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Molecular-Level Dysregulation of Insulin Pathways and Inflammatory Processes in Peripheral Blood Mononuclear Cells by Circadian Misalignment

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Conflict of interest

The authors have declared that no conflict of interest exists.

Abstract

Circadian misalignment due to night work has been associated with elevated risk for chronic diseases. We investigated the effects of circadian misalignment using shotgun protein profiling of peripheral blood mononuclear cells taken from healthy humans during a constant routine protocol, which was conducted immediately after participants had been subjected to a 3-day simulated night shift schedule or a 3-day simulated day shift schedule. By comparing proteomic profiles between the simulated shift conditions, we identified proteins and pathways that are associated with the effects of circadian misalignment, and observed that insulin regulation pathways and inflammation-related proteins displayed markedly different temporal patterns after simulated night shift. Further, by integrating the proteomic profiles with previously assessed metabolomic profiles in a network-based approach, we found key associations between circadian dysregulation of protein-level pathways and metabolites of interest in the context of chronic metabolic diseases. Endogenous circadian rhythms in circulating glucose and insulin differed between the simulated shift conditions. Overall, our results suggest that circadian misalignment is associated with a tug of war between central clock mechanisms controlling insulin secretion and peripheral clock mechanisms regulating insulin sensitivity, which may lead to adverse long-term outcomes such as diabetes and obesity. Our study provides a molecular-level mechanism linking circadian misalignment and adverse long-term health consequences of night work.

Keywords: proteomics, circadian misalignment, glucose regulation, human, sleep, metabolomics

Introduction

The central circadian clock in the hypothalamic suprachiasmatic nuclei (SCN) controls multiple neural and hormonal pathways to orchestrate metabolism and synchronize the timing of peripheral clocks located in a wide variety of tissues. Externally imposed shifts in behavioral rhythms – feeding/fasting, rest/activity, and/or sleep/wake cycles – can misalign peripheral clocks relative to the central clock[1], leading to a state of circadian disruption with potentially adverse impact on metabolism, immunity, and other key aspects of physiology[2, 3]. Night shift workers have been found to be at higher risk for metabolic disorders[4-6], cancer[7], and a host of other chronic diseases[1, 8], for which circadian misalignment may be a mediator[1, 7, 9]. It is essential to understand the molecular mechanisms underlying these effects to be able to implement treatments and regimens to counteract the disease-promoting properties of night shift work.

High-throughput 'omics' methods have been gathered in a number of circadian studies, mainly in mouse models, to study circadian disruption as well as sleep restriction. Studies in human participants have determined that greater than 20% of the transcripts in blood are impacted by circadian misalignment[3, 10]. Previously, we have described the effects of circadian misalignment from simulated night shift work on a subset of the human metabolome through a targeted metabolomics approach[2] and a subset of the transcriptome[11], as well as through the lipidome[12]. Circadian misalignment affected rhythms of the majority of the metabolites and lipids

measured in blood plasma – in particular with respect to their timing – indicating a profound effect on metabolic function[2, 12], and disrupted DNA repair pathways[11].

Mass-spectrometry assisted proteomics has become more prevalent in studying human diseases from cancer[13] to diabetes[14], and has been applied to study circadian rhythms and circadian misalignment. Proteomics studies of rats and mice have identified rhythmic proteins, and determined that between 20-50% of these were not encoded by mRNAs that displayed rhythmic behavior[15, 16]. Using the SomaLogic aptamer-based targeted proteomics approach, one human study identified over 1,000 proteins from the plasma proteome of individuals under simulated night shift conditions and found that 127 (~13%) of these were affected by circadian misalignment[17].

In the current study we analyzed global proteomics from samples of peripheral blood mononuclear cells (PBMCs) taken over time during a 24 h constant routine protocol that followed a 3-day simulated day shift or simulated night shift schedule. We inferred an association network from the proteomics data and the previously described targeted metabolomics data from blood plasma from the same volunteers[2] and used the network to identify proteins of importance in circadian misalignment and to associate metabolites with protein modules[18]. From this analysis we identified patterns of protein expression that are associated with the SCN pacemaker, i.e., those proteins that had similar rhythms following simulated day and night shift indicating that they are driven by central circadian signals, which varied little between the two conditions[2, 19]. We also identified signatures of peripheral clock

signals, which altered the rhythms of proteins following night shift relative to their expression following day shift indicating that they are driven by prior behavior-mediated signals, which were systematically shifted between the two conditions[2, 19].

We report on the circadian behavior of 1,388 proteins from PBMCs and find that circadian rhythmicity of 48% of these were significantly affected by simulated night shift conditions. We show that the temporal profiles of proteins in pathways responsible for insulin secretion and inflammation are profoundly disrupted by circadian misalignment and appear to be driven by dysregulated peripheral clocks. We find that functional implications of this disruption may be reflected in the endogenous circadian rhythms of serum glucose and insulin concentrations.

Methods

Sample acquisition

Fourteen healthy human participants (aged 22–34 y) with normal nighttime sleep schedules were recruited to the sleep laboratory of the Sleep and Performance Research Center at Washington State University Health Sciences Spokane. Participants were randomly assigned to a simulated day shift (i.e., control) condition (n=7) involving three days of daytime wakefulness (06:00–22:00) or to a simulated night shift condition (n=7) involving a daytime nap followed by three days of nighttime wakefulness (18:00–10:00). Following the three days on the simulated day or night shift schedule, participants began a 24 h constant routine protocol during which

they were kept awake under constant laboratory conditions, dim light (< 50 lux), fixed semi-recumbent posture, and evenly distributed food intake (hourly isocaloric snacks).

During the constant routine, blood samples were collected through an intravenous catheter at 2–3 h intervals (Figure 1). These samples were used for targeted plasma metabolomics (n=7 each for day shift [DS] and night shift [NS]) described previously[2]), and untargeted PBMCs proteomics (n=6 each for DS and NS) described here. We note that, due to proteomics sample analysis considerations, only six of the original seven subjects from each condition were analyzed. Metabolomics data were drawn from the same subjects (n=6 per condition). Though metabolomics data were gathered at 2–3 h intervals, for the purposes of the current study only time points that matched with proteomics (6 h intervals) were used. As such, data analyses were limited to four time points in 12 participants (6 in the DS condition, mean age ± SD: 24.2 ± 2.3 y, 4 males; 6 in the NS condition, mean age ± SD: 26.5 ± 1.6 y, 6 males). Further details of the laboratory study and sample collection protocol were reported previously[2].

