Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome

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Insufficient sleep and circadian rhythm disruption are associated with negative health outcomes, including obesity, cardiovascular disease, and cognitive impairment, but the mechanisms involved remain largely unexplored. Twenty-six participants were exposed to 1 wk of insufficient sleep (sleep-restriction condition 5.70 h, SEM = 0.03 sleep per 24 h) and 1 wk of sufficient sleep (control condition 8.50 h sleep, SEM = 0.11). Immediately following each condition, 10 whole-blood RNA samples were collected from each participant, while controlling for the effects of light, activity, and food, during a period of total sleep deprivation. Transcriptome analysis revealed that 711 genes were up- or down-regulated by insufficient sleep. Insufficient sleep also reduced the number of genes with a circadian expression profile from 1,855 to 1,481, reduced the circadian amplitude of these genes, and led to an increase in the number of genes that responded to subsequent total sleep deprivation from 122 to 856. Genes affected by insufficient sleep were associated with circadian rhythms (*PER1, PER2, PER3, CRY2, CLOCK, NR1D1, NR1D2, RORA, DEC1, CSNK1E*), sleep homeostasis (*IL6, STAT3, KCNV2, CAMK2D*), oxidative stress (*PRDX2, PRDX5*), and metabolism (*SLC2A3, SLC2A5, GHRL, ABCA1*). Biological processes affected included chromatin modification, gene-expression regulation, macromolecular metabolism, and inflammatory, immune and stress responses. Thus, insufficient sleep affects the human blood transcriptome, disrupts its circadian regulation, and intensifies the effects of acute total sleep deprivation. The identified biological processes may be involved with the negative effects of sleep loss on health, and highlight the interrelatedness of sleep homeostasis, circadian rhythmicity, and metabolism.

bloodomics | chronobiology | leukocyte | genomics

Insufficient sleep, defined as inadequate or mistimed sleep, is increasingly recognized as contributing to a wide range of health problems (1). Multiple epidemiological studies have shown that self-reported short sleep duration (defined in most studies as ≤6 h) is associated with negative health outcomes, such as all-cause mortality (2), obesity (3), diabetes (4), cardiovascular disease (5), and impaired vigilance and cognition (6). Laboratory studies, in which the sleep of healthy volunteers was restricted, typically to 4 h for 2–6 d, have identified physiological and endocrine variables that may mediate some of these effects (7), but in general the mechanisms by which insufficient sleep leads to negative health outcomes remain unidentified.

Microarray studies designed to investigate the processes underlying sleep regulation in rodents have established that, in brain tissue, sleep deprivation is associated with prominent changes in gene expression, although the number of genes affected varied widely between studies (8) and the mouse strains used (9). Genes up-regulated during sustained wakefulness (i.e., acute total sleep loss) belonged to functional categories, such as synaptic plasticity, heat-shock proteins, and other molecular chaperones, whereas reductions in transcript levels have been reported for genes involved in macromolecular biosynthesis and energy production (10). In the presence of a sleep-wake cycle, ∼8% of the brain transcriptome has been reported to be expressed in a circadian manner (i.e., with an ∼24-h periodicity), whereas during acute sleep loss, the number of rhythmically expressed transcripts is reduced to ∼1.5%, implying a prominent acute effect of the sleep-wake cycle on transcription (9). Although the sleep-wake cycle is generated by the brain, the effects of acute sleep deprivation are not limited to the brain. In fact, the liver transcriptome is affected to a larger extent by sleep loss than the brain transcriptome (9).

Acute sleep loss is a powerful tool to activate sleep regulatory mechanisms, but it is not necessarily the most relevant manipulation to model the kind of sleep loss experienced in society, in which people often get some, but insufficient sleep across every 24-h period. Recently, 2 wk of timed sleep restriction in mice was shown to disrupt diurnal rhythmicity in the liver transcriptome to a much larger extent than in the suprachiasmatic nucleus of the hypothalamus, the site of the master circadian oscillator (11). Biological processes affected included carbohydrate, lipid, and amino acid metabolism, providing clues as to how sleep restriction may lead to some of the reported health problems associated with insufficient sleep in humans. Thus, animal studies have established that both chronic insufficient/mistimed sleep and acute sleep loss lead to changes in the transcriptome, including its circadian modulation, and that these changes are tissue-specific.

Effects of chronic insufficient sleep on the global transcriptome have, to our knowledge, not been reported in humans. One ob-

Significance

Insufficient sleep and circadian rhythm disruption are associated with negative health outcomes, but the mechanisms involved remain largely unexplored. We show that one wk of insufficient sleep alters gene expression in human blood cells, reduces the amplitude of circadian rhythms in gene expression, and intensifies the effects of subsequent acute total sleep loss on gene expression. The affected genes are involved in chromatin remodeling, regulation of gene expression, and immune and stress responses. The data imply molecular mechanisms mediating the effects of sleep loss on health and highlight the interrelationships between sleep homeostasis, circadian rhythmicity, and metabolism.

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vious difficulty with human studies is that the tissues of interest, such as brain or liver, are not accessible for repeated sampling. Several transcriptome profiling studies of different conditions and diseases have, however, indicated that the transcriptome of leukocytes and whole blood may constitute an "accessible window to the multiorgan transcriptome" (12, 13). The blood transcriptome is altered in several neuronal and metabolic disorders and does not merely represent changes in the primary function of leukocytes in the immune system. The use of "bloodomics" thus makes possible the noninvasive and repeated sampling from participants undergoing carefully designed study protocols. This approach will enable the identification of gene-expression differences representative of the whole organism and not only those differences that are specific to immune function, which is in itself well known to be affected by sleep deprivation and circadian rhythmicity (14).

Many physiological and molecular processes are modulated by circadian rhythms. Furthermore, genes involved in the generation of circadian rhythms have been identified and shown to relate intimately to metabolism and other processes associated with health and disease (15, 16). Accurate assessment of circadian rhythmicity requires that confounding factors, such as the sleep-wake cycle, the light-dark cycle, activity, and food intake, which may directly affect the transcriptome, are carefully controlled. Therefore, to assess the effects of insufficient sleep on the whole-blood transcriptome, we conducted frequent RNA sampling over more than one complete circadian cycle, using an established constant routine protocol to control for these confounding factors (17).

Results

Effects of Protocol on Sleep, Waking Performance, and Circadian Phase of the Melatonin Rhythm. In this balanced, cross-over design (Fig. 1), participants obtained on average 5.70 h (SEM = 0.03) of polysomnographically assessed sleep per 24 h during the seven nights of the sleep-restriction condition, and 8.50 h (SEM = 0.11) during the seven nights of the control condition. Sleep obtained in the sleep-restriction condition was not sufficient to maintain alertness and performance. On the last day of sleep restriction, participants were significantly more sleepy, as scored on the Karolinska Sleepiness Scale [4.3 (SEM = 0.2) vs. 3.0 (SEM = 0.2); $P < 0.0001$], and had more lapses of attention [4.9 (SEM = 0.4) vs. 4.0 (SEM = 0.4); $P = 0.0036$] in the Psychomotor Vigilance Task.

The melatonin rhythm, which is a reliable marker of circadian rhythms driven by the hypothalamic circadian pacemaker, was affected by sleep restriction such that the midpoint occurred significantly later after sleep restriction than after the control condition (sleep restriction: 0501 hours, SEM = 19 min; control: 0415 hours, SEM = 19 min; $P < 0.0001$), and the duration of melatonin secretion was nonsignificantly reduced (sleep restriction: 9 h 35 min, SEM = 11 min; control: 9 h 53 min, SEM = 12 min; $P = 0.099$).

