-isolation room during the waking periods. Following the third night on this shifted schedule, subjects underwent a second measurement period ("night shift condition"), consisting of a 16-h constant posture procedure followed by an 8-h sleep period. Throughout the simulated night shifts, subjects were exposed to dim light levels [2.6 ± 0.4 lx (mean ± SD)] during their waking periods to study the effect of a delayed sleep period and shifted feeding behavior in the absence of the confounding effects of light. Under these conditions, melatonin profiles in these subjects remained aligned to the day-oriented schedule, as previously reported (6). Blood samples (10 mL) for microarray analysis were collected in heparin-coated tubes every 4 h during both measurement periods via an indwelling catheter. For further details on the experimental protocol, see Cuesta et al. (6).

Actigraphy-Based Sleep Recording. Throughout the study protocol, actigraphy recordings (Actiwatch-L, Mini-Mitter) were collected. Sleep duration during each of the 8-h sleep opportunities in the study protocol was calculated through automated sleep detection provided by Actiware software (version 6.0.9; Philips Respironics) using default settings. The effect of the study nights on sleep duration was assessed using a linear mixed-effects model with "number of night in study" as fixed effect and subject as random effect (R package *lmerTest*, version 2.0.36). Post hoc pairwise comparisons were performed using R package *lsmeans* (version 2.27.61).

Isolation of PBMCs and mRNA Extraction. PBMCs were isolated from blood samples by centrifugation for 30 min at 370 × *g* on a density gradient (Histopaque-1077; Sigma Aldrich), as described previously (7). PBMCs were washed three times in PBS, lysed in TRIzol (Life Technologies), and stored at −80 °C until further processing. RNA was extracted as follows. Chloroform was added to separate organic and aqueous components. Isopropanol and subsequent ethanol washes were used to precipitate and purify the RNA. RNA was dissolved in RNase-free H_2O (Qiagen). Purity and RNA concentration were verified using a Nanodrop 1000 spectrophotometer (Thermo Fisher).

Microarray Profiling and Data Preprocessing. Out of the planned 112 samples, a total of 103 RNA samples (n = 12–14 samples per subject) was analyzed using Affymetrix Human Clariom S HT microarrays (Fig. S4). One sample was lost due to incorrect sample handling during the collection process; eight other samples were excluded to optimize the microarray plate. All samples passed Affymetrix Quality Control metrics and were used for downstream analysis. A total of 21,448 annotated probe sets was available on each array, corresponding to 19,669 unique transcripts. All downstream analyses were performed in R (version 3.4.1) (8). Background subtraction, quantile normalization, and summarization of the gene expression values were performed with the RMA algorithm using the R package *oligo* (version 1.38.0) (9). Probe sets were included in downstream analysis if they were expressed above background in at least 25% of the samples (n = 11,828 probe sets corresponding to 11,140 unique transcripts).

Individual Time Series Analysis. To assess the proportion of rhythmic transcripts per individual at baseline and during the night shift condition separately, linearized single-component cosinor analysis (10) using a fixed period of 24 h was applied to each individual time series. With eight subjects, two conditions, and 11,828 probe sets, this analysis encompassed 189,248 time series. P values were derived from a zero-amplitude test (10). Statistical significance on the subject level was determined using permutation of 1,000 randomly shuffled time series, using $\alpha = 0.05$. To find significantly rhythmic probe sets across subjects, individual P values obtained from the permutation approach were combined per probe set using Fisher's method using the function *sumlog* from the R package *metap*, version 0.8 (11). This method is typically used in meta-analyses to integrate P values from different studies addressing a similar hypothesis but has been described previously in the context of integrating P values derived from the assessment of rhythmicity of time series from multiple individuals in the MetaCycle package (12).

BIC Model Selection. For differential rhythmicity analysis, model selection was based on Schwarz (BIC) weights, which were computed as follows (13):

$$w_i = \frac{e^{-\frac{1}{2}\Delta(\mathrm{BIC}_i)}}{\sum_{k=1}^{K} e^{-\frac{1}{2}\Delta(\mathrm{BIC}_k)}}.$$

In this equation, $\Delta\mathrm{BIC}_i$ represents the difference between the BIC of model i and the minimum BIC value among the set of models. Schwarz weight w_i can be interpreted as the probability that model i is the optimal model among the set of models. For each probe set, the model with highest w_i was selected if it exceeded the threshold of 0.4.