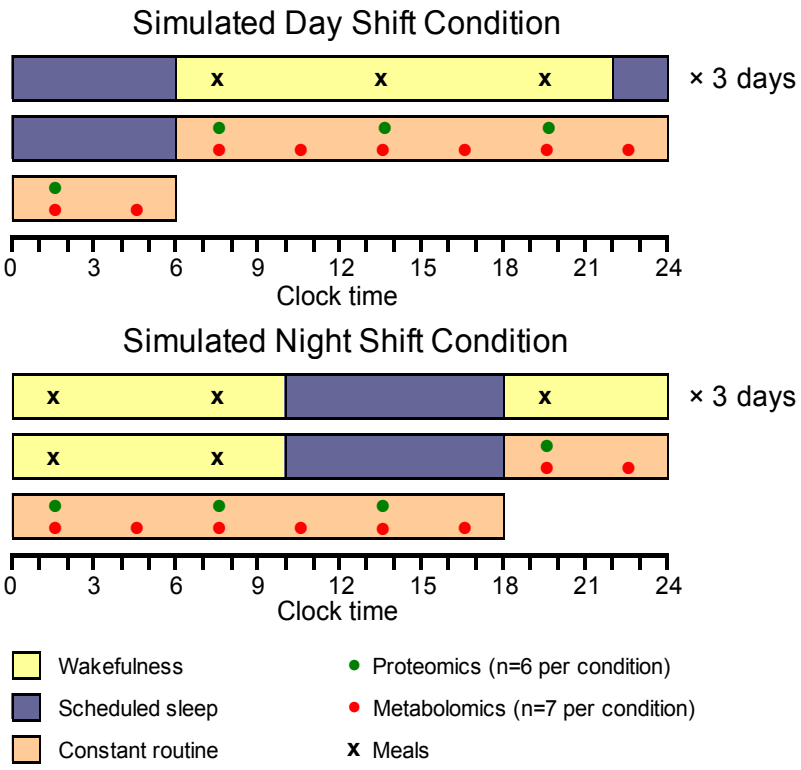

Figure 1. Laboratory study with 3 days on a simulated day shift schedule (top) or night shift schedule (bottom), each followed by a 24 h constant routine protocol (sustained wakefulness under constant conditions, fixed posture, and hourly isocaloric snacks) during which blood samples were taken (n=6 per condition used for this study) and analyzed by targeted metabolomics and mass-spec proteomics. Both conditions were preceded by an adaptation day and night, and the 3-day simulated night shift schedule was also preceded by a 4 h daytime nap to prevent any sleep loss from confounding the data. Both conditions were followed by a recovery period after the constant routine protocol. Ambient temperature was held constant at 21 ± 1 °C, and light levels were fixed at <50 lux during scheduled wakefulness (with lights off during scheduled sleep) throughout the study.

Sample processing and PBMCs isolation

Blood for serum samples was collected in BD Vacutainer Serum Separation Tubes with silica coating and polymer gel for serum separation. Tubes were clotted at 20 °C for 15 min, then

spun down at 1,254 x g for 10 min at 4 °C. Serum was transferred to new tubes kept on ice and subsequently stored at -80 °C until analysis for glucose and insulin concentrations.

Blood for plasma samples was collected in BD Vacutainer tubes coated with dipotassium ethylene diamine tetraacetic acid (K2 EDTA). Tubes were spun down at 300 x g for 10 min at 20 °C with brakes off. Plasma was transferred to new tubes kept on ice and subsequently stored at -80°C until analysis.

PBMCs were isolated using Lymphoprep density gradient and SepMate tubes from STEMCELL Technologies. Following plasma isolation an equal volume of cell sorting buffer (1 mM EDTA in 1x PBS with 2% FBS) was added to the cell pellet and mixed by inverting, then layered onto the Lymphoprep in the SepMate tubes by pipetting down the tube wall. Following centrifugation (1,200 x g at 20 °C for 15 min with brakes on) the top layer containing the PBMCs was collected into a new 50 ml falcon tube. This volume was divided into 1.6 ml tubes for various assays including proteomics as described below. For proteomics use, cell pellets were snap frozen on dry ice and stored in -80 °C following centrifugation (1,000 x g for 5 min at 4 °C) and washing twice with phosphate buffered saline (PBS).

Proteomics reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Ammonium bicarbonate and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA) and Sequencing Grade Modified Trypsin was purchased from Promega

(Madison, WI). Bicinchoninic acid (BCA) assay reagents and standards were obtained from Pierce (ThermoFisher Scientific, Waltham, MA). Purified, deionized water, >18 MW (Nanopure Infinity ultrapure water system, Barnstead, Dubuque, IA) was used to make all aqueous buffers and solutions.

PBMCs protein extraction and trypsin digest

Frozen PBMC samples from the human study were received at Pacific Northwest National Laboratory (PNNL) and resuspended in lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM Potassium Chloride, 0.1 mM EDTA, and 0.1 mM ethylene glycol tetraacetic acid (EGTA).

Estimation of lysate protein concentration was performed with BCA Protein Assay to obtain relative quantitative peptide level information. Proteins were denatured and reduced in this solution with 5 mM dithiothreitol (DTT), followed by alkylation of free sulfhydryl groups with 10 mM iodoacetamide in the dark with each reaction performed for 1 h at 37 °C with constant shaking at 1000 rpm in a Thermomixer R (Eppendorf, Westbury, NY).

Samples were diluted 8-fold with 50 mM Ammonium Bicarbonate buffer pH 7.8 containing 1 mM $CaCl_2$. Trypsin digestion was performed with Sequencing Grade Modified Trypsin prepared according to the manufacturer's instructions. Trypsin was added to protein samples at a 1:50 (wt/wt) trypsin-to-protein ratio and samples were incubated for 3 h at 37 °C in a Thermomixer R. After incubation, the digestion reaction was stopped by acidifying samples to 0.1%

trifluoroacetic acid (TFA) with 10% TFA stock solution. After 15 min 4000 x g centrifugation, samples were transferred to a fresh tube and stored at -80 °C until the next processing step.