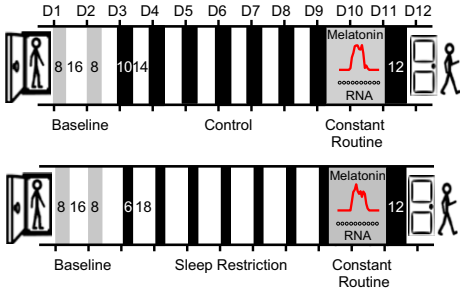

Fig. 1. Study protocol. The study protocol consisted of two 12-d laboratory sessions in a cross-over design. After two baseline/habituation nights, participants were scheduled to seven consecutive sleep opportunities of 6 h in the sleep-restriction condition and seven consecutive sleep opportunities of 10 h in the control condition. Following the final sleep restriction or control sleep opportunity, participants were subjected to a period of extended wakefulness (39–41 h of total sleep deprivation), which included hourly melatonin assessments, a well-established marker of circadian phase, and three hourly RNA samplings, under constant-routine conditions. Following a 12-h recovery sleep opportunity participants were discharged from the study.

Effects of Sleep Restriction on the Blood Transcriptome. ***Main effect of sleep condition.*** For ANOVA, in each participant and for each condition, the transcriptome was analyzed in 10 blood samples collected at three hourly intervals during a period of sustained wakefulness (total sleep deprivation for one day, one night, and the following day) after seven nights of either the sleep restriction or the control condition (Fig. 1). Because sleep restriction affected the melatonin rhythm, and differentially so between subjects, we aligned the transcriptome profiles with the respective individual melatonin profiles.

Mixed-model ANOVA for repeated measures revealed a main effect of sleep condition (sleep restriction vs. control) on the levels of transcripts encoded by 711 genes (~3.1% of the genes determined as present in the arrays) (Fig. 2*A* and Dataset S1). Of these genes, 444 were down-regulated and 267 were up-regulated following sleep restriction. The two genes that were most significantly affected by sleep condition were *MFNG* and *DCAF5* (Fig. 2*B*), which were down-regulated in response to insufficient sleep but had not previously been directly implicated in sleep regulation or circadian rhythms. Genes related to circadian rhythms and sleep, which were down-regulated after sleep restriction, included *RORA* (Fig. 2*B*), *IL6*, *PER2*, *PER3*, *TIMELESS*, and *CAMK2D*; *PRDX5* (Fig. 2*B*), *PRDX2*, *DEC1*, *CSNK1E*, *RHO*, and *OPN1LW* were up-regulated.

Gene-enrichment and functional annotation analyses identified several distinct processes that were significantly associated with the up- and down-regulated genes. For genes down-regulated following sleep restriction compared with control, the associated processes included chromatin modification and organization, gene expression, nucleic acid metabolism, nucleic acid binding, RNA binding, and cellular macromolecule metabolism; those associated with up-regulated genes included cellular response to oxidative stress, cellular response to reactive oxygen species, and response to stress (Fig. 2*C*).

In addition to the main effect of sleep condition, ANOVA also revealed that the effect of circadian time-bin (i.e., the melatonin phase-aligned sampling times) was significant for 22,401 probes that target 17,056 genes (75%), and 252 probes that target 232 genes (1%) showed a significant interaction between sleep condition and circadian time-bin [$P < 0.05$; Benjamini and Hochberg-corrected for multiplicity (18)]. This finding suggests that the expression or processing of many transcripts changed over the sampling period, and that this time course was affected by prior sleep condition (sleep restriction vs. control).

Time-course analysis of gene expression. Because ANOVA does not characterize the nature of the change of gene expression with time, we subjected all transcripts to a time-course analysis that identified those transcripts that exhibited a circadian pattern of expression and/or whose expression increased or decreased with time-awake (data summarized in Fig. 3 and Dataset S2).

Circadian rhythms in gene expression. Prevalent circadian genes were defined as those targeted by probes that showed a significant circadian oscillation in transcript levels in the number of participants that resulted in a false-discovery rate (FDR) of <5% in each condition. Assessment of individual expression profiles for a prevalent oscillatory component with a ~24-h period in the control condition identified 1,855 (8.8%) circadian genes, which included *PER1, PER2, PER3* (Fig. S1*A*), *NPAS2*, *CSNK1E*, *RORA*, *NR1D1* (*REV-ERB-α*) (Fig. S1*B*), *NR1D2* (*REV-ERB-β*), and other genes associated with circadian rhythms, sleep, and metabolism (Figs. 3*A* and 4*A*). After sleep restriction, the total number of circadian genes was reduced to 1,481 (6.9%) (Fig. 3*A* and Dataset S2). Comparing the genes in the two conditions showed that 793 genes were circadian in both conditions, and 688 genes were only circadian following sleep restriction. Gene-enrichment analysis showed that the genes that

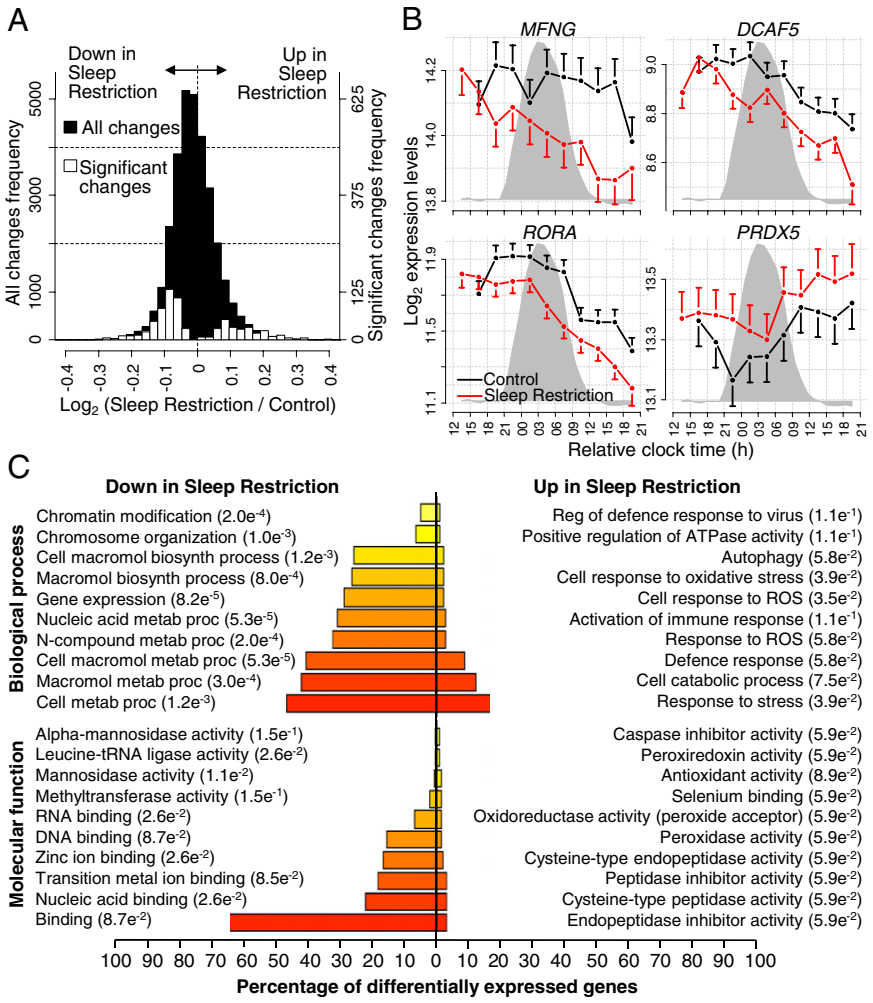

Fig. 2. Effects of chronic sleep restriction on the transcriptome. (*A*) Frequency distribution of expression fold-changes after sleep restriction relative to control. Histogram of changes in all transcripts (filled area; $n = 31{,}685$ probes that target 22,862 genes) and in transcripts identified as having a statistically significant (multiplicity corrected P value < 0.05) main effect of Sleep Condition (open area; $n = 744$ transcript that target 711 genes), plotted separately. (*B*) Example expression plots for genes with a significant main effect of Sleep Condition. *MNFG* (A_24_P224926) ($P < 1 \times 10^{-6}$), *DCAF5* (A_24_P940396) ($P < 1 \times 10^{-6}$), *RORA* (CPID_186) ($P < 1 \times 10^{-6}$), and *PRDX5* (A_24_P155378) ($P < 1 \times 10^{-6}$). Log_2 expression values are least-squares means ± SE (Procedure Mixed, SAS). Greyed area plots represent the melatonin profile averaged for the two conditions. Note that individual data were aligned relative to the individual melatonin rhythm and sorted into discrete circadian phase bins. Because of the shift in circadian phase after sleep restriction and to individual variation, the 10 melatonin samples covered 11 circadian phase bins after sleep restriction. (*C*) The top 10 enriched Gene Ontology Biological Processes and Molecular Functions within the statistically significant differentially expressed gene list as identified by WebGestalt when using the human genome as a background (66). Percentages are based on the number of unique gene symbols annotated as belonging to a specific biological process/molecular function compared with the number of unique gene symbols within the entire gene list. Color bars indicate the enrichment of a process/function, where red is the most enriched (top process/function) and yellow the least enriched (number 10 of the top 10). *P* values are the Benjamini and Hochberg (18) -corrected *P* values as calculated by WebGestalt (62).