The categorization of all probe sets that exceed the threshold is available in Dataset S2. In this dataset, the category (as depicted in Fig. 3) is provided for each probe set, as well as binary codes (0 or 1) to indicate whether the probe set is up-regulated or down-regulated in the night shift condition.

Phase Set Enrichment Analysis. Phase set enrichment analysis (PSEA, version 1.1) was applied as described previously (14) to identify biological processes showing significant temporal clustering across the 24-h period among transcripts that were identified as rhythmic in both conditions using the model selection approach. The "C5 GO biological process set" was downloaded from Molecular Signatures Database, version 6.0 (15). The application's default settings were used. Enrichment of biological processes was tested against a uniform background using Kuiper q value < 0.05.

Functional Interaction Network. To uncover the potential interactions between transcripts that were differentially expressed between baseline and the night shift condition, a functional interaction network of the up-regulated and down-regulated transcripts was created using ReactomeFIViz plugin (version 2016) in Cytoscape, version 3.5.1 (16). Default settings of the application were used. Only transcripts that have a functional interaction with at least one other transcript in the list are displayed in the network.

1. Archer SN, et al. (2014) Mistimed sleep disrupts circadian regulation of the human transcriptome. *Proc Natl Acad Sci USA* 111:E682–E691.
2. Möller-Levet CS, et al. (2013) Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci USA* 110: E1132–E1141.
3. Atger F, et al. (2015) Circadian and feeding rhythms differentially affect rhythmic mRNA transcription and translation in mouse liver. *Proc Natl Acad Sci USA* 112:E6579–E6588.
4. Hughes ME, et al. (2017) Guidelines for genome-scale analysis of biological rhythms. *J Biol Rhythms* 32:380–393.
5. James FO, Boivin DB, Charbonneau S, Bélanger V, Cermakian N (2007) Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles. *Chronobiol Int* 24:1009–1034.
6. Cuesta M, Boudreau P, Dubeau-Laramée G, Cermakian N, Boivin DB (2016) Simulated night shift disrupts circadian rhythms of immune functions in humans. *J Immunol* 196:2466–2475.
7. Cuesta M, Cermakian N, Boivin DB (2015) Glucocorticoids entrain molecular clock components in human peripheral cells. *FASEB J* 29:1360–1370.
8. R Core Team (2016) *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna).
9. Carvalho BS, Irizarry RA (2010) A framework for oligonucleotide microarray preprocessing. *Bioinformatics* 26:2363–2367.
10. Cornelissen G (2014) Cosinor-based rhythmometry. *Theor Biol Med Model* 11:16.
11. Dewey M (2016) *metap*: Meta-analysis of significance values. R package, Version 0.7. Available at https://CRAN.R-project.org/package=metap. Accessed April 17, 2018.
12. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB (2016) MetaCycle: An integrated R package to evaluate periodicity in large scale data. *Bioinformatics* 32: 3351–3353.
13. Wagenmakers EJ, Farrell S (2004) AIC model selection using Akaike weights. *Psychon Bull Rev* 11:192–196.
14. Zhang R, Podtelezhnikov AA, Hogenesch JB, Anafi RC (2016) Discovering biology in periodic data through phase set enrichment analysis (PSEA). *J Biol Rhythms* 31: 244–257.
15. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545–15550.
16. Wu G, Dawson E, Duong A, Haw R, Stein L (2014) ReactomeFIViz: A Cytoscape app for pathway and network-based data analysis. *F1000 Res* 3:146.