Tryptic peptides were desalted, first, via reversed-phase SCX Solid-Phase Extraction (SPE) columns (Discovery-SCX, SUPELCO, Bellefonte, PA) using 80:20 acetonitrile (ACN):500 mM triethylammonium bicarbonate (TEAB) for elution of peptides. Peptide samples were acidified to 0.1% TFA and sample volume was adjusted to 5 ml for each sample after concentration of peptide samples in a SpeedVac vacuum concentrator (Thermo Savant, Holbrook, NY). Next, sequential desalting was performed via appropriately selected C18 Strata C18-E SPE columns (Phenomenex, Torrance, CA). Peptides were eluted from the SPE column with 80% ACN : 20% water and concentrated in the SpeedVac vacuum concentrator[20].

A BCA Protein Assay was performed at this step to determine relative peptide concentration and 100 µg cleaned peptide aliquot from each sample was dried in the SpeedVac and then stored at -80 °C until needed for further analysis.

LC-MS/MS analysis

Global samples were subjected to a custom high mass accuracy Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system as previously described[21, 22], at 2.5 µg total peptide injection amount. The LC component consisted of automated reversed-phase columns prepared in-house by slurry packing 3 µm Jupiter C18 (Phenomenex) into 35 cm x 360 µm o.d. x 75 µm i.d. fused silica (Polymicro Technologies , Phoenix, AZ). The MS component consisted of a

Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) outfitted with a custom electrospray ionization interface. Electrospray emitters were custom made using 360 µm o.d. x 20 µm i.d. chemically etched fused silica capillary. The ion transfer tube temperature and spray voltage were 325 °C and 2.2 kV, respectively. Data were collected for 100 min following a 15 min delay from sample injection. FT-MS survey spectra were acquired from 400 to 2000 m/z at a resolution of 30k (automatic gain control, AGC, target 3e6), and data-dependent FT-HCD-MS/MS spectra were acquired for the top 12 most abundant ions in each survey spectrum with an isolation window of 2.0 m/z and at a resolution of 15k (AGC target 1e5), using a normalized collision energy of 30% and a 60 s exclusion time[21].

Proteomics data analysis

Identification and quantification of the detected peptide peaks was performed utilizing the AMT tag approach (25). Briefly, the generated LC-MS/MS data were searched using MS-GF v6432 (2011-9-8) against Human UniPROT (2015-4-22) over 20,204 protein entries, filtered with a score ≥ 10^{-11} and then used to populate their respective AMT tag databases[23]. Multiple in-house developed informatics tools (publicly available at ncrr.pnnl.gov/software) were used to process the LC-MS/MS data and correlate the resulting LC-MS/MS features to an AMT tag database that contained accurate mass and LC separation elution time information for peptide tags generated from PBMC proteins. Among the tools used were algorithms for peak-picking and for determining isotopic distributions and charge states[24]. Further downstream data analysis incorporated all possible detected peptides into a visualization program, VIPER, to correlate LC-MS/MS features to the peptide identifications in the AMT tag database[25].

Measurement of glucose and insulin concentrations

Serum glucose and insulin were measured using commercially available kits according to manufacturers' instructions. For glucose, a colorimetric detection assay was used (Glucose Colorimetric Detection Kit, EIAGLUC, Invitrogen Corporation, Camarillo, CA). For insulin, a solid-phase sandwich ELISA was used (Human Insulin ELISA Kit, KAQ1251, Invitrogen Corporation, Camarillo, CA). All samples were assessed in duplicate along with standards using a BioTek Synergy Neo Microplate Reader. The mean intra-assay coefficients of variation were 4.73% for glucose and 4.68% for insulin.

Network inference

Association networks were inferred from the combined metabolomics and proteomics data using the GENIE-3 algorithm[26]. GENIE-3 constructs random forest models for each protein and metabolite in the network trained on how well its abundance can be predicted by other proteins and metabolites. We have previously shown that GENIE-3 outperforms many other methods when integrating different kinds of data in networks[27]. A threshold z-score of 2.5 was used to create the network, chosen as a fairly conservative cutoff based on previous work[27]. Communities were detected from the resulting association network using the Girvan-Newman fast greedy algorithm as implemented in the community cluster tool (GLay) in the clusterMaker app in Cytoscape (version 3.7.1).

Functional enrichment

To identify functional pathways that were significantly enriched from the cosinor analysis, we applied a Fisher's exact test for the members of each pathway from the KEGG, Reactome and BioCarta databases[28] using the proteins that were significantly rhythmic in the day and/or night shift conditions, relative to all the proteins observed in our study. This approach was also used to calculate enrichment for the protein modules identified in the network analysis. We next tested for the enrichment of pathways in circadian phase to identify pathways for which the rhythm peak times of members are significantly clustered during particular times of the day. To do so we applied a Kolmogorov-Smirnov rank test to the cosinor-determined acrophases for proteins that were significantly rhythmic. Multiple hypothesis correction was applied using Benjamini-Hochberg and an FDR adjusted $p < 0.05$ was considered to be significant. The leapR package was used to perform all functional enrichment analyses[29].

Statistical and temporal analysis

Generated peptide relative abundance values were $\log_2$ transformed and combined using VIPER[25] to obtain protein-level abundance values.

Proteins were analyzed to determine potential rhythmicity using mixed-effects cosinor analysis[2, 30]. This approach uses mixed-effects regression analysis[31] to estimate amplitude and acrophase (i.e., peak timing) of a cosine curve to data on a specified periodicity (here 24 h). Significance of rhythm was determined by testing the amplitude for each condition against zero using t test, with a one-sided type I error threshold of 0.05. We subsequently performed

analyses with acrophase values for pathway enrichment using the Layered Enrichment Analysis of Pathways in R (leapR) package[29]. The set of significantly rhythmic ($p < 0.05$) proteins were then used to assess temporal patterns.

We compared participants in the day shift and night shift conditions at each time point referenced to time of day. To identify those proteins that are associated with the timing of the central circadian clock we considered proteins that were rhythmic in both conditions and had a small difference in acrophase < 3 h. To identify those proteins that are associated with the timing of shifted behavioral patterns, we considered proteins that were rhythmic in both conditions and had a difference in acrophase > 6 h.

Study approval

The study was approved by the Institutional Review Board of Washington State University. Participants gave written, informed consent and had to meet defined inclusion criteria to be deemed eligible for the study.

Data availability

All data are available as supplemental tables for this publication. Proteomics data have been deposited in the MassIVE database under accession MSV000093054.