were circadian after sufficient sleep but were no longer circadian following sleep restriction were significantly associated with biological processes that included inositol triphosphate kinase activity ($P = 0.033$), phospholipid transporter activity ($P = 0.033$), transferase activity ($P = 0.033$), nucleotide binding ($P = 0.033$), and catalytic activity ($P = 0.044$). In contrast, the 688 genes that became circadian after sleep restriction were associated with processes such as alanyl-tRNA aminoacylation ($P = 0.029$), alanine-tRNA ligase activity ($P = 0.0076$), and translational elongation ($P = 0.037$). Biological processes and molecular functions associated with the 793 genes that were classified as circadian in both conditions included those related to T-cell activation ($P = 2.9 \times 10^{-5}$), lymphocyte activation ($P = 2.6 \times 10^{-6}$), leukocyte activation ($P = 7.5 \times 10^{-7}$), inflammatory response ($P = 3.6 \times 10^{-6}$), immune response ($P = 1.3 \times 10^{-6}$), response to external stimuli ($P = 2.0 \times 10^{-6}$), cytokine receptor activity ($P = 0.016$), cytokine binding ($P = 0.037$), and hydrolase activity ($P = 0.011$).

Circadian phase, amplitude, and waveform of gene expression. We analyzed the phase, amplitude and waveform of circadian transcripts in the two conditions. We first used a circular self-organizing map (SOM) to identify distinctive temporal patterns within the set of prevalent circadian profiles in the control condition (Fig. 4*A*). This analysis identified median circadian expression profiles that separated into five clusters (C1–C5 in Fig. 4*A*), with peak times ranging from late in the biological day/early night (cluster 1), the biological night (clusters 2 and 3), and the early and middle of the biological day (clusters 4 and 5). Well-known circadian/sleep genes in cluster 1 included *NFKB2*, *CSNK1E*, and *RORA*. Genes whose transcripts peaked during the biological night (clusters 2 and 3) included known sleep- and circadian-related genes, such as *PER1*, *PER2*, *PER3*, *NR1D1*, *NR1D2*, and *NPAS2*. Genes with maximum transcript levels during the biological day (clusters 4 and 5) included kinases (*MAPKAPK2*, *MAP3K3*, *CMAKK2*), interleukin-related transcripts (*IL1B*, *IL1R2*, *IL1RN*, *IL1RAP*, *IL8RBF*, *IL13RA1*), tumor necrosis factor receptors (*TNFA1P6*, *TNFSF4*, *TNFRSF1DC*, *TNFRSF1A*, *TNFRSF9*), and lipid metabolism transcripts (*ABCA1*, *ABCD1*, *ABCG1*), in addition to genes of known circadian or sleep interest, such as *ARNTL* (*BMAL1*), *GHRL*, *STAT3*, and *PROK2*.

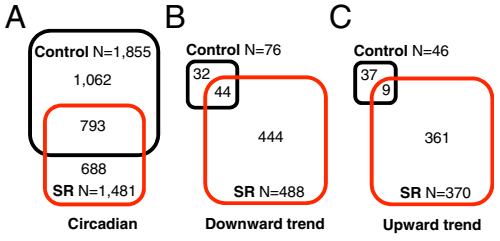

Fig. 3. Intersection of genes identified as circadian and time-awake–dependent in control and sleep-restriction (SR) conditions. (*A*) Venn diagram of prevalent circadian genes. (*B*) Venn diagram of genes identified as having a prevalent time-awake upward trend. (*C*) Venn diagram of genes identified as having a prevalent time-awake downward trend.