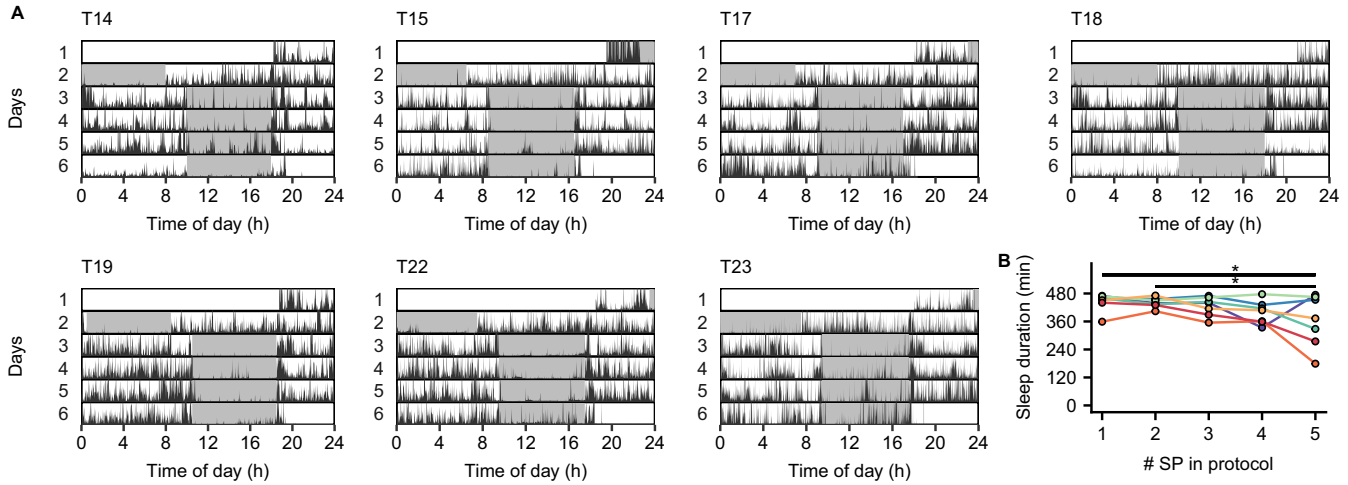

Fig. S1. (*A*) Actigraphy recordings collected during the study protocol relative to actual time of day in each of the subjects. Data from one subject (T21) is missing due to technical issues with the Actiwatch. Shaded areas indicate the protocol sleep times. (*B*) Sleep duration during the baseline sleep period (SP) and during each of the four sleep periods during the night shift condition, calculated as the minutes of sleep detected in the actigraphy recordings during each of the 8-h sleep opportunities. Different colored lines correspond to different subjects. A main effect of study night on actigraphy-based sleep duration was found ($P = 0.013$; mixed-effects model with subject as random effect), with significantly less sleep during the last (fifth) sleep opportunity compared with the first ($P = 0.019$; post hoc comparison using Tukey method) and second ($P = 0.027$) sleep periods (Fig. S1).

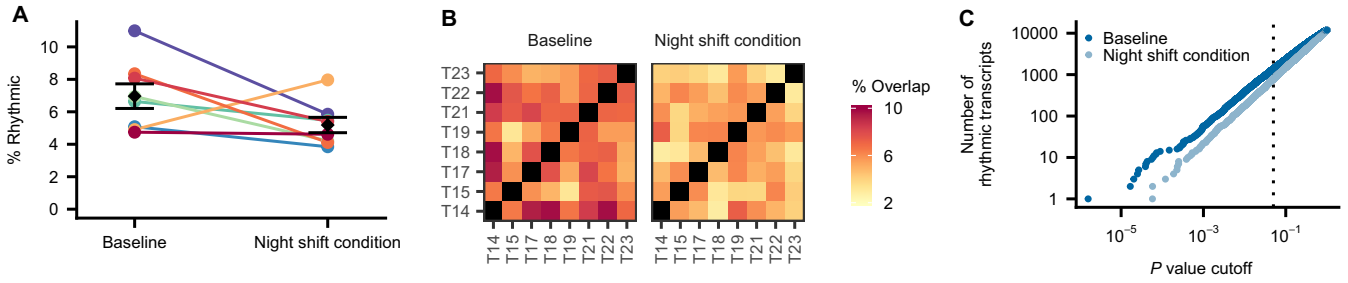

Fig. S2. Rhythms of gene expression at baseline and during the night shift condition using individual time series analysis. (*A*) Percentage of significantly rhythmic probe sets per subject at baseline and during the night shift condition ($P < 0.05$). Different colored lines correspond to different subjects. Individual *P* values were corrected for multiple testing using permutations. (*B*) Degree of overlapping rhythmic probe sets for each combination of subjects. Percentage overlap was calculated by dividing the number of overlapping significantly rhythmic probe sets by mean number of total rhythmic probe sets for each pair of subjects. The percentage overlap was significantly lower in the night shift condition (4.9 ± 1.2%; mean ± SD) compared with baseline (6.8 ± 1.5%) [$t_{(27)} = 5.4$, $P < 0.0001$, paired *t* test]. (*C*) Number of rhythmic transcripts identified by individual level cosinor analysis at baseline and during the night shift condition across the continuum of *P* value cutoffs. *P* values from the individual analyses (corrected for multiple testing using a permutation-based approach) were integrated per probe set using Fisher's method to obtain a combined *P* value across all subjects per condition. Dotted vertical line represents a *P* value cutoff of 0.05.