Results

Changes in endogenous protein rhythmicity after simulated night work

To assess the effects of circadian misalignment on protein temporal profiles in humans we subjected 14 volunteers to a simulated day shift schedule (n=7) or a simulated night shift schedule (n=7) for three days, then collected sequential blood samples during a 24 h constant routine protocol (Figure 1). Accurate mass tag (AMT) proteomics was performed on PBMCs taken from 12 of the volunteers (n=6 in each condition) at 6 h intervals during the constant routine protocol. This analysis identified a total of 32,455 unique peptides for quantification. These peptides were rolled up to 1,388 proteins with two or more unique peptides. Targeted metabolomics data acquired at higher temporal resolution but filtered to match the same volunteers and time points, assessed using a targeted liquid-chromatography mass-spectrometric (LC-MS/MS) approach, were available from a previous study[2], and used here to link the protein responses to metabolite responses.

We first examined the overall patterns of protein expression throughout the sampling period for the participants following the simulated day shift and night shift conditions. We assessed the coefficient of variation within each treatment and time point sampled among the 12 volunteers (six within each group) and found variation to be between 2-4%. To simplify visualization, we averaged the protein abundance measures from each time point across participants in each of the two conditions, but individual measurements, and coefficients of variation, can be found in Supplemental Table 1. The observed temporal patterns in both shift

conditions are shown as heatmaps in Figure 2, revealing profound differences between the two conditions.

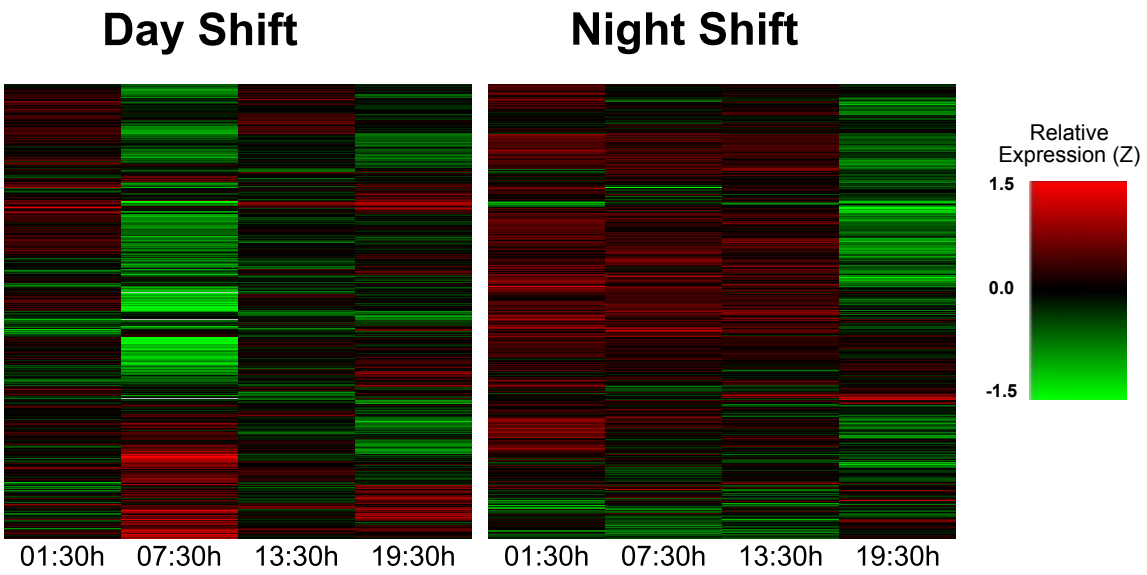

Figure 2. Z-normalized abundance of all of the proteins detected across participants, time points, and day shift (DS) / night shift (NS) experimental conditions, averaged by clock time point across the participants in each of the two study conditions.

To ascertain which proteins exhibited 24 h rhythmic behavior, we analyzed protein abundance changes using the cosinor method in both the day shift and night shift conditions. Results from this analysis are provided in Supplemental Table 1. They indicate that a significant amount (30-50%) of the human proteome follows a circadian rhythm, in agreement with previous estimates from transcriptome[11] and proteome[16] studies. We found that 415 proteins (30%) exhibited significantly rhythmic behavior following day shift, 419 proteins (30%) exhibited significantly rhythmic behavior following night shift, and 131 proteins (9%) maintained rhythmicity under

both conditions, with 36 of those (3%) maintaining a similar phase following both conditions. Overall, 703 proteins were significantly rhythmic in one or both conditions, which is 51% of the proteins observed, and 667 proteins (48%) showed significant alteration of endogenous circadian rhythmicity following simulated night shift.

To examine if rhythmic proteins were enriched in particular pathways, we performed pathway enrichment analysis on each of the sets using the Layered Enrichment Analysis of Pathways in R (leapR) package[29]. We found that proteins rhythmic following simulated day shift were enriched in the glycolysis/pentose phosphate, extracellular matrix interactions, platelet degranulation, and RAC pathways. Proteins rhythmic following simulated night shift were enriched in the caspase apoptosis-related, CD28 T cell activation, and FAS/TNFR1 pathways, which are involved in multiple aspects of the immune response, suggesting that circadian misalignment leads to immune dysregulation. Proteins rhythmic following both the day and night shift conditions were not significantly enriched for any pathways, in part due to the lower number of proteins (138) showing rhythmicity in both conditions. See Supplemental Table 2.

Identification of proteins governed by central versus peripheral clocks

The acrophase (peak time) of the significantly rhythmic proteins varied widely across the 24 h day. Figure 3 shows all rhythmic proteins plotted according to the time of their acrophase in the day shift versus night shift conditions. To identify proteins most affected by circadian misalignment, indicating that their endogenous rhythmicity is associated with peripheral clock timing, we compared temporal patterns between the day and night shift conditions. Starting

with the set of 131 proteins found to exhibit 24 h rhythms in both conditions, we identified those with an acrophase that varied less than 3 h between the two conditions as likely to be associated with the central SCN circadian clock – that is, the timing of their rhythmicity was minimally affected by misaligned circadian and behavioral rhythms. By contrast, those with an acrophase that varied more than 6 h between the two conditions, while measured under constant routine and thus showing their endogenous rhythmicity, were considered to be associated with peripheral clock timing, dysregulated in the night shift condition due to the shifted behavioral time cues[2] (Figure 3). From this analysis 19 proteins were determined to be strongly centrally regulated and 96 proteins strongly peripherally regulated, as shown in Supplemental Table 1.