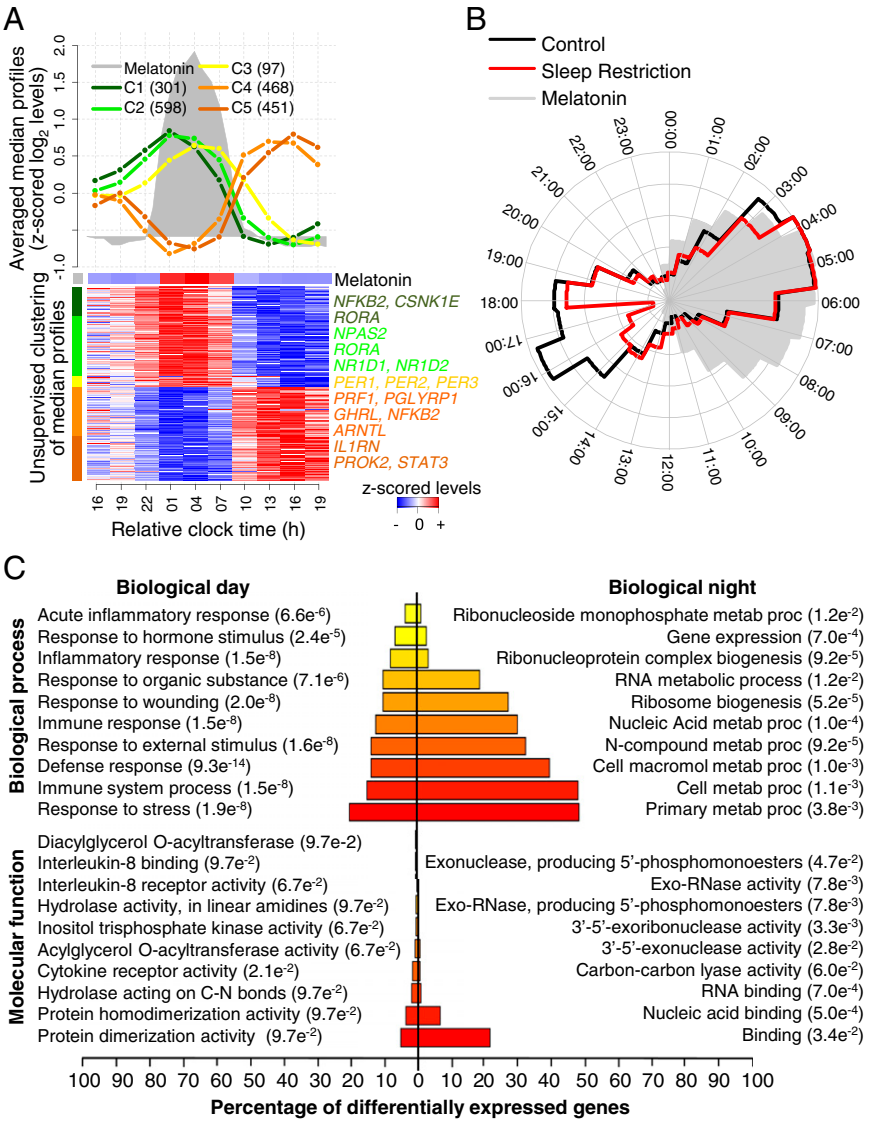

Fig. 4. Effect of sleep restriction on the phase of circadian genes. (*A*) Genes with a prevalent circadian variation during the constant routine/total sleep deprivation after the control condition (2,103 probes that target 1,855 genes, FDR <5%). Heatmap rows correspond to the median of the melatonin-aligned probe values across all participants in the control condition. Rows are clustered based on a circular self-organizing map. Cluster means are plotted above as time-series and the number of genes per cluster (C1–C5) is indicated in parenthesis (genes belonging to multiple clusters are counted in each cluster independently). Color codes to the left of the heatmap correspond to the colors of the clusters. Sampling times and melatonin profile shown correspond to the average values across all participants in the control condition. Genes related to circadian rhythmicity and sleep (according to Gene Ontology) are indicated in the heatmap (colors indicate cluster location). (*B*) Phase histogram of melatonin-aligned peak times of prevalent circadian genes following control (black contour; 2,103 probes circadian in an average of 11.56 participants, n = 24,311) and sleep restriction (red contour; 1,644 probes circadian in an average of 10.50 participants, n = 17,276). The distribution of the phases is significantly different between conditions (on 1-h binned data, $\chi^2 = 1305.785$, $df = 23$, $P < 2.2 \times 10^{-16}$). Histogram bins are 1-h-wide and bin heights are normalized to the maximum bin height per variable. The relative clock times and melatonin profile shown correspond to the average values across all participants and sleep conditions. (*C*) The top 10 enriched Gene Ontology Biological Processes and Molecular Functions within the circadian gene list of the control condition as identified by WebGestalt when using the human genome as a background (62). Percentages are based on the number of unique gene symbols annotated as belonging to a specific biological process/molecular function compared with the number of unique gene symbols within the entire gene list. Color bars indicate the enrichment of a process/function, where red is the most enriched (top process/function) and yellow the least enriched (number 10 of the top 10). *P* values are the Benjamini and Hochberg (18) -corrected *P* values as calculated by WebGestalt (62).

For each circadian transcript, we next assessed the melatonin-aligned peak times of the circadian component of each time course and plotted the distribution of these phases (Fig. 4*B*). The distribution was bimodal with peaks during the night (in-phase with melatonin; 1,072 circadian genes, 5.1% of all genes, 57.8% of circadian genes) and the day (out-of-phase with melatonin; 591 genes, 2.8% of all genes, 31.9% of circadian genes). Gene Ontology analysis of the circadian genes in the control condition showed that the genes whose transcripts had a circadian maximum during the biological night were significantly associated with processes and functions related to gene expression, RNA metabolic processes, cellular metabolic processes, and nucleic acid binding (Fig. 4*C*). In contrast, the circadian genes whose transcripts peaked during the biological day were associated with responses to hormones and stress, inflammatory, immune and defense responses, interleukin and cytokine activity, and protein dimerization (Fig. 4*C*). Gene Ontology analysis on genes from the individual clusters described above showed that genes associated with RNA processing and gene expression were present in cluster 2 with an average expression peak at 0130 hours; those genes associated with responses to stress, hormone, external stimuli, immune and inflammatory responses, cytokine activity, and NF-κB signaling were present in clusters 4 and 5, with average peak expression times of 1600 and 1906 hours, respectively.

In the sleep-restriction condition, the distribution of phases was also bimodal (Fig. 4*B*) (see Fig. S2*B* for the corresponding clusters and heatmap). In this condition, there were 905 genes (4.2% of all genes, 61.1% of circadian genes) in-phase and 281 genes (1.3% of all genes, 19.0% of circadian genes) in antiphase with melatonin. Therefore, the reduction in the number of genes whose transcripts were classified as having a circadian expression profile after sleep restriction is mainly a result of the elimination from the list of genes whose transcripts peaked during the biological day, which have more than halved. Gene enrichment and functional annotation analyses were applied to the circadian gene clusters following sleep restriction. The distribution of biological processes within the clusters was broadly similar to the control condition (see above), but the average expression peak time of cluster 2 (processes associated with regulation of gene expression) had moved later to 0250 hours, and the average peaks for clusters 4 and 5 (immune, inflammatory, stress responses, and so forth) had moved earlier in the day to 1430 and 1710 hours, respectively. Thus, a further effect of sleep restriction was to narrow the window of circadian gene expression and to increase the temporal separation of clusters 4 and 5, which can also be seen in the distribution of biological day genes in Fig. 4*B*.

Because the analyses of the melatonin rhythm suggested that sleep restriction could alter the waveform of circadian variables,

we analyzed the circadian profiles of transcripts in several ways. We used a circular SOM to generate a clustered heatmap for transcripts that were circadian in the control and/or the sleep-restriction condition (Fig. 5*A*), and for transcripts that were circadian in both conditions (Fig. S3*A*). We also plotted the time course of several transcripts separately for the two conditions (Fig. 5*B*). It can be seen from the heatmap that after sleep restriction, the nocturnal time window of peak expression has narrowed for the genes whose transcripts peaked during the night, whereas the nocturnal time window of minimal expression has narrowed for the genes that peaked during the day. This finding was quantified by comparing the width at mid-amplitude for the crest of the night-active genes ($P < 2.2 \times 10^{-16}$) and the width at mid-amplitude for the trough of the day-active genes, ($P < 1.4 \times 10^{-5}$) (Fig. 5*C*). This effect remained significant when the width at mid-amplitude change induced by sleep restriction was compared for those time-series that were identified as circadian in both conditions (Fig. S3*B*).

Individual transcript expression profiles suggested a reduction in amplitude following sleep restriction (Fig. 5*B*). Analysis of the amplitude of all individual time-series that were classified as circadian in the control and/or sleep-restriction conditions revealed that sleep restriction led to a significant reduction of the circadian amplitude ($P < 2.2 \times 10^{-16}$) compared with the control condition (Fig. 5*D*). This effect remained significant when the amplitude was compared for those time-series that were identified as circadian in both conditions (Fig. S3*C*).

Response of gene expression to time awake (acute total sleep deprivation). To identify transcripts that responded to total sleep deprivation we calculated a cumulative trend for each individual time-series and identified probes with statistically significant upward or downward trends during the constant routine after both the control and sleep-restriction conditions. The prevalent time-awake–dependent genes are defined as those targeted by probes whose transcript levels showed a significant trend in response to total acute sleep deprivation in a number of participants that resulted in a FDR of <5%.

The analysis identified 122 time-awake–dependent genes during the total sleep deprivation following the control condition (Fig. 3*B* and Dataset S2). Following the sleep-restriction condition, there was a sevenfold increase in the number of prevalent time-awake–dependent genes (856 genes; 3.8%) compared with the control condition (122 genes; 0.5%) ($P < 2.2 \times 10^{-16}$). In both conditions, the number of genes with a downward trend exceeded the number of genes with an upward trend (in the control, 46 genes up and 76 genes down, and in sleep restriction, 368 up and 488 down) (Fig. 3 *B* and *C*, and Dataset S2) (see *SI Methods* for an explanation of how gene numbers were derived).

Gene-enrichment and functional annotation analyses were carried out on the genes whose transcripts responded to time-awake with an increased or decreased expression (i.e., upward and downward trends were analyzed separately) in the control condition. For upwardly regulated transcripts, these included processes associated with phagocytosis ($P = 4.5 \times 10^{-2}$), and for the downward transcripts significant associations were with protein trimerization ($P = 1.4 \times 10^{-2}$), regulation of striated muscle differentiation ($P = 2.0 \times 10^{-4}$), histone H3 acetylation ($P = 1.5 \times 10^{-2}$), nucleic acid metabolism ($P = 2.3 \times 10^{-2}$), and H3 histone acetyltransferase complex ($P = 5.0 \times 10^{-4}$). Processes and functions associated with genes whose expression increased after sleep restriction included IL-6 signaling ($P = 0.044$), phagocytosis ($P = 0.044$), inflammatory response ($P = 0.044$), response to wounding ($P = 0.044$), and response to external stimuli ($P = 0.044$). Processes and functions associated with genes whose expression decreased during total sleep deprivation after sleep restriction included RNA processing ($P = 0.0023$), chromosome organization ($P = 8.3 \times 10^{-5}$), protein transport ($P = 0.0021$), gene expression ($P = 0.0017$), nucleic acid metabolism ($P = 2.0 \times 10^{-4}$), cellular macromolecule metabolism ($P = 6.0 \times 10^{-5}$), protein binding ($P = 0.0051$), and nucleic acid binding ($P = 0.0069$). We also conducted an enrichment analysis for those 361 genes and 444 genes that did not respond to time-awake in the control condition but became responsive after sleep restriction (Fig. 3 *B* and *C*). Processes and functions associated with these genes are very similar to those identified above (Fig. S4) and this is not unexpected, given that only 9 and 44 genes had an upward or downward expression trend, respectively, in both conditions.