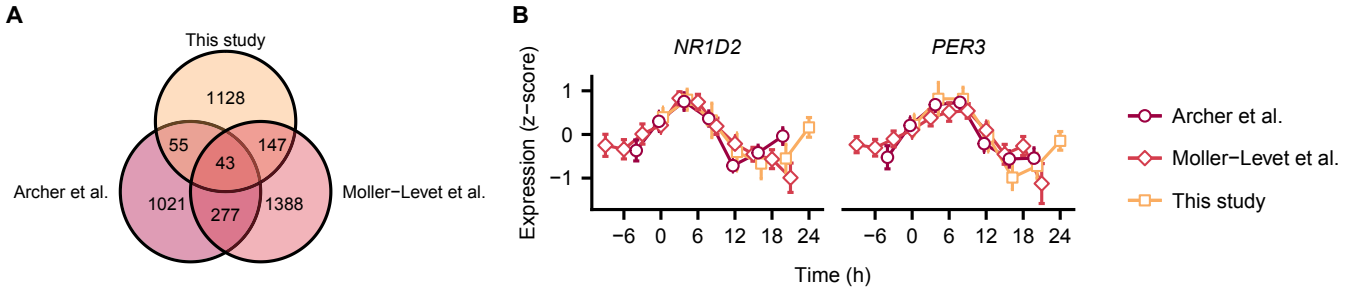

Fig. S3. Comparison of our results with results from Archer et al. (1) and Möller-Levet et al. (2). (*A*) Venn diagram showing the number of transcripts identified as significantly rhythmic during the control or baseline condition in each of the three studies. For this comparison, our results from the individual-based cosinor analysis shown in Fig. S2*C* is used ($P < 0.05$), as this analysis method is most comparable with those used in the other two studies. (*B*) Gene expression profiles of two clock genes that were identified as rhythmic in all three studies. Gene expression levels were obtained from GSE48113 (1) and GSE39445 (2) and subsequently normalized using *z* scores per study per probe set. In case multiple probe sets matched to a transcript, the one with the highest mean expression levels is shown here.

1. Archer SN, et al. (2014) Mistimed sleep disrupts circadian regulation of the human transcriptome. *Proc Natl Acad Sci USA* 111:E682–E691.
2. Möller-Levet CS, et al. (2013) Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci USA* 110:E1132–E1141.