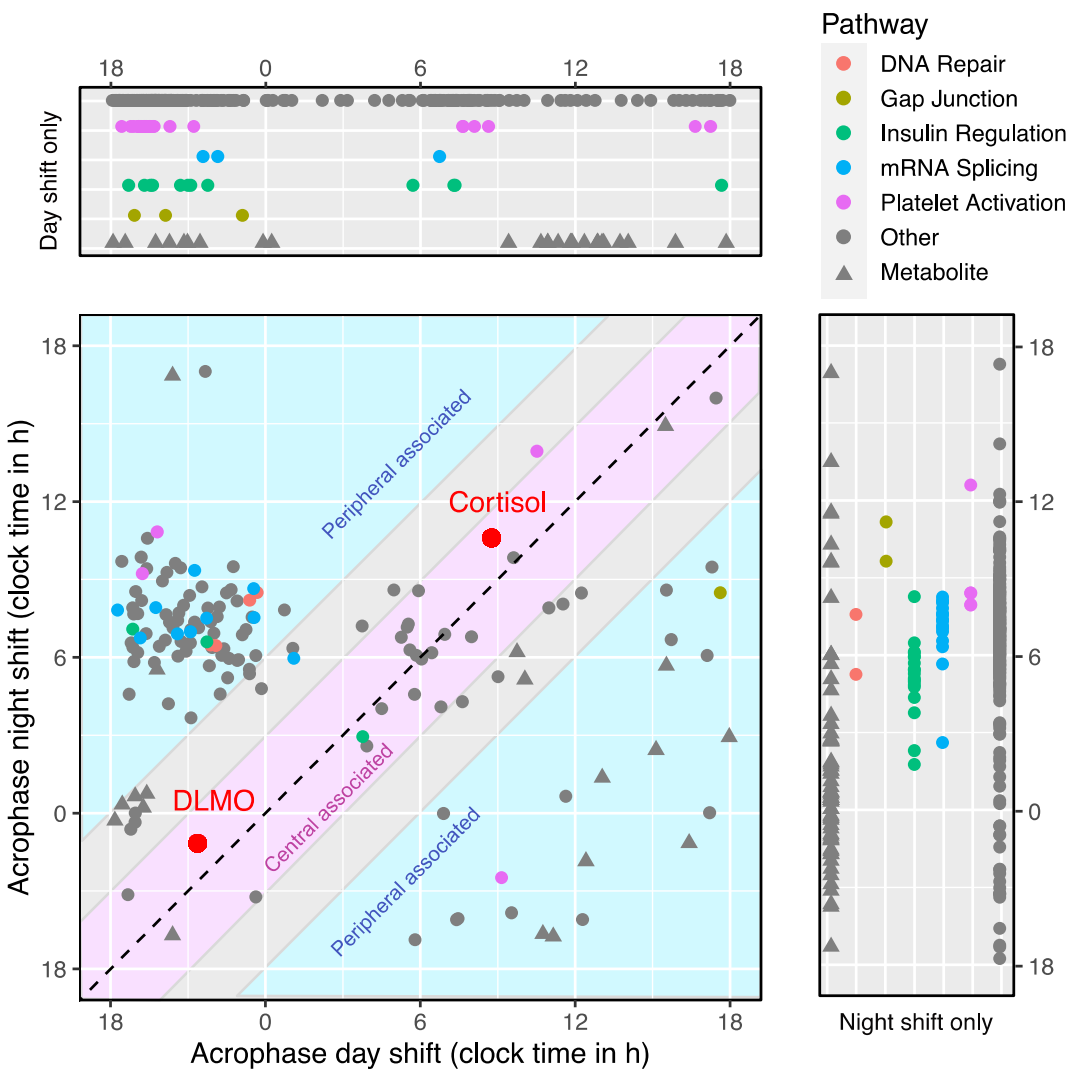

Figure 3. Timing of the acrophase (i.e., peak of the 24 h rhythm in clock time) during constant routine following three days on a day shift schedule (horizontal axes) versus three days on a night shift schedule (vertical axes) in the proteins found to be rhythmic in either or both conditions. Dim light melatonin onset (DLMO) and cortisol acrophase, markers of the timing of the central SCN circadian clock, are shown for reference. The dashed line represents where proteins would appear if the timing of their rhythmicity during constant routine was completely unperturbed by the preceding night shift condition relative to the day shift condition. Proteins (circles) and previously assessed metabolites [2] (triangles) are shown for those found to be rhythmic significantly ($p < 0.05$) only following the day shift schedule (top panel); those found to be rhythmic following both the day and night shift schedules (central panel); and those found to be rhythmic only following the night shift schedule (side panel). Proteins are colored by selected pathways (legend) if those pathways were found to be significantly enriched for rhythmicity in the day shift or night shift condition.

To determine if specific pathways were expressed differentially between the two conditions in terms of timing, we performed further pathway enrichment analyses using the acrophase of the rhythms for identified pathway members (Supplemental Table 2). We found that following simulated day shift, no significant pathways were enriched in proteins that were rhythmic with similar acrophases. Following simulated night shift, however, a number of pathways were significantly enriched – including energy metabolism, focal adhesion, glucose regulation of insulin secretion, and leukocyte transendothelial migration (cf. data points colored by pathway in Figure 3). Following simulated day shift, energy metabolism and insulin regulation had mean acrophases occurring at around 20:00 (clock time), near the end of the daytime waking period just before sleep would normally occur. Following simulated night shift, the mean acrophases were found to be at around 06:00, closer to the end of the prior nighttime waking period. Each of these pathways appeared to uncouple from the central circadian pacemaker and thus appeared to be peripherally regulated (Figure 4).