We next applied a circular SOM to describe the time course of the transcripts that had a significant upward or downward trend following the control (Fig. 6*A*) or sleep-restriction conditions in more detail (Fig. S2*D*). The five clusters differ with respect to the overall trend (upward: clusters 1 and 2; downward: clusters 3, 4, and 5) and the extent of the rhythmic component (e.g., cluster 1 vs. 2). Genes with an upward expression trend following the control condition included *PROKR2*, *NTSR1*, *PTEN*, and *ABCA1*; those with a downward expression trend included *LSG1* and *NCOR1* (Fig. S5*A*). Genes with an upward trend following sleep restriction included *IL6*, *IL1RN*, *OPN4*, *STAT3*, *PER2*, *UCP3*, *ABCA1*, *KCNV2*, *CEACAM3*, *CEACAM4*, *CEACAM20*, *SLC2A3*, *SLC2A5*, *KSR1*, and *IMPDH1*. Genes with a downward trend following sleep restriction included *RORA*, *CRY2*, *CREM*, *CAMK2D*, *ENOX2*, *ZNF696*, *LAX1*, *POP1*, *PPM1K*, *NCOR1* (Fig. S5*A*), *SETD2* (Fig. S5*B*), *MLL* (Fig. S5*C*), and *MYST4* (Fig. S5*D*). (See Fig. S2*D* for heatmap and clusters).

To examine the change in the time course of gene expression after sleep restriction in more detail, we performed an analysis on genes whose transcripts became responsive to acute total sleep deprivation (i.e., those that had an upward or downward profile only after sleep restriction). The time course of the transcripts associated with these genes following sleep restriction was compared with their time course in the control condition (Fig. 6*B*). The heatmap shows clusters of downward transcripts that after sleep restriction had higher expression at the beginning of the sleep-deprivation period and lower expression at the end of the sleep-deprivation period, compared with their equivalent profiles in the control condition. The opposite was true for transcripts with upward expression profiles. This result is confirmed when averaged median profiles for the upward and downward clusters were compared for the two conditions (Fig. 6*C*). Statistical evaluation of this phenomenon by comparing trend angles for the upward and downward probes between the conditions revealed significant reductions in the cumulative trend angle for downward genes ($P < 2.2 \times 10^{-16}$), and significant increases for upward genes ($P < 2.2 \times 10^{-16}$) (Fig. 6*D*). A similar conclusion was reached when this analysis was applied to the transcripts which had a significant upward or downward trend in the control and sleep restriction condition (Fig. S6).

Discussion

This study has demonstrated that insufficient sleep to an extent frequently or chronically experienced by many individuals in industrialized societies altered the temporal organization of the human blood transcriptome, including its circadian regulation and the response to acute total sleep loss. The biological processes encoded by the genes most affected by sleep restriction included chromatin organization and modification, gene expression, inflammatory and stress responses, as well as cellular macromolecule metabolism, and oxidative stress responses (see Fig. S7 for a summary of all results).

Relevance of the Protocol. The sleep-restriction protocol led to a reduction of polysomnographically assessed total sleep time (5.7 h hours per 24 h), which is insufficient for this age group, as evident from the increase in sleepiness and lapses of attention, compared with the well-rested control condition (19). Insufficient sleep of this magnitude and duration may also lead to changes in many metabolic and endocrine variables (20) and may be relevant to many people living in industrialized societies worldwide. For example, according to the Centers for Disease Control and Prevention, 30% of civilian adults in the United States (~40.6 million workers) report an average sleep duration of 6 h or less (21). The current protocol was designed to assess effects of sleep restriction

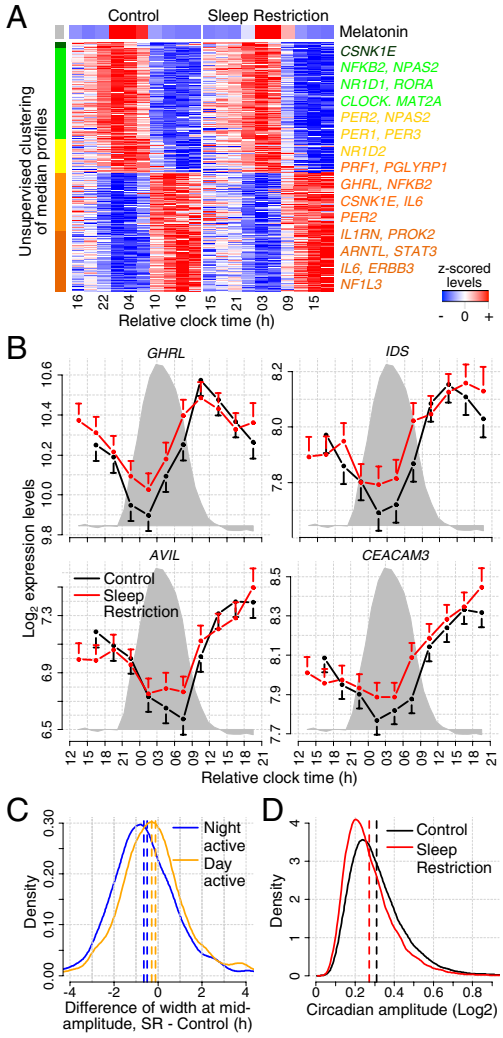

Fig. 5. Circadian variations in the transcriptome following control and sleep restriction. Genes with a prevalent circadian variation during the constant routine/total sleep deprivation after control and/or sleep restriction (n = 2,859 probes that target 2,510 genes). (*A*) Heatmap rows correspond to the median of the melatonin-aligned probe values across all participants per sleep condition. Rows are clustered based on a circular SOM. Color codes on the left side of the heat map identify the clusters. Relative clock times and melatonin profiles are average values across all participants per sleep condition. Genes related to circadian rhythmicity and sleep (according to Gene Ontology) are indicated in the heatmap (gene colors indicate cluster location). (*B*) Examples of genes with a significant difference in circadian amplitude: *GHRL* (A_23_P40956) (pair-wise comparison across participants: P = 0.0040), *IDS* (A_24_P285032) (P = 0.0042), *AVIL* (A_23_P390157) (P = 0.0109), and *CEACAM3* (A_23_P358244) (P = 0.0004). Log_2 expression values are least-squares means ± SE (Procedure Mixed, SAS). (*C*) Comparison of width at mid-amplitude for the night hours (trough) in the melatonin-aligned median profiles of day-active probes [n = 1,356 paired values, estimated mean of the difference –0.199 h (95% CI -0.288, –0.109), $P < 1.416 \times 10^{-5}$; density of the paired differences and 95% CI are shown in orange] and comparison of width at mid-amplitude for the night hours (crest) in the night-active probes [n = 1,469 paired values, estimated mean of the difference –0.572 h (95% CI –0.656, –0.489), $P < 2.2 \times 10^{-16}$; density of the paired differences and 95% CI are shown in blue]. (*D*) Density plot of circadian amplitudes per participant for the prevalent circadian genes in control and sleep restriction [control n = 29,568 (black solid line) corresponding to 2,859 probes circadian in an average of 10.34 participants; sleep restriction n = 24,354 (red solid line) corresponding to 2,859 probes circadian in an average of 8.51 participants]. The estimated mean for the circadian amplitude is 0.313 in control (black broken line) and 0.273 in sleep restriction (red broken line), 95% CI of the difference (–0.042, –0.037), $P < 2.2 \times 10^{-16}$.

on the temporal organization of the blood transcriptome, independent of the direct masking effects of longer light exposure and more intense sleep, which are present during the imposition of restricted sleep. To quantify effects of insufficient sleep on the endogenous temporal organization of the transcriptome, we assessed this during a period of sustained wakefulness and aligned the transcriptome time series with the rhythm of plasma melatonin. The melatonin rhythm provides an internal circadian-phase reference point that is generally relevant because of the circadian variation in a considerable proportion of the transcriptome, and particularly relevant because sleep restriction has been reported to shift circadian rhythms (22), as was also observed in this study.