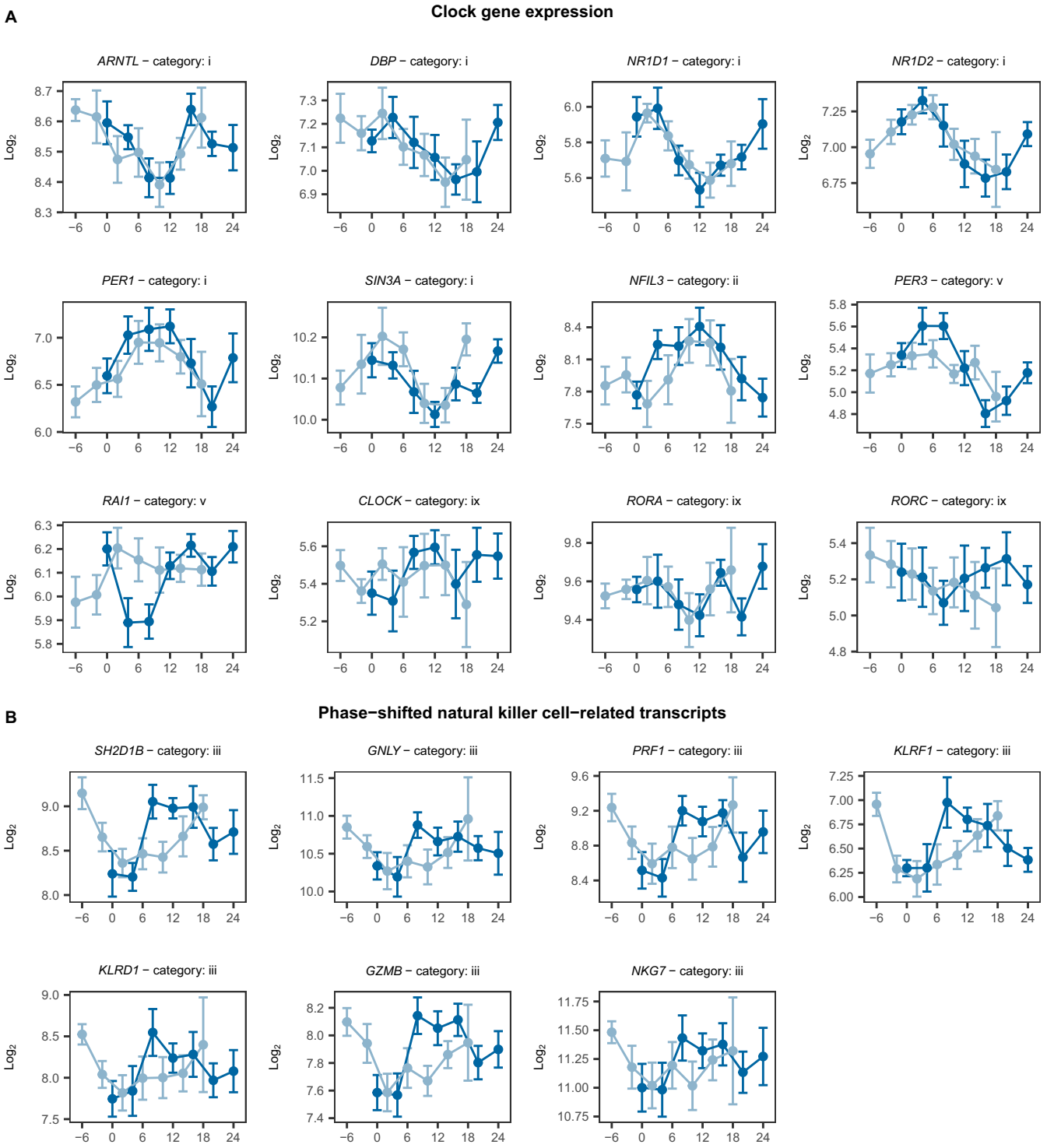

Fig. S4. Expression profiles of (*A*) clock genes and (*B*) phase-shifted transcripts related to the natural killer cell-mediated immune response during baseline (dark blue) and night shift conditions (light blue). Category numbers correspond to the numbers used in the main text of the manuscript.

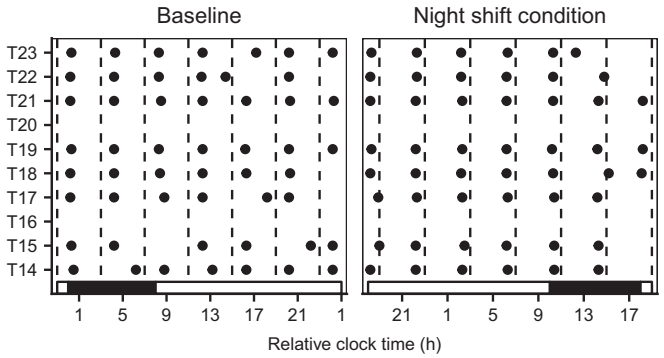

Fig. S5. Overview of the samples included in the microarray analysis per subject and sampling time point (total $n = 103$), including the two subjects (T16 and T20) that were excluded from analysis as explained in *SI Methods*. Dashed vertical lines show the borders of the time bins that were used to group the samples. Relative clock time refers to the time relative to the individual habitual bedtimes during baseline (from $t = 0$ h until $t = 8$ h). Black horizontal bar beneath the plot shows the sleep period during the experimental protocol.

Dataset S1. Transcripts identified as significantly rhythmic at baseline and/or during the night shift condition by the group and individual-level cosinor analysis

Dataset S1

Dataset S2. Categorization of transcripts according to model selection approach

Dataset S2

Dataset S3. Biological processes that show significant temporal clustering and their corresponding magnitudes, phases ("vector average value"), and (corrected) *P* values as shown by phase set enrichment analysis

Dataset S3