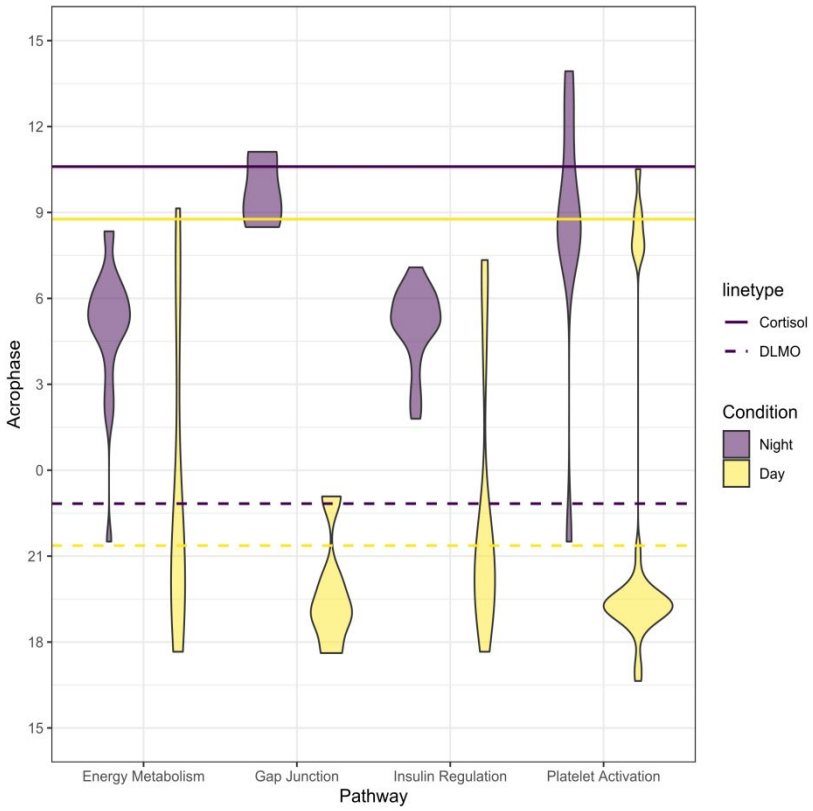

Figure 4. Comparison of the acrophase distribution after night shift condition (purple) and day shift condition (yellow) for pathways that were found to be significantly enriched in either condition. Each distribution consists of the acrophase of all proteins associated with the enriched pathway that were significantly rhythmic ($p < 0.05$) in the given condition. Acrophase is represented on the vertical axis (time of day); wider sections of the plot indicate more proteins had an acrophase in that region while thinner sections indicate a lower quantity. The night and day shift condition acrophase for cortisol and the Dim light melatonin onset (DLMO) are represented by solid and dashed lines, respectively. The general shift in acrophase greater than 6 h following the night shift condition compared to the day shift condition indicates uncoupling from the central circadian pacemaker and thus implies peripheral regulation.

To explore possible functional consequences of the dysregulation we observed in insulin regulation-related proteins from the PBMCs, we assessed the endogenous circadian rhythms of circulating glucose and insulin in serum from the blood samples drawn during the 24 h constant

routine protocol, using mixed-effects cosinor analysis (Supplemental Table 3). As seen in Figure 5, glucose levels showed endogenous circadian rhythmicity under both conditions, with a phase shift of 8.5 ± 2.8 h (mean ± SE) in the night shift condition compared to the day shift condition ($p = 0.010$) indicating a peripheral-driven pattern. By contrast, insulin did not display rhythmicity following simulated day shift, but had a pronounced and altered circadian pattern following simulated night shift, consistent with our proteomics findings.

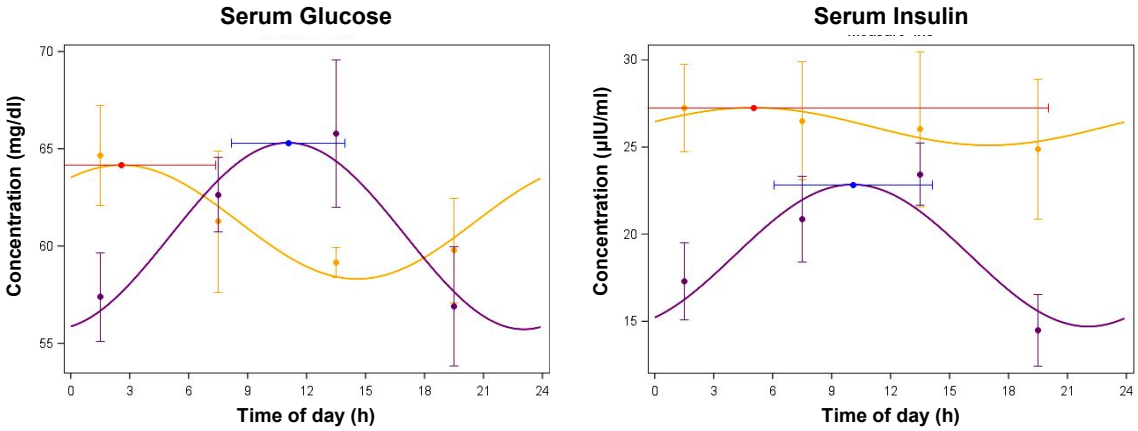

Figure 5. Circulating glucose and insulin. Glucose (left) and insulin (right) were measured in blood serum from all samples and analyzed using the cosinor method. Patterns observed during constant routine following simulated day shift are shown in yellow, and those following simulated night shift are shown in purple. Dots with vertical error bars indicate group means ± SE. Dots with horizontal error bars indicate timing of the circadian peak with 95% confidence interval (red for after day shift, blue for after night shift). The results show differential regulation of endogenous circadian rhythmicity in glucose and insulin following a simulated day versus night shift.

Network data integration

To gain further insight into the mechanisms driving the effects of circadian misalignment, we integrated the rhythms observed in the proteomics and previously collected metabolomics

datasets through network analysis. While network inference can be used to generate integrated association networks linking disparate datasets[32], we recently showed that a random-forest based method, GENIE3, performs best to link different data modalities[33]. Accordingly, we applied GENIE3 to the combined proteomics and metabolomics datasets to infer relationships between components. The results of this analysis are presented in Figure 6.