Because the constant routine conditions involve a period of sustained wakefulness, the protocol also allowed the assessment of the effects of acute total sleep loss on the transcriptome, which has been the focus of many rodent studies on the homeostatic regulation of sleep (8). Importantly, our analyses of the transcriptome revealed that the effects of sleep history, circadian phase, and acute total sleep loss interact. For example, following sufficient sleep, the expression of only 122 genes changed in response to time awake, whereas after insufficient sleep, 856 genes changed expression. In other words, one major effect of sleep restriction is that it affects the impact of sustained wakefulness on the transcriptome. Similarly, the data show that circadian phase and amplitude are affected by sleep history. The fold-change differences in gene expression that we report are relatively small. Nevertheless, the within-subject, cross-over design of our protocol and the consequent large number of arrays (>500 providing >2 million probe time series) and the robust and stringent data quality control and statistical analyses (*Methods*) has allowed the detection of highly significant differential gene expression.

Blood Transcriptome. We found changes in the whole-blood transcriptome, some specifically related to leukocyte functions (e.g., an up-regulation of immune and inflammatory responses), and others related to more general processes (e.g., down-regulation of processes associated with chromatin modification, RNA processing, and gene expression), or in genes thought to be specific to particular tissues (e.g., opsins). The changes in expression of genes not typically implicated in leukocyte function are unlikely to be statistical artifacts because we used robust statistical procedures, correcting for multiplicity by generally accepted methods. These changes are also unlikely to be related to low expression levels because the quality-control procedures implemented safeguard against this potential artifact (*Methods*). Leukocytes, which are the only nucleated components of blood, are the main source of RNA in our samples. Other sources may include erythrocytes and platelets, and RNA from other tissues that have been shed into circulating blood may also be present. A previous study has shown that ∼80% of the blood transcriptome was shared with nine other tissues, including brain, heart, kidney, prostate, and lung, leading the authors to propose that peripheral blood can act as a "sentinel" for diagnosis or prognosis of conditions in a range of tissues (12). It is known that peripheral blood cells express genes normally associated with other tissue types, such as neurotransmitter receptors and transporters, notably GABA (23), but also opsins such as *RHO*, *OPN1LW*, and *OPN4* (Gene Expression Omnibus database). Indeed, circulating levels of *RHO* mRNA have been proposed as an assessment tool for diabetic retinopathy (24). In our study, GABA transporters, as well as *RHO*, *OPN1LW*, and *OPN4* were also differentially expressed. The presence of these neurotransmitter-related or opsin-related transcripts in blood may not necessarily have functional consequences within blood. However, their presence in the blood transcriptome may inform on the effects of external manipulation (including sleep deprivation) on the expression of genes in central and peripheral organs (13), as well as the processes and molecular functions associated with these genes.

Circadian Modulation of the Blood Transcriptome After Sufficient Sleep. Our observation that after sufficient sleep under constant

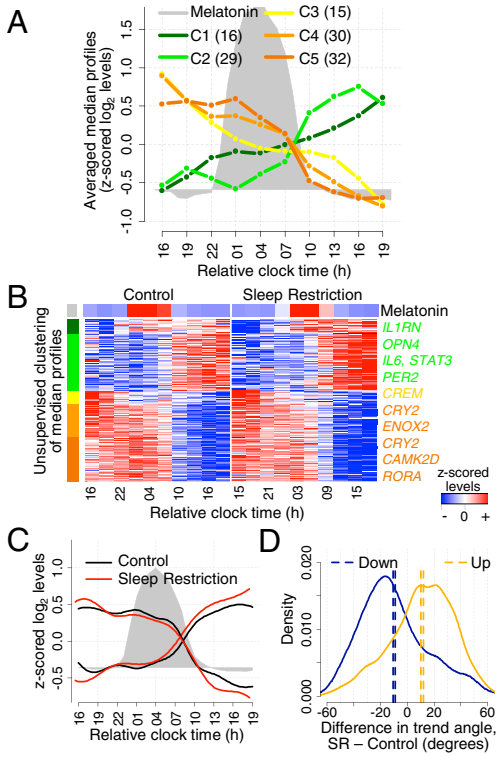

Fig. 6. Time-awake–dependent variations in the transcriptome. (*A*) Genes with a prevalent time-awake–dependent variation during the constant routine/total sleep deprivation following the control condition (124 probes that target 122 genes, FDR <5%). Medians of the melatonin-aligned probe values across all participants in the control condition are clustered based on a circular SOM. Cluster means are plotted as time-series and the number of genes per cluster is indicated in parenthesis (genes belonging to multiple clusters are counted in each cluster independently). Sampling times and melatonin profile shown correspond to the average values across all participants in the control condition. (*B–D*) Data are based on genes with a prevalent time-awake variation during the constant routine/total sleep deprivation after sleep restriction and not significantly prevalent after control (363 probes that target 361 genes with cumulative upward trend, and 470 probes that target 444 genes with cumulative downward trend; see Fig. 3 *B* and *C*). (*B*) Heatmap rows correspond to the median of the melatonin-aligned probe values across all participants per sleep condition. Rows are clustered based on a circular SOM of the sleep-restriction profiles. Color codes to the left of the heat map identify the clusters. Relative clock time and melatonin profile are the average values across all participants per condition. Genes related to circadian rhythmicity and sleep (according to Gene Ontology) are indicated in the heatmap (gene colors indicate cluster location). (*C*) Smoothing spline (64) of the average of melatonin-aligned median profiles (shown in *B*) of probes with an increasing trend and of probes with a decreasing trend. (*D*) Density plot of cumulative trend angle differences between sleep restriction and control. A total of 1,838 paired trend angles (363 probes significant in an average of 5.06 participants) were used for the comparison of upward trends, and a total of 2,454 paired trend angles (470 probes significant in an average of 5.22 participants) were used for the comparison of downward trends. For upward trend (orange), the estimated mean of the differences is 20.1085° [95% CI (19.3552, 20.8618), indicated by orange broken lines; *t* test $P < 2.2 \times 10^{-16}$]. For downward trend (blue), the estimated mean of the differences is −21.1062° [95% CI (−21.7344, −20.4781), indicated by blue broken lines; *t* test $P < 2.2 \times 10^{-16}$].

routine conditions, ∼9% of genes expressed in peripheral blood had a circadian expression profile is a unique demonstration that, even in the absence of a 24-h sleep-wake, light-dark, and fasting-feeding schedule, circadian rhythmicity is a major characteristic of the temporal landscape of the blood transcriptome in humans. This finding also agrees well with previous data for the prevalence of circadian genes in other tissues (for review, see ref. 25). Circadian genes included the core clock genes (*PER1*, *PER2*, *NR1D1*, *NR1D2*, and *ARNTL*), as well as genes involved in metabolism (e.g., *SLC2A3*, *SLC2A14*, *PYGL*, *ABCA1*) and the regulation of gene expression (e.g., *TCEA3*, *ELP2*, *ELAV1*, *SNRPF*, *HNRNPU*). Functional annotation analyses of the rhythmic genes associated with different temporal profiles revealed a marked temporal segmentation of biological processes and functions.

Genes whose transcripts peaked during the biological night (∼58%) included most of the canonical clock genes, and those whose transcripts peaked during the biological day (∼32%) included the antiphase clock gene *ARNTL* and *PROK2*. All three *PER* genes were rhythmic, confirming previous clock-gene expression data from human blood cells and hair follicles (26, 27), and were confined to a small cluster with a peak close to the melatonin offset. Thus, the peripheral blood circadian clock, assessed in the absence of a sleep-wake cycle, appears to operate in concordance with what is known for other tissues. Biological processes and molecular functions associated with biological night genes were predominantly related to nucleic acid binding, gene expression, RNA metabolic processes, RNA binding, ribosome biogenesis, and cellular macromolecular metabolism, which peaked at around 0130 hours. Biological processes, such as immune, defense, stress and inflammatory responses, cytokine receptor activity, IL-1 receptor activity, and NF-κB signaling were more prominent during the biological day, which generally agrees with previous animal studies if we take into account that most animal studies concerned nocturnal rodents (for review, see ref. 8). This biphasic organization of gene expression and associated processes also agrees well with recent data on the temporal, circadian organization, and epigenetic regulation of gene expression (28, 29). One particular characteristic of our protocol is that it demonstrates that this temporal order in the transcriptome persists in the absence of sleep. The large number of transcripts reported here as showing circadian modulation may make it possible to construct circadian phase markers from the blood transcriptome, similar to that suggested for the blood metabolome (30).

Effects of Insufficient Sleep on the Circadian Modulation of the Blood Transcriptome. Comparison of the circadian organization of the blood transcriptome after 1 wk of insufficient or sufficient sleep revealed both stability and change. Stable aspects of the circadian organization included the rhythmic oscillation of 793 genes, which included some of the core clock genes and genes related to specific leukocyte functions. In general, the phase of these oscillations was not changed dramatically, although some subtle changes were observed. More importantly, changes induced by sleep restriction included the marked reduction in the number of transcripts and associated genes that were classified as having a circadian expression profile. In particular, genes whose transcripts peaked during the biological day during the control condition were no longer circadian after sleep restriction. This implies that even in the absence of a sleep-wake cycle, sleep restriction leads to a change in the control of functions and processes such as immune function, response to inflammation and stress. Of particular interest is that sleep restriction also led to a set of genes that became classified as circadian. These genes were associated with functions and processes, such as alanyl-tRNA aminoacylation, alanine-tRNA ligase activity, and translational elongation. Previously, a study in mice reported that mistimed sleep (6 h of sleep deprivation during the light phase) induced rhythmic expression of a large set of genes (11). Whereas in that study the rhythmicity could be related to an acute response to activity in the sleep-deprivation condition, this is unlikely to be the case in the present study because the transcriptome was assessed under constant routine conditions. In the present study, the effects of sleep restriction were, however, not limited to changes in the number of genes classified as circadian or noncircadian. Within the set of genes classified as circadian, sleep restriction also led to a reduction of circadian amplitude and a reduction in the width of the period of expression. Whereas the former finding may be interpreted as a weakening of circadian organization, the effects on the waveform of ex-

pression could be interpreted as a response to the altered duration of the night and associated dark period during sleep restriction. Changes in photoperiod are well known to alter circadian organization and some of the effects of sleep restriction in humans have been interpreted within this framework (31). The observed reduction in amplitude and changes in waveform are unlikely to reflect interindividual differences in changes of circadian phase after sleep restriction because the time-series were aligned with the melatonin rhythm.

Effects of Insufficient Sleep on the Changes in the Blood Transcriptome in Response to Acute Total Sleep Deprivation. The observation that in the control condition only very few genes were affected by acute total sleep loss was unexpected in view of the animal data for acute total sleep loss (8–10, 32, 33). However, it should be noted that there are no comparable blood transcriptome data from animals, nor are there brain or liver data from humans. Thus, it is possible that this difference is related to the different tissues sampled. It is also possible that the effects of sleep deprivation on gene expression are larger in other tissues, or that a longer period of sleep deprivation is required to see the same magnitude of effect in humans. In addition, more genes were up-regulated than down-regulated during acute sleep loss, although less so after sleep restriction compared with the control condition. This result is also different to the findings of previous animal studies and may also be related to differences in the tissues or the sleep deprivation protocols used, or because of limitations in the comparison of diurnal humans with nocturnal animals.

After sufficient sleep, acute total sleep deprivation led to changes in gene expression that were significantly associated with up-regulated processes related to phagocytosis, and down-regulated processes related to protein trimerization, histone H3 acetylation, and striated muscle development. The large (sevenfold) increase in the number of genes responding to total sleep deprivation after sleep restriction was related to biological processes associated with "upward trend" genes, such as IL-6 signaling, inflammatory and defense responses, and responses to external stimuli and wounding; "downward trend" genes were associated with chromosome organization, RNA processing, gene expression, nucleic acid metabolism, macromolecule metabolism, and RNA, nucleotide, and protein binding. The down-regulation with time-awake following sleep restriction of many genes associated with the regulation of gene transcription and translation [e.g., *HIST1H4J*, *HIST1H4F*, *HIST2H2AC*, *NCOR1* (Fig. S5*A*), *SETD2* (Fig. S5*B*) (cf. ref. 34), *MLL* (Fig. S5*C*) (cf. refs. 35 and 36), *SMARCE1, and MYST4* (Fig. S5*D*)] is of particular interest in view of the temporal organization that has been described for these processes (28, 29), but also underlines the significant impact of sleep restriction.

Up-regulation with time-awake after sleep restriction of genes associated with processes such as stress, immune, and inflammatory responses, agrees with what has been observed previously for sleep-deprivation studies designed to assess the correlates of sleep homeostasis. Animal studies have demonstrated the existence of reliable brain-specific markers of sleep homeostasis, such as *Homer1a* (9, 37–40), but also extremely robust cytokine markers with known functional roles, such as IL-1 and TNF, which show increased levels in response to sleep loss (41, 42). As expected, expression of *HOMER1* in blood did not show a significant effect of sleep restriction and remained unchanged with time awake in both conditions of our study of the blood transcriptome. However, during sleep deprivation after sleep restriction we did observe increased expression of *IL6* and *IL1RN* together with up-regulation of *PER2* and the inflammatory response genes *NFKB1D* and *STAT3*.

Our observation that *IL6*, *STAT3*, and *PER2* were up-regulated in response to total sleep deprivation after sleep restriction is in accordance with total sleep deprivation studies that have implicated these genes in sleep homeostasis (33, 43). The fact that these genes were not detected as being up-regulated in the control condition underlines how 1 wk of restricted sleep has exacerbated the effects of acute total sleep deprivation, which is a well-documented phenomenon for cognitive performance measures (44). We also observed up-regulation of three members of the *CEACAM* gene family, which code for Ig-related glycoproteins. Two members of this family were significantly up-regulated after 60 h of prolonged wakefulness in a recent human study (22).

Interaction of Circadian Regulation, the Response to Acute Total Sleep Loss, and the Effects of 1 wk of Insufficient Sleep. The transcriptome, assessed in blood, liver, or brain, is highly dynamic. Our data demonstrate that the history of sleep and wakefulness affects these dynamics in such a manner that the deduced circadian components and responses to acute sleep loss are altered. This finding implies that when only a single sample is measured, the effects of sleep restriction may depend on where in the circadian cycle these effects are assessed, which is why we assessed the effects of sleep restriction by both analyzing the time course of the transcriptome in the two conditions, and also by assessing the overall main effects through ANOVA. Because all of these analyses make use of the same data, the various results should be interrelated. For example, the processes identified as down-regulated by ANOVA are remarkably similar to the processes associated with the genes whose transcripts exhibited a downward trend during acute total sleep deprivation following sleep restriction. Among the most prominent of these processes were chromosome organization, gene expression, nucleic acid metabolism, and cellular macromolecule metabolism. Therefore, the conclusion that these processes are affected by insufficient sleep is justified. The interpretation of the reduction in the number of circadian day active genes may be more complex. The biological processes and functions associated with the day genes that were no longer circadian after sleep restriction included many leukocyte-specific processes, such as immune and inflammatory responses. These were the same processes that were associated with genes whose expression showed an upward trend during time-awake following sleep restriction. Our interpretation of this observation is that circadian rhythmicity was lost because of an increased response to time-awake, although the mechanisms underlying this enhanced response remain unclear. Overall, the data show robust effects of sleep restriction on the human blood transcriptome that are comparable to animal studies. The overlap between the transcripts affected by 1 wk of sleep restriction in our bloodomics study and the transcripts identified as differentially expressed in the mouse liver after 2 wk of sleep restriction (11) was 344 transcripts (46%). Of particular interest, there was overlap with probes targeting circadian genes (*PER2*, *PER3*, *CRY2*, *RORA*, *RHO*) and genes involved in the response to oxidative stress (*PRDX2* and *PRDX5*). The latter two are of specific interest because they form part of the oxidative stress response, and peroxiredoxins have also been reported to be a universal marker of transcription/translation-free circadian rhythmicity (21). This demonstrates that the effects of sleep restriction translate across species and tissues/organs.