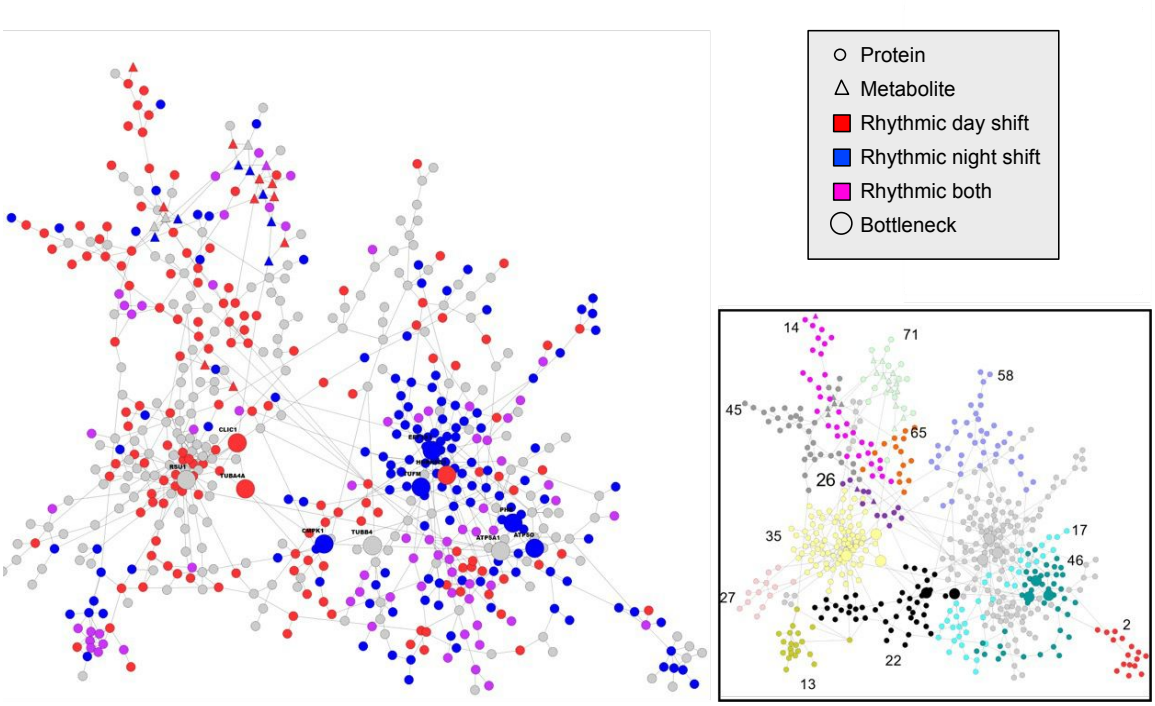

Figure 6. Association network of proteins and metabolites with similar temporal expression profiles across both experimental conditions. Symbols represent proteins (circles) and metabolites (triangles), colored by presence of rhythmicity in either or both simulated shift conditions, and edges represent the inferred relationships between them. Bottleneck proteins, which rank high on betweenness centrality as determined by network topology, are shown by large circles. The inset network shows features clustered into modules (indicated by colors) using a community detection algorithm.

We then applied community detection to identify clusters of proteins and metabolites that exhibit similar behavior. We also analyzed the topology of this network to identify proteins that occupy central locations. Proteins in the network were ranked by betweenness centrality, calculated as the number of shortest paths between all pairs of proteins in the network that pass through that protein – where proteins with higher betweenness represent proteins that link disparate regions of the network, like bridges across a river dividing a busy city[34, 35]. The top ranked proteins, termed bottlenecks, are indicated in Supplemental Table 1 and include ATP5A1, ATP5O, CLIC1, CMPK1, EEF1A1, HNRNPA3, PHB, RSU1, TUBA4A, TUBB4, and TUFM; we discuss some of these below.

Discussion

Circadian misalignment due to simulated night shift work, as compared to simulated day shift work, caused widespread changes in the 24 h rhythms of proteins (Figures 2–4), similar to what we observed previously for metabolites[2], transcripts[11], lipids[12], hormones[19], and cytokines[36]. Notably, these changes involved endogenous circadian rhythms – observed during the 24 h constant routine protocol immediately after the three days on simulated day or night shift, in the absence of any exogenous drivers of rhythmicity. This allowed us to distinguish rhythms associated with behavioral cycles (light/dark exposure, sleep/wakefulness, rest/activity, and/or feeding/fasting) during the simulated shift schedules versus the central circadian pacemaker located in the SCN (which showed little change in timing in the night shift condition compared to the day shift condition[2]. While some proteins (n=19) maintained rhythmicity in

synchronization with the central circadian clock, our data indicate that a substantial portion of the human proteome in PBMCs (~50%) exhibited rhythms impacted by behavioral cycles, uncoupled from the central SCN clock under conditions of circadian misalignment induced by a simulated night schedule.

A previous study examined the response of the plasma proteome to circadian misalignment in six healthy men *during* a simulated night shift schedule, including both wake and sleep periods, and found 127 proteins with 24 h temporal patterns altered between the day and night shift conditions[17]. Of the 1,129 proteins analyzed in that study, 196 were also identified in our study. Comparing the previous results in human blood plasma[17] with the current results in human PBMCs, we found no overlap in classification of central versus peripheral regulation; however, the study did find that immune and inflammatory pathways were dysregulated by circadian misalignment[17], corroborating our observations in PBMCs. Importantly, the difference between studies may reflect the masking effect of sleep and feeding on the observed protein rhythms[37] in the previous study[17]. In the present study, we used a global proteomics approach allowing a more complete assessment of protein abundance in PBMCs, rather than blood plasma. Our study also controlled for masking effects and other potential confounds by taking measurements during a 24 h constant routine protocol[38] immediately *after* a simulated night shift schedule, which exposed the effects of the night shift schedule on *endogenous* protein rhythmicity and thereby revealed the likely underlying central or peripheral clock regulation.

The results we present show that a majority of the proteins measured were rhythmic following the simulated day and/or simulated night shift schedules. Additionally, we identified a subset of these proteins that were rhythmic following both schedules, which could be identified as either primarily associated with the central clock or rather affected by the prior, externally imposed shifts in behavioral rhythms presumably reflected in peripheral oscillators. Relevant to our objective to understand the molecular mechanisms underlying the role of circadian misalignment in the elevated risk of chronic diseases in night shift workers[1], we identified a number of pathways that were misaligned following the simulated night shift schedule, including insulin regulation and inflammation-related pathways.

The pathway for regulation of insulin showed a marked shift from peaking late at night following the day shift schedule to peaking in the early morning following the night shift schedule, indicating that the pathway uncoupled from the central circadian pacemaker and followed a peripheral clock signal. Insulin levels measured in blood serum obtained from our samples indicated insulin dysregulation as well, as evident from internal desynchrony between endogenous circadian rhythms of insulin versus glucose (Figure 5). This suggests there may be functional relevance to the uncoupling of central versus peripheral regulation at the level of the proteome and metabolome[39]. Our findings appear to be consistent with a report that four days of simulated shift work dampens insulin sensitivity[40], a marker of risk for metabolic disease[41], which may be mediated by internal desynchronization between circadian- and behavior-driven rhythms[42] like that found in our study. Data from experiments focused specifically on meal timing[43] suggest a specific role for the prior schedule of food intake in our study, potentially

mediated by shifted circadian rhythmicity in functional enzymatic activity of the gut microbiome[44].