These data are important for understanding the mechanisms of how sleep deprivation can lead to circadian disruption and misalignment, and consequent negative effects on health and well-being in general, and with respect to immune function and metabolism in particular. Interactions between sleep restriction and circadian disruption have previously been reported to adversely affect metabolic processes (45).

Sleep Restriction and Health. Our data suggest several pathways by which sleep restriction and circadian rhythmicity may be linked to negative health outcomes associated with insufficient sleep. The baseline circadian data underscore the pronounced rhythmic variation in classic circadian genes, (e.g., *PER1*, *PER2*, *PER3*, *ARNTL*, *CSNK1E*), and genes implicated in metabolism (e.g., *RORA*, *NR1D1*, *NR1D2*, *GHRL*) and sleep homeostasis (e.g., *PER2*) (for review, see refs. 16 and 46), but also in immune function (e.g., *IL6*, *IL1RN*, *STAT3*, *TNFSF4*) (47). Circadian organization of the transcriptome and physiology are often implicated in health and disease (30). Our data show that this

circadian organization is altered and this could be one general pathway by which sleep restriction leads to health problems. In addition, our data show that specific processes are down-regulated or up-regulated by sleep restriction. These processes may affect the temporal organization of gene expression through chromatin modification and remodeling (28), or may simply affect the overall level of specific processes (e.g., immune function) and thereby affect health. In addition, the intensified response to acute sleep deprivation following sleep restriction may imply that insufficient sleep increases the response to challenges and stressors, and in this way negatively affects health. Finally, sleep restriction led to changes in the expression of a number of genes that may be linked to specific health outcomes. For example, the observed changes in *NR1D1* and *NR1D2* are of interest in view of recent animal data, which have shown that *Nr1d1* and *Nr1d2* liver-specific double-knockout mice showed disruption to over 90% of the liver circadian transcriptome and increased circulating levels of glucose and triglycerides (48).

The data emphasize the temporal organization of the human blood transcriptome and identify processes primarily active during the biological day or the biological night. Overall, the results show that sleep debt effects can be readily studied in the blood transcriptome, and imply several mechanisms for its effect on health. The data presented in this study will form an important resource for research on sleep and chronobiology and their interface with health outcomes of insufficient sleep.

Methods

Ethics and Participants. The protocol received a favorable opinion from the University of Surrey Ethics Committee and was approved by the Institutional Review Board of the Air Force Research Laboratory. The study was conducted in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent after receiving a detailed explanation of the aims and procedures of the study and before any procedures described in the study. Individuals were recruited as reported in ref. 49. Data from 26 participants (14 males; mean ± SD of age = 27.5 ± 4.3 y) are presented in this report. The subjects were in general good health as assessed by medical history, physical examination, and standard biochemistry and hematology; they did not suffer from sleep disorders as assessed by self-report questionnaires [Pittsburgh Sleep Quality Index ≤5 (50)] and a clinical polysomnographic recording. The participating individuals were predominantly white (19 of 26) and homozygous for the *PER3* VNTR (rs57875989), with 12 participants carrying the shorter allele. Their habitual sleep duration was 8.2 ± 1.7 h (SD).

Study Protocol. Participants were resident in the clinical research center of the University of Surrey for 12 d on two occasions in a balanced, cross-over design. The interval between the two legs of the study was at least 10 d. Following two baseline nights, participants were scheduled for a sleep-restriction condition (6-h sleep opportunity per night for seven consecutive nights) or a control condition (10-h sleep opportunity for seven consecutive nights), which allowed sufficient sleep for this age group to maintain alertness and performance. Both conditions were followed immediately by a 39- to 41-h constant routine (17), followed by 12-h recovery sleep episode. The midpoint of the 10- and 6-h sleep opportunities coincided with the midpoint of the participant's habitual sleep-wake schedule. Sleep was recorded polysomnographically during all sleep episodes. Waking performance was assessed five times per wake episode during the sleep-restriction and control segments and every 2 h during the constant routine, using a battery of tests. Assessments of sleepiness were based on the well-validated and sensitive Karolinska Sleepiness Scale (51), which is a 9-point Likert scale (1 = very alert to 9 = very sleepy, great effort to keep awake). We also report lapses of attention (reaction time > 500 ms) on the psychomotor vigilance task, which are also among the most sensitive indicators of effects of sleep loss (52, 53). Please see *SI Methods* for a description of the constant routine protocol.

Melatonin Assay and Assessment of Circadian Phase. The onset of melatonin secretion is considered a reliable marker of circadian phase (54) and was determined for each participant in each condition. Each RNA sample was assigned a circadian phase (*SI Methods*).

RNA Extraction from Whole Blood, Labeling, and Microarray Hybridization. See *SI Methods*.

Microarray Statistical Analysis. For details of the quality control and preprocessing of the microarray data, see the *SI Methods*.

ANOVA. For the primary analyses aimed at identifying effects of sleep restriction, we used a mixed-model ANOVA approach as implemented in Procedure Mixed in SAS v9.1. To adjust for multiplicity, we used the Benjamini and Hochberg approach (18). For more details of the ANOVA, see *SI Methods*.

Time-series analyses. To characterize changes over time, we subjected the time-series to analyses aimed at identifying rising or falling trends with time awake or rhythmic components with a 24-h period. We defined a time-series $X_{psc} = \{X_1, X_2, X_3, .. X_{nt}\}$ as the set of n_t time-ordered expressions levels detected by probe *p*, in participant *s*, in sleep condition *c*. A total of 42,119 probes, 26 participants and two sleep conditions, generating over 2 million different time-series, were analyzed. We characterized the time-series based on their time-awake-dependent and circadian properties (Fig. S8).

Identification of time-awake–dependent transcripts. A derivative-based analysis was used to calculate a time-awake cumulative trend for each time-series. Briefly, the cumulative upward and downward trends (*CuT* and *CdT*, respectively) were calculated as the sum of weighted median-normalized slopes of resampled and smoothed *z*-scored series. Time-series with a cumulative trend angle [*arctan(CuT/CdT)*] *P* value of less than 0.05 and a coefficient of variation in the top 90th percentile were classified as being time-awake–dependent. *P* values were based on random resampling of original data, which is a common approach in this area (55). Genes targeted by probes identified as time-awake–dependent in a minimum number of participants (n_s) were defined as having a prevalent time-awake–dependent expression in the associated sleep condition. We determined n_s such that the FDR was 5% or less, by comparing the observed distribution (number of participants in which a probe is identified as time-awake–dependent) to the distribution obtained when time-awake–dependent probes were randomly assigned to participants, but keeping the total number of time-awake–dependent probes per participant constant.

Identification of circadian transcripts. We defined genes whose expression levels have a circadian rhythmicity as those that showed one full oscillation every ~24 h. To identify the set of genes with circadian profiles, we followed a time-domain analysis similar to ref. 56, which fitted a sinusoidal function to the data and set a threshold to the R^2 value of the fit. Comparable to other pattern-matching time-series methods (57–61), this approach allows the characterization of each time-series based on their phase, amplitude, and period. Although frequency-based approaches have proved to be effective [e.g., GeneTS (62) or ARSER (63)], these are better suited for a larger number of sampling time points or higher sampling frequencies. See *SI Methods* for further details.

Comparison of Width at Midcircadian Amplitude. The sine function used for the identification of circadian profiles of gene expression (described above) was fitted to the smoothing spline (64) of the melatonin-aligned median profiles. The resulting sine wave was zero-centered and de-trended based on the linear component of the fit. The width at the midcircadian amplitude of the circadian wave during the biological night was then calculated.

Clustering Analysis. Unsupervised clustering analysis was performed on the prevalent circadian and prevalent time-awake–dependent genes to identify main profile patterns and the biological processes with which they are associated. The median of the melatonin-aligned probe values across all participants was entered in the clustering analysis. The coexpression coefficient-based circular SOM (65) was used to partition the data into distinct groups according to their temporal properties.

Gene-Enrichment and Functional Annotation Analyses. See *SI Methods* for details of the methods used to perform these analyses.

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