Indeed, recent studies have shown that the temporal pattern of food intake during the prior shift days may serve as a peripheral clock source[2, 43]. In our study, food intake was equally distributed across the 24 h of the constant routine when the blood samples were taken but was shifted to the night in the prior simulated night shift days, thus providing a shifted peripheral clock signal. Notably, some members of the insulin regulation pathway were identified as central clock regulated (IDH3A) or by topological analysis as being bottleneck proteins having high betweenness centrality (ATP5A1, ATP5O). We and others have used betweenness centrality as a proxy of the importance of genes and proteins for function of the system being studied[34]. Taken together, our findings indicate that circadian misalignment causes internal desynchrony and dysregulates insulin regulation at the protein level.

Previous work in human volunteers has shown that insulin regulation and insulin secretion are all affected by circadian misalignment[45] and that insulin sensitivity follows a circadian rhythm in adipose tissues[46], which can be disrupted by circadian misalignment[5, 47]. Additionally, earlier analyses of samples from the present study showed evidence that the cargo of circulating exosomes in the night and day shift conditions differentially affects cellular clock function and metabolic pathways in peripheral tissues, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways PI3K-Akt signaling and insulin resistance[48]. Furthermore, exosomes extracted from samples of the night shift condition caused a substantial shift in rhythmicity of the Akt

phosphorylation response to insulin in naïve differentiated human adipocytes, providing a putative mechanism for the underlying peripheral signaling.

We identified several bottleneck proteins that have been implicated in mitochondrial dysfunction and insulin resistance. This includes prohibitin (PHB), which has been reported to play an important role in adipocyte function[49]. PHB-overexpressing mice have been shown to be insulin resistant[50], and to develop impaired glucose homeostasis and obesity[51]. Another such bottleneck is chloride intracellular channel protein 1 (CLIC1), an ion channel that has been identified as a downstream effector of insulin signaling, though the mechanism is not understood[52]. Further, the mRNA expression of mitochondrial protein ATP synthase subunit O (ATP5O) has been linked to glucose uptake in skeletal muscle[53]. Yet another mitochondrial protein situated as a bottleneck, Tu Elongation Factor (TUFM), can assist with metabolic regulation and was shown to have reduced expression in obese, insulin-resistant mice[54]. Overall, our results suggest that circadian misalignment is associated with a tug of war between central clock mechanisms controlling insulin secretion and peripheral clock mechanisms regulating insulin sensitivity. This may lead to adverse outcomes such as diabetes and obesity, which are prevalent among shift workers[1].

We also identified platelet activation as being dysregulated by circadian misalignment, and this pathway contains a number of proteins involved in inflammation and immune response. Although not much is known about platelet-related abnormalities associated with night shift work, a recent study investigating platelet function reported increased serum COX-metabolized

mediators of eicosanoids after night shift work[55]. Gap junctions, also dysregulated by circadian misalignment, play multiple roles in coordination of immune cell activity for both innate and adaptive responses[56], among other functions. Other research has connected night shift work and circadian misalignment with alterations in inflammatory markers more broadly[5, 36, 57].

Plasma glycine was found to have an acrophase concomitant with inflammation in our study, as well as with insulin regulation discussed above. The connections of glycine with inflammation have previously been reported[58], and glycine has been found to have a cytoprotective effect following cell damage[59]. In our network analysis, glycine was found to be associated with protein phosphatase 1F (PPM1F/POPX2), which promotes apoptosis through Cam kinase[60]. Since our metabolites come from plasma and our protein information from PBMCs, we believe that this observed association likely represents an indirect association of action, which nonetheless could be important in regulation of inflammation and its relationship to circadian misalignment.

Previously, we had used transcriptome data from the same study to show that the DNA repair pathway was disrupted by circadian misalignment[11]. In the current study we observed 6 key proteins of the DNA repair pathways, namely PCNA, APEX1, PARP1, HMGB1, XRCC5 and XRCC6. These DNA repair proteins were identified as associated with peripheral clock regulation, consistent with the earlier transcriptome findings[11]. Interestingly, it is known that DNA damage in adipocytes induced by inflammation and recruitment of inflammatory cells can induce insulin

resistance[61], suggesting a possible connection between the three main pathways identified as dysregulated in our two studies.

We note that although the number of participants in the current study was limited (n=6 for each condition), we were able to show a number of proteins and pathways that exhibited significant rhythmicity and were different following simulated night shift versus day shift conditions. It is possible that increasing the study size would improve resolution and indicate other proteins and pathways that have similar behavior. The cell type we assessed, PBMCs, is a limitation – though a necessary one given the nature of our study. We validated our observation of the dysregulation of insulin pathways in PBMCs by measuring insulin from serum, which showed similar dysregulation.

A previous plasma proteome study of circadian misalignment showed dysregulation of immune and inflammatory pathways, similar to our findings[17]. Previous studies have found sex differences in circadian timing[62], and our study included two female participants in the DS group. To assess whether sex differences were a significant factor in our results we repeated our cosinor analyses with an additional difference parameter for female versus male on circadian phase. No proteins showed a significant phase difference by sex after correction for multiple comparisons (Supplemental Table 4), which does not rule out sex differences, but suggests that this was not a significant factor in our findings.

Collectively, these effects point to dysregulation of insulin regulation and inflammatory processes, which may contribute to the established association between night shift work and cardiovascular disease[4, 63], type 2 diabetes, metabolic syndrome, and other chronic health conditions that have been associated with long-term low-grade inflammation[1]. Though the adverse effects of circadian misalignment from night work on health have long been recognized, our study provides a platform for further understanding and eventually mitigating these effects. Future efforts may involve detailed investigations of the molecular mechanisms suggested by this study, to more fully characterize the important pathways and temporal relationships involved.

Supporting Information

The following supporting information is available free of charge at ACS website https://pubs.acs.org.

Table S1. Processed proteomics data from MS/MS analysis and results of the cosinor rhythmicity analysis.

Table S2. Functional enrichment results from the study.

Table S3. Processed data from glucose and insulin measurements and cosinor analysis.

Table S4. Cosinor analysis of proteomics data for sex differences.

Author Contributions

H.P.A.V.D., D.J.S., and S.G. designed the study. B.C.S. performed the sample collection and processing. R.P.G., K.I.P., and S.G. performed sample processing. J.M.J. performed the mass-

spectroscopy-assisted proteomics. K.R.L. performed glucose and insulin assays. J.E.M., N.J.M., H.M., O.A.A., R.M., and H.P.A.V.D. performed the statistical and network analyses. J.T., H.P.A.V.D., S.G., and D.J.S. provided funding. J.E.M. wrote the paper. All authors reviewed and edited the paper.

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